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In vivo activity of co-trimoxazole combined with colistin against *Acinetobacter baumannii* producing OXA-23 in a *Galleria mellonella* model

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Abstract

Objectives. *Acinetobacterbaumannii is* a critical nosocomial pathogen. *A. baumannii* infections have become a grave challenge due to their ability to develop resistance to different antimicrobial agents. The current study aimed to evaluate the potential synergism and bactericidal activity of a combination of colistin andcotrimoxazole against carbapenem-resistant*A. baumannii* (CRAB) in a *Galleria mellonella* model.

Methods. Four clinical *A. baumannii* isolates were biochemically and molecularly identified. Their antimicrobial susceptibility levels were established and the molecular characterization of the carbapenemase-encoding genes was performed. The synergism and bactericidal effect of the colistin/cotrimoxazole combination was assessed using the checkerboard assay and time–kill experiments. An *in vivo* evaluation of the activity of the combination was performed using the *Galleria mellonella* model.

Results. A fractional inhibitory concentration index (FICI) of $\leq$0.5 was found for all strains, indicating that the colistin/cotrimoxazole combination exhibited powerful synergistic activity. The combination displayed both synergistic and bactericidal activity at sub-breakpoint concentrations for all strains. Cotrimoxazole monotherapy showed the least protective activity in the *G. mellonella* model. The survival rate ranged from 66.7–79.2 % at 24 h and was 29.2–60.4 % at 96 h for the tested isolates. Colistin monotherapy performed better than cotrimoxazole monotherapy; the *G. mellonella* survival rate ranged from 77.1–97.9 %, at 24 h and from 64.5–72. % at 96 h. The colistin/cotrimoxazole combination improved *G. mellonella*'s survival rate at 96 h remarkably in comparison to colistin or cotrimoxazole monotherapy.

Conclusions. Finally, the combination of colistin and cotrimoxazole appears to be a promising therapeutic option for the management of CRAB-associated infections. It is essential to assess the clinical application and the dose–response relationships of combinations such as colistin plus cotrimoxazole.

INTRODUCTION

Acinetobacter baumannii is considered to be a problematic healthcare-associated pathogen. It is accountable for several infections, including bloodstream infections, ventilator-associated pneumonia, surgical site infections and urinary tract infections [1]. The restricted therapeutic options for *A. baumannii* have been linked to a growing mortality rate, which is as high as 50 % in some series [2–4]. *A. baumannii* infections have become a grave challenge due to their ability to develop resistance to many antimicrobial classes, including carbapenems.

A. baumannii resistance to carbapenem is attributed to carbapenem-hydrolyzing enzymes, decreased permeability or the alteration of penicillin-binding proteins. Class D carbapenemases are the most prevalent cause of carbapenem resistance among *A. baumannii*. They are categorized into four subgroups: the OXA-23, OXA-24, OXA-58 and OXA-51 families. The OXA-51 family is inherent in *A. baumannii*. These four subgroups have many variants [5].

Limited newer antimicrobial agents for multidrug-resistant *A. baumannii* (MDRAB) infections, render their control a great concern. Such challenge attracted attention to recall older antibiotics to the treatment arsenal until new options

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Sequence accession numbers: LC382026, LC38207, LC382028 and LC382029.

Table 1. *In vitro* evaluation of the checkerboard and time–kill assays for cotrimoxazole/colistin combinations

