Ultrastructural changes caused by polymyxin B and meropenem in multiresistant Klebsiella pneumoniae carrying bla_{KPC-2} gene

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The ultrastructural alterations caused by polymyxin B and meropenem and by the association between polymyxin B and meropenem were investigated in two multiresistant isolates of Klebsiella pneumoniae (K3-A2 and K12-A2) carriers of bla_{KPC-2}, coming from infection and colonization in patients in a public hospital in Recife, Brazil. The ultrastructural changes were detected by transmission electron microscopy and scanning. The susceptibility of the isolates to antimicrobials was tested by the disc diffusion method and microdilution in broth. The analysis by electron microscopy showed that the isolates presented morphological and ultrastructural cellular changes when subjected to a clinically relevant concentration of antimicrobials alone or in combination. When subjected to meropenem, they presented retraction of the cytoplasmic material, rupture of the cell wall and extravasation of the cytoplasmic content. When submitted to polymyxin B, the isolates showed condensation of the ribosomes, DNA clotting, cell wall thickening and the presence of membrane compartment. When subjected to polymyxin B and meropenem in combination, the isolates showed a higher intensity of the ultrastructural changes visualized. This is the first report of the ultrastructural changes caused by polymyxin B and meropenem in multiresistant isolates of K. pneumoniae carriers of the bla_{KPC-2} gene. It should be noted that even when the K. pneumoniae isolates were multiresistant carriers of the bla_{KPC-2} gene, they underwent important structural change owing to the action of polymyxin B and meropenem.

INTRODUCTION

Carbapenems are the treatment of choice for severe infections caused by Gram-negative nosocomial pathogens, including extended-spectrum β-lactamase-producing Enterobacteriaceae. The increased use of carbapenems to treat multidrug-resistant bacteria was followed by the emergence of resistance to these antimicrobial agents (Cabral et al., 2012; Jain et al., 2013). The Klebsiella pneumoniae carbapenemase (KPC) enzyme confers resistance to all β-lactam antibiotics, including carbapenems (Lolans et al., 2010; Melo et al., 2014).

The high prevalence of bla_{KPC-2}-producing K. pneumoniae causing colonization and infection among intensive care unit (ICU) patients associated with significant mortality has demonstrated the importance of identification and isolation of the aetiological agent, as well as the early treatment of patients (Lin et al., 2013; Papadimitriou-Olivgeris et al., 2013).

Polymyxins are commonly used for the remaining therapy of infections caused by KPC-producing bacteria when these strains are still susceptible to this antimicrobial drug (Dubrovskaya et al., 2013). The usual treatment of choice for KPC-producing organisms, including in Brazil, is polymyxin B or polymyxin E combined with carbapenem, aminoglycoside or tigecycline (ANVISA–Agência Nacional de Vigilância Sanitária, nota técnica n° 01/2013). This therapy scheme has recently been related to improving survival in patients with bacteraemia (Lee & Burgess, 2013; Munoz-Price et al., 2013).

The increasing development of antibiotic resistance and the lack of new drugs highlight the potential role of combination antimicrobial therapy (Lee & Burgess, 2013) and

Abbreviations: ERIC-PCR, enterobacterial repetitive intergenic consenssus PCR; ICU, intensive care unit; KPC, Klebsiella pneumoniae carbapenemase; MIC, minimum inhibitory concentration; PBP, penicillin-binding protein; TEM, transmission electron microscopy.
recently have led to the revival of interest in the use of polymyxin B (Bassetti et al., 2013).

Bacteria use multiple to protect themselves from adverse environmental conditions, including avoidance of the inhibitory effects of antimicrobial cationic peptides, such as polymyxin B. These strategies include modifications of their lipopolysaccharides, which have overall negative charges and are the initial targets of polymyxins (Moffatt et al., 2010), in addition to the formation of capsules and the use of efflux pump AcrAB–TolC (Campos et al., 2004; Padilla et al., 2010).

When the bacteria divide in the presence of a β-lactam, deficient forms of the cell wall are produced. Since the bacterial intracellular environment is hyperosmotic, the cells swell and lyse (Whitfield & Naismith, 2008). To date, there are no published studies that evaluate and compare the ultrastructural alterations in bacterial cells induced by antibiotics used to treat infections by K. pneumoniae possessing blaKPC, such as polymyxin.

