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Modified time–kill assay against multidrug-resistant *Enterococcus faecium* with novel antimicrobial combinations

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This study used a modified time–kill assay to compare the in-vitro activity of chloramphenicol and quinopristin/dalfopristin combined with vancomycin, ampicillin or gentamicin against multidrug-resistant *Enterococcus faecium*. The assay uses standardized time–kill methods with the following modifications: centrifugation of the test tubes at 1–2 h intervals, removal of supernatant and resuspension of bacteria in media containing antibiotic concentrations corresponding to simulated steady-state serum concentrations. None of the agents, alone or in combination, produced bactericidal or synergic activity. The modified time–kill assay more closely simulates in-vivo conditions and may provide a better qualitative assay to determine the interaction between antimicrobial agents and bacteria.

Introduction

In-vitro studies are valuable in determining whether antibiotic combinations are active against resistant organisms. Current methodology is often limited in its ability to provide clinically relevant assessments of the activity of combination regimens. Static concentrations of antibiotics used in most bactericidal assays differ markedly from the in-vivo environment, where bacteria are exposed to fluctuating serum antibiotic concentrations and continual growth of organisms.

The primary objective of this study was to develop further the current standardized time–kill method to better simulate in-vivo conditions without increasing the expense of the method or sacrificing its simplicity. The secondary objective was to use the modified procedure to study novel combinations of chloramphenicol and quinopristin/dalfopristin for the treatment of multidrug-resistant *Enterococcus faecium* (MDR-EF).

Materials and methods

Modified time–kill assays were performed in duplicate with six clinical isolates of MDR-EF. Suspensions were prepared with organisms in log phase growth and diluted to obtain final inoculums of *c.* 5×10^5 cfu/mL.

Chloramphenicol, ampicillin, gentamicin (United States Pharmacopeia, Rockville, MD, USA), quinopristin/dalfopristin (Rhône-Poulenc Rorer Laboratories, Collegeville, PA, USA) and vancomycin (Eli-Lilly, Indianapolis, IN, USA) were used in the susceptibility studies. Antibiotics were prepared according to NCCLS guidelines or manufacturer's recommendations.[1] MICs were determined according to NCCLS guidelines for the microbroth dilution procedure.[1]

The activities of chloramphenicol and quinopristin/dalfopristin were compared alone and in combinations of chloramphenicol plus quinopristin/dalfopristin with or without vancomycin, ampicillin or gentamicin. Using a pharmacokinetic computer software package (PK Model 2 from WinNonLin Version 1.1, Apex, NC, USA), steady-state serum concentrations were simulated for a 70 kg patient (Table I). The simulation assumed a one-compartment model for each antibiotic. Dosing intervals, rates of elimination (K_e) and volumes of distribution (V_d) were obtained from Rhône-Poulenc Rorer (for quinopristin/dalfopristin) and from primary literature.[2–4]

NCCLS guidelines for the standardized time–kill assay were incorporated in the modified time–kill procedure.[5] At time 0, the inoculum and antibiotics were combined in cation-supplemented Mueller–Hinton broth and initial colony counts performed (WASP Spiral Plater, Microbiol-

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Table I. Simulated steady-state serum concentrations (mg/L) at specific sampling times

Antibiotic and dosage simulated	Time (h)[a]							
	0	1	2	4	6	8	10	12
Chloramphenicol 500 mg q6 h	16	13	11	21	16	11	21	16
Quinopristin/dalfopristin 525 mg (7.5 mg/kg) q8 h	2	0.125	5.0	2.0	0.75	0.3	5.0	2.0
Vancomycin 1 g q12 h	15	13	11	7	25	21	17	15
Gentamicin 140 mg q8 h	0.5	7.0	5.0	2.0	1.0	0.5	5.0	2.0
Ampicillin 1 g q6 h	12	8.0	5.0	35	12	5.0	35	12

[a]Time (h) refers to sampling times and may not correspond with time of dosing.