Strain	Sample type	Carbapenamase gene	Type of oxa51-like	Accession no.	MIC (μg ml^{-1})[a]		FICI	SBPI	Time–kill assay results		
					Cotrimoxazole	Colistin			Cotrimoxazole	Colistin	Cotrimoxazole/colistin
AB1	WI	$bla_{oxa\text{-}23}$, $bla_{oxa\text{-}51}$	$bla_{oxa\text{-}115}$	LC382026	32/608	1	0.28	10	Bactericidal, regrowth	Bactericidal, regrowth	Bactericidal, no regrowth
AB2	Bacteraemia	$bla_{oxa\text{-}23}$, $bla_{oxa\text{-}51}$	$bla_{oxa\text{-}80}$	LC382027	8//152	1	0.5	9	Bactericidal, regrowth	Bactericidal, regrowth	Bactericidal, regrowth
AB3	RTI	$bla_{oxa\text{-}23}$, $bla_{oxa\text{-}51}$	$bla_{oxa\text{-}378}$	LC382028	32/608	2	0.38	4.5	Bactericidal, regrowth	Bactericidal, regrowth	Bactericidal, no regrowth
AB4	UTI	$bla_{oxa\text{-}23}$, $bla_{oxa\text{-}51}$	$bla_{oxa\text{-}336}$	LC382029	128/1216	2	0.16	8.5	Bactericidal, regrowth	Bactericidal, regrowth	Bactericidal, no regrowth

a, as observed in three independent experiments.

FICI, fractional inhibitory concentration index; MIC, minimum inhibitory concentration; SBPI, susceptible breakpoint index.

WI, wound infection; RTI, respiratory tract infection; UTI, urinary tract infection.

appear. Additionally, the use of combination therapies has broadened for combating such infections [6, 7].

Many reports documented the importance of colistin as an effective therapy for management of carbapenems resistant *A. baumannii* (CRAB) [6]. Colistin is a cyclic polypeptide with detergent-like effect. It binds and interacts with lipopolysaccharides and phospholipids at the surface of the bacterial outer membrane, disturbing the cytoplasmic membrane permeability [8]. This interaction may result in osmotic imbalance, disruption and leakage of the cellular contents. Various reports have mentioned the potential synergistic effect of unusual colistin combinations [8, 9]. Colistin effect on the outer membrane can promote permeability to large and/or hydrophobic molecules like cotrimoxazole.

Apart from the rising need to reuse old and existing antibiotics, the potential *in vivo* activity of a combination of cotrimoxazole and colistin versus clinical MDRAB has not been reported. Cotrimoxazole is a combination of trimethoprim/sulfamethoxazole (TMP-SMX) in a 1/19 concentration ratio to achieve maximal synergistic activity between both drugs. It inhibits the synthesis of bacterial DNA through dihydrofolate pathway inhibition. Currently, cotrimoxazole has not been a good choice for the treatment of MDRAB [10].

Animal models have frequently been used to evaluate the possible value of antimicrobial combinations, as they provide data that are relevant to human infections. However, these models are costly and time-consuming and present real ethical concerns [6]. Recently, invertebrate infection models such as *Galleria mellonella* (the larvae of the wax moth), have been used for primary confirmation of the *in vivo* efficacy of antimicrobial agents [11, 12]. The aim of this study was to evaluate the probable synergy and bactericidal activity of a combination of colistin and cotrimoxazole against clinical MDRAB isolated from Egypt using a *G. mellonella* infection model.

METHODS

Bacteria and antimicrobial susceptibility testing

Four clinical isolates of *A. baumannii* designated as (AB1–4) were defined as MDRAB isolates. Isolates were identified as *A. baumannii* by conventional microbiological methods and confirmed by species-specific PCR for the $bla_{OXA\text{-}51}$-like gene [13]. The characteristics of the isolates used are shown in Table 1. Carbapenemase-encoding genes [$bla_{OXA\text{-}23}$-like, $bla_{OXA\text{-}24}$-like, $bla_{OXA\text{-}51}$-like, $bla_{OXA\text{-}58}$-like andthemetallo β-lactamase (MBL) genes$bla_{\text{VIM-2}}$, $bla_{\text{IMP-1}}$ and bla_{NDM}] were tested by PCR and sequencing as previously described [14–16]. Positive control strains with a known content of carbapenemase (OXA, MBL enzymes) were supplied from the Department of Microbiology, Faculty of Pharmacy, Misr University for Science and Technology.

