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Efficacy of colistin alone and in various combinations for the treatment of experimental osteomyelitis due to carbapenemase-producing *Klebsiella pneumoniae*

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Objectives: In a new experimental model of carbapenemase-producing *Klebsiella pneumoniae* osteomyelitis we evaluated the efficacy of colistin alone and in various combinations and examined the emergence of colistin-resistant strains and cross-resistance to host defence peptides (HDPs).

Methods: KPC-99YC is a clinical strain with intermediate susceptibility to meropenem (MIC=4 mg/L) and full susceptibility to gentamicin, colistin and tigecycline (MICs=1 mg/L) and fosfomycin (MIC=32 mg/L). Time–kill curves were performed at 4× MIC. Osteomyelitis was induced in rabbits by tibial injection of 2×10^8 cfu. Treatment started 14 days later for 7 days in seven groups: (i) control; (ii) colistin; (iii) colistin + gentamicin; (iv) colistin + tigecycline; (v) colistin + meropenem; (vi) colistin + meropenem + gentamicin; and (vii) colistin + fosfomycin.

Results: *In vitro*, colistin was rapidly bactericidal, but regrowth occurred after 9 h. Combinations of colistin with meropenem or fosfomycin were synergistic, whereas combination with tigecycline was antagonistic. *In vivo*, colistin alone was not effective. Combinations of colistin with meropenem or fosfomycin were bactericidal ($P<0.001$) and the addition of gentamicin enhanced the efficacy of colistin + meropenem ($P=0.025$). Tigecycline reduced the efficacy of colistin ($P=0.007$). Colistin-resistant strains emerged in all groups except colistin + fosfomycin and two strains showed cross-resistance to HDP LL-37.

Conclusions: In this model, combinations of colistin plus meropenem, with or without gentamicin, or colistin plus fosfomycin were the only effective therapies. The combination of colistin and tigecycline should be administered with caution, as it may be antagonistic *in vitro* and *in vivo*.

Introduction

The dramatic spread of carbapenemase-producing Enterobacteriaceae (CPE) is a global health problem and has been recently categorized by the WHO as a critical priority.[1] These organisms are mostly responsible for urinary tract, bloodstream and surgical site infections and pneumonia in hospitalized patients.[2] Post-traumatic or post-operative osteomyelitis and prosthetic joint infection (PJI) due to CPE have been more recently described in countries where CPE is endemic or in hospitals receiving patients from endemic areas.[3,4] Treatment of these infections is especially difficult because they present a combination of the main current challenges in the field of antibiotics. First, despite the recent approval of new compounds, such as ceftazidime/avibactam, therapeutic options remain scarce and could rely on

old antibacterial agents with limited clinical data on their efficacy and a high risk of adverse events. Second, osteomyelitis is a difficult-to-treat infection because of the presence of biofilm[5] and impaired diffusion of most antibiotics within bone tissues.[6]

Colistin is often one of the remaining options for carbapenemase-producing *Klebsiella pneumoniae*.[7,8] However, parenteral administration of colistin may be a source of adverse effects, including nephrotoxicity and neurotoxicity.[7] Moreover, the emergence of colistin-resistant strains has been described in patients treated with colistin,[9] possibly promoted by suboptimal drug exposure due to the low colistin therapeutic index,[10,11] and this outcome may leave patients with no antibiotics left to treat life-threatening infections, as recently reported by the CDC.[12]

Based mostly on cohort studies, experts recommend a combination of two active agents for the management of severe CPE infections, primarily colistin combined with meropenem if the MIC is $\leq$8 mg/L and/or an aminoglycoside, tigecycline or fosfomycin, based on *in vitro* susceptibility tests.[13,14]

Optimal treatment is poorly defined and it is critical to develop experimental models that closely reproduce human infections to provide direct comparisons of therapeutic regimens currently available for these difficult-to-treat infections with limited therapeutic options. Most models of CPE infections described in the literature are acute murine models of pneumonia, thigh infection or septicaemia.[15–17] Our primary objective was to test the efficacy of various colistin-based combinations in a new subacute osteomyelitis model due to a KPC-producing *K. pneumoniae*.

In addition, given that (i) cross-resistance to colistin and host defence peptides (HDPs) LL-37 and lysozyme has been previously described[18] and (ii) we previously demonstrated the emergence of cross-resistance to HDPs and daptomycin in an experimental model of PJI in rabbits, [19,20] we investigated the emergence of colistin-resistant strains in untreated and treated rabbits with osteomyelitis and cross-resistance to HDPs.

Materials and methods

Bacterial strain

K. pneumoniae KPC-99YC is a clinical strain with intermediate susceptibility to meropenem (MIC=4 mg/L) and full susceptibility to gentamicin, colistin and tigecycline (MICs=1 mg/L) and fosfomycin (MIC=32 mg/L). This strain produces KPC-2 carbapenemase and belongs to the epidemic clone ST-258.

Before induction of the animal model of infection, the KPC-producing strain was cultured in casein hydrolysate-yeast extract-containing (CCY) broth medium at 37°C for 18 h. After centrifugation, the pellets were washed twice and resuspended in PBS before inoculation. All inocula were quantified by plating serial dilutions on tryptic soy agar (bioMérieux, La Balme-les-Grottes, France).

Time–kill curve studies

The bactericidal activities of colistin, meropenem, gentamicin and fosfomycin, alone or in combination, were determined in duplicate or triplicate. Overnight cultures were diluted in 10 mL of fresh Mueller–Hinton broth to yield an inoculum of $\sim 10^5$ cfu/mL as often recommended for macrodilution time–kill assays and corresponding to the bacterial load in bones.[21–23] The antibiotic concentrations used were equivalent to 4× MIC.[24] After 0, 3, 6, 9 and 24 h of incubation in a shaking water bath at 37°C, serial dilutions of 0.1 mL samples were subcultured on Mueller–Hinton agar plates (Bio-Rad, Marnes la Coquettes, France) and incubated at 37°C for 24 h before the numbers of cfu were counted. A bactericidal effect was defined as a $>3 \log_{10}$ cfu decrease compared with the initial inoculum. Synergy was defined as a decrease of $>2 \log_{10}$ cfu/mL for the combination compared with that of its most active constituent. Antagonism was defined as an increase of $>2 \log_{10}$ cfu/mL for the combination compared with that of its most active component.