The aim of this study was to investigate and compare the ultrastructural alterations caused by polymyxin B and meropenem, as well as by the association of polymyxin B and meropenem in multidrug-resistant blaKPC-producing K. pneumoniae isolates, obtained from colonization and infection in a public hospital in Recife, Pernambuco, Brazil.

METHODS

Bacterial isolates. For this study, we selected two isolates of K. pneumoniae (K3-A2 and K12-A2). Both were resistant to 16 antimicrobials and were blaKPC positive and were obtained, respectively, from colonization (rectal swab) and urinary tract infection of patients from a public hospital in Recife, PE, Brazil. The isolates were biochemically identified by the automated system BACTEC 9120/BD Phoenix (Table 1). The isolate K3-A2 was tested regarding the antimicrobial profile by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) as well as the presence of K3-A2 was tested regarding the antimicrobial profile by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) as well as the presence of blaKPC, whereas the isolate K12-A2 was characterized in this study. The bacterial cultures were preserved with glycerol (20%) at −70°C, previously the analysis cultures having been grown in brain–heart infusion broth at 37°C for 18 h.

ERIC-PCR. For the ERIC-PCR method, the primers described by Yigit et al. (2001) were used. A negative control was included in each round of amplification. Amplification conditions were described by Melo et al. (2014). The analysis of the amplified fragments by ERIC-PCR and construction of the dendrogram were performed using the Darwin 5.0 software.

Antimicrobial susceptibility testing. The K. pneumoniae isolates were seeded on Mueller–Hinton agar (Bioline), and the susceptibility of the isolates to different classes of antimicrobials was detected by the automated BD Phoenix system and the disc diffusion method proposed by Kirby and Bauer, according to the CLSI (2014). The following antimicrobials were tested: amikacin, aztreonam, ceftazidime, cefotaxime, cefepime, piperacillin/tazobactam, polymyxin B, trimethoprim/sulfamethoxazole, gentamicin, ciprofloxacin, norfloxacin, nalidixic acid, levofloxacin, imipenem, meropenem, and ertapenem. The results were interpreted according to the criteria of CLSI (2014).

Broth microdilution testing for polymyxin B (EuroPharma) and meropenem (European Pharmacopoeia Reference Standard; Sigma) was performed according to the CLSI (2014) recommendations. Previously, to determine the minimum inhibitory concentrations (MICs), the quality control test was performed using the reference strain Escherichia coli ATCC 25922, in accordance with CLSI (2014). For the interpretation of MICs of polymyxin B and meropenem, the following breakpoints against Acinetobacter baumannii (sensitivity, ≤2 µg ml⁻¹; resistance, ≥4 µg ml⁻¹) were adopted when CLSI (2014) did not provide interpretive criteria for polymyxin B against Enterobacteriaceae.

Growth curves. To verify growth inhibition caused by the combination of meropenem and polymyxin B (4 µg ml⁻¹ + 4 µg ml⁻¹), the growth curve was determined at 0, 2, 4, 6 and 8 h. Both isolates were analysed for polymyxin B (4 µg ml⁻¹), meropenem (4 µg ml⁻¹), polymyxin B (4 µg ml⁻¹ + 4 µg ml⁻¹) and a negative control without the antibiotic. Statistical analysis was performed using GraphPad Prism 5 (GraphPad). Analyses were derived from at least three independent biological replicates. The numbers of c.f.u. were analysed using two-way ANOVA, with Bonferroni post-tests, and error bars representing the SEM. Significance was assessed at P<0.05.

Transmission electron microscopy and scanning electron microscopy. The two K. pneumoniae isolates were analysed by transmission electron microscopy (TEM) in the presence of sub-MICs (defined as antibiotic concentrations that allow susceptible strains to continue to grow) for polymyxin B (4 µg ml⁻¹) and meropenem (4 µg ml⁻¹) and between polymyxin B and meropenem combination (4 µg ml⁻¹ + 4 µg ml⁻¹).