Table II. Changes in $\log_{10}$ cfu/mL of individual isolates over 12 h with different antibiotics alone and in combination

Antibiotic(s) used	Isolate No.						
	2	3	5	7	9	11	mean
Control 1[a]	3.23	4.35	4.05	4.21	4.23	4.37	4.07
Control 2[b]	3.31	4.02	3.82	3.85	3.96	4.16	3.85
C	–0.08	–0.58	–0.55	–0.24	–0.25	–0.14	–0.31
R	–0.41	–1.40	–2.45	–0.68	–0.27	–0.98	–1.03
CR	–0.45	–1.13	–2.11	–0.48	–0.43	–1.30	–0.98
CRV	–0.37	–1.30	–1.57	0.40	–0.15	–0.90	–0.65
CRA	–0.90	–1.19	–1.52	–0.37	–0.36	–0.57	–0.82
CRG	–0.40	–1.30	–2.37	–0.57	0.20	–0.83	–0.88

[a]Control of standardized time–kill procedure.
[b]Control of modified time–kill procedure.
C, chloramphenicol; R, quinopristin/dalfopristin; V, vancomycin; A, ampicillin; G, gentamicin.

ogy International, Frederick, MD, USA). The test tubes were then placed on a platform shaker in a 35°C incubator. After 1 h of incubation, viable counts were performed, and the tubes were centrifuged at 5520**g** for 15 min at room temperature, which resulted in the formation of a bacterial pellet at the bottom of the test tube. The supernatant was removed from each tube and the pellet resuspended with fresh medium containing the required antibiotic concentration to correspond to pharmacokinetically achievable levels for that time point. Viable counts were repeated to verify that the bacterial inoculum was not significantly reduced during the process of centrifugation and changing antibiotic concentrations. This procedure was performed for each sampling period at 0, 1, 2, 4, 6, 8, 10 and 12 h. Two test tubes containing no antibiotics were included in each assay as a growth control. One control test tube was used in the modified procedure described above. Standard time–kill methods[5] were used for the second control tube to verify the lack of significant loss of bacteria.

Colony counts were read (Protos Colony Counter, Microbiology International) after 48 h incubation in humidified room air at 35°C. The activity of the antimicrobials alone and in combination was determined by plotting $\log_{10}$ colony counts (cfu/mL) against time. Bactericidal activity was defined as a 3 $\log_{10}$ decrease in cfu/mL from the most active single agent. Synergy and antagonism were defined as a greater than 2 $\log_{10}$ cfu/mL decrease or increase, respectively, from the original inoculum.

Results and discussion

Modifications of the standardized time–kill procedures were used in this study to more closely simulate in-vivo conditions in an effort to predict more accurately the clinical activity of the antibiotics. We used agents and novel combinations of agents based on results of previous investigations and regimens that are used to treat resistant enterococcal infections.[6]

All isolates were susceptible to chloramphenicol (4.0 mg/L) and quinopristin/dalfopristin (0.5 mg/L). The isolates demonstrated varying susceptibility patterns to

vancomycin, ampicillin and gentamicin with MICs (mg/L) of 16–512, 16–128 and 250–≥2000, respectively. Given the varying susceptibility patterns, it is likely that the isolates are unique strains.

Table II shows the individual and mean $\log_{10}$ cfu/mL change with each regimen. Only minor changes were observed with quinopristin/dalfopristin and chloramphenicol, with mean $\log_{10}$ decreases of –1.03 and –0.31, respectively. No bactericidal or synergic activity was noted with any of the regimens studied. The mean $\log_{10}$ decreases with three-drug combinations (–0.65, –0.82, –0.88) were less than with the regimen of chloramphenicol plus quinopristin/dalfopristin (–0.98). However, these differences are too small to predict antagonistic or synergic trends.

The interaction between chloramphenicol and the other agents did not result in antagonism as previously reported and as might possibly be expected. Antagonism has been reported with the pneumococcus when bacteriostatic and bactericidal compounds such as chloramphenicol and penicillin are combined.[7] Antagonism was also noted with the combination of chloramphenicol and ampicillin against *Haemophilus influenzae* and group B streptococci.[8] In a preliminary study, we did not find antagonism with combinations containing chloramphenicol against vancomycin-resistant enterococci.[6] The combination of chloramphenicol plus vancomycin, quinopristin/dalfopristin or ampicillin resulted in additive activity in 21%, 63% and 15% of the isolates, respectively.

The primary objective of this study was to modify current time–kill methodology using simulated serum concentrations to mimic in-vivo concentrations. The modified time–kill methodology is a refinement of earlier methods published by Bauernfeind *et al.*[9] who used declining serum concentrations of ceftriaxone and netilmicin to assess synergic activity against *Pseudomonas aeruginosa*. Antibiotic concentrations were altered by centrifugation and resuspension in broth containing the desired concentration. Serum concentrations were deduced from curves showing mean values with the curves extended to 14 h to include the effect of a second dose. Using the model, this group concluded that synergy between these antibiotics was likely to occur *in vivo*.