Selection of antibiotic doses in rabbits

Plasma antibiotic concentrations were measured in uninfected rabbits to select doses leading to plasma concentrations equivalent to those obtained in humans. Each antibiotic was tested on one or several groups of four uninfected rabbits (n=16 for colistin and 12 for meropenem). After 24 h of treatment (steady-state concentrations), blood samples were drawn 15 min and 1, 2, 4, 6, 8 and 12 h after the injection and centrifuged, and plasma was frozen until assayed.

Gentamicin concentrations were determined using an enzyme immunoassay, kinetic interaction of microparticles in solution (KIMS), on a Roche Cobas system (Roche Diagnostics, Mannheim, Germany). Meropenem, fosfomycin, tigecycline and colistin were analysed by HPLC-tandem MS using an electrospray ionization method. For meropenem, fosfomycin and tigecycline, the HPLC system was interfaced with a triple-stage quadrupole spectrometer Finnigan TSQ Quantum Discovery Max from Thermo Fisher (Waltham, USA). Reversed-phase chromatography was performed on an Atlantis® T3 dC18 column (3 μm, 2.1×100 mm; Waters, Milford, USA). The lower limits of quantification were 1, 2 and 0.25 mg/L for meropenem, fosfomycin and tigecycline, respectively. Colistin was analysed using a triple-stage quadrupole spectrometer Acquity H Class-Quattro Premier XE from Waters. The samples were purified by solid-phase extraction on OASIS HLB Cartridges (Waters) and separation was performed on a C_{18} XBridge™ column (3.5 μm, 2.1×100 mm; Waters). The transitions of the $[M + 3H]^{3+}$ precursors to the product ions were m/z 390.82>100.73, m/z 386.18>100.73 and m/z 402.16>100.85 for colistin A, colistin B and polymyxin B, respectively. The concentration range was 62.5 to 8000 ng/mL for colistins A + B.

Pharmacokinetic parameters were calculated using Monolix version 2016R1 (Lixoft SAS, Antony, France).

Rabbit osteomyelitis model

The method of Norden[25] was used to induce osteomyelitis in female New Zealand white rabbits, each weighing 2 to 3 kg. The rabbits were housed in individual cages and received food and water *ad libitum*. The experimental protocol complied with French legislation on animal experimentation and was approved by the Animal Use Committee of Maisons-Alfort Veterinary School. The animals were anaesthetized by intramuscular (im) injection of ketamine (25 mg/kg; Vibrac, Carros, France) and 2% xylazine (25 mg/kg; Bayer Santé, Puteaux, France). Infection was induced by tibial intramedullary injection of a sclerosing agent, 0.1 mL of 3% sodium tetradecyl sulphate (Kreussler Pharma, La Chaussée-Saint-Victor, France), followed by 0.2 mL of a KPC-99YC inoculum (10^9 cfu/mL) and 0.1 mL of saline. Patch analgesia with fentanyl (Janssen-Cilag, Issy-les Moulineaux, France) was given for 7 days following surgery. A 10^9 cfu/mL inoculum was selected because preliminary experiments (data not shown) revealed that it induced persistent osteomyelitis in 90% of animals, with low sepsis-related early mortality (<10%).

Treatment and its evaluation

Rabbits were randomly assigned to one of seven groups of 12 rabbits: (i) control; (ii) colistin 150 000 IU/kg q8h im (equivalent to 3 million IU q8h in humans); (iii) colistin + gentamicin 30 mg/kg im q24h (5 mg/kg q24h in humans); (iv) colistin + tigecycline 14 mg/kg im q12h (50 mg q12h in humans); (v) colistin + meropenem 80 mg/kg subcutaneously (sc) q8h (250 mg q8h in humans); (vi) colistin + meropenem + gentamicin; and (vii) colistin + fosfomycin

150 mg/kg q12h im (4 g q8h in humans). The same dose of colistin was used in all groups. Each regimen was started 14 days after surgery and administered for 7 days. Rabbits with no osteomyelitis and those that died before surgery, due to anaesthesia, or before the start of treatment were not included (Table S1, available as Supplementary data at *JAC* Online).

Rabbits were euthanized by intravenous (iv) injection of pentobarbital 3 days after the end of therapy (day 24 post-infection) to allow for bacterial regrowth after treatment discontinuation. Control rabbits were also euthanized on day 24. At the time of death, the upper third of the tibia (3 cm long), including compact bone and marrow, was isolated, split with a bone crusher, weighed, cut into small pieces, frozen in liquid nitrogen and crushed in an autopulverizer (Spex 6700; Freezer/Mill Industries Inc., Metuchen, USA). The pulverized bone was suspended in 10 mL of sterile saline. Serial dilutions were made and plated on tryptic soy agar. After 24 h of incubation at 37°C, the number of viable microorganisms was determined. The results were expressed as the median (IQR) $\log_{10}$ cfu/g of bone and as the percentage of animals with sterile bones. Bone was considered sterile when the culture showed no growth after incubation for 48 h at 37°C and the number of cfu was recorded as the lowest detectable bacterial count (1.10 to 1.30 cfu/g of bone, depending on the weight of the sample). Analyses were performed including all animals that received at least one dose of the treatment assigned.

