Antimicrobial Combinations against Pan-Resistant Acinetobacter baumannii Isolates with Different Resistance Mechanisms

Gleice Cristina Leite¹,², Maura Salaroli Oliveira¹,³, Lauro Vieira Perdigão-Neto¹,²,³, Cristiana Kamia Dias Rocha², Thais Guimarães⁴, Camila Rizek², Anna Sara Levin¹,²,³, Silvia Figueiredo Costa¹,²,³*

¹ Department of Infectious Diseases, University of São Paulo, São Paulo, Brazil, ² Laboratory of Medical Investigation 54 (LIM-54), São Paulo, Brazil, ³ Hospital Das Clínicas FMUSP, São Paulo, Brazil, ⁴ Department of Infection Control, Hospital das Clínicas, University of São Paulo, São Paulo, Brazil

* costasilvi@ig.com.br

Abstract

The study investigated the effect of antibiotic combinations against 20 clinical isolates of A. baumannii (seven colistin-resistant and 13 colistin-susceptible) with different resistance mechanisms. Clinical data, treatment, and patient mortality were evaluated. The following methods were used: MIC, PCRs, and outer membrane protein (OMP) analysis. Synergy was investigated using the checkerboard and time-kill methods. Clonality was evaluated by PFGE. Based on clonality, the whole genome sequence of six A. baumannii isolates was analyzed. All isolates were resistant to meropenem, rifampicin, and fosfomycin. OXA-23 and OXA-143 were the most frequent carbapenemases found. Four isolates showed loss of a 43kDa OMP. The colistin-susceptible isolates belonged to different clones and showed the highest synergistic effect with fosfomycin-amikacin. Among colistin-resistant isolates, the highest synergistic effect was observed with the combinations of colistin-rifampicin followed by colistin-vancomycin. All colistin-resistant isolates harbored blaOXA-23-like and belonged to CC113. Clinical and demographic data were available for 18 of 20 patients. Fourteen received treatment and eight patients died during treatment. The most frequent site of infection was the blood in 13 of 14 patients. Seven patients received vancomycin plus an active drug against A. baumannii; however, mortality did not differ in this group. The synergistic effect was similar for colistin-susceptible isolates of distinct clonal origin presenting with the same resistance mechanism. Overall mortality and death during treatment was high, and despite the high synergism in vitro with vancomycin, death did not differ comparing the use or not of vancomycin plus an active drug against A. baumannii.

Introduction

Infections caused by multidrug-resistant Acinetobacter baumannii have emerged as a serious problem throughout the world [1]. Old antibiotics, such as fosfomycin and polymyxins, are
now considered potential treatment alternatives to overcome the lack of new antibiotics [2–4]. Studies have demonstrated that fosfomycin is a promising drug, particularly in combination with other antimicrobials for the treatment of infections due to multidrug-resistant (MDR) Gram-negative bacilli. However, there is concern about its use against A. baumannii, due to intrinsic resistance to fosfomycin [5–6]. On the other hand, although polymyxins B and E (colistin) are generally active against multidrug-resistant A. baumannii (MDRAB) [3] and have been used to treat infections, colistin resistance among A. baumannii has been reported and has clearly increased in the last years [4]. In this scenario, treatment with combination therapy, using two or more antibacterial drugs, appears to be the only remaining option [7].

Two of the most frequent in vitro methods used to evaluate interactions between drugs are the checkerboard technique and time-kill kinetics. The checkerboard method only evaluates the inhibitory activity, not bactericidal activity. Additionally, it shows different results when different methods of interpretation are used [8]. Thus, its results may require confirmation using a more dynamic interaction method such as time-kill kinetic studies. To date, few studies have evaluated the antimicrobial combinations against pan-resistant A. baumannii isolates using both methods, and correlations between in vitro and in vivo results are often controversial. There is also some concern as to whether the synergistic effect of antibiotics is related to the resistance mechanism or to the clonality of isolates, or both [9]. Thus, data on the synergistic effect of antibiotic combinations and their efficacy in the treatment of infections caused by Acinetobacter are needed.

The objective of this study was to evaluate the in vitro activity of antibiotic combinations against twenty MDRAB, including pan-resistant isolates with different resistance mechanisms and clonal origins. In addition, clinical and demographic data of patients submitted to different treatments against these infections were evaluated.

Methods

Bacterial Isolates

Twenty A. baumannii isolates were obtained from the bacterial collection of the Laboratory of Bacteriology (LIM-54) of the Department of Infectious Diseases of the School of Medicine, University of São Paulo. Thirteen isolates were colistin-susceptible (at 0.5 mg/L to 2 mg/L) and seven were colistin-resistant (at 8 mg/L to 64 mg/L). Isolates had been stored at -80°C and were subcultured on 5% sheep blood agar before being tested.

Susceptibility testing

Minimal Inhibitory Concentrations (MICs) of colistin (USP-Reference Standard, Rockville, MD, USA), rifampicin, imipenem, gentamycin, amikacin, tigecycline, fosfomycin, vancomycin (Sigma-Aldrich, St Louis, MO, USA), and meropenem (Astra Zeneca, Cotia, SP, Brazil) were determined using the broth microdilution method in duplicate according to the Clinical and Laboratory Standards Institute (CLSI) protocol [10]. Breakpoints for fosfomycin were used according to EUCAST criteria for Enterobacteriaceae [11]. These antibiotics were selected based on local therapeutic protocols used at the Hospital das Clínicas of the University of São Paulo. Pseudomonas aeruginosa ATCC 27853 and Escherichia coli ATCC 25922 were used as controls for susceptibility testing. Pan-resistance was defined as resistance to all antimicrobials tested, and multidrug-resistance was defined as resistance to at least three antimicrobials tested.
Polymerase chain reaction (PCR)

The PCR techniques for carbapenemase genes (bla\textsubscript{OXA-23-like}; bla\textsubscript{OXA-51-like}; bla\textsubscript{OXA-58-like}; bla\textsubscript{OXA-24-like}; bla\textsubscript{OXA-143-like}; bla\textsubscript{IMP}; bla\textsubscript{SPM}; bla\textsubscript{VIM}; bla\textsubscript{SIM}; bla\textsubscript{NDM}), and the genes that encode the omp33-36 porin and carO were performed in duplicate; other genes described in A.\textit{baumannii} were verified by whole genome sequence. DNA were extracted using Illuma bacteria genomicPrep Mini Spin Kit (GE Healthcare Bio-Sciences Corp, USA). For each reaction, a positive internal control was used. The primers used in the study are listed on Table 1.