The modified time–kill methodology used pharmacokinetic software that allowed simulation of steady-state serum concentrations of multiple antibiotics with varying half-lives. While we chose kinetic parameters of healthy male volunteers, the kinetic parameters of others such as the critically ill, obese or paediatric populations could be used in this model.

Bauernfeind *et al.*[9] used an initial inoculum of 10^8 cfu/mL in the synergy study against *P. aeruginosa*. Unlike Bauernfeind *et al.*,[9] however, we incorporated the 10^5 cfu/mL inoculum used in standard time–kill methodology. Bacterial counts in the body often exceed the inoculum used in this study. However, the use of a standard inoculum allows comparisons with other assays.

The process of centrifugation, changing media and antibiotic concentrations was of concern owing to potential loss of inoculum, death of bacterial cells and subsequent effect on the overall results. In the earlier study,[9] the control increased <1 $\log_{10}$ after 14 h of incubation. Although no explanation was given, this could be due to centrifugation and a resulting loss of bacteria while changing antibiotic concentrations. Since the speed of centrifugation or an evaluation of bacterial loss was not reported in earlier experiments, our procedure was tested before the primary investigation to determine its effect on the inoculum. In addition, two controls as described were run with each isolate. We found that relatively high-speed centrifugation resulted in sedimentation of bacterial cells at the bottom of the test tube without noticeable cell loss. When viable colony counts of the centrifuged control tubes were compared with those of the non-centrifuged control tubes the growth curves were identical (data not shown).

The modified time–kill assay simulates the in-vivo interaction that may occur by using steady-state simulated serum concentrations and providing fresh medium for growth of the organism. Although the agents and combinations of agents did not result in bactericidal activity or synergy, no antagonism was detected. We feel the methodology used in this study is superior that of the standardized time–kill and chequerboard titration assays and allows the clinician to predict more accurately the interaction likely to occur between antimicrobial agents and bacteria.

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References

1. National Committee for Clinical Laboratory Standards. (1997). *Methods for Antimicrobial Susceptibility Testing for Bacteria That Grow Aerobically—Third Edition: Approved Standard M7-A4*. NCCLS, Villanova, PA.

2. Kirby, W. M. & Kind, A. C. (1967). Clinical pharmacology of ampicillin and hetacillin. *Annals of the New York Academy of Sciences* **145**, 291–7.

3. Mandell, G. L. & Sande, M. A. (1990). Antimicrobial agents. In *The Pharmacological Basis of Therapeutics*, 8th edn, (Gillman, A. G., Rall, T. W., Nies, A. S. & Taylor, P., Eds), pp. 1065–145. Pergamon Press, New York.

4. Moellering, R. C. (1984). Pharmacokinetics of vancomycin. *Journal of Antimicrobial Chemotherapy* **14**, *Suppl. D*, 43–52.

5. National Committee for Clinical Laboratory Standards. (1992). *Methods for Determining Bactericidal Activity of Antimicrobial Agents: Tentative Guideline M26-T* (ISBN 1-56238-144-X). NCCLS, Villanova, PA.

6. Messick, C. R. & Pendland, S. L. (1997). In vitro activity of chloramphenicol alone and in combination with vancomycin, ampicillin, or RP 59500 (quinupristin/dalfopristin) against vancomycin-resistant enterococci. *Diagnostic Microbiology and Infectious Disease* **29**, 203–5.

7. Wallace, J. F., Smith, R. H., Garcia, M. & Petersdorf, R. G. (1967). Studies on pathogenesis of meningitis. VI. Antagonism between penicillin and chloramphenicol in experimental pneumococcal meningitis. *Journal of Laboratory and Clinical Medicine* **70**, 408–18.

8. Lapointe, J.-R., Lavallée, C., Michaud, A., Chicoine, L. & Joncas. J.-H. (1986). In vitro comparison of ampicillin-chloramphenicol and ampicillin-cefotaxime against 284 *Haemophilus* isolates. *Antimicrobial Agents and Chemotherapy* **29**, 594–7.

9. Bauernfeind, A., Jungwirth, R. & Petermüller, C. (1982). Simultaneous simulation of the serum profiles of two antibiotics and analysis of the combined effect against a culture of *Pseudomonas aeruginosa*. *Chemotherapy* **28**, 334–40.

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