A negative control in the same experimental conditions, but without antibiotic, was included in all the procedures. All isolates were inoculated into cation-adjusted Mueller–Hinton broth for 6 h at 37°C in the

Table 1. Origin, ERIC-PCR profile, meropenem and polymyxin B MICs and resistance profile by disc diffusion method of selected nosocomial isolates of K. pneumoniae carriers of the blaKPC gene in Recife, Brazil

<table>
<thead>
<tr>
<th>Identification of isolates</th>
<th>Origin</th>
<th>ERIC</th>
<th>Meropenem (µg ml⁻¹) MIC</th>
<th>Polymyxin B (µg ml⁻¹) MIC</th>
<th>Resistance profile by disc diffusion method</th>
</tr>
</thead>
<tbody>
<tr>
<td>K3-A2</td>
<td>Rectal swab</td>
<td>5E</td>
<td>128</td>
<td>16</td>
<td>AMC, AMO, ATM, CAZ, CFO, CPM, CTX, CIP, LEV(I), NAL, NOR, ERT, GEN, IMP, MPM, PIT, SUT</td>
</tr>
<tr>
<td>K12-A2</td>
<td>Urine</td>
<td>9E</td>
<td>8</td>
<td>128</td>
<td>AMC, AMO, ATM, CAZ, CFO, CPM, CTX, CIP, LEV(I), NAL, NOR, ERT, GEN, IMP, MPM, PIT, POL, SUT</td>
</tr>
</tbody>
</table>

Identification of isolates: K, K. pneumoniae; A2, public hospital; AMC, amoxicillin–clavulanic acid; AMO, amoxicillin; AMI, amikacin; ATN, aztreonam; CAZ, ceftazidime; CFO, cefotaxime; CTX, cefotaxime; CPM, cefepime; PIT, piperacillin/tazobactam; POL, polymyxin B; SUT, trimethoprim/sulfamethoxazole; GEN, gentamicin; CIP, ciprofloxacin; NOR, norfloxacin; NAL, nalidixic acid; LEV, levofloxacin; IPM, imipenem; MPM, meropenem; ERT, ertapenem.
presence or absence of antimicrobial agents. After growth, isolates were fixed and processed in duplicate, the cultures were washed in sterile 0.1 M phosphate buffer (pH 7.2–7.4) and then centrifuged at 3500 r.p.m. for 2 min for post-fixation in 0.1 M phosphate buffer, 2.5 % glutaraldehyde and 4 % paraformaldehyde (Sigma-Aldrich) (Brayner et al., 2005). After fixation, three washes were performed in 0.1 M phosphate buffer; specimens were post-fixed for 1 h with 1 % osmium tetroxide in 0.1 M phosphate buffer (Sigma-Aldrich).

For analysis by transmission microscopy, the isolates were contrasted in the block with 5 % uranyl acetate (Electron Microscopy Science). Subsequently, two washes were performed in 0.1 M phosphate buffer, and one wash was performed in distilled water for 10 min each. Dehydration was performed in a series of increasing acetone concentrations (Sigma-Aldrich) at 30 %, 50 %, 70 % and 90 % and three times at 100 %, for 10 min each. Infiltration of material was performed for replacement of acetone by Epon 812 resin. Therefore, the samples were observed in a triple-blinded study using a transmission electron microscope (Tecnai G2 Spirit BioTWIN; FEI Company).

For scanning electron microscopy analysis, isolates were washed three times, twice in 0.1 M phosphate buffer and once in distilled water, for 10 min each. Subsequent dehydration was performed using an increasing ethanol series of 30 %, 50 %, 70 %, 90 % and 100 %, three times for 10 min each time (Sigma-Aldrich). Thereafter, critical point drying was performed, and ethanol was replaced by carbon dioxide. The dry material was mounted on metal stubs using a double-sided carbon adhesive tape. Then, metallization was performed covering the material with a thin layer of gold, and the bacterial isolate surfaces were observed in a triple-blinded study using a scanning electron microscope (Zeiss EVO LF-15).