Analysis of outer membrane proteins

Bacterial cells were obtained from overnight Brain Heart Infusion (BHI) cultures of A.\textit{baumannii}. Extraction of outer membrane proteins as well as the analysis of PAGE was performed in duplicate, using a previously described method [12]. \textit{Acinetobacter baumannii} ATCC 19606 was used as a control.

Pulsed-field gel electrophoresis (PFGE)

Clonality of A.\textit{baumannii} isolates was evaluated using PFGE with Apal endonuclease (Applied Biosystems, Foster City, California, USA) and chromosomal DNA Ultrapure Agarose (Invitrogen\textsuperscript{™}, Life Technologies) [13]. Restriction fragments were obtained by separation using a CHEF DR\textsuperscript{®} III system (Bio-Rad, Hercules, California, USA). Patterns were interpreted according to Bionumerics version 7.1 (Applied-Maths, Sint-Martens-Latem, Belgium). Results from our previous study [12] allowed us to select distinct clones of colistin-susceptible isolates, and to examine the possible different synergistic effects. \textit{Acinetobacter baumannii} ATCC 19606 were used as controls.

Whole genome sequencing

Genomic DNA was extracted using the Illuma bacteria genomicPrep Mini Spin Kit (GE Healthcare Bio-Sciences Corp, USA). An Ion Torrent adapter-ligated library was made following the manufacturer’s Ion AmpliSeq Library Kit (Life Technologies). The whole-genome sequence was determined using Ion Torrent Personal Genome Technology Machine\textsuperscript{™} (PGM) system with a 318 chip (Life Technologies, Foster City, CA). Raw sequencing reads were quality-controlled using Trimmomatic [14]. Draft genomes were \textit{de novo} assembled using MIRA [15]. Genome annotation was performed using Prokka [16], and rRNA was identified using RNAmer [17]. The ResFinder 2.0 server (http://cge.cbs.dtu.dk/services/ResFinder/) was used to identify antibiotic resistance genes. Comparative analysis were made using BLAST (www.ncbi.nlm.nih.gov/blast/) by alignment and by MAFFT (http://mafft.cbrc.jp/alignment/server/) to verify amino acid replacement [18].

MLST (Multilocus sequence typing)

MLST was determined by \textit{in silico} analysis of the draft genomes using the A.\textit{baumannii} MLST database (http://pubmlst.org/abaumannii), and Clonal Complexes were analyzed by eBURST software (http://eburst.mlst.net/) [19].

Checkerboard microdilution

The MDR A.\textit{baumannii} isolates were exposed to combinations of two drugs, and checkerboard microdilution testing was performed in duplicate and evaluated after 20–24 hours of incubation at 35°C. Growth and sterility controls were tested in all plates. Colistin, imipenem, fosfomycin, and tigecycline were combined with amikacin, gentamycin, rifampicin,
vancomycin, and meropenem at the respective minimum inhibitory concentrations determined by microdilution. The antimicrobial agents were diluted from the stock solution, and left at concentrations 4 times higher than the final concentration in plate, and then a serial dilution was performed. FICI and 2-well interpretation methods were used as described by Eliopoulos et al. [20]. FICIs were calculated as \((\text{MIC of drug A in combination})/\text{MIC of drug A alone}) + (\text{MIC of drug B in combination})/\text{MIC of drug B alone})\). Synergy was defined as a FICI of \(<0.5\), indifference as a FICI of \(0.5<4\), and antagonism as a FICI of \(\geq4\). The second method used was the 2-well method, with synergy defined as the absence of growth in wells containing 0.25 x MIC of both drugs and 2 x MIC of both drugs [8, 20].

### Time-Kill assay

Time-kill assays were performed in duplicate for all isolates at concentrations based on the MIC determined from the checkerboard testing of isolates as follows: drugs alone and combined at 1 x MIC and 0.5 x MIC. For isolates with indifferent effect and for colistin-resistant isolates, they were performed combined at 0.125 x MIC, 0.25 x MIC, 2 x MIC, and 4 x MIC. Time-kill analysis was performed based on a previously published method [9]. The tests were repeated for the third time with the isolates that had grown at 24 hours. Synergism was interpreted as a \(\geq 2 \log_{10}\) decrease in colony count with the antimicrobial combination when compared to the most active single agent. The drug combination was considered to be antagonistic if there was a \(\geq2 \log_{10}\) increase in counts, and the combination was considered to be indifferent.

### Table 1. DNA sequences of oligonucleotides used in polymerase chain reactions to detect carbapenemase and outer-membrane proteins of A. baumannii isolates.

| Oligonucleotides | Sequence (5'-3') | Size (bp) | Reference |
|------------------|------------------|-----------|-----------|
| blaOXA51—F       | TAATGCTTTGATCGGCCTTG | 353       | [22]      |
| blaOXA51—R       | TGGATTGACTTTCATCTTGG |           |           |
| blaOXA23—F       | GATGGATTGGAAGACGAGA | 501       | [22]      |
| blaOXA23—R       | ATTCTGACCGCATTTCCAT |           |           |
| blaOXA24—F       | GTTAGCTTGGCGCCCTTTAAA | 246       | [22]      |
| blaOXA24—R       | AGTTGAGGCAAAGGGGATT |           |           |
| blaOXA58—F       | AAGTATTGGGCTTGTGCTG | 599       | [22]      |
| blaOXA58—R       | CCCCTCTGCGCTCTACATAAC |       |           |
| blaOXA143—F      | AGTTAACCTTTCAATTAAT | 604       | [23]      |
| blaOXA143—R      | TTGGAAATTATATATAATCCC |         |           |
| blaSPM—F         | CCTTTTCGCGGACTCTTGATC | 798       | [24]      |
| blaSPM—R         | ATGGCCTCTATCTACGCA |           |           |
| blaSIM—F         | GTACAAGGGTTCGCGCATG | 569       | [24]      |
| blaSIM—R         | GTACAAGGGTGTCGCGCATG |           |           |
| blaIMP—F         | TTGGAAATTATATAATCCC | 188       | [24]      |
| blaIMP—R         | CCAAAACACTAGTGTATC |           |           |
| blaSIM—F         | TTGGTGCAGCATACGCAAG | 382       | [24]      |
| blaSIM—R         | CCAATTCAGCGCCGATGGCAT |     |           |
| blaNDM—F         | GGCAGGGGTTCGCTACGGA | 375       | [25]      |
| blaNDM—R         | GGCAGGCTGCGGCTCTTTC |           |           |
| omp33-36—F       | CATGGAGGCTAATCAGGG | 175       | [25]      |
| omp33-36—R       | AGTTGACCCACCCCAACAT |           |           |
| carO—F           | GTTAGCTTGGCTTGTGCTT | 118       | [25]      |
| carO—R           | GTGTCGAAACCACCAATCA |           |           |