Repetitive element palindromic (REP)-PCR genotyping

PCR reaction was performed using REP1(5′-IIIGCGCCGI-CATCAGGC-3′) and REP2 primers (5′ACGTCTTATCAGGCCTAC-3′) (50 pmol μl^{-1}), dNTP mixture (0.2 mM), MgCl2 (3 mM), 1 U of *Taq* DNA polymerase (Thermo Fisher Scientific), 1× PCR buffer and 50 ng of DNA in a total volume of 25 µl. The amplification reaction was conducted using a thermal cycler (Bio-Rad C1000, USA). The first step encompassed denaturation at 95 °C for 10 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 45 °C for 1 min and an elongation step at 72 °C for 3 min, with a final extension at 72 °C for 16 min [17]. The PCR products underwent separation using agarose gel electrophoresis. The phylogenetic trees were outlined using PyElph version 1.4

Antimicrobial agents

Trimethoprim, sulfamethoxazole and colistin sulfate (Sigma-Aldrich) were prepared in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines in the proper solvent (Clinical and Laboratory Standards Institute, 2014).

Susceptibility testing

The minimum inhibitory concentrations (MICs) for cotrimoxazole (at a ratio of 1 : 19) and colistin were determined in duplicate according to the CLSI broth microdilution method. Susceptibility was determined using the CLSI breakpoints. The results for colistin and trimethoprim/sulfamethoxazole susceptibility were interpreted in accordance with the CLSI criteria (susceptible, ≤ 2 mg l^{-1}; resistant, ≥ 4 mg l^{-1} and susceptible, $\leq 2/38$ mg l^{-1}; resistant, $\geq 4/76$ mg l^{-1}, respectively) [18]. *Escherichia coli* (ATCC 25922) and *A. baumannii* (ATCC 19606) were used as quality controls in all assays.

Synergy testing by the checkerboard assay

The synergy between cotrimoxazole (1/19) and colistin was assessed using the checkerboard microtitre plate assay as described previously. Briefly, 96-well microtitre plates were prepared with increasing concentrations of cotrimoxazole (1/19) at concentrations varying from 0.25 to 128 mg ml^{-1} in the horizontal wells and increasing concentrations of colistin (0–4 mg l^{-1}) in the vertical wells and were inoculated with 10^5 c.f.u. ml^{-1} of *A. baumannii* prepared in Luria–Bertani (LB) broth. Visual assessment of the plates for turbidity was performed after 24 h of incubation at 35 °C to determine growth. The synergy was measured by the fractional inhibitory concentration index (FICI) and the susceptible breakpoint index (SBPI) [15]. The interpretation of FICI values was performed according to the following criteria: the potential for bacteriostatic effect or the potential for synergy when the FICI was ≤ 0.5 and the SBPI was >2. All experiments were performed in triplicate, and the results were reported as the median values [19].

Time–kill assays and bactericidal activity

Time–kill assays were conducted for each strain using cotrimoxazole (1/19), colistin and a combination of both drugs as previously described [6]. Briefly, colistin was added at a final concentration of 1 mg l^{-1} and cotrimoxazole (1/19) was added at 2 mg l^{-1}. The concentrations of colistin and cotrimoxazole (1/19) were selected according to the steady-state plasma concentration achieved when the optimum dose is used [20]. LB broth was inoculated with 5×10^5 c.f.u. ml^{-1} of the *A. baumannii* isolate and was incubated at 37 °C. Colonies were counted at 0, 2, 4, 8 and 24 h to verify the viable c.f.u. ml^{-1}. For all experiments, serial dilutions of aliquots (100 µl) in normal saline were performed. Bacterial counts were reported by applying three spots of 10 µl of proper dilutions on Muller–Hinton agar plates followed by incubation at 35 °C for 18 to 24 h. Then, time–kill curves were created by plotting the mean colony counts ($\log_{10}$ c.f.u. ml^{-1}) against time. The results were interpreted in accordance with previously described approaches [21].