In vivo selection of mutants

Each undiluted bone homogenate (50 μL) of untreated and treated rabbits was plated onto Mueller–Hinton II agar and onto Mueller–Hinton II agar supplemented with colistin (0.125, 0.25, 0.5, 1, 2, 4 or 16 mg/L) to detect resistant mutants after 24 h of incubation at 37°C. When bacterial growth was observed, *K. pneumoniae* identification was confirmed using MALDI-TOF MS (VITEK MS; bioMérieux, La Balme-les-Grottes, France). The MIC values of colistin were determined by liquid broth dilution methods based on the UMIC kit (Biocentric, Bandol, France). Resistant mutants were defined by an MIC value >2 mg/L (i.e. MIC increased by at least 2-fold compared with that for the initial strain).

Genetic characterization of colistin-resistant strains

Mgr protein is involved in the regulation of LPS biosynthesis in *K. pneumoniae*.[26] Mutations in the *mgrB* gene are key elements as a source of acquired resistance to colistin in *K. pneumoniae*.[27] Therefore, alterations in the *mgrB* gene sequences were sought in colistin-resistant strains that emerged in control or treated rabbits. The *mgrB* genes of four colistin-resistant isolates were amplified with pre-*mgrB*F and *mgrB*-extR primers and sequenced as reported.[27]

Cross-resistance to human antimicrobial peptides (AMPs)

Colistin-resistant bacteria were tested for their susceptibility to specific AMPs produced by human cells in response to infections or wounds and by infiltrating neutrophils: LL-37, human α-defensin 5 (HDA5) and human β-defensin 3 (HDB3).[26]

Approximately 1000 cfu of mid-logarithmic phase bacteria were incubated with 0, 0.3125, 0.625, 1.25, 2.5, 5, 10 and 20 mg/L diluted peptide for 2 h in a 96-well plate at 37°C in PBS supplemented with 1% LB broth. The upper limit of 20 mg/L peptide was based on previous studies,[28,29] which showed less than 20% survival at this concentration. Samples were plated in triplicate on LB agar plates to quantify the remaining bacteria by cfu counts and the assays were run at least in duplicate for each peptide concentration. The means and standard deviations were calculated for each condition.

Data analysis

The data were analysed with R software.[30] Due to the small sample size and asymmetric distributions of variables, exact non-parametric tests implemented in the coin package were used.[31] Data of numeric variables were summarized by the minimum, maximum, median, range, IQR, mean and standard deviation, and data of categorical variables by numbers and percentages. Between-group comparisons were performed using appropriate statistical tests. The χ^2 test, Fisher's exact test and the Kruskal–Wallis non-parametric method were used. The effect of antibiotics and their 95% CIs on $\log_{10}$ cfu criteria were calculated by the Hodges–Lehmann estimator and the Bauer algorithm, respectively. The effect of the antibiotics on the sterility criteria was estimated by the difference in the proportion of sterile bones and the 95% exact CI. A P value <0.05 was considered statistically significant.

Results

In vitro bactericidal effect

Time–kill curves obtained at 4× MIC (Figure 1) showed that colistin alone was rapidly bactericidal during the first 6 h. However, regrowth occurred after 9 h of incubation. The MICs for isolates obtained after regrowth were similar to the MICs for the initial strain. Meropenem alone was slowly bactericidal, with a decrease in the initial inoculum by only 2 $\log_{10}$ at 9 h. Fosfomycin and gentamicin alone exhibited early bactericidal activity, but regrowth was observed at 3 and 6 h, respectively.

For the combination of gentamicin and colistin, rapid bactericidal activity was observed (with a complete bactericidal effect up to 2 h), but regrowth was observed after 3 h. When fosfomycin was added to colistin, rapid bactericidal activity was observed (as with colistin alone). However, this combination of antibiotics prevented regrowth. The combination of colistin, meropenem and gentamicin was the most effective combination, with a fast and complete bactericidal effect at 3 h and no subsequent regrowth.

Tigecycline alone was not bactericidal and slight regrowth was observed at 9 h (Figure 2). The combination of tigecycline and colistin was antagonistic, with a loss of the initial bactericidal effect observed with colistin alone. A dose-dependent decrease of bactericidal activity for the combination was observed when a range of tigecycline concentrations (2-fold serial dilutions, from 32 to 4 mg/L) with a fixed concentration of colistin (4× MIC) were tested (data not shown).

Serum concentrations of meropenem and colistin in rabbits

For colistin, the pharmacokinetic/pharmacodynamic target (AUC/MIC ratio of 25–50) was achieved with the dosing regimen of 150 000 IU three times daily im, equivalent to 3 M IU q8h in humans. For meropenem, the initial target ($T_{>MIC}$ 50%) was achieved with 250 mg/kg q8h, but the treatment induced severe diarrhoea and lethality of >50%. With a dosage of 80 mg/kg q8h sc ($T_{>MIC}$ 20%), the plasma mean C_{max} and mean C_{min} were 17.9±12.4 and 0.35±0.11 mg/L, respectively. These values were comparable to the C_{max} and C_{min} values observed with 250 mg q8h in humans. For gentamicin, the pharmacokinetic/pharmacodynamic parameter target ($C_{max} = 10\times MIC$) was achieved with a dosage of 30 mg/kg q24h im. For fosfomycin, C_{max} and AUC values of 400 mg/L and 1128 mg·h/L, equivalent to 4 g q8h in humans and allowing a $T_{>MIC}$ of >50%, were obtained with 150 mg/kg q12h im dosage. For tigecycline, the pharmacokinetic/