doi:10.1371/journal.pone.0151270.t001

Antimicrobial Combinations against Pan-Resistant A. baumannii

PLOS ONE | DOI:10.1371/journal.pone.0151270 March 21, 2016 4 / 16
if there was a \(< 2 \log_{10}\) increase or decrease in colony count with the combination when compared with the most active drug alone [9].

Clinical and demographic data

The following clinical and demographic data from the medical records of patients hospitalized at Hospital das Clínicas of the University of São Paulo, Brazil, were registered: age, gender, underlying diseases, site of infection, length of stay in the Intensive Care Unit, APACHE II score, and treatment of *A. baumannii* infection (with at least 48 hours of use of a specific antibiotic with activity against *A. baumannii*). Definitions for the infections were those used by the Centers for Disease Control and Prevention [21].

The *in vitro* antimicrobial combinations of vancomycin plus colistin, and vancomycin plus imipenem or meropenem against *A. baumannii* were compared using demographic data. We also evaluated patient mortality during treatment (if the patient was receiving specific treatment for *A. baumannii*) and during hospitalization as outcomes. An Epi Info™ database was built, and results were expressed as means (standard deviation) or median (interquartile range), depending on normality. Statistical analysis was not done due to the limited number of patients. All data were analyzed anonymously and confidentially, with approval by the Research Ethics Committee of the FM-USP (School of Medicine, University of São Paulo).

Results

All 20 isolates of *A. baumannii* were resistant to meropenem, rifampicin, and fosfomycin. All isolates of *A. baumannii* harbored \(\text{bla}_{\text{OXA-51-like}}\), 10 carried the \(\text{bla}_{\text{OXA-23-like}}\) gene, seven carried \(\text{bla}_{\text{OXA-143-like}}\), and three carried the \(\text{bla}_{\text{IMP}}\) gene. The \(\text{bla}_{\text{SPM}}, \text{bla}_{\text{VIM}}\) and \(\text{bla}_{\text{SIM}}\) genes were not identified by PCR. The \(\text{bla}_{\text{SPM}}, \text{bla}_{\text{VIM}}\) and \(\text{bla}_{\text{SIM}}\) genes were not identified. Sixteen isolates carried the \(\text{opm33-36}\) gene and all isolates carried the \(\text{carO}\) gene. For all the isolates harboring \(\text{bla}_{\text{OXA-23-like}}\), the combination of colistin-vancomycin was synergistic. Synergism was present in 80% with the colistin-imipenem combination, and in 80% with fosfomycin-amikacin. For all the isolates that harbored \(\text{bla}_{\text{OXA-143-like}}\), the fosfomycin-amikacin combination was synergistic, and 85.7% presented synergism for the tigecycline-amikacin combination.

The absence or decrease of outer-membrane proteins of 43 kDa, 33–36 kDa, and 29 kDa of the 20 isolates were compared with *A. baumannii* ATCC 19606. In four colistin-susceptible isolates, a total absence of the protein of 43 kDa was observed. These four isolates presented with high MICs for carbapenem and harbored \(\text{bla}_{\text{OXA-51-like}}, \text{bla}_{\text{OXA-143-like}}\) and \(\text{bla}_{\text{IMP}}\). Table 2 shows the MIC of the antibiotics tested and the resistance mechanisms of *A. baumannii* isolates determined by PCR and OMP evaluation. The interpretation criteria for antimicrobial susceptibility established by CLSI and EUCAST are shown on S1 Table.

The colistin-susceptible *A. baumannii* isolates showed eight distinct profiles and the colistin-resistant isolates showed three distinct profiles by PFGE.

There were two distinct ST profiles among colistin-susceptible isolates; two colistin-susceptible isolates were assigned to ST236 (CC103), and one to ST406 (singleton). The three colistin-resistant isolates were assigned to ST233, a member of the CC113. These ST are unrelated to international clones I, II, and III (Table 3).

Based on the FICI method, the synergistic effect was observed only in one isolate for the colistin-vancomycin combination, and in all colistin resistant isolates (\(n = 7\)) for the colistin-rifampicin combination.

All the isolates harboring \(\text{bla}_{\text{OXA-23-like}}\) displayed a synergistic effect of the colistin-vancomycin combination. Four isolates of *A. baumannii*, in which the protein of 43 kDa was absent, had a high MIC for imipenem, and the combinations with carbapenems were
indifferent. Also, all isolates presented synergism for the fosfomycin-amikacin and tigecycline-amikacin combinations.

Table 4 shows the effect of antimicrobial combinations by the checkerboard (FICI and 2-well) and time-kill methods of the combinations tested that showed the largest number for synergic effect. In these antimicrobial combinations, synergism was observed in 100% of isolates by the time-kill assay. Antagonism was not noted.

The percentage of synergistic effect using FICI and 2-well for all antimicrobial combinations against A. baumannii isolates is shown on Table 5.

The time-kill results confirmed a synergistic effect for all isolates with a synergistic effect by checkerboard. The synergistic effect was observed for almost all isolates by time-kill assay, except for combinations with tigecycline, whose synergistic effect was observed with tigecycline and colistin combinations only for susceptible-colistin isolates. The tigecycline with amikacin combination occurred against 17/20 isolates, and tigecycline with meropenem against ten isolates. For fosfomycin combinations, the synergistic effect was not observed when tested with vancomycin and with meropenem. Fig 1 shows the time-kill curve results.