G. mellonella treatment assays

The *G. mellonella* infection model for *A. baumannii* was modified from that suggested by Peleg *et al.* Batches of *G. mellonella* caterpillars in their final instar stage (supplied by the Pests and Plant Protection Department, NRC, Egypt) were stored in the dark on wood shavings at 15 °C prior to use. The caterpillar weights were typically 250 mg with slight variations. We calculated the treatment doses according to this value. In order to prepare the inoculum needed to kill *G. mellonella* in 24–96 h, we inoculated 16 caterpillars with 10 µL of suspensions containing 10^5 c.f.u. of bacteria/larva in phosphate-buffered saline (PBS). The caterpillars were incubated at 37 °C and were observed daily for 4 days. Ten microlitres of antibiotics were injected within 2 h of bacterial inoculation. Treatment doses that simulated human ones were given only once (2.5 mg kg^{-1} for colistin and 10 mg kg^{-1} for cotrimoxazole) [11]. Sixteen uninoculated and sterile PBS-inoculated caterpillars were prepared and used as negative controls. The caterpillars were examined every 24 h for 4 days. The experiments were carried out in triplicate at separate times.

Statistics

All statistical analyses were performed using the Statistical Package for Social Sciences software (SPSS version 17), with a *P*-value of ≤ 0.05 considered statistically significant. The survival data were plotted using the Kaplan–Meier method and the log rank test was run for *G. mellonella* survival analysis.

Ethical conduct of research

All of the procedures performed in the current study were approved by the Ethical Committee, Faculty of Medicine, Fayoum University and were in accordance with the 1964 Helsinki Declaration and its amendments.

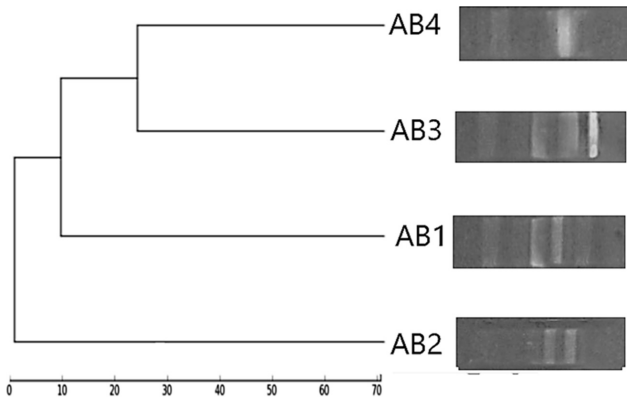

Fig. 1. Dendrogram of the four clones of *Acinetobacter baumannii* isolates. Gel images of the fingerprints generated from REP-PCR of the *Acinetobacter baummanni* were analysed using a gel documentation system. The cluster homology of the isolates was designed using PyElph version 1.4 based on the unweighted pair group method using arithmetic averages algorithm (UPGMA).

RESULTS

Phenotypic and genotypic characterization of *A. baumannii* strains

Four clinical *A. baumannii* isolates were selected for testing in this study and were designated as AB1, AB2, AB3 and AB4. All isolates were resistant to cotrimoxazole and carbapenems but were susceptible to colistin. Susceptibility testing of the *A. baumannii* isolates revealed resistance to cefotaxime, ceftazidime, cefepime, imipenem, meropenem, ciprofloxacin, gentamicin and amikacin. The MICs of colistin and cotrimoxazole against the four *A. baumannii* isolates are shown in Table 1. All *A. baumannii* isolates carried the $bla_{\text{OXA-51}}$ and $bla_{OXA\text{-}23}$ genes (Table 1). $bla_{OXA\text{-}24\text{-like}}$, $bla_{OXA\text{-}58\text{-like}}$, $bla_{\text{VIM-2}}$, $bla_{\text{IMP-1}}$ and bla_{NDM} were not detected in the four isolates. Sequencing of the $bla_{\text{oxa-51}}$-like genes was performed for all isolates. The sequencing data were published and their accession numbers are given in Table 1. REP-PCR showed that the four *Acinetobacter* that were isolated were clonally unrelated (Fig. 1).