RESULTS

Antimicrobial susceptibility

The isolates of *K. pneumoniae* obtained from colonization and infection, without clonal relationship by ERIC-PCR, showed the same resistance profile, including resistance to polymyxin B and meropenem, sensitivity only to amikacin and intermediate resistance to levofloxacin (Table 1). The MICs of meropenem obtained by microdilution method have confirmed the resistance rate as determined by the disc diffusion method (Table 1).

Considering the difference between the MICs of meropenem and polymyxin B for the two isolates, we chose a concentration that would allow growth of cells at the time of the drug association; otherwise, we would not have a sufficient number of cells for microscopic assays, since many cells may be lost during processing. We used sub-MICs; therefore the concentration of meropenem used (4 µg ml⁻¹) represents 1/32 of the MIC for isolate K3-A2 and half the MIC for isolate K12-A2, similarly to polymyxin B, one-quarter of the MIC for K3-A2 and only 1/32 of the MIC for K12-A2.

Growth curves

The growth curves demonstrated that the combination of meropenem and polymyxin B was able to strongly inhibit the growth of both isolates after 6 and 8 h, with a significant decrease of c.f.u. (*P<0.001*), when compared with growth control wells without antibiotics (Fig. 1a).
Ultrastructural and morphological analyses

Control cells of the two isolates of *K. pneumoniae* analysed by TEM, without the addition of antimicrobials, had preserved morphology, maintenance of cell wall integrity, electron-dense cytoplasmic content, the presence of ribosomes and genetic material distributed in the bacterial cytoplasm (Figs 2a and 3a). The analysis of control cells by scanning electron microscopy showed bacterial morphology preserved as rod shaped and a significant number of cells with length from 1 to 5 µm (Figs 4a and 5a).

**K3-A2 isolate.** The isolate K3-A2, despite carrying *bla*KPC-2 and exhibiting resistance to meropenem and polymyxin B, showed ultrastructural cell alterations comparable to the control cells, when in the presence of the cited antimicrobials.

The TEM analysis showed that all cells from isolate K3-A2 when treated with meropenem showed several morphological alterations, such as increased periplasmic space with better visualization of the cellular ends, suggesting cytoplasmic retraction (Fig. 2b). Also, small cytoplasmic compartments and structural disruption of the cytoplasmic membrane and cell wall, without maintenance of the regular bacterial cellular shape (Fig. 2c), were observed. Morphological modifications were also reproduced in the analysis by scanning electron microscopy, where different cell shapes of isolate K3-A2 were found, including oval and spherical shapes as well as aberrant or indefinite forms.

According to TEM analysis, when cells of this strain were treated with polymyxin B, all cells of the K3-A2 isolate showed condensation of ribosomes and coagulation of DNA (Fig. 2d). No morphological alterations were observed by TEM, as the cytoplasmic membrane and cell wall were undamaged; however, cell wall thickening was verified (Fig. 2e). By scanning electron microscopy, it was observed that the cells of isolate K3-A2 maintained their morphology, but 4 µg ml\(^{-1}\) of polymyxin B was enough to greatly reduce the number of cells (Fig. 4c).

The TEM analysis also showed that all cells of K3-A2 when treated with a combination of polymyxin B and meropenem demonstrated more intense cellular damage than when antimicrobials were used separately, presenting cell wall thickening, coagulation of DNA, rupture of cytoplasmic membrane and cell wall, with the loss of cytoplasmic material (Fig. 2f, g). According to scanning electron microscopy, the cells treated with polymyxin B combined with meropenem showed no morphological changes, although some ruptured cells were still evident, with cytoplasmic leakage of content and a reduced number of cells (Fig. 4d).

**K12-A2 isolate.** The isolate K12-A2 carrying presented *bla*KPC-2, having efflux pump and exhibiting resistance to polymyxin B and meropenem, has demonstrated ultrastructural cell modifications in comparison to control cells, when treated with the previously mentioned antimicrobials alone or in combination.

By TEM analysis, all cells treated with meropenem showed rupture of membrane and cell wall, with loss of cytoplasmic material (Fig. 3b, d), the presence of large electrolucent spaces and prominent cell membrane compartments without cytoplasmic material and the presence of ghost cells (Fig. 3c). TEM observations showed that isolate K12-A2 suffered more alterations caused by meropenem than the isolate K3-A2. Scanning electron microscopy analysis revealed that K12-A2 bacterial cells treated with meropenem showed no morphological modifications (Fig. 5b). The differences observed between the TEM and scanning electron microscopy images most likely occurred because scanning electron microscopy only shows morphological alterations in bacterial cells.