For all isolates there was a synergistic effect of antibiotic combinations at 2 x MIC and 4 x MIC, except for one isolate using tigecycline-gentamicin and one isolate using colistin-gentamicin. For combinations at 0.125 x MIC and 0.25 x MIC, the synergistic effect was observed only for colistin-vancomycin and colistin-rifampicin.
A wide diversity of OXA-genes (OXA-51, OXA-23, OXA-72, OXA-88, and OXA-182) was found by whole genome sequencing as well as aminoglycoside resistance genes and genes encoding a series of proteins for the resistance-nodulation-division (RND) family and their regulators (Table 3). This Whole Genome Shotgun project was deposited at the DDBJ/EMBL/GenBank under the accession LMZH00000000 (Ab03), LMBM00000000 (Ab06), LMBN00000000 (Ab18), LMBO00000000 (Ab23), LMBP00000000 (Ab25), and LMBQ00000000 (Ab28). The carbapenem-resistant isolate with the highest MIC for carbapenem co-harbored OXA types and IMP. On the other hand, less diversity of OXA genes was found among colistin-resistant isolates.

Clinical, demographic, and treatment data of 18 patients were evaluated. Of these, three patients were colonized, and one died before the culture result; thus, a total of 14 patients received antibiotic therapy. Eight patients died during treatment and five during hospitalization; therefore, five continued in the investigation. The most frequent site of infection was the blood, in 13/14 patients. Seven patients received vancomycin plus specific therapy (colistin and or ampicillin/subactam) against A. baumannii; however mortality did not differ for this group compared with the group that did not receive vancomycin, despite the high in vitro synergism of colistin-vancomycin (Table 6).

**Discussion**

The combinations of colistin plus rifampicin, and colistin plus vancomycin showed the highest synergistic effect against colistin-resistant A. baumannii isolates. The synergistic effect in vitro by time-kill analysis occurred at a concentrations lower (0.5 and 1.0 x of the MIC values) than those used in clinical treatment (2.0 to 8.0 x the MIC values). Mortality among patients during treatment of infections due to MDRAB was high, despite high in vitro synergism with vancomycin, considering the use of vancomycin plus an active drug against A. baumannii.
Different interpretation procedures can be used to determine the effect of the antimicrobial combinations, but these methods may lead to different results. Therefore, we chose FICI and 2-well, the most frequently used methods in literature, and the time-kill method, which is considered the gold-standard [26]. We found different results comparing 2-well and FICI as demonstrated by other authors [27]. Bonapace et al. [27] demonstrated a 72% (range 42–97%) agreement between the time-kill test and Etest, and 51% (range 30–67%) between the time-kill and checkerboard tests. Thus, it seems that for clinical purposes, it may be important to confirm checkerboard results with time-kill testing.

To the best of our knowledge, this is the first study that evaluated the synergistic effect of fosfomycin using the time-kill assay against well-characterized colistin-susceptible and resistant \textit{A. baumannii} isolates. We found a synergistic effect with fosfomycin and aminoglycoside against colistin-susceptible and resistant \textit{Acinetobacter} isolates. However, we could not evaluate the use of fosfomycin in our patients because it is not yet approved to be used as systemic treatment in Brazil.

A recent study showed that patients who received combination therapy with colistin and fosfomycin had a significantly more favorable microbiological response and a trend towards more favorable clinical outcomes and lower mortality than those who received colistin alone.

### Table 4. Results of the three different methods (FICI, 2-well, and time-kill assay) used to evaluate \textit{in vitro} synergism of antibiotic combinations against 20 \textit{A. baumannii} isolates.

| Isolates | \textbf{Col+Imi} | \textbf{Col+Van} | \textbf{Col+Mer} | \textbf{Col+Rif} | \textbf{Fos+Gen} | \textbf{Fos+Ami} |
|----------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|          | \textit{aFICI}  | \textit{bTK}    | \textit{aFICI}  | \textit{bTK}    | \textit{aFICI}  | \textit{bTK}    |
| 1        | 1.5/I           | 2/2h            | 0.75/I          | 3/2h            | 1.25/I          | 2/2h            |
| 2        | 2/I             | 2/2h            | 1/I             | 4/2h            | 2/I             | 2/2h            |
| 3        | 3/I             | 6/4h            | 1.5/I           | 3/2h            | 1/I             | 2/2h            |
| 4        | 1.5/I           | 2/2h            | 0.75/I          | 3/2h            | 2/I             | 2/2h            |
| 5        | 2/I             | 6/2h            | 0.75/I          | 3/2h            | 2/I             | 2/2h            |
| 6        | 1.5/I           | 2/2h            | 1.5/I           | 4/2h            | 1.25/I          | 2/2h            |
| 7        | 1.5/I           | 2/2h            | 1.5/I           | 4/2h            | 2/I             | 2/2h            |
| 8        | 1.5/I           | 2/2h            | 1.5/I           | 4/2h            | 2/I             | 2/2h            |
| 9        | 2/I             | 6/2h            | 0.75/I          | 3/2h            | 2/I             | 2/2h            |
| 10       | 1.5/I           | 2/2h            | 1.5/I           | 4/2h            | 2/I             | 2/2h            |
| 11       | 1.5/I           | 6/2h            | 2/I             | 2/2h            | 0.75/I          | 2/2h            |
| 12       | 2/I             | 2/2h            | 1/I             | 4/2h            | 1/I             | 2/2h            |
| 13       | 2/I             | 2/2h            | 1.5/I           | 3/2h            | 1/I             | 2/2h            |
| 14       | 2/I             | 2/2h            | 1.5/I           | 3/2h            | 1/I             | 2/2h            |
| 15       | 2/I             | 2/2h            | 1.5/I           | 3/2h            | 1/I             | 2/2h            |
| 16       | 2/I             | 2/2h            | 1/I             | 4/2h            | 1.5/I           | 3/2h            |
| 17       | 2/I             | 2/2h            | 1/I             | 4/2h            | 1.5/I           | 3/2h            |
| 18       | 2/I             | 2/2h            | 1/I             | 4/2h            | 1.5/I           | 3/2h            |
| 19       | 2/I             | 2/2h            | 1/I             | 4/2h            | 1.5/I           | 3/2h            |
| 20       | 2/I             | 2/2h            | 1/I             | 4/2h            | 1.5/I           | 3/2h            |
| 21       | 2/I             | 2/2h            | 1/I             | 4/2h            | 1.5/I           | 3/2h            |
| 22       | 0.75/I          | 3/2h            | 0.625/I         | 4/2h            | 1.25/I          | 6/2h            |
| 23       | 0.625/I         | 2/2h            | 0.5/I           | 4/2h            | 1.25/I          | 5/2h            |
| 24       | 0.625/I         | 2/2h            | 0.5/I           | 4/2h            | 1.25/I          | 6/2h            |
| 25       | 0.75/I          | 2/2h            | 0.625/I         | 4/2h            | 1.25/I          | 6/2h            |
| 26       | 0.75/I          | 2/2h            | 0.625/I         | 4/2h            | 1.25/I          | 6/2h            |
| 27       | 0.75/I          | 2/2h            | 0.625/I         | 4/2h            | 1.25/I          | 6/2h            |
| 28       | 0.75/I          | 2/2h            | 0.625/I         | 4/2h            | 1.25/I          | 6/2h            |