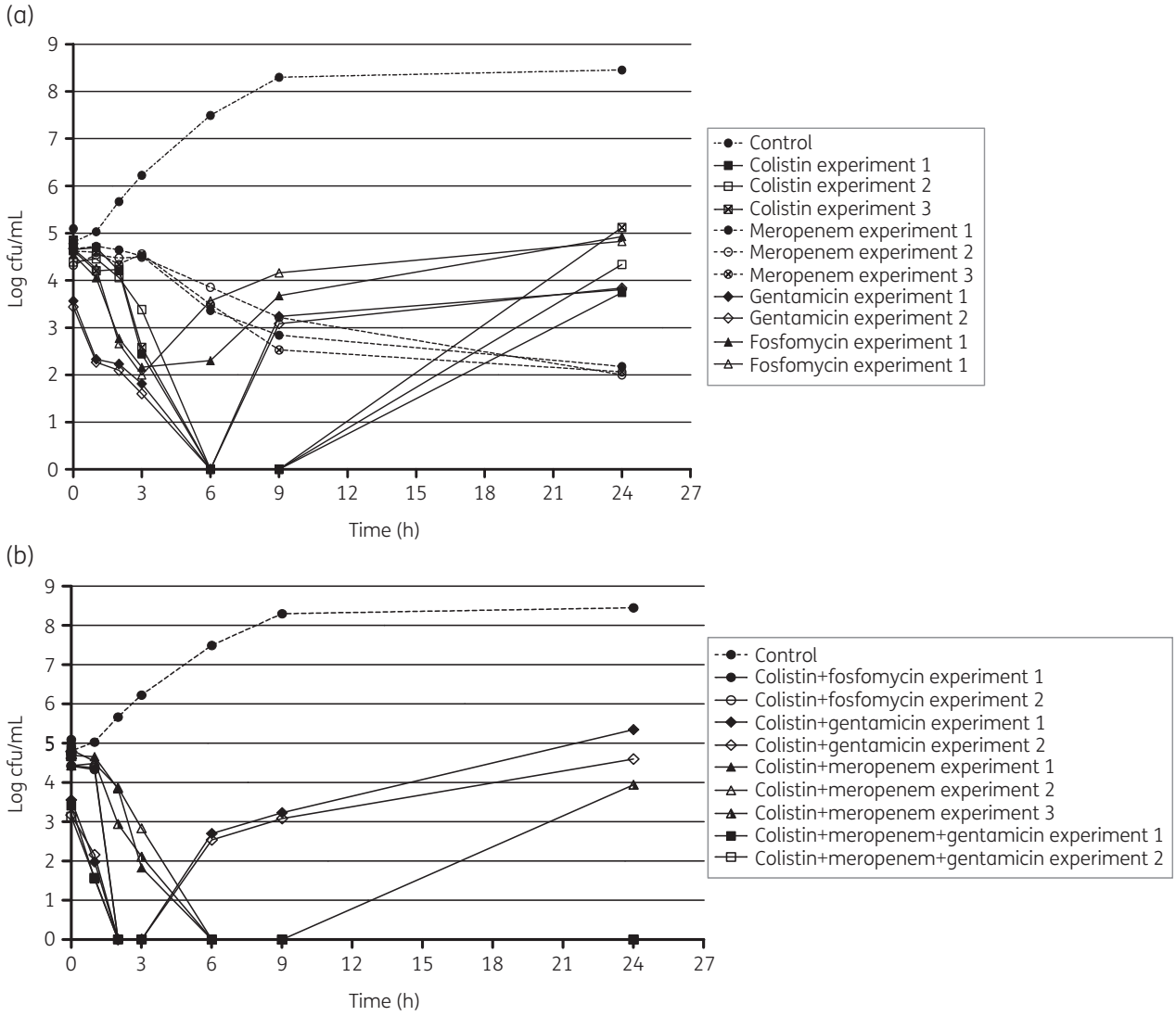

Figure 1. *In vitro* killing curves for carbapenemase-producing *K. pneumoniae* strain KPC-99YC using various antibiotics alone (a) or in combination (b) at concentrations equivalent to 4× MIC. Experiments were performed in duplicate or triplicate.

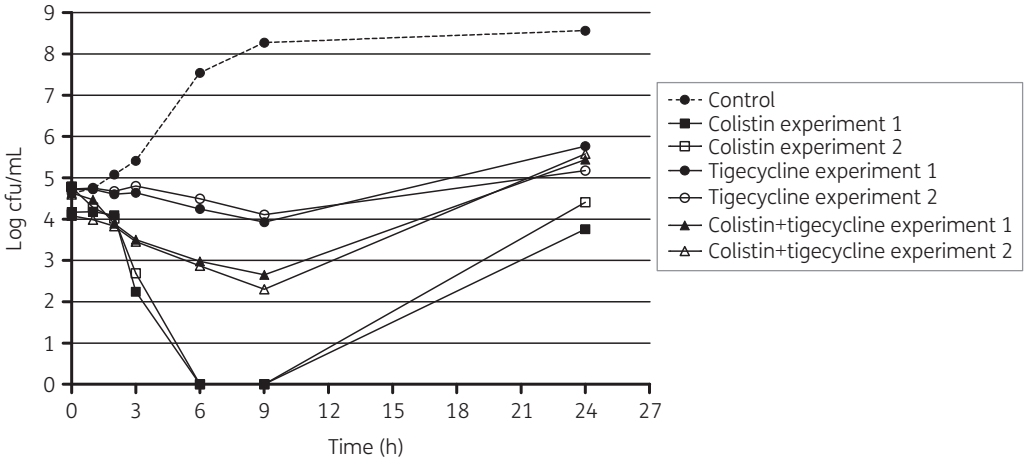

Figure 2. *In vitro* killing curves for carbapenemase-producing *K. pneumoniae* strain KPC-99YC using colistin and tigecycline alone and in combination at concentrations equivalent to 4× MIC. Experiments were performed in duplicate.

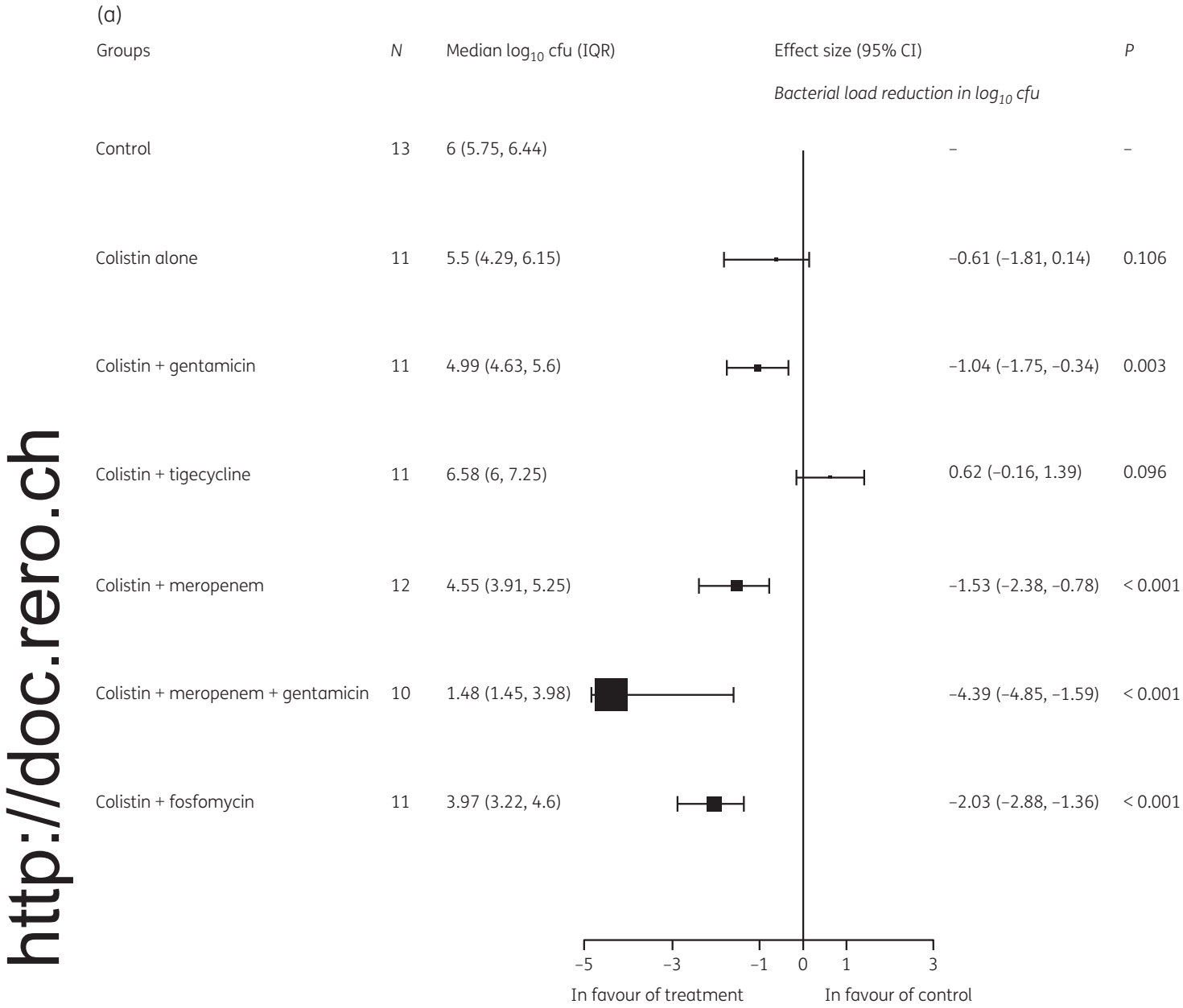

Figure 3. Effect of colistin alone or in combination with various antibiotics on $\log_{10}$ cfu/g of bone (a) and on percentage of sterile bones (b) in animals with carbapenemase-producing *K. pneumoniae* (strain KPC-99YC) experimental osteomyelitis.

pharmacodynamic target (AUC/MIC ratio >18) was achieved with a dosage of 14 mg/kg q12h im.

Therapeutic studies

In control rabbits at day 24, the median (IQR) of bacterial counts was 6 (5.8–6.4) $\log_{10}$ cfu/g of bone.

Compared with those of controls (Figure 3a and Figure 4), bacterial counts in rabbits treated with colistin alone were not different, with a median (IQR) of 5.5 (4.3–6.2) $\log_{10}$ cfu/g of bone (P=0.106) and no sterile bones at day 24 (Figure 3b). Colistin combined with meropenem or gentamicin or fosfomycin or combined with meropenem + gentamicin significantly decreased bone bacterial concentrations. In accordance with time–kill curves, the triple combination of colistin plus meropenem plus gentamicin was more effective than colistin plus meropenem and colistin plus gentamicin on bone bacterial density (Table 1) and was the only effective regimen for bone sterilization (Figure 3b). Notably, as observed *in vitro* with time–kill curves, an antagonist effect was observed with colistin plus tigecycline, which was significantly less effective than colistin alone on bone bacterial concentrations (P=0.007) (Table 1).

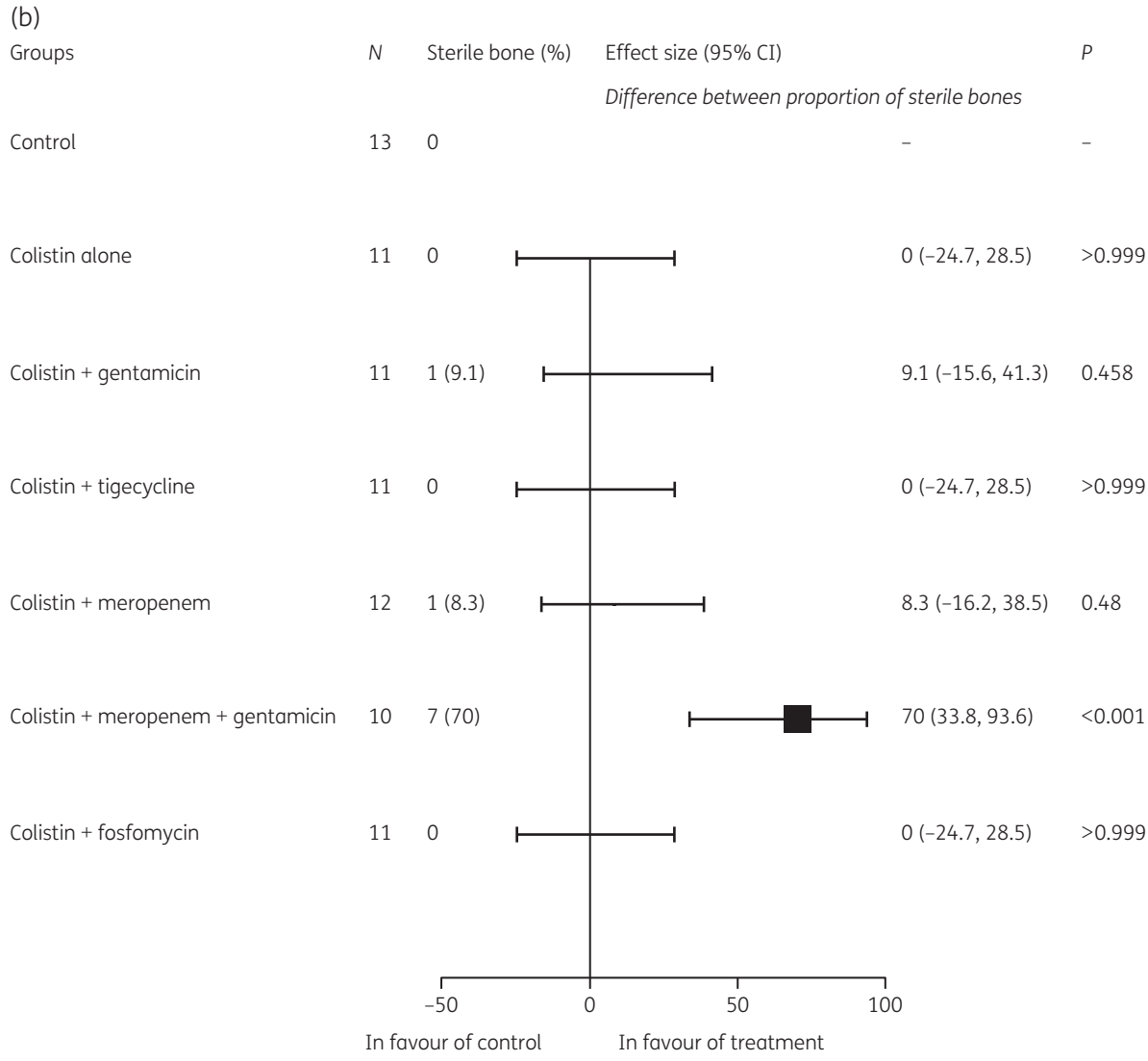

Figure 3. Continued.

Colistin-resistant mutants

Colistin-resistant mutants emerged in rabbits treated with colistin [*n*=2; L31 and L35 (MICs=64 mg/L)], colistin + gentamicin [*n*=3; L37, L43 and L48 (MIC=64, 64 and 16 mg/L, respectively)], colistin + tigecycline [*n*=2; L57 (MIC=16 mg/L) and L59 (MIC=64 mg/L)] and colistin + meropenem + gentamicin [*n*=1; L74 (MIC=32 mg/L)]. Notably, colistin-resistant strains were also detected in one untreated rabbit at day 24 [L17 (MIC=8 mg/L)].

Characterization of colistin-resistant strains

Mutation in the mgrB gene

As colistin resistance was unstable *in vitro*, selected colistin-resistant strains were subcultured on Mueller–Hinton II agar with 2 mg/L colistin before amplification of the *mgrB* gene by PCR and sequencing. MgrB proteins from mutant L35 (from a rabbit treated with colistin alone) and mutant L59 (from a rabbit treated with colistin + tigecycline) were truncated. For strain L35, the truncation was due to insertion of an *IS*Kpn26-like sequence in the coding sequence of the gene, whereas for strain L59 the truncation was due to a nucelotide substitution responsible for a premature stop codon (Table 2). For strain L37 (from a rabbit treated with colistin + gentamicin), no amplicon was obtained with *mgrB* primers, suggesting either a complete deletion of the *mgrB* gene or mutations in regions corresponding to the binding of the primers used for amplification of the *mgrB* gene (Table 2). Finally, the *mgrB* gene was partially deleted in the L17 control rabbit. Other resistant strains (L31 and L57) had a WT *mgrB* gene, suggesting other chromosomally encoded resistance mechanisms (Table 2).

Cross-resistance to host AMPs

Colistin-resistant strains that emerged in control rabbits [L17 (MIC=8 mg/L)] had a slightly increased resistance to LL-37 with decreased susceptibility at 10 mg/L compared with the initial strain

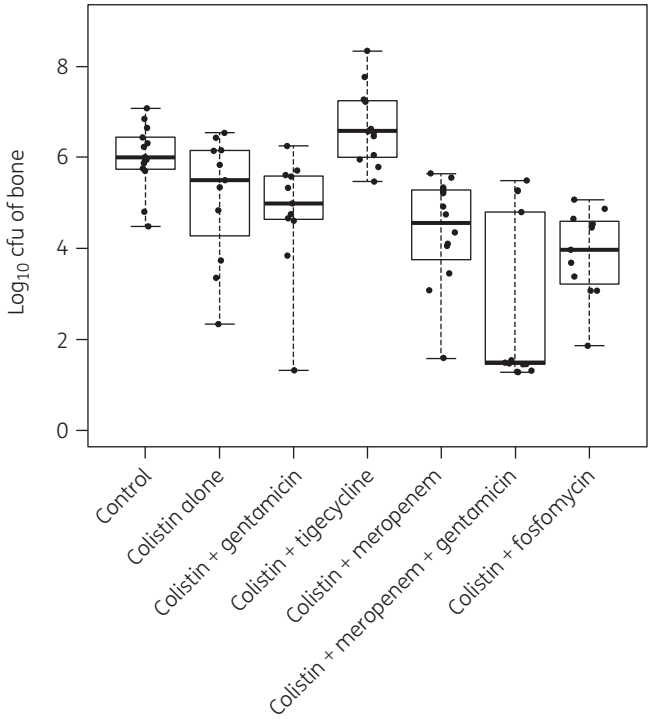

Figure 4. Bacterial density (log_{10} cfu/g of bone) of colistin alone or in combination with various antibiotics for the treatment of carbapenemase-producing *K. pneumoniae* experimental osteomyelitis due to strain KPC-99YC. Whiskers are the minimum and the maximum values and the horizontal line in each box plot, which covers the IQR, is the median.

Table 1. Pairwise comparisons of colistin in combination with various antibiotics in carbapenemase-producing *K. pneumoniae* (strain KPC-99YC) experimental osteomyelitis

Antibiotics	Difference in log_{10} cfu (95% CI)	*P*
Colistin + meropenem + gentamicin compared with:		
colistin + gentamicin	−3.2 (−4.07 to −0.09)	0.024
colistin + tigecycline	−4.58 (−5.73 to −2.43)	<0.001
colistin + meropenem	−2.58 (−3.61 to −0.1)	0.025
colistin + fosfomycin	−1.86 (−3.07–0.34)	0.095
Colistin + gentamicin compared with:		
colistin + tigecycline	−1.63 (−2.63 to −0.87)	<0.001
colistin + meropenem	0.5 (−0.32–1.49)	0.267
colistin + fosfomycin	1.04 (0.14–1.92)	0.019
Colistin + tigecycline compared with:		
colistin + meropenem	2.23 (1.28–3.17)	<0.001
colistin + fosfomycin	2.72 (1.93–3.59)	<0.001
colistin alone	1.24 (0.33–2.6)	0.007
Colistin + meropenem compared with:		
colistin + fosfomycin	0.52 (−0.37–1.37)	0.184

(Figure 5). HDB3 and HDA5 showed lower toxicity, as previously observed, and no difference was observed between the parental strain KPC-99YC and the colistin-resistant KPC-99YC derivatives (Figure 5). The colistin-resistant strain L31, with a colistin MIC of 64 mg/L, that emerged in a rabbit with osteomyelitis treated by colistin alone was not susceptible to LL-37 even at a high concentration, in contrast to the initial parental strain (Figure 5).

Discussion

The efficacy of colistin in bone infections due to Gram-negative bacilli is poorly known. Our work showed that the efficacy of colistin alone or in combination, in a new subacute stringent model of KPC-producing *K. pneumoniae* osteomyelitis, was limited. Monotherapy with colistin was ineffective and colistin-resistant strains emerged after 14 days of treatment. The addition of gentamicin, fosfomycin or meropenem was the only effective therapy. The triple combination of colistin + meropenem + gentamicin was the only regimen able to sterilize most animals. However, colistin-resistant strains emerged in all groups, except in rabbits treated with colistin + fosfomycin. Notably, the suppression of colistin-resistant strains by fosfomycin has been described in time–kill experiments by others and this effect could be due to the killing of colistin-resistant strains by fosfomycin.[32]

The findings regarding osteomyelitis are in line with clinical observations and with recommendations for the treatment of other CPE infections.[14,33] Indeed, those expert recommendations, based mostly on retrospective or prospective non-comparative clinical studies, state that: (i) colistin should always be used in combination with other drug(s); and (ii) penems should be included in the combination when the MIC is $\leq$8 mg/L.

The combination of colistin and tigecycline is often used for treating severe KPC-producing *K. pneumoniae* infections, as tigecycline often remains active *in vitro*. Notably, our data highlighted that this combination was less bactericidal against KPC-99YC than colistin alone, suggesting an antagonistic effect *in vivo*. These data are in agreement with those provided by *in vitro* time–kill curves with KPC-99YC. The antagonistic effect of the tigecycline + colistin combination has already been described *in vitro* with other CPE, including NDM1-producing Enterobacteriaceae,[34] and *in vivo* in a thigh infection model of KPC-producing Enterobacteriaceae.[35] This may be due to the mechanisms of action of tigecycline and colistin, i.e. inhibition of protein synthesis, interaction with LPS and DNA biosynthesis, which may be antagonistic. The experiments performed with two other KPC-producing *K. pneumoniae* (data not shown) belonging to distinct clonal complexes suggested that the antagonist effect may be strain-dependent and deserves to be tested when a clinical strain is isolated before the use of such a combination.

An interesting finding of this work is that our *in vivo* results mirror *in vitro* time–kill curves performed with logarithmic-growth phase bacteria. This result, which was also found by others in a foreign-body experimental model due to ESBL-*Escherichia coli*,[36] contrasts with results obtained with MRSA experimental implant-related infections, where the *in vivo* efficacy of antibiotics on MRSA is correlated with the bactericidal activity of antibiotics on stationary-growth phase bacteria.[37] This finding suggests that in subacute infections such as osteomyelitis, the *in vivo* adaptation of the organisms to the local environment depends on the bacterial

Table 2. Analysis of the *mgrB* gene sequences in the parental KPC-99YC strain that is susceptible to colistin inoculated into rabbits and some colistin-resistant strains that emerged in controls and treated rabbits with KPC-99YC osteomyelitis sacrificed 24 days post-infection

Isolate	Treatment	Colistin MIC (mg/L)	*mgrB* gene
KPC-99YC parental strain		0.25	WT
L17	none (control)	8	partial deletion
L31	colistin	64	WT
L35	colistin	64	insertion of an IS*Kpn26*-like sequence into the coding sequence
L37	colistin + gentamicin	64	total deletion
L57	colistin + tigecycline	16	WT
L59	colistin + tigecycline	64	truncated (stop codon)

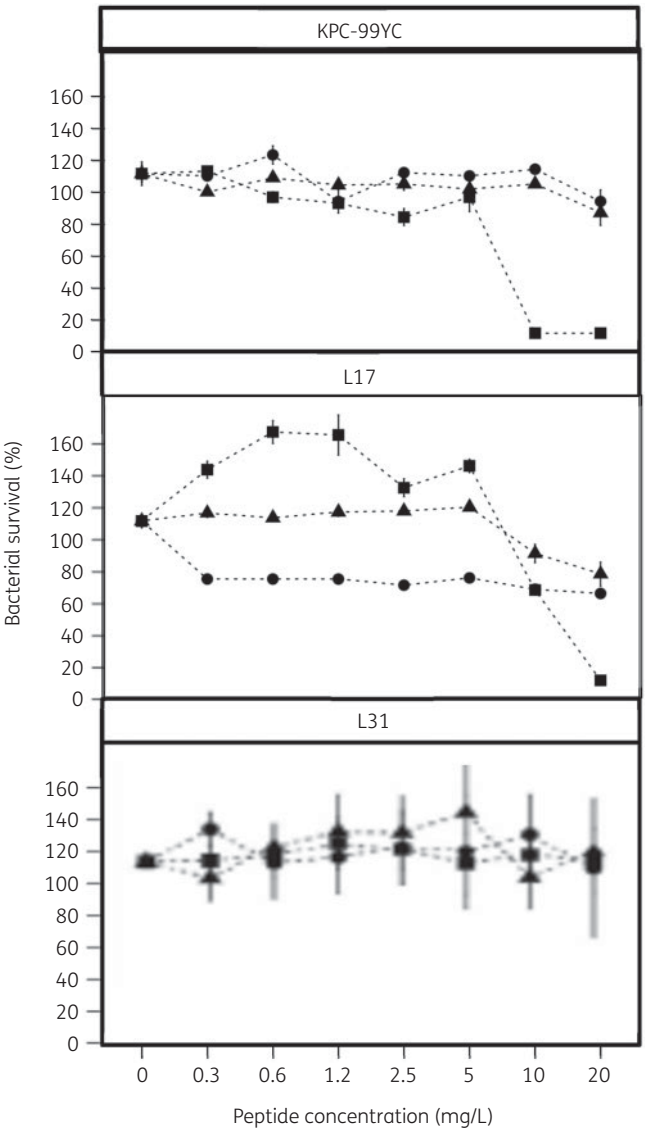

Figure 5. Percentage of bacterial survival as a function of peptide concentration. Panels show data for the initial strain (KPC-99YC) and colistin-resistant *K. pneumoniae* strains. Data represent the experimental means (symbols) ± standard errors of the mean (vertical bars) for cationic AMPs LL-37 (squares), HDA5 (circles) and HDB3 (triangles).

species. The concordance between *in vitro* and *in vivo* results, as well as the strain-dependent antagonistic effect of tigecycline combined with colistin, suggests that *in vitro* time–kill curves could be a useful tool to select the best therapeutic combination for severe KPC-producing *K. pneumoniae* infections.

Finally, we documented the treatment-induced emergence of colistin-resistant strains. As already described for several *K. pneumoniae* strains from humans treated with colistin,[27] this colistin resistance trait was mostly due to mutation/deletion in the *mgrB* gene involved in LPS biosynthesis. Interestingly, resistance to colistin was also observed in one untreated rabbit. As cross-resistance to colistin (i.e. a cationic peptide) and host antimicrobial cationic peptides has already been described in Gram-negative bacilli,[26,38] we investigated the co-occurrence of resistance to HDPs among those strains. The data showed that, in contrast to previous findings,[26] some colistin-resistant strains isolated in animals with osteomyelitis 24 days post-infection have slightly increased resistance to LL-37, which was enhanced after colistin treatment. This adaptation to the local host environment has already been described with staphylococci in our PJI model.[19,20] The spontaneous emergence of resistance to AMPs could play a role in PJI persistence, as HDPs are the first line of host defence against pathogens.[39] This cross-resistance may contribute to the emergence of colistin resistance in patients treated with colistin, as colistin exposure amplifies this phenomenon.

Our study has limitations. First, we used a low dose of meropenem (80 mg/kg im q8h, equivalent to only 250 mg iv q8h in humans) due to poor tolerability of higher doses in rabbits. Hence, we could not replicate pharmacokinetic/pharmacodynamic targets for meropenem in humans as initially planned and the efficacy of meropenem *in vivo* may have been underestimated. As meropenem enhances the efficacy of colistin *in vivo* at this suboptimal dosage, this limitation strengthens the potential interest of this combination, as meropenem doses used in humans are much higher. The second major limitation of our study is that we used only a single strain. Hence, our findings may not be extrapolated to any CPE, especially strains with penem MICs >8 mg/L.

In conclusion, our findings highlight that colistin monotherapy should not be recommended for treating KPC-producing Enterobacteriaceae osteomyelitis owing to its poor *in vivo* efficacy and the emergence of resistant strains. For strains with MIC ≤8 mg/L, combinations of colistin and meropenem, with or without gentamicin, could be effective, but did not completely prevent the emergence of colistin-resistant strains. The combination of

colistin and fosfomycin appears promising and should be further evaluated *in vivo*. The combination of colistin and tigecycline should be administered with caution, as it may be antagonistic *in vitro* and *in vivo*. Experimental stringent models such as bone and joint infections may improve our knowledge for designing optimal treatment of CPE, including KPC-producing Enterobacteriaceae, and better inform clinical recommendations. The concordance between *in vitro* and *in vivo* bactericidal activity may be used as a tool to screen optimal combinations for selected clinical isolates and design individualized regimens for the treatment of difficult-to-treat CPE infections.

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Transparency declarations

None to declare.

Supplementary data

Table S1 is available

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