It was observed by TEM that all K12-A2 bacterial cells treated with polymyxin B had become elongated, with...
multiple membranes remaining due to the antibiotic action which led to the disruption and destruction of cytoplasmic cell membrane (Fig. 3e–g). The TEM analysis revealed cytoplasmic retractions, with cell the membrane compartment containing cytoplasmic material between the membrane and the cell wall (Fig. 3e). Cell wall thickening as well as disruption of the cytoplasmic membrane and cell wall with the loss of cytoplasmic material, may be seen in Fig. 3(f).

In general, the isolate K12-A2 showed more severe alterations when treated with meropenem than when treated with polymyxin B. Scanning electron microscopy showed elongated cells in the presence of polymyxin B (Fig. 5c), which were also observed by TEM analysis.

However, according to TEM analysis, cells of isolate K12-A2 treated with polymyxin B combined with meropenem showed more intense cellular damage than when the antimicrobials were used separately. Disruption of the cytoplasmic membrane and cell wall, with loss of cytoplasmic material, cell wall thickening, condensation of ribosomes and coagulation of DNA were observed, as well as irregularities in the cell wall (Fig. 3h, i). Scanning electron microscopy observation revealed that cells of this strain in the presence of polymyxin B associated with meropenem apparently showed no morphological changes; however, the combination of meropenem (4 µg ml⁻¹) and polymyxin B (4 µg ml⁻¹) was able to reduce the number of viable cells (Fig. 5d).

**DISCUSSION**

This is the first study on the ultrastructural alterations caused by polymyxin B, meropenem, and polymyxin B in combination with meropenem in multiresistant blaKPC-2-harbouring *K. pneumoniae* isolates.

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**Fig. 3.** Electron micrographs of isolate K12-A2 from *K. pneumoniae*. (a) Untreated control bacterial cells have preserved morphology, with cytoplasmic membrane (M), cell wall (P) and cytoplasmic contents (C) intact. (b–d) Cells treated with meropenem (4 µg ml⁻¹) show membrane and cell wall disruption (RMP) with loss of cytoplasmic material, membrane compartments (CM) and electroluscent space (EE). (e–g) Cells treated with polymyxin B (4 µg ml⁻¹) are elongated (CA), show destruction of the cell membrane (DM), membrane compartments (CM), cytoplasmic shrinkage (asterisk) and membrane and cell wall disruption (RMP) with loss of cytoplasmic material. (h, i) Cell treated with meropenem+polymyxin B in combination (4 µg ml⁻¹+4 µg ml⁻¹) show membrane and cell wall disruption (RMP) with loss of cytoplasmic material, cell wall thickening (EP), condensation of ribosomes (CR), DNA coagulation (CD) and irregularities in the cell wall (arrowheads).
In this study, it was observed that the two isolates showed different responses when treated with the antibiotics. By scanning electron microscopy, the K3-A2 isolate showed morphological changes when exposed to meropenem; in contrast, the K12-A2 isolate demonstrated morphological changes when treated with polymyxin B. In the presence of meropenem and polymyxin, the number of cells observed was reduced because of loss due to poor technique, which limited further analysis.

Isolate K12-A2 showed more alterations after treatment with meropenem than K3-A2, which can be explained since K12-A2 had an MIC of 8 µg ml⁻¹. However, the isolate K3-A2 also showed morphological alterations when treated with meropenem, despite the fact that its MIC was higher.

The β-lactams present bactericidal activity by covalent binding and inactivation of penicillin-binding proteins (PBPs), resulting in deficient synthesis and peptidoglycan remodelling which promotes different morphological alterations (Tang et al., 2014). PBPs of Gram-negative bacteria may be responsible for peptidoglycan synthesis, maintaining the rod shape or septation during bacterial division.

Each PBP has a specific role in the proliferation and maintenance of bacterial morphology, for example, the blocking of PBP-1 leads to rapid bacterial lysis, whereas that of PBP-2 causes spherical cells. Binding of antibiotic agents to PBP-3 results in long filaments due to inhibition of division septa formation after bacterial cell mass replication (Buijs et al., 2008). The efficiency of carbapenems is due to the high affinity for PBP-2 (Walther-Rasmussen & Høiby, 2007). The isolate K3-A2 presented spherical cells after treatment with meropenem, by blocking of PBP-2, which was not observed in K12-A2. However, the latter isolate presented more intense alterations, especially that of the cell compartment membrane without cytoplasmic material; membrane and cell wall disruption, with the loss of cytoplasmic material; and the presence of ghost cells, probably by inhibition of PBP-1.

The ultrastructural cell changes were different among isolates in response to polymyxin B and meropenem, which was expected since the target of polymyxins is the cell membrane (Olaitan et al., 2014), whereas the target of carbapenems is the cell wall (Tang et al., 2014).

The isolate K12-A2 also showed further damage when treated with polymyxin B, even presenting a higher MIC than K3-A2, with disruptions in the cell membrane and loss of cytoplasmic material. This may be explained by the action of polymyxin B, which has a long hydrophobic tail that breaks down the structure of the bacterial cell membrane by interaction with its phospholipids and lipopolysaccharides, with similar action to simple cationic detergents, competitively displacing ions Ca²⁺ and Mg²⁺ that act as membrane stabilizers, causing its rupture, leading to loss of cellular contents and causing bacterial death (Mendes & Burdmann, 2009).

In bacteria, cell wall thickening and condensation of ribosomes indicate mechanisms of defence to maintain the osmotic pressure after antibiotic activity; therefore, the bacteria produce more peptidoglycan to protect themselves.

**Fig. 4.** Electron micrographs of isolate K3-A2 from *K. pneumoniae*. (a) Untreated control bacterial cells with preserved morphology. (b) Cells treated with meropenem (4 µg ml⁻¹) showed morphological changes (asterisks). (c) Cells treated with polymyxin B (4 µg ml⁻¹) that did not exhibit morphological alterations were found in small numbers. (d) Cells treated with meropenem + polymyxin B in combination (4 µg ml⁻¹ + 4 µg ml⁻¹) did not show morphological changes, but disrupted cells with leakage of cytoplasmic contents may be visible (arrow).

**Fig. 5.** Electron micrographs of isolate K12-A2 from *K. pneumoniae*. (a) Untreated control bacterial cell with preserved morphology. (b) Cells treated with meropenem (4 µg ml⁻¹) did not show morphological changes. (c) Cells treated with polymyxin B (4 µg ml⁻¹); show elongated features (asterisks). (d) Cell treated with to meropenem + polymyxin B in combination (4 µg ml⁻¹ + 4 µg ml⁻¹) apparently show no morphological changes but are very small in number (arrow).
(Santhana et al., 2007), which has occurred in isolate K3-A2 when treated with polymyxin B and isolate K12-A2 when treated with polymyxin B in combination with meropenem. Both isolates also showed coagulation of DNA.

Coagulation of DNA means that the DNA gyrase has been inhibited, and therefore DNA replication would be compromised (Dimech et al., 2013; Romano et al., 2013). Isolate K3-A2 when treated with of polymyxin B and meropenem in combination also showed coagulation of DNA.

In the present study, ultrastructural modifications caused by antibiotics used to treat infections caused by K. pneumoniae isolates possessing bla\textsubscript{KPC-2} were evaluated. Polymyxin B in combination with meropenem showed increased efficacy when compared to the antimicrobials used separately. However, the antimicrobials used alone also resulted in ultrastructural damage to the isolates. This is the first study that has investigated and compared the ultrastructural alterations caused by polymyxin B, meropenem, and polymyxin B combined with meropenem against multiresistant K. pneumoniae isolates harbouring the bla\textsubscript{KPC-2} gene.

It is noteworthy that although the isolates of K. pneumoniae were multidrug resistant and carried the bla\textsubscript{KPC-2} gene, they suffered structural modifications due to the action of polymyxin B and meropenem.

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