Synergy testing by the checkerboard assay

In the microtitre checkerboard assays, the presence of 0.25 MIC of colistin caused a 4–32-fold reduction in the cotrimoxazole MIC. The presence of 0.25 MIC of cotrimoxazole caused a four- to eightfold reduction in the colistin MIC in all experiments. An FICI of $\leq$0.5 was found for all strains, indicating powerful synergistic activity (Table 1). An SBPI of >2 was also seen for all isolates, adding further evidence of the effect and clinical importance of the combination (Table 1).

Time–kill assays and bactericidal activity

In the time–kill assays, both colistin alone and cotrimoxazole alone were used at a certain sub-concentration (1 and 2 μg ml^{-1} respectively). Cotrimoxazole's breakpoint concentration was expressed as the trimethoprim concentration. They were initially bactericidal in the four experiments but sustained killing did not continue over 24 h, even with the apparent sensitivity of strains to colistin in static assays. With colistin and cotrimoxazole monotherapy, all isolates showed bactericidal activity with regrowth.

The colistin/cotrimoxazole combination initially exhibited both synergistic and bactericidal activity at sub-breakpoint concentrations for all strains. However, this activity was followed by bacterial regrowth at 24 h to come close to the level of the control after 24 h in the AB2 (Fig. 2). Synergy was identified as a 2 $\log_{10}$ c.f.u. ml^{-1} decrease in the bacterial count between the combination and the most active agent alone at 24 h, while the bactericidal activity was characterized as a $\geq$3 $\log_{10}$ c.f.u. ml^{-1} reduction in cell counts in comparison to the initial inoculum count after incubation for 24 h.

Activity of a combination of colistin and cotrimoxazole in the *G. mellonella* infection model

Cotrimoxazole monotherapy showed the least protective activity in *G. mellonella* with all *A. baumannii* isolates. The survival rate ranged from 66.7–79.2 % at 24 h and was 29.2–60.4 % at 96 h for the tested isolates. Colistin monotherapy performed better than cotrimoxazole monotherapy; the survival rate for *G. mellonella* ranged from 77.1–97.9 % at 24 h and from 64.5–72. % at 96 h. The colistin/cotrimoxazole combination improved the survival rate for *G. mellonella* against *A. baumannii* isolates at 96 h remarkably in comparison to colistin or cotrimoxazole monotherapy (Fig. 3). The difference in the survival percentage between cotrimoxazole alone and the colistin/cotrimoxazole combination was significant for all strains ($P\leq$0.05). The colistin/cotrimoxazole combination significantly improved the survival rate in comparison with colistin alone with the two strains AB3 and AB4 (P=0.44 and 0.17 respectively). The colistin/cotrimoxazole combination had the same protective activity against AB3 as colistin alone at 24 h, but this activity was maintained for the combination and reduced for colistin alone at 96 h (Fig. 3). AB2 had a borderline FICI and showed regrowth in the time–kill curves and in the *in vivo* model the protective effect of the colistin/cotrimoxazole combination was not significantly different from that for colistin alone (P=0.078). The combination showed good protective activity against AB1, although this effect was not significantly different from that for colistin monotherapy (P=0.068).

DISCUSSION

Carbapenems used to be the most valuable therapeutic option for the treatment of MDRAB. Recently, the worldwide dissemination of carbapenem-resistant *A. baumannii* has limited its use [22]. The current study involved four carbapenem-resistant *A. baumannii*. The isolates were clinically recovered from Egyptian patients with different infections. Susceptibility testing of these strains revealed