\textbf{Abbreviations:} Col: colistin; Imi: imipenem; Mer: meropenem; Gen: gentamycin; Ami: amikacin; Rif: rifampicin; Fos: fosfomycin; I: indifferent; S: synergism; h: hour.

\textit{aFICI} (Fractional Inhibitory Concentration Index): value/effect;

\textit{bTK} (Time-Kill): log decrease/bactericidal effect hour.

\textbf{doi:10.1371/journal.pone.0151270.t004}
Nevertheless, the use of fosfomycin to treat infections due to *Acinetobacter* is controversial. Data using intravenous fosfomycin are scarce, *Acinetobacter* usually shows high MICs, and breakpoints are not well defined by CLSI [10] or EUCAST [11]. Few studies have evaluated the synergistic effect of fosfomycin combined with colistin against *A. baumannii* [30–31], which may vary from 37.5% to 75% [30–31]. Despite all limitations, our data demonstrated a high rate of synergy of fosfomycin-aminoglycoside combinations against colistin-susceptible and resistant *Acinetobacter* isolates. This suggests that these combinations may be clinically useful.

### Table 5. In vitro synergistic effect, according to two different methods (FICI and 2-well) of antimicrobial combinations against 20 isolates of multidrug resistant *Acinetobacter baumannii*.

| Antibiotic | Antibiotic tested in combination | Colistin-Susceptible (n = 13) | Colistin-Resistant (n = 7) |
|------------|---------------------------------|------------------------------|---------------------------|
|            |                                 | **Synergism (%)**            | **Synergism (%)**         |
|            |                                 | **FICI** 2-Well               | **FICI** 2-Well            |
| Colistin   |                                  |                              |                           |
|            | Imipenem                         | 0 15                         | 0 100                     |
|            | Tigecycline                      | 0 15                         | 0 0                       |
|            | Gentamycin                       | 0 23                         | 0 0                       |
|            | Amikacin                         | 0 15                         | 0 14                      |
|            | Meropenem                        | 0 23                         | 0 100                     |
|            | Vancomycin                       | 0 69                         | 14 100                    |
|            | Rifampicin                       | 23 77                        | 100 100                   |
|            | Fosfomycin                       | 0 54                         | 0 0                       |
| Imipenem   | Tigecycline                      | 0 31                         | 0 43                      |
|            | Gentamycin                       | 0 54                         | 0 29                      |
|            | Amikacin                         | 0 46                         | 0 14                      |
|            | Vancomycin                       | 0 0                          | 0 0                       |
|            | Rifampicin                       | 0 39                         | 0 0                       |
|            | Fosfomycin                       | 0 23                         | 0 0                       |
| Tigecycline| Gentamycin                       | 0 8                          | 0 29                      |
|            | Amikacin                         | 0 46                         | 0 14                      |
|            | Meropenem                        | 0 8                          | 0 29                      |
|            | Vancomycin                       | 0 8                          | 0 0                       |
|            | Rifampicin                       | 0 0                          | 0 0                       |
|            | Fosfomycin                       | 0 0                          | 0 0                       |
| Fosfomycin | Gentamycin                       | 0 77                         | 0 43                      |
|            | Amikacin                         | 0 100                        | 0 71                      |
|            | Meropenem                        | 0 23                         | 0 29                      |
|            | Vancomycin                       | 0 23                         | 0 14                      |
|            | Rifampicin                       | 0 39                         | 0 0                       |

*Synergism is a positive interaction; the combined effect of the drugs being examined is significantly greater than the expected result. The isolates that showed no synergistic effect showed indifference; antagonism was not observed for any combination against any isolate tested.

**FICI**—Fractional Inhibitory Concentration Index.
In the present study, all colistin-resistant *A. baumannii* isolates harboring *bla*OXA-23-like belonged to CC113 (ST233). This differed from prior reports that identified colistin-resistance in ST2 of the international clone II [32] and ST375 [33] in Italy and in Korea [32–33]. On the other hand, the colistin-susceptible *A. baumannii* isolates in our study that harbored...
bla\textsubscript{OXA-23}-like belonged to CC103 (ST236). This CC has already been reported by Coelho-Souza et al. [34] in 2003 in another state of Brazil. Most OXA-23-producing A. baumannii isolates in Latin America belong to CC113, in contrast with studies in many countries around the world that showed that A. baumannii harboring OXA-23 isolates were related to specific clones belonging to CC92 [35–42].

OXA-23 and OXA-143 were the most frequent resistance mechanisms found in our isolates. A. baumannii harboring OXA-23 has been reported in many countries including Brazil, and it is associated with resistance to imipenem [43–47]. This is the first report of OXA-88 and OXA-182 among clinical isolates of A. baumannii in Brazil. OXA-88 sequences differ from OXA-51 by five to eight amino acids. It has been described among clinical isolates in Singapore [48]. OXA-182 has been reported among A. baumannii isolates in Korea and revealed 93% nucleotide identity with \textit{bla\textsubscript{OXA-143}} [49]. Other carbapenemase found in our isolates was OXA-72, already described in Brazil [50–52].

Outer-membrane proteins of 43 kDa previously associated with carbapenem resistance in \textit{Acinetobacter} spp. [53] were absent in four isolates analyzed in the present study. These isolates showed a high MIC for imipenem and an indifferent effect of combinations with carbapenem. However, in all isolates, fosfomycin-amikacin and tigecycline-amikacin combinations were synergistic.

In our study, the combination of colistin with rifampicin showed the highest synergistic effect against colistin-resistant A. baumannii. This combination has already been suggested for treatment of MDRAB by both \textit{in vitro} and \textit{in vivo} studies, mainly in case series [54]. There is only one prospective small randomized trial that showed a higher rate of microbiological cure.

| Table 6. Clinical, demographic, and vancomycin time-kill synergism data of 18 patients colonized and infected by multidrug-resistant \textit{Acinetobacter baumannii}. |
|-----------------|-----------------|-----------------|
| Age (years), mean (range) | 43 (15–68) | 38 (16–59) | 44 (15–69) |
| Female | 6 | 5 | 1 |
| Underlying diseases |
| Hematologic cancer | 4 | 0 | 4 |
| Cirrhosis | 3 | 3 | 0 |
| Abdominal surgery | 2 | 1 | 1 |
| Trauma | 2 | 2 | 0 |
| Kidney transplant | 1 | 1 | 0 |
| Others | 5 | 2 | 3 |
| Intensive Care Unit | 6 | 4 | 2 |
| Apache II, median | 16 | 15 | 13 |
| Colonization | 3 | - | - |
| No treatment | 4\* | - | - |
| Infection | 14 | 6 | 8 |
| Blood | 13 | 6 | 7 |
| Tracheal secretion | 1 | - | 1 |
| Treated A. baumannii infection | 14 | 6 | 8 |
| In \textit{vitro} synergism with vancomycin plus colistin by time-kill | 14 | 6 | 8 |
| In \textit{vitro} synergism with vancomycin plus imipenem by time-kill | 9 | 4 | 5 |
| Received vancomycin plus A. baumannii treatment | 7 | 2 | 5 |

*Three colonization (one blood specimen collected by central venous catheter and two fecal specimens) and one patient with a bloodstream infection that died before treatment.

doi:10.1371/journal.pone.0151270.t006
in the colistin and rifampicin combination group (71% of 15 patients) compared to the colistin group (59% of 13 patients) [55]. However, due to the high incidence of tuberculosis infection in our country, the use rifampicin is restricted and not used to treat A. baumannii infections.

In this study, we detected a synergistic effect of colistin associated with vancomycin in 80% of isolates by using the 2-well method. These results were confirmed by the time-kill assay, considered the gold standard. Three previous studies reported the activity of a combination of colistin with vancomycin against MDRAB; however, they analyzed a very small number of A. baumannii isolates [56–58]. One study evaluated six A. baumannii isolates by the checkerboard method [56], and synergism was detected in four. The other study included only three colistin-resistant A. baumannii isolates and showed a synergistic effect for all [58]. A study conducted by Vidaillac et al. [57] showed that all tested combinations including colistin-vancomycin were synergistic against four isolates of A. baumannii. According to these authors, colistin would disrupt the outer membrane and could facilitate glycopeptide penetration across the outer membrane, thus exposing the target site in the cell wall. The synergistic effect of colistin and vancomycin may be clinically useful in the intensive care setting because the empiric combination for septic patients usually includes a beta-lactam plus vancomycin. In hospitals with high MDR rates, polymyxin is added. On the other hand, this combination may involve risks such as acute kidney damage, and its impact on mortality has not yet been clearly demonstrated [58]. Although we found a high in vitro rate of synergism with vancomycin, death among patients who received vancomycin did not differ from that of those who did not receive it. These results could be due to the small number of patients evaluated, as well as the site of infection. The types of resistance mechanism among A. baumannii isolates could explain, at least in part, why combination regimens reported as successful in literature were not successful in our assays.

In our study, although we found a high rate of susceptibility to tigecycline (only 5% of isolates were resistant to this antibiotic), few antibiotic combinations using tigecycline showed a synergistic effect. The highest synergistic effect achieved with tigecycline was in combination with amikacin, similar to a prior study reported by Petersen et al. [9]. Thus, it would seem that tigecycline is not a good option to use in a combination to treat infection due to A. baumannii harboring OXA-23 and OXA-143, the most frequent resistance mechanisms identified in our isolates.

Our study has several limitations, such as the retrospective design of the study and the number of patients evaluated. We tried to evaluate the impact of “in vitro synergism” of vancomycin on death; however, we only had information on 18 patients, and only seven received vancomycin plus specific treatment against A. baumannii. The colistin-resistant isolates were closely related, the colistin-susceptible isolates belonged to different clones, but the synergistic effect was similar for isolates showing the same resistance mechanism.

In conclusion, our study demonstrates that colistin plus rifampicin, and colistin plus vancomycin showed the highest synergistic effect against colistin-resistant A. baumannii isolates. Among colistin-susceptible isolates, the highest synergistic effect was achieved with fosfomycin combined with amikacin or with gentamycin, and colistin combined with rifampicin or with vancomycin. Moreover, the synergistic effect against MDRAB appears to be related to the resistance mechanism and not to the clonality of isolates. Despite the high synergism in vitro with vancomycin, mortality of patients did not differ when comparing the use or not of vancomycin plus an active drug against A. baumannii.

**Supporting Information**

S1 Table. Antimicrobials susceptibility established by CLSI and EUCAST.

(DOCX)
Author Contributions
Conceived and designed the experiments: GCL SFC ASL. Performed the experiments: GCL CKDR CR. Analyzed the data: MSO LVPN TG SFC. Contributed reagents/materials/analysis tools: GCL MSO LVPN CKDR TG CR SFC ASL. Wrote the paper: GCL SFC. Technical support: CKDR CR.

