**INTRODUCTION**

*Acinetobacter baumannii* is commonly associated with nosocomial infections. This organism is usually multi-drug-resistant (MDR) and the therapeutic options to treat *A. baumannii* infections are very limited (Peleg *et al.*, 2008). Unfortunately, no new antibiotics will be available for MDR Gram-negative pathogens in the near future. Old antibiotics, such as colistin (COL) and minocycline (MIN), are generally the most active against MDR *A. baumannii* strains (Peleg *et al.*, 2008). MIN resistance is widespread, mainly due to the presence of the tetB gene (Huys *et al.*, 2005). However, isolates that produce it present a wide range of MIC values (Huys *et al.*, 2005). The reintroduction of intravenous MIN to the market provides an additional agent to the limited options for the treatment of infections by MDR *A. baumannii* (Goff & Kaye 2014). Experience with intravenous MIN to treat infections by *A. baumannii*, particularly nosocomial pneumonia, is scarce and the majority of the patients reported in the literature received more than one antibiotic (Wood *et al.*, 2003; Ritchie & Garavaglia-Wilson, 2014). Only a few reports have described interactions between MIN and other antibiotics in *A. baumannii*, none of them involving strains harbouring the tetB gene; the renewed clinical interest in MIN requires a re-evaluation of these associations (Tan *et al.*, 2007; Liang *et al.*, 2011).

The aim of this study was to assess the *in vitro* antibacterial activity of MIN alone or in combination with rifampicin (RIF), imipenem (IMI) and COL against five *A. baumannii* clinical isolates, selected on the basis of their resistance profiles.

**METHODS**

Five clinical isolates of MDR *A. baumannii* were selected on the basis of different antimicrobial resistance profiles from routine clinical samples of patients admitted to the intensive care unit of a university hospital in Buenos Aires city during the period August–December 2014. Strains were identified using matrix-assisted laser desorption ionization-time-of-flight MS Biotyper software version 3.1 (Bruker Daltonik). Susceptibility was determined using a VITEK 2 system (bioMérieux) and interpreted according to the clinical breakpoints established by the Clinical and Laboratory Standards Institute (CLSI, 2012). PFGE was performed with *Apa* I as described previously (Seifert *et al.*, 2005). The detection of OXA-type carbapenemases and *bla* _AOB_ genes was performed using PCR amplification followed by sequencing, as described previously (Woodford *et al.*, 2006; Poirel *et al.*, 2011).

MICs of *A. baumannii* to MIN, COL, RIF and IMI were determined by the agar dilution method, and interpreted according to the CLSI and Societe Française de Microbiologie for RIF (Members of the SFM...
Antibiogram Committee, 2003; CLSI, 2012). Antibiotic powders were obtained from their respective manufacturers (COL, RIF, MIN: Sigma; IMI: Merck, Sharp & Dohme). The activities of COL, RIF, IMI and their combinations with MIN were evaluated by killing curves. These antimicrobials are either the most active or the most utilized ones, alone or in combination, against A. baumannii infections. The following concentrations were used, COL, 2 µg ml⁻¹; RIF, 4 µg ml⁻¹; IMI, 8 µg ml⁻¹; MIN, 4 µg ml⁻¹. Briefly, tubes containing cation-supplemented Mueller–Hinton broth (Laboratorios Britannia) with and without antibiotic were inoculated with A. baumannii strains in an exponential-phase inoculum of ~5 × 10⁵ c.f.u. ml⁻¹. Tubes were incubated in an ambient atmosphere at 35 °C. At time 0, 4 and 24 h after inoculation, serial 10-fold dilutions were performed and aliquots were plated onto nutrient agar; the number of c.f.u. were counted after 24 h of incubation at 37 °C. To avoid the in vitro carry-over effect, the sample was allowed to be absorbed by the agar until the plate surface appeared dry and then it was spread over the plate. The time-kill studies were performed twice and thus results were analysed by using mean c.f.u. values from the duplicate plates for each isolate.

The 24 h-time-kill curves were produced in exponential-growth phase with two of the clinical isolates (3AAb and 543Ab). Exponential-growth phase studies were performed using a tube macrodilution method in Mueller–Hinton broth with 10⁵ c.f.u. ml⁻¹ inoculum and multiple antibiotic concentrations. The final antibiotic concentrations were: MIN (0.125–64 µg ml⁻¹) with fixed subinhibitory concentrations of COL (0.25 µg ml⁻¹), RIF (4 µg ml⁻¹) and IMI (8 µg ml⁻¹). Each tube was incubated for 24 h at 37 °C, and then 0.1 ml from each tube was diluted and plated onto brain heart infusion agar; finally, the number of c.f.u. was counted after 24 h of incubation at 37 °C. The studies were performed twice and analysed using mean c.f.u. values. The lower limit of detection of this in vitro method was 20 c.f.u. ml⁻¹.

The bactericidal activity of single antibiotics or combinations was defined as a ≥ 3 log₁₀ cf.u. ml⁻¹ decrease in the viable count compared with the initial inoculum, whereas bacteriostatic activity was defined as a < 3 log₁₀ cf.u. ml⁻¹ decrease. Synergism and antagonism were respectively defined as ≥ 2 log₁₀ cf.u. ml⁻¹ decrease or increase in the viable count with the combination compared with the most active agent alone at different time points (Rodriguez et al., 2010).

The time required to decrease the viable count to half the initial inoculum (t₅₀) for each antimicrobial agent and their combinations was assessed by the time-kill curves.

The change in log₁₀ cf.u. ml⁻¹ over the 24 h incubation (Δlog cf.u.24 h) for each association was calculated according to the equation: Δlog cf.u. = 24 h log₁₀ cf.u. ml⁻¹ – 0 h log₁₀ cf.u. ml⁻¹, where cf.u. counts were mean values for duplicate experiments in two isolates (with and without the tetB gene). In addition, the Δlog cf.u.24 h for each association was converted to an effect by the equation: effect = Δlog cf.u.24 h (max) – Δlog cf.u.24 h (for each MIN concentration), where Δlog cf.u.24 h (max) refers to the MIN concentration with the largest positive Δlog cf.u.24 h.

**RESULTS**

All isolates were resistant to ampicillin/sulbactam, piperacillin/tazobactam, ceftriaxone, ceftazidine, imipenem, meropenem, amikacin, gentamicin, trimetoprim/sulphamethoxazole and ciprofloxacins. All the strains harboured *bla_XX2,A3* Carbapenemase, and 3AAb and 1416Ab also carried *bla_HORB*. Strain 543Ab was resistant to COL and RIF by undefined mechanisms, and strain 971Ab showed COL heteroresistance, which was previously defined by Li *et al.*

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**Table 1. Epidemiological and microbiological characteristics of five clinical A. baumannii isolates**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Source</th>
<th>Patient age [years, sex]</th>
<th>Type of infection</th>
<th>PFGE type</th>
<th>Carbapenem-hydrolysing class D oxacillinase</th>
<th>MIC (µg ml⁻¹)</th>
<th>Susceptibility to COL</th>
</tr>
</thead>
<tbody>
<tr>
<td>3AAb</td>
<td>46, male</td>
<td>Bronchoalveolar lavage</td>
<td>Ventilator-associated pneumonia</td>
<td>A</td>
<td>Colistin-hydrolysing</td>
<td>32</td>
<td>No</td>
</tr>
<tr>
<td>1416Ab</td>
<td>71, female</td>
<td>Blood</td>
<td>Ventilator-associated pneumonia</td>
<td>E</td>
<td>Ralstonia-hydrolysing</td>
<td>32</td>
<td>Yes</td>
</tr>
<tr>
<td>46Ab</td>
<td>80, male</td>
<td>Blood</td>
<td>Ventilator-associated pneumonia</td>
<td>C</td>
<td>K. pneumoniae-hydrolysing</td>
<td>32</td>
<td>Yes</td>
</tr>
<tr>
<td>543Ab</td>
<td>51, male</td>
<td>Bronchoalveolar lavage</td>
<td>Ventilator-associated pneumonia</td>
<td>D</td>
<td>K. oxytoca-hydrolysing</td>
<td>32</td>
<td>Yes</td>
</tr>
<tr>
<td>971Ab</td>
<td>83, female</td>
<td>Bronchoalveolar lavage</td>
<td>Ventilator-associated pneumonia</td>
<td>D</td>
<td>K. oxytoca-hydrolysing</td>
<td>32</td>
<td>Yes</td>
</tr>
</tbody>
</table>

**Table 1.** Epidemiological and microbiological characteristics of five clinical *A. baumannii* isolates
in three isolates (2 or 16 × MIC) and bactericidal activity in the other two isolates (64 × MIC). The kill curve for the isolate (1416Ab) studied with subinhibitory concentrations was similar to the growth control curve. In isolates harbouring tetB, the associations studied were always indifferent, whereas in isolates which did not carry the tetB gene, the use of MIN in combination showed a rapid synergistic effect (at 4 h) in four out of nine combinations (two with RIF and one each with IMI and COL). At 24 h this effect was observed in six out of nine combinations (two in each association) (Table 2).

In the 24 h-time-kill curves obtained for strains 3AAb and 543Ab, MIN alone exhibited bactericidal activity at concentrations up to 16 μg ml⁻¹. However, no synergy was observed in isolate 3AAb, which was tetB-positive. In the isolate not harbouring tetB, the MIN combinations showed a synergistic activity, and the bactericidal effect was extended to MIN concentrations in the range 2–8 μg ml⁻¹ in the case of COL and 0.5–8 μg ml⁻¹ for RIF and IMI (Fig. 1).

The t50 values of MIN in combination with COL, IMI or RIF were lower than for MIN alone against tetB-negative isolates, whereas they were similar in isolates harbouring tetB (Table 2).