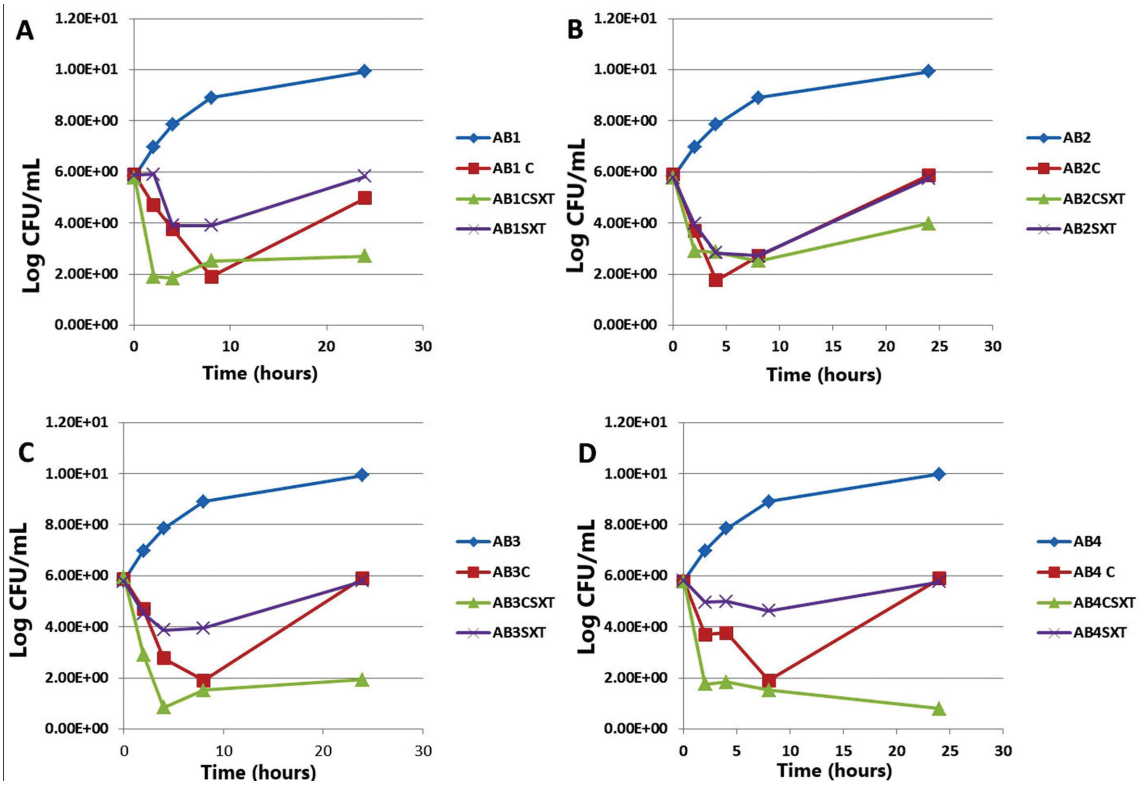

Fig. 2. Time-kill assay performed on *Acintobacter baumannii*. (a) AB1, (b) AB2, (c) AB3 and (d) AB4 using colistin (C), cotrimoxazole (STX) and a combination of colistin and cotrimoxazole (CSTX). Colonies were counted at 0, 2, 4, 8 and 24 h. Time–kill curves were created by plotting the mean colony counts (log_{10} c.f.u. ml^{-1}) against time.

resistance to the tested antimicrobials, including cefotaxime, ceftazidime, cefepime, imipenem, meropenem, ciprofloxacin, gentamycin and amikacin. PCR examination of the OXA-type carbapenemase and MBL genes showed that the four strains harbour both bla_{OXA-51} and bla_{OXA-23}. The sequences of the *blaoxa*-$_{51}$-like genes were analysed. They belonged to *blaoxa-115, blaoxa-80, blaoxa-378* and *blaoxa-336* for AB1– 4, respectively. In Egypt, most of the CRABs were principally linked with bla_{OXA-23}. Reports from Egypt have addressed the widespread prevalence of bla_{OXa-23} among the studied CRABs (23, 24). In the Mediterranean region, the Middle East and North Africa, bla_{OXA-23} has been reported to be the most common carbapenemase-encoding gene [25–27].

The failure of carbapenems to control infections with bla_{OXA-23}-producing *A. baumannii* represents a true healthcare problem. This has restored colistin as a therapy for the management of CRAB infections. Colistin is considered to be a hopeful option for CRAB treatment.

However, several reports have described the emergence of *A. baumannii* resistance to colistin and polymyxin B, with resistance rates ranging from 0.9 to 3.3 % in the USA [28, 29]. In addition, studies from Europe have reported rates of colistin resistance among *A. baumannii* of as high as 19.1 and 16.7 % (from Spain and Bulgaria, respectively) [28, 30].

It is noteworthy that antibiotic combination therapy is regularly required for severe CRAB infections. Combination therapy would reduce the potential for the development of resistance and also improve clinical outcomes [31]. Colistin can disturb the permeability of the outer membrane. This property was considered to evaluate the potential synergism of combining colistin with hydrophobic compounds, such as trimethoprim, or glycopeptides [32].

At this time, only scanty clinical data on cotrimoxazole activity against *Acinetobacter* infections are available. In Greece in 2014, the national resistance surveillance data reported that cotrimoxazole was the most active antibiotic against *A. baumannii* isolated from blood in intensive care units (ICUs) [33]. It is obvious that cotrimoxazole can be the treatment of choice for CRAB infections, especially when it is combined with other drugs [34]. Moreover, the anti-inflammatory activity of cotrimoxazole provides additional benefits from the use of such compounds. Cotrimoxazole decreases the production of toxic metabolites from neutrophils and removes reactive oxygen species, which decreases tissue damage [35]. This anti-inflammatory activity, together

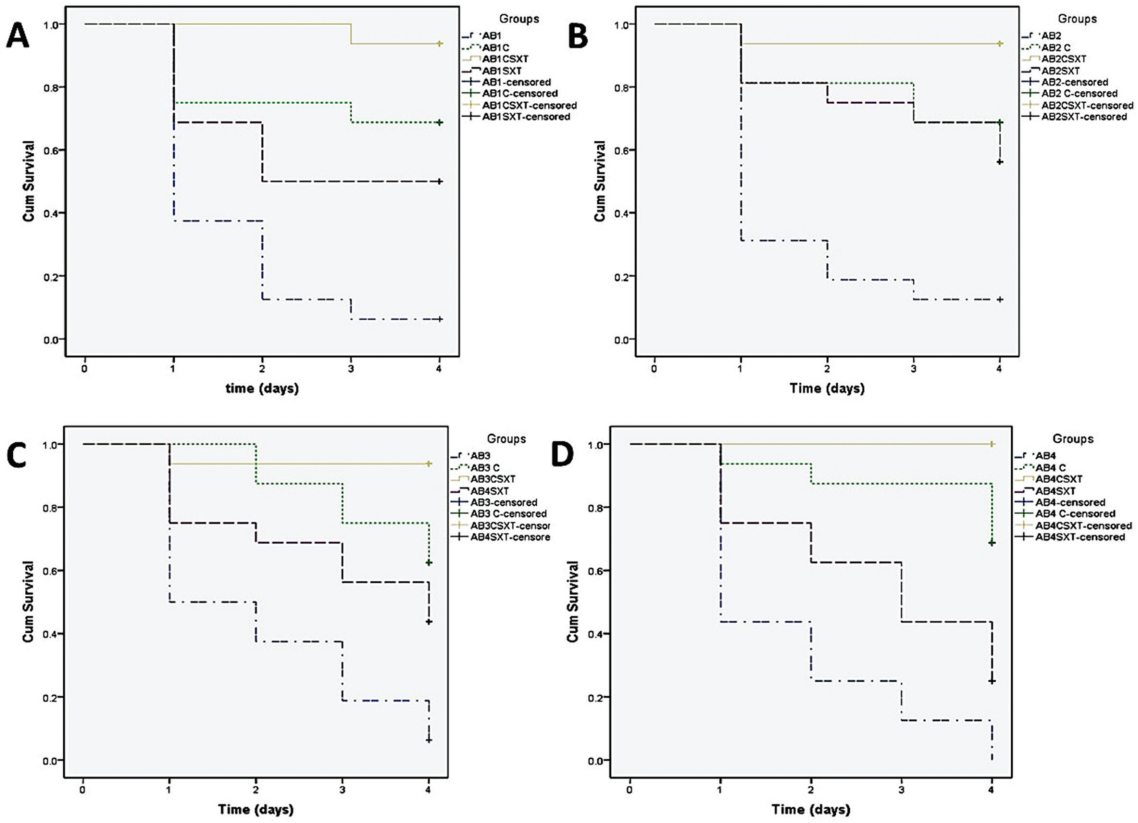

Fig. 3. Survival curves for *Galleria mellonella* larvae inoculated with *Acinetobacter baumannii*. Groups of 16 larvae were inoculated with *A. baumannii* (a) AB1, (b) AB2, (c) AB3 and (d) AB4 following treatment with colistin (C) (2.5 mg kg^{-1}), cotrimoxazole (STX) (10 mg kg^{-1}), or a combination of cotrimoxazole and colistin (SXT/C). The treatment was only given once. Caterpillars were observed daily for 4 days. Sixteen uninoculated and sterile PBS-inoculated caterpillars were prepared and used as negative controls. The data are from three representative experiments.

with its antimicrobial activity, makes it a good option for the treatment of MDRAB.

In the current study, the FICI for colistin/cotrimoxazole combination ranged from 0.16 to 0.5, which means that the combination exhibited synergistic activity. This agrees with a recent *in vitro* study that reported some potency of the combination against colistin-resistant *A. baumannii* [9].

Nepka *et al.* evaluated the combination of colistin and cotrimoxazole (1/19) against cotrimoxazole -susceptible CRAB. They found that this combination was bactericidal for all the tested isolates and that the killing activity was maintained for 24 h, with no bacterial regrowth. Furthermore, synergism was observed in 83.3 % of isolates [33]. In the current work, the colistin/cotrimoxazole combination initially exhibited both synergistic and bactericidal activity at sub-breakpoint concentrations for all strains. However, although this activity was maintained for three strains (AB-1, 3 and 4), there was regrowth with AB2 after 24 h to a level that was close to that of the control. The variations between both studies can be attributed to the differences in the tested strains. In our study, cotrimoxazole-resistant CRAB were investigated, in comparison to the cotrimoxazol-susceptible strains that were studied by Nepka and co-workers [33].