References
1. Custovic A, Smajlovic J, Tihic N, Hadzic S, Ahmetagic S, Hadzagic H. Epidemiological Monitoring of Nosocomial Infections Caused by Acinetobacter baumannii. Med Arh. 2014; 68(6):402–406.
2. Falagas ME, Kastoris AC, Karageorgopoulos DE, Rafailidis PI. Fosfomycin for the treatment of infections caused by multidrug-resistant non-fermenting Gram-negative bacilli: a systematic review of microbiological, animal and clinical studies. Int J Antimicrob Agents. 2009; 34(2):111–120. doi:10.1016/j.ijantimicag.2009.03.009 PMID: 19403273
3. Bishburg E, Bishburg K. Minocycline an old drug for a new century: emphasis on methicillin-resistant Staphylococcus aureus (MRSA) and Acinetobacter baumannii. Int J Antimicrob Agents. 2009; 34(5):395–401. doi: 10.1016/j.ijantimicag.2009.06.021 PMID: 19665876
4. Gales AC, Jones RN, Sader HS. Global assessment of the antimicrobial activity of polymyxin B against 54 clinical isolates of Gram-negative bacilli: report from the SENTRY Antimicrobial Surveillance Programme (2001–2004). Clin Microbiol Infect. 2006; 12(4):315–321. PMID: 16524407
5. Lu CL, Liu CY, Huang YT, Liao CH, Teng LJ, Turnidge JD, Hsueh PR. Antibacterial Susceptibilities of Commonly Encountered Bacterial Isolates to Fosfomycin Determined by Agar Dilution and Disk Diffusion Methods. Antimicrob Agents and Chemother. 2011; 55(9):4295–4301.
6. Sirijatuphat R, Thamlikitkul V. Preliminary study of colistin versus colistin plus fosfomycin for treatment of carbapenem-resistant Acinetobacter baumannii infections. Antimicrob Agents Chemother. 2014; 58(9):5598–601. doi: 10.1128/AAC.02435-13 PMID: 24982065
7. Principe L, D’Arezzo S, Capone A, Petrosillo N, Visca P. In vitro activity of tigecycline in combination with various antimicrobials against multidrug resistant Acinetobacter baumannii. Annal Clin Microbiol and Antimicrob. 2009; 8:18.
8. Pillai SK, Moellering RC, Eliopoulos GM. In: Antimicrobial combinations. Antibiotics in laboratory medicine. (5 th edn). Lorian V, Lippincott Williams and Wilkins, Philadelphia, PA, 2005.
9. Petersen PJ, Labthavikul P, Jones CH, Bradford PA. In vitro antibacterial activities of tigecycline in combination with other antimicrobial agents determined by checkerboard and time-kill kinetic analysis. J Antimicrob Chemother. 2006; 57(3):573–6. PMID: 16431863
10. Clinical and Laboratory Standard Institute. Performance standards for antimicrobial susceptibility testing. Ninth informational supplement. CLSI document M100 S19. CLSI, Wayne, PA, USA. 2013.
11. European Committee on Antimicrobial Susceptibility Testing. Clinical Breakpoints. Available: http://www.eucast.org/clinical_breakpoints. 2013.
12. Mostachio AK, van der Heidjen I, Rossi F, Levin AS, Costa SF. High prevalence of OXA-143 and alteration of outer membrane proteins in colistin-susceptible Acinetobacter spp. isolates in Brazil. Int J Antimicrob Agents. 2012; 39(5):396–401. doi: 10.1016/j.ijantimicag.2012.01.021 PMID: 22455794
13. Shahcheraghi F, Abbassaliou M, Feizabadi M, Ebrahimipour G, Akbari N. Isolation and genetic characterization of metallo-β-lactamase and carbapenemase producing strains of Acinetobacter baumannii from patients at Tehran hospitals. Iran J Microbiol. 2011; 3(2):68–74. PMID: 22347585
14. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014; 30(15):2114–20. doi: 10.1093/bioinformatics/btu170 PMID: 24695404
15. Chevreux B, Wetter T, Suhai S. Genome Sequence Assembly Using Trace Signals and Additional Sequence Information. 1999:45–56.
16. Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics. 2014; 30(15):2114–20. doi: 10.1093/bioinformatics/btu153 PMID: 24642063
17. Lagesen K, Hallin P, Redlund EA, Staerfeldt HH, Rognes T, Ussery DW. RNAmer: consistent and rapid annotation of ribosomal RNA genes. Nucleic Acids Res. 2007; 35(9):3100–8. PMID: 17452365
18. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Molecular Biology and Evolution. 2013; 30(4):772–780. doi: 10.1093/molbev/mst010 PMID: 23329690
19. Maiden MCJ. Multilocus Sequence Typing of Bacteria. Annu. Rev. Microbiol. 2006; 60:561–88. PMID: 16774461
20. Eliopoulos GM, Moellering RC. In: Antimicrobial combinations. Antibiotic in Laboratory Medicine (4th edn). Lorian V, Williams and Wilkins. Baltimore, MD. 1996:330–96.

21. CDC/NHSN surveillance definition of health care–associated infection and criteria for specific types of infections in the acute care setting. Horan TC, Andrus M, Dudeck MA. Am J Infect Control 2008 (36 (3)): 309–32.

22. Woodford N, Ellington MJ, Coelho JM, Turton JF, Ward ME, Brown S, Amyes SG, Livermore DM. Multiplex PCR for genes encoding prevalent OXA carbapenemases in Acinetobacter spp. Int J Antimicrob Agents. 2006; 27(4):351–3. PMID: 16564159

23. Higgins PG, Poirel L, Lehmann M, Nordmann P, Seifert H. OXA-143, a novel carbapenem-hydrolyzing class D beta-lactamase in Acinetobacter baumannii. Antimicrob Agents Chemother. 2009; 53 (12):5035–8. doi: 10.1128/AAC.00856-09 PMID: 19770279

24. Mostachio AK, van der Heijden I, Rossi F, Levin AS, Costa SF. Multiplex PCR for rapid detection of genes encoding Oxacillinases and metallo-b-lactamases in colistin-susceptible Acinetobacter spp. J Med Microbio. 2009; 58(11):1522–4.

25. Bonapace CR, Bosso JA, Friedrich LV, White RL. Comparison of methods of interpretation of checkerboard synergy testing. Diagn Microbiol Infect Dis. 2002; 44(4):363–6. PMID: 12453452

26. Bonapace CR, White RL, Friedrich LV, Bosso JA. Evaluation of antibiotic synergy against Acinetobacter baumannii: a comparison with Etest, time-kill, and checkerboard methods. Diagn Microbiol Infect Dis. 2000: 38(1):43–50. PMID: 11025183

27. Zhang Y, Chen F, Sun E, Ma R, Qu C, Ma L. In vitro antibacterial activity of combinations of fosfomycin, minocycline and polymyxin B on pan-drug-resistant Acinetobacter baumannii. Experimental Therapeutic Med. 2013; 5(6):1737–1739.