The relationship between MIN concentration and effect in tetB-negative isolates showed a maximum effect at MIN concentrations of ~4 μg ml⁻¹ for MIN/COL and 16 μg ml⁻¹ for MIN/RIF and MIN/IMI (Fig. 2).

**DISCUSSION**

Tetracyclines show time-dependent killing and have an increased post-antibiotic effect. Their activity is mainly bacteriostatic. Free drug area under the concentration-time curve/MIC appears to be the parameter which was most closely linked to the antibacterial effect. Pharmacokinetic studies have indicated that peak serum concentrations following a 200 mg intravenous dose of MIN range from 2.52 to 6.63 μg ml⁻¹ (mean 4.18 μg ml⁻¹) (Castanheira et al., 2014).

Unlike other previously published studies, we included in the analysis two strains harbouring the tetB gene and the synergistic activity of associations with different concentrations of MIN was evaluated (Tan et al., 2007; Liang et al., 2011).

Although the clinical relevance of this in vitro synergy is difficult to determine, it is logical to think that reaching and maintaining bactericidal MIN concentrations at the infection site would lead to successful therapeutic results.
As was mentioned earlier, MIN (and tetracyclines in general) typically have a bacteriostatic activity; however, some authors have reported a bactericidal effect in A. baumannii isolates (Bantar et al., 2008). In our study, MIN showed bactericidal activity only at concentrations ≥32 μg ml⁻¹ or when the related concentration/MIC in killing curves was ≥64 μg ml⁻¹. However, these concentrations cannot be achieved with therapeutic doses (Fig. 1).

The associations studied in this work have modified the bacteriostatic activity of MIN alone for a rapid initial bactericidal effect in the majority of the isolates without tetB and this synergy occurred at various concentrations of MIN, including the peak concentration and trough at habitual doses. The major effect (>Δlog c.f.u.24 h) was detected at concentrations similar to or slightly higher than the peak level (Table 2, Figs 1 and 2).

In the literature, the MIN/IMI association showed a synergistic effect in isolates both susceptible and resistant to carbapenems (Rodríguez-Hernández et al., 2000). Similar to a report by Liang et al. (2011), we detected synergy in IMI-resistant isolates working with subinhibitory concentrations of carbapenems (4–8 μg ml⁻¹) (Liang et al., 2011). In 24 h-time-kill curves, we confirmed the synergy observed in time-kill curves with one concentration of MIN (4 μg ml⁻¹) in isolates without tetB and this synergy was determined using similar concentrations to those achieved therapeutically (peak 6.6 μg ml⁻¹; trough 1.4 μg ml⁻¹) (Fig. 1). In addition, the t₅₀ values showed a quicker decrease (at 12 h) (Table 2, Figs 1 and 2).
The bactericidal activity of MIN/RIF was observed irrespective of the RIF MIC value (4 or 128 µg ml⁻¹). One study reported the activity of RIF combined with MIN not including isolates with high RIF MIC values (Liang et al., 2011). The features of this synergy are similar to that obtained with IMI, except for the value of t₅₀, which in the isolate 543Ab (MIC RIF 128 µg ml⁻¹) is slightly lower than the t₅₀ corresponding to MIN alone (Table 2, Figs 1 and 2).

MIN/COL synergy was observed in heteroresistant COL strains by suppression of regrowth of the resistant subpopulations. In the COL-resistant strain we observed bactericidal synergy at 24 h despite the high MIC value to both antimicrobials. The COL/RIF synergy in COL-resistant A. baumannii isolates has been described previously (Rodriguez et al., 2010), but to the best of our knowledge, this is the first study reporting the MIN/COL synergistic activity in COL-resistant A. baumannii isolates. The mechanism remains unknown, but the instability of COL-resistant isolates could play an important role in the synergistic activity obtained. Surprisingly, the COL t₅₀ value of the COL-resistant strain is lower than that obtained with the heteroresistant isolate, presumably due to the regrowth of the resistant subpopulations; nevertheless, in the associations with MIN the t₅₀ value is close to 2 h irrespective of the susceptibility to COL (Table 2, Figs 1 and 2).

The activity of MIN and combinations in isolates with tetB were indifferent, irrespective of the MIN MIC value and the concentrations of MIN utilized in the experiments (1/8 × or 8 × MIC). Moreover, the higher Δlog c.f.u.24 h was detected at concentrations not achieved under therapeutic conditions (Table 2, Figs 1 and 2).

Only Tan et al. (2007) have previously reported the performance of MIN-resistant strains. Contrary to our results, they reported synergy in three of four isolates studied, by inhibition of regrowth of the COL-resistant subpopulations.

CONCLUSIONS

MIN has proved to be one of the best antimicrobial agents against MDR A. baumannii; however, the fact that MIN usually presents bacteriostatic activity hinders its use for the treatment of severe infections. The combinations with COL, RIF or IMI showed bactericidal activity in both susceptible and resistant A. baumannii isolates. However, the lack of synergy in isolates with tetB should prevent its use as an empiric treatment in hospitals with a high presence of these strains.

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REFERENCES