Recently, *G. mellonella* larvae, an invertebrate model, have been recommended as another option to test bacterial as well as fungal pathogens [36, 37]. These larvae have complicated humeral and cellular immune responses that are similar to those in mammals., making them suitable for the study of acute bacterial infections [38]. We used these insects to study the *in vivo* synergy and bactericidal activity of a combination of colistin and cotrimoxazole against *A. baumannii* to predict their appropriateness for therapeutic use. To the best of our knowledge, this is the first study to have evaluated the *in vivo* activity of the colistin/cotrimoxazole combination against CRAB using a *G. mellonella* model. In the current study, the combination of cotrimoxazole and colistin was more effective than either drug alone when assessed using this model. The combination of cotrimoxazole and colistin improved the survival of *G. mellonella* infected with the studied *A. baummaiii* isolates. This was in accordance with the data from our *in vitro* experiments and provided primary *in vivo* support for the useful therapeutic activity of this combination.

The small FICI values (FICI $\leq$0.5) recorded for all of the tested strains in the current study suggest that lower clinically relevant doses might be used. This was supported by the use of antibiotic therapeutic doses in *G. mellonella* model that were similar to those used in the treatment of human infections. Accordingly, the preliminary data obtained from this *in vivo* model indicate that this combination is a promising treatment option for MDRAB that can be studied in clinical trials before full consideration for clinical use.

Conclusion

The combination of colistin and cotrimoxazole is an encouraging therapeutic alternative to improve the survival rates for and clinical responses to life-threatening *A. baumannii* infections. It is now important to assess the clinical application and the dose–response relationships of the colistin/cotrimoxazole combination. Because it is an old drug, many pharmacokinetic/pharmacodynamic data are available, including the phase IV safety profile data. In this respect, and in view of the the results of this study, this combination might be considered to be appropriate for the treatment of MDRB or CRAB infections. This would expand the arsenal for the treatment of these infections. Further, antimicrobials that have both anti-inflammatory and antibacterial effects are expected to be most useful at combating bacterial infection-induced inflammatory reactions.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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