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In Vitro and *In Vivo* Activities of Tigecycline-Colistin Combination Therapies against Carbapenem-Resistant *Enterobacteriaceae*

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We assessed the activity of tigecycline (TGC) combined with colistin (COL) against carbapenem-resistant enterobacteria. Synergy occurred *in vitro* against the majority of isolates, with the exception of *Serratia marcescens*. In a simple animal model (*Galleria mellonella*), TGC-COL was superior ($P < 0.01$) in treating *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter* infections, including those with TGC-COL resistance. Clinical studies are needed to determine whether TGC-COL regimens may be a viable option.

Resistance to antimicrobial agents is an ongoing problem that consistently undermines our ability to treat bacterial infections (1). The emergence of multidrug-resistant (MDR) *Enterobacteriaceae* producing extended-spectrum β-lactamases (ESBLs) has led to the increased use of carbapenems. Carbapenem resistance in *Enterobacteriaceae* (CRE) may be mediated by mutations affecting membrane permeability (porin loss), overexpression of intrinsic β-lactamases (AmpC), and/or broad-spectrum resistance-nodulation-division (RND)-type efflux pumps (2) but also via the acquisition of carbapenem-hydrolyzing enzymes, including members of the KPC, IMP, VIM, OXA-48, and NDM families (3, 4). Although CRE strains are often reported to be susceptible to polymyxins (polymyxin B, colistin) and tigecycline (TGC) (5–7), there are concerns with the use of these drugs for treatment with debate over how to safely dose colistin (COL) (8) and warnings over the efficacy of TGC in the treatment of bloodstream and other serious infections (9). Rapid emergence of resistance has also been documented with these two agents if either one is used alone in the treatment of MDR Gram-negative infections (10). Due to the lack of alternatives, clinicians are increasingly using antimicrobials in combination. The mechanism of action of COL is not entirely clear, although it has been previously shown to disrupt the integrity of the Gram-negative outer membrane (11), thereby increasing its permeability by drugs that are typically excluded (12). This may improve the activity of a number of antibiotics which would otherwise have little effect (13). This approach is validated by reports of better clinical outcomes with unorthodox therapies for an increasing range of carbapenem-resistant bacteria (14–16). In this study, we assessed the activity of TGC in combination with COL against a range of CRE both *in vitro* and *in vivo* by using standard checkerboard and time-kill assays and a simple invertebrate model (*Galleria mellonella*) of infection and therapeutics (17–19) (we do acknowledge that currently the *G. mellonella* model lacks the required validation with regard to comparability with human therapy).

Enterobacteriaceae isolates used in this study ($n = 18$) consisted of susceptible type strains and clinical isolates exhibiting resistance to β-lactams (including carbapenems), TGC, or COL (Table 1). MICs of TGC and COL were determined by Etest (bioMérieux, France), and mechanisms of resistance were confirmed by genetic and phenotypic tests as previously described (20).

Synergy testing was conducted in IsoSensitest broth in 96-well microtiter plates. Assays were set up in checkerboard style with 2-fold-decreasing concentrations of COL (16 to 0 μg/ml) and TGC (32 to 0 μg/ml) and bacterial inocula of 10^5 CFU per well. Plates were read after 24 h of incubation at 37°C. Synergy between TGC and COL was quantified by calculation of the fractional inhibitory concentration index (FICI) and the susceptibility breakpoint index (SBPI) (21, 22). A FICI of ≤0.5 was defined as synergy, a FICI of 0.5 to ≤4.0 as indifferent or additive, and values of ≥4.0 as antagonistic. An SBPI of >2 was deemed to represent therapeutically useful synergy. Time-kill assays were performed for each isolate with a FICI of ≤0.5 by using starting inocula of 1×10^6 CFU/ml TGC (1 μg/ml) and COL (2 μg/ml) alone and in combination. Time-kill curves were plotted from serial viable counts collected over 24 h, and synergy between TGC and COL was defined as a difference of ≥2 $\log_{10}$ CFU/ml between single and combination therapies (23). All isolates for which synergy was observed in time-kill assays were then used for treatment assays with *G. mellonella*.

To establish the optimal inocula (50% lethal dose [LD_{50}]) required for staggered killing of *G. mellonella* over 96 h, 10 caterpillars (KJ Reptile Supplies, Nuneaton, United Kingdom) were inoculated with bacterial suspensions containing final concentrations of 10^2 to 10^5 CFU/larva. Suspensions were injected directly into the *G. mellonella* hemocoel, and larvae were incubated at 37°C for 96 h. Treatment assays with TGC and COL alone and in combination were assessed using 16 animals as previously described (24). Drug doses were selected to be representative of those used to treat human infection and consisted of TGC at 1 mg/kg and COL at 2.5 mg/kg. Phosphate-buffered saline (PBS) injections (10 μl) were used to control for both inoculation injury and no antimicrobial treatment. Survival curves were plotted over 96 h and an-

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TABLE 1 Characteristics of bacterial strains investigated, including resistance determinants (10, 20), MICs of TGC and COL, FICIs and SBPIs for TGC/COL combinations, and results from time-kill assays

Strain	Feature(s)	TGC MIC (μg/ml)	COL MIC (μg/ml)	FICI[a]	SBPI	Time-kill assay results[a]
E. coli NCTC 12241	Antibiotic-susceptible type strain	0.064	0.75	0.625 (I)	40	
E. coli EC5	ST131 CTX-M-15 producer	0.064	1	0.5 (S)	39.95	S
E. coli EC204	Carbapenem-resistant NDM-1 producer	0.25	0.38	0.374 (S)	24.13	S
E. coli EC421	Carbapenem-resistant NDM-1 producer	0.16	8	0.748 (I)	20.13	
E. coli EC405	Carbapenem-resistant NDM-5 producer	0.16	0.75	1 (I)	10	
E. aerogenes NCTC 9753	Antibiotic-susceptible type strain	0.16	0.75	0.56 (I)	40	
E. aerogenes EA2	Carbapenem-susceptible CTX-M-1 producer	0.25	3	0.188 (S)	39.95	S
E. aerogenes EA1	Carbapenem -resistant (porin loss, AmpC hyperproducer); *in vivo* derivative of EA2	0.125	0.38	0.75 (I)	24.13	
E. cloacae NCTC 13380	Antibiotic-susceptible type strain	0.16	0.5	0.56 (I)	39	
E. cloacae TGC-R	TGC-resistant efflux mutant	8	1.5	0.5 (S)	8.5	S
K. pneumoniae NCTC 9633	Antibiotic-susceptible type strain	0.125	0.38	0.125 (S)	12	I
K. pneumoniae KP52	Carbapenem-resistant OXA-48 producer	0.5	1	0.375 (S)	12	S
K. pneumoniae KPC-3	Carbapenem-resistant KPC-3 producer	1	0.5	0.75 (I)	9	
K. pneumoniae KP51	Carbapenem-susceptible CTX-M-15 producer	4	0.5	0.313 (S)	9	S
K. pneumoniae KP96	Carbapenem-resistant VIM-1 and KPC-2 producer	0.5	0.38	0.531 (I)	39.95	
S. marcescens NCTC 13382	Antibiotic-susceptible type strain	0.125	>256	9 (A)	0.125	
S. marcescens	Carbapenem-resistant NDM-1 producer	1.5	>256	9 (A)	0.09	
S. marcescens SM346	TGC-resistant efflux mutant	6	2	5 (A)	0.125	

[a] S, synergy; I, intermediate or additive; A, antagonism.

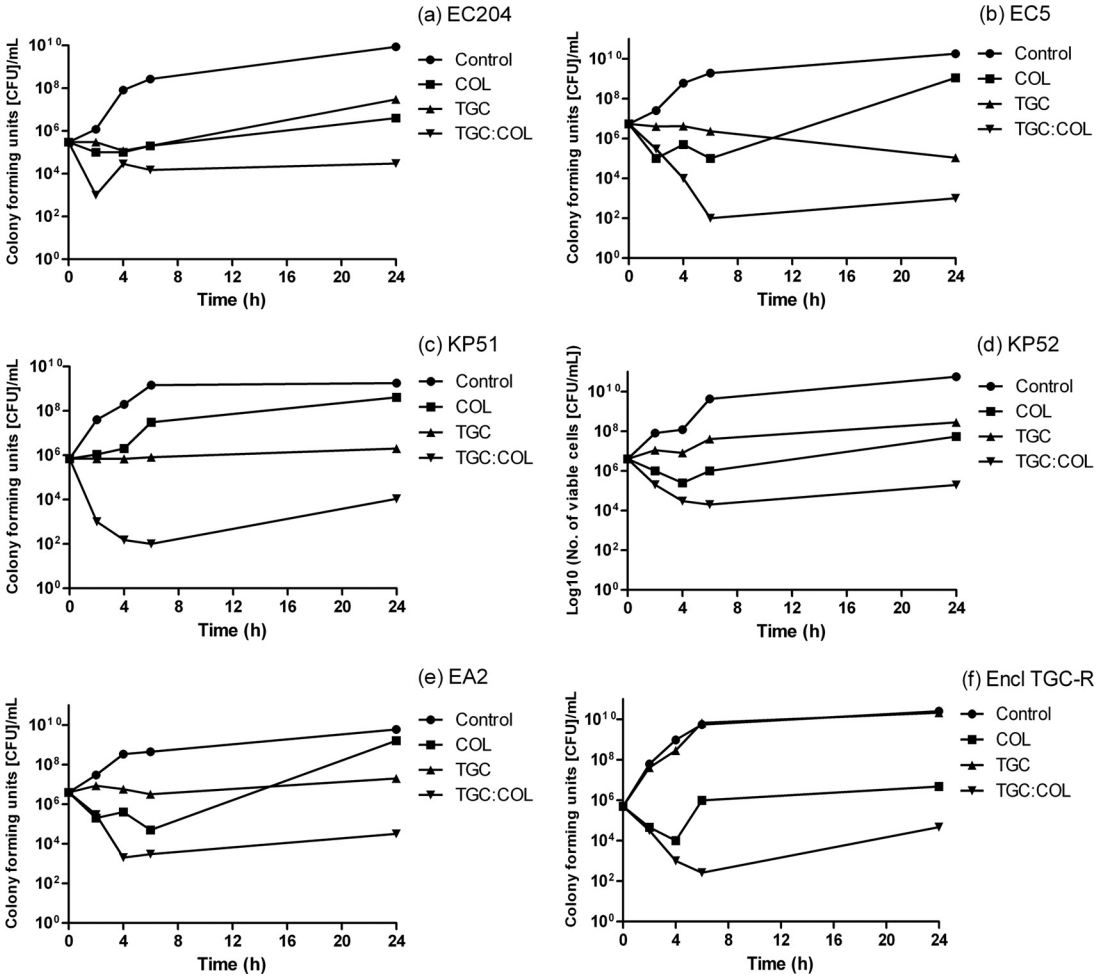

FIG 1 Time-kill graphs showing antimicrobial synergy between TGC and COL against MDR isolates of *Enterobacteriaceae*, namely EC204 (a), EC5 (b), KP51 (c), KP52 (d), EA2 (e), and Encl TGC-R (f).

alyzed using the log rank test. All *in vitro* and *in vivo* experiments were performed three times on separate occasions to ensure reproducibility.

The combination of TGC and COL was active against susceptible and resistant strains of *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter* spp., with synergy (FICI, ≤0.5) noted against 7 of 15 (47%) strains and an indifferent or additive effect (FICI, >0.5 and <4.0) produced against a further 8 strains (53%). In contrast, marked antagonism (FICI, ≥4.0) was observed against all *Serratia marcescens* strains (Table 1). Synergy was largely independent of resistance profiles and was seen against producers of CTX-M-1 (strain EA2), CTX-M-15 (EC5 and KP51), NDM-1 (EC204), and OXA-48 (KP52) enzymes and a TGC-resistant efflux mutant (Encl TGC-R). Indifferent or additive effects seen using the FICI as the definition of synergy contrasted with the SBPI data for *E. coli*, *K. pneumoniae*, and *Enterobacter* isolates with carbapenem resistance due to KPC (strains KPC-3 and KP96), VIM-1 (KP96), hyper-AmpC (EA1), NDM-5 (EC405), and/or NDM-1 (EC421) production, whereby values of 8.5 to 40 indicated that the interaction may still be clinically relevant.

Synergy in checkerboard analysis was confirmed in time-kill assays for all isolates except *K. pneumoniae* NCTC 9633 (Fig. 1; see also Fig. S1 in the supplemental material). TGC alone was bacteriostatic against all 7 strains, as expected for a glycylcycline. Although COL was initially bactericidal, regrowth was observed with COL at 2 μg/ml against isolates NCTC 12241, EC5, NCTC 9633, KP51, KP52, and *Enterobacter cloacae* TGC-R. Previous studies have shown that regrowth after COL exposure can occur at concentrations many times above the MIC and may be indicative of a heteroresistant phenotype, detectable when investigated by population analysis profiling (25). MICs of colistin sulfate can also be overestimated due to its affinity to bind to laboratory plastic and glassware used in *in vitro* susceptibility tests (26). Although regrowth with the TGC-COL combination was also seen with 5 isolates, TGC-COL was still significantly more effective than either agent alone (≤3 log different from the control), supporting other reports of sustained bactericidal synergy with the TGC-COL combination *in vitro* (27, 28).

Of interest, TGC-COL was not effective against *S. marcescens*, with marked antagonism of the combination observed against all the strains tested. The mechanism of synergy between the two antibiotics against other species tested is likely to be linked to COL-mediated membrane permeabilization, allowing entry of TGC into bacterial cells. The intrinsic colistin resistance of *S.*

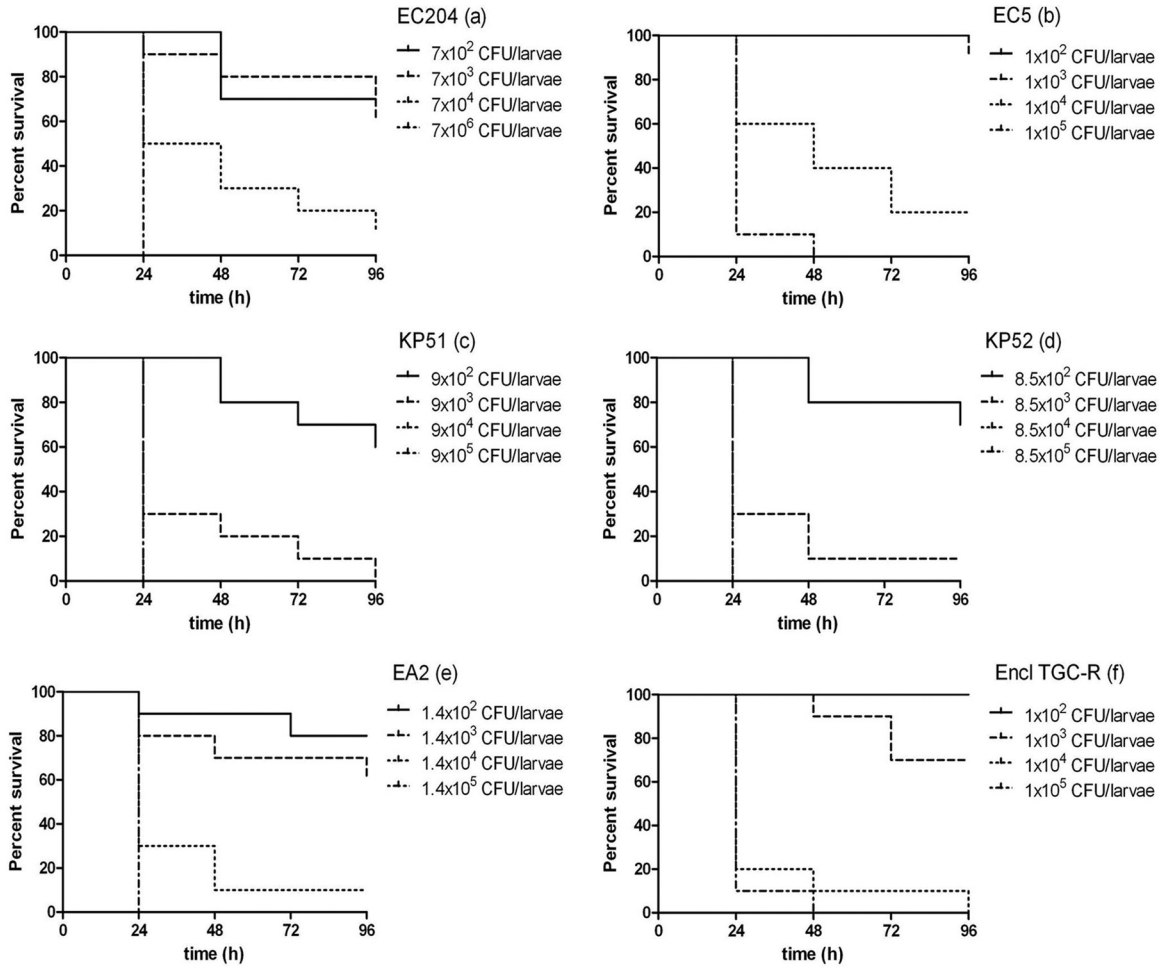

FIG 2 Kill kinetics of strains EC204 (a), EC5 (b), KP51 (c), KP52 (d), EA2 (e), and Encl TGC-R (f) at various numbers of CFU/ml in *G. mellonella* over 96 h. Curves were plotted from single experiments using 10 insect larvae.

marcescens indicates that its membrane is not permeabilized in this fashion. The mechanism of antagonism in *S. marcescens* remains to be elucidated, although it was not abolished by the addition of broad-spectrum inhibitors of resistance-nodulation-division (RND) pumps (*para*-aminobenzoic acid [PaβA]) (unpublished data), suggesting that dysregulation of antibiotic efflux is unlikely to be involved.

All of the isolates tested were pathogenic to *G. mellonella* when inocula of $\geq 10^2$ CFU/larva were used (Fig. 2). The optimal inoculum able to promote staggered killing of >50% of larvae over 96 h for use in treatment assays varied from 10^3 to 10^4 CFU/larva (Fig. 3). Monotherapy with TGC was superior to COL in the treatment of EC204, EC5, EA2, and *K. pneumoniae* (KP52) infections ($P < 0.01$) but not against *K. pneumoniae* (KP51). TGC monotherapy of caterpillars infected with the *E. cloacae* TGC-R strain was ineffective, as predicted from *in vitro* susceptibility tests (Fig. 3).

Overall, the TGC-COL combination was significantly more effective ($P < 0.01$) than monotherapy against all of the CRE isolates studied *in vivo*. Treatment with TGC-COL resulted in survival of 99% (±1 percentage point [pp]) and 96% of EC204- and EC5-infected larvae (Fig. 3a and b), respectively, and was significantly more effective than TGC alone ($P < 0.01$). The TGC-COL combination was also significantly ($P < 0.01$) more effective against *K. pneumoniae* KP51 (67% survival ± 33 pp), KP52 (81% survival ± 19 pp) (Fig. 3c and d), *E. aerogenes* EA2 (88% ±13 pp), and the *E. cloacae* (85% ± 4 pp) TGC-R strain than either agent given as monotherapy ($P < 0.01$) (Fig. 3e and f).

In vitro data on the potential for TGC-COL regimens to be beneficial in treatment were correlated by the *in vivo* studies using *G. mellonella* larvae. In *Galleria* organisms, TGC and COL given together at humanized doses significantly improved survival against infections with *E. coli*, *K. pneumoniae*, and *E. cloacae*, including those producing KPC and NDM-1 carbapenemases. Although the relevance of an invertebrate model to human therapeutics can be questioned, there is increasing evidence that virulence, therapeutic outcomes, and pharmacokinetic parameters (29) can be predicted in this system. However, it should be noted that recent studies using murine models of sepsis (30), pneumonia (31), and soft tissue infection (32) have not found TGC-COL combination treatment to be consistently superior to treatment with either drug alone against carbapenemase-producing *K. pneumoniae* and *A. baumannii* isolates.

In summary, a synergistic or additive effect between TGC and COL was observed *in vitro* and *in vivo* against a number of MDR *Enterobacteriaceae* isolates, although not against *S. marcescens*. For

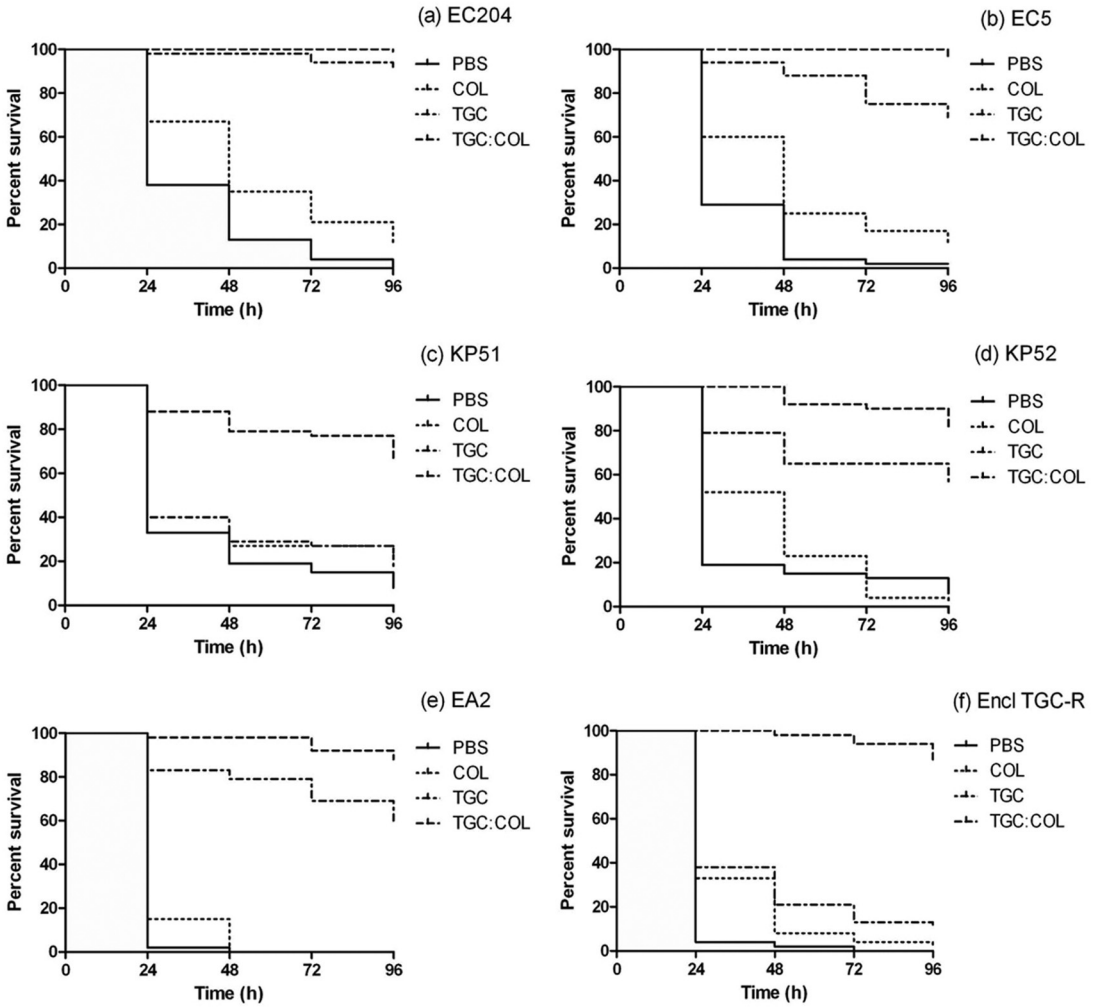

FIG 3 *G. mellonella* survival curves over 96 h when larvae were infected with strains EC204 (a), EC5 (b), KP51 (c), KP52 (d), EA2 (e), and Encl TGC-R (f) and treated with PBS (control), TGC, COL, or the TGC-COL combination at humanized doses (mg/kg). Inoculum for strains KP51 and KP52 was 10^3 CFU/larva; that for strains EC204, EC5, EA2, and Encl TGC-R was10^4 CFU/larva.

TGC-COL combination therapy to be employed effectively and safely, further work on the pharmacokinetic parameters of TGC-COL *in vivo* and its effects on clinical outcomes from MDR Gram-negative infections will be needed.

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