28. Sirijatuphat R, Thamlikitkul V. Preliminary study of colistin versus colistin plus fosfomycin for treatment of carbapenem-resistant Acinetobacter baumannii infections. Antimicrob Agents Chemother. 2014; 58(9):5598–601. doi: 10.1128/AAC.02435-13 PMID: 24982065

29. Santimaleeworagun W, Wongpoowarak P, Chayakul P, Patharachayakul S, Tansakul P, Garey KW. In vitro activity of colistin or sulbactam in combination with fosfomycin or imipenem against clinical isolates of carbapenem-resistant Acinetobacter baumannii producing OXA-23 carbapenemases. Southeast Asian J Trop Med Public Health. 2011; 42(4):890–900. PMID: 22299471

30. Wei W, Yang H, Liu Y, Ye Y, Li J. In vitro synergy of colistin combinations against extensively drug-resistant Acinetobacter baumannii producing OXA-23 carbapenemase. J Chemother. 2015 May. doi: 10.1179/1973947815Y.0000000030

31. Agodi A, Voulgar E, Barchitta M, Quattrocchi A, Bellocchi P, Poulou A et al. Spread of a carbapenem-and colistin-resistant Acinetobacter baumannii ST2 clonal strain causing outbreaks in two Sicilian hospitals. J Hosp Infect. 2014; 86(4):260–6. doi: 10.1016/j.jhin.2014.02.001 PMID: 24680473

32. Kim UJ, Kim HK, An JH, Cho SK, Park KH, Jang HC. Update on the Epidemiology, Treatment, and Outcomes of Carbapenem-resistant Acinetobacter infections. Chonnam Med J. 2014; 50(2):37–44. doi: 10.4068/cmj.2014.50.2.37 PMID: 25229014

33. Coelho-Souza T, Reis JN, Martins N, Martins IS, Menezes AO, Reis MG et al. Longitudinal surveillance for meningitis by Acinetobacter in a large urban setting in Brazil. Clin Microbiol Infect. 2013; 19(5):E241–4. doi: 10.1111/1469-0691.12145 PMID: 23398654

34. Lee Y, Bae IK, Kim J, Jeong SH, Lee K. Dissemination of ceftazidime resistant Acinetobacter baumannii clonal complex 92 in Korea. J. Appl. Microbiol. 2012; 112(6):1207–11. doi: 10.1111/j.1365-2672.2012.05283.x PMID: 22404202

35. Nigro SJ, Hall RM. Tn6167, an antibiotic resistance island in an Australian carbapenem-resistant Acinetobacter baumannii GC2, ST92 isolate. J. Antimicrob Chemother. 2010; 65(4):644–50. doi: 10.1093/jac/dkq027 PMID: 20154023

36. Grosso F, Carvalho KR, Quinteira S, Ramos A, Carvalho-Assel AP, Asensi MD, Peixe L. OXA-23-producing Acinetobacter baumannii: a new hotspot of diversity in Rio de Janeiro? J Antimicrob Chemother. 2011; 66(1):62–7. doi: 10.1093/jac/dko406 PMID: 21051372

37. Martins N, Martins IS, de Freitas WV, de Matos JA, Giário VB, Coelho-Souza T, et al. Imported and Intensive Care Unit-Born Acinetobacter baumannii clonal complexes: one-year prospective cohort
40. Ramírez MS, Vilacoba E, Stietz MS, Merkier AK, Jeric P, Limansky AS, et al. Spreading of AbaR-type.

41. Stietz MS, Ramírez MS, Vilacoba E, Merkier AK, Limansky AS, Centrón D, Catalán M. Acinetobacter baumannii extensively drug resistant lineages in Buenos Aires hospitals differ from the international clones I–III. Infect Genet Evol. 2013; 14:294–30. doi: 10.1016/j.meegid.2012.12.020 PMID: 23318331

42. Clímaco EC, Oliveira ML, Pitondo-Silva A, Oliveira MG, Medeiros M, Lincopan N, da Costa Darini AL. Clonal complexes 104, 109 and 113 playing a major role in the dissemination of OXA-carbapenemase-producing Acinetobacter baumannii in Southeast Brazil. Infect Genet Evol. 2013; 19:127–33. doi: 10.1016/j.meegid.2013.06.024 PMID: 23838284

43. Grosso F, Carvalho KR, Quinteira S, Ramos A, Carvalho-Assel AP, Asensi MD, Peixe L. OXA-23-producing Acinetobacter baumannii: a new hotspot of diversity in Rio de Janeiro? J Antimicrob Chemother. 2011; 66(1):62–65. doi: 10.1093/jac/dkq406 PMID: 21051372

44. Aydemir H, Akduman D, Piskin N, Comert F, Horuz E, Terzi A, et al. Colistin vs colistin and rifampicin combination in the treatment of multidrug-resistant Acinetobacter baumannii ventilator-associated pneumonia. Epidemiol Inf. 2013; 141(6):1214–1222.

45. Gordon NC, Prq K, Wareham DW. Potent Synergism and Sustained Bactericidal Activity of a Vancomycin-Colistin Combination versus Multidrug-Resistant Strains of Acinetobacter baumannii. Antimicrobial Agents Chemother. 2010; 54(12):3516–22. doi: 10.1128/AAC.00922-10 PMID: 20876375

46. O’Hara JA, Ambe LA, Casella LG, Townsend BM, Pelletier MR, Ernst RK, et al. Activities of vancomycin-containing regimens against colistin-resistant Acinetobacter baumannii clinical strains. Antimicrobial Agents Chemother. 2013; 57(5):2103–8. doi: 10.1128/AAC.02501-12 PMID: 23422916

47. Vidalicac C, Benichou L, Duval RE. In vitro synergy of colistin combinations against colistin-resistant Acinetobacter baumannii, Pseudomonas aeruginosa, and Klebsiella pneumoniae isolates. Antimicrobial Agents Chemother. 2012; 56(9):4856–61. doi: 10.1128/AAC.00996-11 PMID: 22751540
58. Garnacho-Montero J, Amaya-Villar R, Gutiérrez-Pizarro A, Espejo-Gutiérrez de Tena E, Artero-González ML, Corcia-Palomo Y, Bautista-Paloma J. Clinical Efficacy and Safety of the Combination of Colistin plus Vancomycin for the Treatment of Severe Infections Caused by Colistin-susceptible Acinetobacter baumannii. Chemotherapy. 2013; 59(3):225–31. doi:10.1159/000356004 PMID: 24356297