Title: Colistin resistance in a clinical Acinetobacter baumannii strain appearing after colistin treatment: effect on virulence and bacterial fitness.

Running title: Cost of colistin resistance in clinical A. baumannii.

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Abstract.
The fitness and virulence costs associated with the clinical acquisition of colistin resistance by *Acinetobacter baumannii* were evaluated. The growth of strain CR17 (colistin-resistant) was lower than the strain CS01 (colistin-susceptible) when grown in competition (72h competition index, 0.008). In a murine sepsis model, CS01 and CR17 reached spleen concentrations when co-infecting of 9.31 and 6.97 log$_{10}$CFU/g, respectively, with an *in vivo* competition index of 0.016. Moreover, CS01 was more virulent than CR17 with respect to mortality and time to death.

Keywords: *Acinetobacter baumannii*, colistin resistance, fitness cost, virulence, PmrAB mutations.
Acinetobacter baumannii is increasingly clinically relevant due to the rising number of nosocomial infections that it causes, and its ability to develop resistance to all antimicrobials, including colistin (CST) (1). Although the rate of outbreaks of CST-resistant strains remains low, their incidence is increasing due to the rise in the use of this antibiotic (2).

CST-resistance in A. baumannii may occur via mutations in the PmrAB two-component system (3), which leads to the addition of phosphoethanolamine to the lipid A molecule (4, 5), or by the loss of the bacterial lipopolysaccharide due to mutation or insertional inactivation of the genes responsible for lipid A biosynthesis (lpxA, lpxC, and lpxD)(6, 7). In previous reports, the in vitro acquisition of non-stable CST-resistance in the A. baumannii ATCC 19606 strain selected by growth under increasing pressure of the antibiotic, associated with mutations in the PmrAB system, was associated with changes in the expression of numerous proteins (8). This phenotype was associated with decreased fitness and virulence compared to its parental susceptible strain (9). This decrease in virulence could explain the low prevalence of CST-resistance in clinical settings. To illustrate, a report from Rolain et al., described the colonizing nature of a strain that acquired CST-resistance after the clinical administration of CST (10), which was also associated with a mutation in the PmrAB system (11).

We have previously reported the acquisition of CST-resistance in a strain from a CST-treated patient that maintained its ability to cause infection (12). The objective of the present work was to study the cost in terms of fitness and virulence of the CST resistance in this clinical A. baumannii strain.

Two previously described clinical A. baumannii isolates were used, the CST-susceptible CS01 strain (CST MIC: <0.03 mg/L), isolated from the cerebrospinal fluid (CSF) from a patient with meningitis prior to CST treatment, and its CST-resistant derivative (CR17, CST MIC: >16 mg/L), which was isolated from CSF 9 days after initiation of treatment with CST (12). The MIC of CST for CR17 strain was maintained after ten serial passages on plates in the absence of CST, and spontaneous reversion
of CR17 to the susceptible phenotype was not seen during the course of the experiment, indicating that the CST-resistant phenotype was stable. In order to characterize mutations in the PmrAB system, genomic DNA from CS01 and CR17 strains was extracted by resuspending a single colony in 25 µl of water and then lysing the cells by incubation at 100°C for 10 min. After centrifugation, the genomic DNA in the supernatant was used to amplify the pmrA and pmrB genes with specific primers (9), the amplified sequences were cloned into the pGEM-T Easy vector (Promega Biotech Ibérica SL, Madrid, Spain), and sequenced using standard methods. In the resistant CR17 strain, no mutations were found in pmrB, the sensor kinase element. In the response regulator element pmrA, a Met to Lys substitution was found at position 12.

For in vitro growth, bacterial duplication time, and competition index (CI) experiments, growth curves were performed for both strains separately and growing together. Briefly, bacteria at a concentration of 5x10^5 CFU/mL were grown in 20 mL of Mueller-Hinton broth (MH, Becton Dickinson Microbiology Systems, Cockeysville, MD). At 2, 4, 8, 24, 48, and 72 h, 100 µL aliquots were taken and susceptible and resistant CFUs were determined by plating serial log_{10} dilutions on MH agar or MH agar plus 8 mg/L of CST (Sigma Chemical Co., St Louis, MO, USA). The CI was defined as the number of CR17 CFUs recovered/number of CS01 CFUs recovered, divided by the number of CR17 CFUs inoculated/number of CS01 CFUs inoculated. Duplication times were 43 min for CS01 and 40.7 min for CR17. Significant differences in in vitro growth between strains were not observed when grown separately. However, CR17 growth was reduced compared to CS01 when both strains were grown in competition (Figure 1A), with CI at 24h of 0.097 and at 72h of 0.008. These results are similar to those obtained with a CST-resistant strain derived in vitro by growth of the A. baumannii ATCC 19606 strain in the presence of CST, but with unstable resistance to CST (9).

For in vivo bacterial growth and CI experiments in an animal model of peritoneal sepsis, three groups of 19 C57BL/6 female mice (University of Seville, Seville, Spain)
were inoculated intraperitoneally with 0.5 ml containing $5 \log_{10}$ CFU/mL (LD$_{100}$, see below) of each strain, CS01 and CR17, separately and with a mixed inoculum (50% of each strain). Subgroups of three mice were sacrificed at 2, 4, and 8h, and 10 mice at 24h (for calculation of the CI). Spleens were removed aseptically and homogenized (Stomacher 80 Tekmar Co., Cincinnati, OH). CFUs were determined after plating in MH agar with or without CST and the CI calculated as above. For the duration of this experiment (24h), the in vivo growth of CS01 reached a maximal concentration in the spleen of $10 \log_{10}$ CFU/g, whereas CR17 reached a maximal concentration of $9.17 \log_{10}$ CFU/g (Figure 1B). Growing in competition, the maximum concentration of CS01 decreased to $9.31 \log_{10}$ CFU/g (0.69 log$_{10}$ decrease), while that of CR17 decreased to $6.97 \log_{10}$ CFU/g (2.2 log$_{10}$ decrease). The in vivo CI at 24h was 0.016. These results show a lower fitness of the CR17 strain in vivo, suggesting a lower infecting ability than its CST-susceptible parental strain. These results are also in concordance with the experiments performed previously with an in vitro CST-resistant induced strain (9). The in vivo studies were approved by the Ethics and Clinical Research Committee of the University Hospital Virgen del Rocío, Seville, Spain.

The virulence of both strains was assessed in a murine peritoneal sepsis model by measuring mortality and lethal doses following the Reed and Muench method (13), as well as by measuring survival time of infected mice. Briefly, for each strain, groups of 10 animals were infected i.p. with an inoculum of 0.5 mL starting at $8 \log_{10}$ CFU/mL, and serially 10-fold diluted until 100% survival was reached. Bacteria were mixed with porcine mucin (Sigma-Aldrich, Madrid, Spain) at 5% w/v prior to inoculation. The higher mortality of CS01 with respect to CR17 in the peritoneal sepsis model is shown in Table 1, and lethal doses (LD) 100, 50, and 0 are shown in Table 2. In animals inoculated with the minimal lethal dose that produced a 100% of mortality for both strains ($5 \log_{10}$ CFU/mL) the survival time for CS01 was lower than for CR17 (mean ± standard deviation, $23.6 \pm 3.29$h vs. $28.4 \pm 2.19$h, $p<0.026$, Student t test, Figure 2).

These results show a loss in virulence of the clinical strain associated with the
acquisition of CST-resistance produced by clinical treatment with CST, and are consistent with the lower virulence seen in strains that acquired CST resistance in vitro (9). However, it should be noted that a large difference was seen between the virulence of the A. baumannii ATCC 19606 strain (LD50, 6.4 log10 CFU) (6) and the clinical strain CS01 (LD50, 3.29 log10 CFU), highlighting the strain-dependant virulence in A. baumannii that has been described previously (14).

In summary, we have shown decreased fitness and lower virulence associated with the acquisition of CST resistance due to antibiotic pressure during clinical administration of CST. However, although these results could in part explain the low spread of CST-resistance in clinical settings, there are many more factors (1) that may influence the infective ability of clinical strains (14), leading to the emergence of CST-resistant strains able to produce severe infections (12), causing a major clinical concern.

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The nucleotide sequence of the A. baumannii CR17 pmrA gen was submitted to the EMBL database (GeneBank accession number KC776915).
Table 1. Mortality in a murine model of peritoneal sepsis after infection with *Acinetobacter baumannii* CS01 and its colistin resistant mutant CR17 clinical strains.

<table>
<thead>
<tr>
<th>Inoculum (log_{10} CFU/mL)*</th>
<th>8</th>
<th>7</th>
<th>6</th>
<th>5</th>
<th>4</th>
<th>3</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS01</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CR17</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>10</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

* Inoculum volume 500 µL.
Table 2. Lethal doses of *Acinetobacter baumannii* CS01 and its colistin resistant mutant CR17 clinical strains in a murine model of peritoneal sepsis.

<table>
<thead>
<tr>
<th>Lethal Doses (log$_{10}$ CFU/mL)</th>
<th>LD$_{100}$</th>
<th>LD$_{50}$</th>
<th>LD$_{0}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS01</td>
<td>4</td>
<td>3.29</td>
<td>2</td>
</tr>
<tr>
<td>CR17</td>
<td>5</td>
<td>4.39</td>
<td>3</td>
</tr>
</tbody>
</table>

* Inoculum volume 500 µL.
Figure 1. *In vitro* (1A) and *in vivo* (1B) growth of *A. baumannii* CS01 and CR17, separately and in competition.
Figure 2. Time of survival with the minimal lethal dose (5 log_{10} CFU/mL) of A. baumannii CS01 and CR17 that produced a 100% of mortality (survival was assessed every 2 hours).

*p=0.029, Log Rank Test.
References:


