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In vitro synergy and time-kill assessment of interaction between kanamycin and metronidazole against resistant bacteria

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This study assessed the influence of combining kanamycin and metronidazole against Gram positive and Gram negative bacteria by agar diffusion, checkerboard and time-kill assays. The test isolates were highly resistant with minimum inhibitory concentrations (MICs) ranging between 15.63 and >250 µg/ml for kanamycin and between 15.63 and 125 µg/ml for metronidazole. The antibacterial combinations resulted in drastic decreases in the MICs with an increased antibacterial activity that indicated synergistic interaction against all the bacteria except *Acinetobacter calcaoceuticus* UP, *Enterobacter cloacae* ATCC 13047 and *Shigella flexneri* KZN. The fractional inhibitory concentration indices (FICIs) showed synergy ranging from 0.31 to 0.50, additive interaction with FICI ranging from 0.53 to 1.25 and no antagonistic interaction. *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Bacillus cereus* ATCC 10702, *E. cloacae* ATCC 13047, *Klebsiella pneumoniae* ATCC 10031, *A. calcaoceuticus* UP and *Micrococcus luteus* were totally eliminated by the antibacterial combinations within 24 h of incubation. The lack of antagonism between these antibacterial agents in checkerboard and time-kill assays suggests that kanamycin may prove to be effective in monotherapy and combination therapy. The study indicates the potential beneficial value of combining kanamycin and metronidazole in the treatment of microbial infections in clinical settings.

Key words: Drug-drug interactions, synergy, time-kill, FICI, microbial infections.

INTRODUCTION

Infectious diseases are a significant cause of morbidity and mortality accounting for approximately 50% of all deaths in tropical countries (Mahady, 2005; Khosravi and Behzadi, 2006) and a leading cause of death worldwide (Ahmad and Aqil, 2009). Due to indiscriminate use of antibacterial agents in infectious diseases (Davies, 1994; Service, 1995), multidrug resistance in bacteria has become a great challenge to human health (Peters et al., 2008). With the increasing prevalence of multi-drug resistant bacteria and appearances of strains with reduced susceptibility to antibiotics (Boucher et al., 2009) as well as the inexorable invasion of hospitals and communities, there are increases in health care costs (Gums, 2002), many untreatable bacterial infections and the need to search for new infection-fighting strategies and novel antibacterial agents (Zy et al., 2005; Rojas et al., 2006; Ymele-Leki et al., 2012).

Although previous studies have indicated interactions between other aminoglycosides or nitroimidazole (metronidazole) and other antibacterial agents, combining kanamycin and metronidazole against bacteria of clinical importance has not been reported. For the aminoglycosides,

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Burgess and Hastings (2000), Song et al. (2003) and CLSI (2006) reported the effectiveness of combining β-lactam with aminoglycoside in the treatment of *Pseudomonas aeruginosa*. Kelesidis et al. (2008) and Petrosillo et al. (2008) showed that polymyxins and tigecycline were combined for treating infections caused by carbapenem-resistant Enterobacteriaceae and Cottagnoud et al. (2003) reported the synergy of vancomycin and gentamicin against penicillin-resistant pneumococci. While synergistic clinical efficacies of amoxicillin and metronidazole have been reported (Winkel et al., 2001; Yek et al., 2010), Pulkkinen et al. (1993) and Sanchez et al. (2004) showed that metronidazole-nystatin combination produced better prevention against bacterial vaginosis in women using intra uterine drug (IUD) as a contraceptive method than the respective drug. Azithromycin combined with metronidazole was more effective in treating pelvic inflammatory diseases (Bevan et al., 2003), symptomatic bacterial vaginosis (Schwebke and Desmon, 2007) and pediatric Crohn's disease (Levine and Turner, 2011). The use of combinations of antimicrobials that together achieve synergistic activities against targeted micro-organisms is one potential strategy for overcoming bacterial resistance (Allen et al., 2002). Theoretically, it is aimed at broadening antimicrobial empirical coverage, improving efficacy against isolates with a minimum inhibitory concentration (MIC) at or approaching the breakpoint for susceptibility as well as preventing the further emergence of resistant organisms (Rybak and McGrath, 1996; Walsh and Howe, 2002). Drug combinations are characterized by an increased activity and tolerability compared to that of monotherapy and those used to increase the killing of single-drug resistant strains or mutants. While preventing the emergence of reduced susceptibility, it achieves bactericidal synergy and provides activity against stationary-phase organisms and organisms growing in biofilm. The use of drug combinations is an excellent strategy to avoid or delay drug resistance since different drug targets are attacked simultaneously. This study was, therefore, aimed at assessing the effect of combining kanamycin and metronidazole, having different mechanisms of action, against bacteria of clinical relevance.

MATERIALS AND METHODS

Bacterial strain

The bacteria used in this study included *Acinetobacter calcaoceuticus* UP, *Bacillus cereus* ATCC 10702, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Enterobacter cloacae* ATCC 13047, *Klebsiella pneumoniae* ATCC 10031, *Shigella flexneri* KZN, *Micrococcus luteus, E. faecalis* KZN and *Staphylococcus aureus* OK_{2b}. These organisms were obtained from the Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa. The antibacterial assays were carried out using Mueller Hinton II Agar (Biolab) and broth.

Antibiotics used in this study

Antibiotic powders of Kanamycin (Duchefa) and Metronidazole (Duchefa) were used. Stock antibiotic solutions were prepared and dilutions made according to the CLSI (Clinical Laboratory Standardization Institute) method or manufacturer's recommendations (NCCLS, 1997; Richard et al., 2007).

Antibiotic susceptibility testing - (Agar diffusion method)

Each of the isolates was standardized using colony suspension method (EUCAST, 2012). Each strain's suspension was matched with 0.5 McFarland standards to give a resultant concentration of 1×10^6 cfu/ml. The antibiotic susceptibility testing was determined using the modified Kirby-Bauer diffusion technique (Cheesbrough, 2002) by swabbing the Mueller-Hinton agar (MHA) (Oxoids U.K) plates with the resultant saline suspension of each strain. Wells were then bored into the agar medium with heat sterilized 6 mm cork borer. The wells were filled with 100 µl of different concentrations (62.5, 125 and 250 µg/ml) of each of the antibiotics taking care not to allow spillage of the solutions onto the surface of the agar. To determine the combinatorial effect of the antibiotics, different solutions containing combined concentrations (62.5, 125 and 250 µg/ml) of kanamycin and metronidazole were prepared and used. The plates were allowed to stand for at least 30 min before being incubated at 37°C for 24 h (BSAC, 2002). The determinations were done in duplicate. After 24 h of incubation, the plates were examined if there is any zone of incubation (Bauer et al., 1966). The diameter of the zone of inhibition produced by the respective antibiotic alone and their combinations were measured and interpreted using the CLSI zone diameter interpretative standards (CLSI, 2008).

Determination of minimal inhibitory concentration (MIC)

The minimum inhibitory concentrations (MICs) for the two antibiotics under study were determined in duplicate by the macrobroth dilution method in Mueller Hinton broth according to CLSI (Clinical Laboratory Standardization Institute) (Richard et al., 2007). To determine the MICs of each antibiotic, different concentrations of each of the antibiotics (0.0019 - 500) µg/ml were prepared by serial dilution in Mueller Hinton broth. To determine their combinatorial effects, combinations of different concentrations used in the determination of the MICs of each of the antibiotics were used. The tubes were inoculated with 100 µl of each of the bacterial strains. Blank Mueller Hinton broth was used as negative control. The bacterial containing tubes were incubated at 37°C for 24 h. Each combination assay was performed two times. The MIC was defined as the lowest dilution that showed no growth in the Mueller Hinton broth.

Checkerboard assay

The interactions between the two antibiotics were determined using the checkerboard as previously described (Petersen et al., 2006). The range of drug concentration used in the checkerboard assay was such that the dilution range encompassed the MIC for each drug used in the analysis. The fractional inhibitory concentration (FIC) was derived from the lowest concentrations of the two antibiotics in combination permitting no visible growth of the test organisms in the Mueller Hinton broth after an incubation for 24 h at 37°C (Mandal et al., 2004). FIC indices were calculated using the formula FIC index = (MIC of kanamycin in combination/MIC of kanamycin alone) + (MIC of metronidazole in combination/MIC of metronidazole alone). While Eliopoulos and Eliopoulos (1988),

Isenberg (1992) and Petersen et al. (2006) defined synergy by the checkerboard method as $\sum FIC \leq 0.5$, additivity as $0.5 < \sum FIC \leq 1$, indifference as $1 < \sum FIC \leq 4$ and antagonism as $\sum FIC > 4$, Giertsen et al. (1988), Grytten et al. (1988), and Kamatou et al. (2006) defined synergy to occur when $\sum FIC < 1.0$, additivity occur when $\sum FIC = 1.0$ and antagonism when $\sum FIC > 1.0$. In this study, synergy was defined as $\sum FIC \leq 0.5$, additivity as $0.5 < \sum FIC \leq 1$, indifference as $1 < \sum FIC \leq 4$. Concentrations within the FIC panel were such that the MIC of each antibiotic was in the middle of the range of concentrations tested but lower than the MICs of the respective antibiotics.

Determination of rate of kill

Assays for the rate of killing bacteria by the combined antibiotics were carried out using a modified plating technique of Eliopoulos and Eliopoulos (1988) and Eliopoulos and Moellering (1996). The combined antibiotics were incorporated into 10 ml Mueller Hinton broth in McCartney bottles at ½ MIC and MIC. Two controls, one Mueller Hinton broth without combined antibiotics inoculated with test organisms and Mueller Hinton broth incorporated with the combined antibiotics at the test concentrations without the test organisms, were included. Inoculums density, approximately 10^{10} cfu/ml further verified by total viable count, was used to inoculate 10 ml volumes of both test and control bottles. The bottles were incubated at 37°C on an orbital shaker at 120 rpm. A 100 µl aliquot was removed from the culture medium at 0, 24 and 48 h for the determination of cfu/ml by the plate count technique (Cruishank et al., 1975) by plating out 25 µl of each of the dilutions. The problem of antibiotics carryover was addressed by dilution as described previously by Pankuch et al. (1994). After incubating at 37°C for 24 h, emergent bacterial colonies were counted, cfu/ml calculated and compared with the count of the culture control without antibiotic.

RESULTS

In this study, *A. calcaoceuticus* UP and *E. faecalis* KZN were highly resistant to kanamycin while other isolates exhibited concentration dependent susceptibility to the varied concentrations of this antibiotic. To metronidazole, *E. faecalis* KZN was susceptible to the different concentrations, *E. faecalis* ATCC 29212, *K. pneumoniae* ATCC 10031 and *A. calcaoceuticus* UP were slightly inhibited by the highest concentration. Other isolates were not affected by the different concentrations used. On combining different concentrations of the two antibiotics, concentration dependent significant synergistic interactions were observed. The resultant zones of inhibition from the combined antibiotics were wider than those obtained from the antibacterial activities of each antibiotic used (Table 1). Though the bacteria showed varied resistance to both antibiotics, resistant colonies were not isolated within the zones of inhibition and fuzzy zones were not found around the edges of the zones of inhibition.

The macrobroth assay indicated that the test isolates were highly resistant to the two antibiotics by exhibiting minimum inhibitory concentrations (MICs) ranging between 15.625 and >250 µg/ml for kanamycin and 15.625 to 125 µg/ml for metronidazole. On combining the two antibiotics against these bacteria, the MICs of both antibiotics were drastically reduced in the range between ½ MIC and $^1/_8$ MIC with a simultaneous increase in the antibacterial activity of the combined antibiotics (Table 2). The results of both assays were complementary. The significant reduction in the MICs and the observed increase in the zones of inhibition from combined antibiotics showed that the resultant effect of combining these two antibiotics was synergy. In the checkerboard assay, combining the two antibiotics showed synergistic interaction against most of the bacteria except *Acinetobater calcaoceuticus* UP, *E. claoacae* (ATCC 13047) and *S. flexneri* KZN. While the fractional inhibitory concentration indices (FICIs) showing synergy ranged from 0.3125 – 0.5, an additive/indifference interaction was indicated with FICI ranging between 0.5313 and 1.25 and no antagonism was recorded from the antibacterial combinations. The combined antibiotics indicated ability to improve the bactericidal effects of each other on both Gram-negative and Gram-positive bacteria.

In the time-kill assay, the results presented in terms of the changes in the $\log_{10}$ cfu/ml of viable colonies showed that the antibacterial combinations exhibited a significant bactericidal activity. The bactericidal activity was defined as being equal to 3 $\log_{10}$ cfu/ml or greater reduction in the viable colony count relative to the initial inoculum (Scheetz et al., 2007). *E. coli* ATCC 25922, *E. faecalis* ATCC 29212, *B. cereus* ATCC 10702, *E. cloacae* ATCC 13047 and *M. luteus* were completely annihilated by the combination of kanamycin and metronidazole at ½ MICs. In addition to these bacteria, *K. pneumoniae* (ATCC 10031) and *A. calcaoceuticus* UP were totally killed by the combined activity of both antibiotics at the MICs within 24 h of incubation. *S. flexneri* KZN, *E. faecalis* KZN and *Staphylococcus aureus* OK_{2b} were not totally inhibited at the combined MICs, despite the degree of the observed synergism because each of these isolates exhibited a very high level of resistance to either or both antibiotics. Average log reduction in viable cell count in time-kill assay for *K. pneumoniae* ATCC 10031, *A. calcaoceuticus* UP, *S. flexneri* KZN, *E. faecalis* KZN and *Staphylococcus aureus* OK_{2b}, not totally eliminated, however, ranged between 3.4472 Log_{10} to 5.7782 Log_{10} cfu/ml after 24 h of interacting the bacteria with the combined antibiotics at the ½ MIC and MIC values (Table 3). A post-antibiotic treatment bioassay done after 48 h showed that all isolates not totally inhibited within 24 h incubation period had an increase in cfu/ml.

DISCUSSION

Due to the frequent development of resistance during monotherapy treatment of infected patients, multiple combinations of antibacterial agents are being proposed (Campbell et al., 1996; El Solh and Alhajhusain, 2009). This is to effectively treat mixed and severe infections,

Table 1. Zones of inhibition produced by each antibiotic and their combinations at different concentrations.

Zone of inhibition	Kanamycin alone (± 1.0 mm)			Metronidazole (± 1.0 mm)			Kan-Met combinations (± 1.0 mm)		
	250	125	62.5	250	125	62.5	250/250	125/125	62.5/62.5
	(µg/ml)			(µg/ml)			(µg/ml)		
Escherichia coli ATCC 25922	25	20	18	0	0	0	29	26	20
Enterococcus faecalis ATCC 29212	26	24	21	16	0	0	27	24	21
Bacillus cereus ATCC 10702	27	24	22	0	0	0	29	25	23
Enterobacter cloacae ATCC 13047	22	20	19	0	0	0	25	22	20
Klebsiella pneumoniae ATCC 10031	28	25	21	13	0	0	29	27	22
Acinetobacter calcaoceuticus UP	0	0	0	14	0	0	26	24	20
Shigella flexneri KZN	28	25	23	0	0	0	28	26	22
Micrococcus luteus	24	21	20	0	0	0	28	25	21
Enterococcus faecalis KZN	0	0	0	20	19	14	31	28	23
Staphylococcus aureus OK_{2b}	28	24	22	0	0	0	26	24	20

Table 2. Fractional inhibitory concentration values for the antibiotics alone and their combinations against resistant bacterial isolates.

Tested bacteria	Minimum inhibitory concentration (µg/ml)			Fractional inhibitory concentration index			
	Kanamycin	Metronidazole	KAN-MET	FICI Kan	FICI Met	FICI	Remarks
Escherichia coli ATCC 25922	125	31.25	15.63/7.81	0.13	0.25	0.38	Synergistic
Enterococcus faecalis ATCC 29212	125	31.25	15.63/7.81	0.13	0.25	0.38	Synergistic
Bacillus cereus ATCC 10702	125	31.25	7.81/7.81	0.06	0.25	0.31	Synergistic
Enterobacter cloacae ATCC 13047	62.5	31.25	15.63/15.63	0.25	0.5	0.75	Additive
Klebsiella pneumoniae ATCC 10031	31.25	31.25	7.81/7.81	0.25	0.25	0.5	Synergistic
Acinetobacter calcaoceuticus UP	> 250	15.63	7.81/7.81	0.03	0.5	0.53	Additive
Shigella flexneri KZN	15.63	62.25	15.63/15.63	1.0	0.25	1.25	Indifference
Micrococcus luteus	250	31.25	15.63/7.81	0.06	0.25	0.31	Synergistic
Enterococcus faecalis KZN	> 250	62.5	15.63/15.63	0.06	0.25	0.31	Synergistic
Staphylococcus aureus OK_{2b}	62.5	125	15.63/15.63	0.25	0.125	0.38	Synergistic

enhance antibacterial activity, reduce the time needed for long-term antimicrobial therapy and prevent the emergence of resistant micro-organisms (Hugo, 1993; Levinson and Jawetz, 2002). Combining existing antimicrobial agents can improve delivery of safe and cost effective patient care in an era where research into discovery of new agents is limited and expensive. In this study, examination of synergy by the checkerboard method demonstrated synergy between kanamycin and metronidazole for the majority of the strains while antagonism was not observed. This is in agreement with previous reports on interaction between aminoglycosides and other antibacterial agents (Tessier and Quentin, 1997; Hayami et al., 1999). Their combination in chemotherapy could decrease resistance development, broaden antibacterial spectrum and

Table 3. In vitro time-kill activity of Kanamycin – Metronidazole combinations at ½ X MIC and MIC against test bacteria.

Tested bacteria	Reduction in bacterial counts (Log_{10}CFU/ml) for the combined antibiotics					
	½ × MIC			MIC		
	0 h	24 h	48 h	0 h	24 h	48 h
Escherichia coli ATCC 25922	7.18	0	0	6.90	0	0
Enterococcus faecalis ATCC 29212	8.90	0	0	8.91	0	0
Bacillus cereus ATCC 10702	7.62	0	0	7.26	0	0
Enterobacter cloacae ATCC 13047	12.15	0	0	12.38	0	0
Klebsiella pneumoniae ATCC 10031	11.51	5.78	5.97	11.61	0	0
Acinetobacter calcaoceuticus UP	12.70	3.45	3.94	12.81	0	0
Shigella flexneri KZN	12.43	3.92	4.32	12.51	3.86	3.96
Micrococcus luteus	11.93	0	0	11.99	0	0
Enterococcus faecalis KZN	10.42	4.11	4.54	11.15	3.90	4.28
Staphylococcus aureus OK_{2b}	9.38	5.13	5.27	9.66	3.94	4.08

encourage synergistic antibacterial activity (den Hollander and Mouton, 2007). As determined by Eliopoulos and Moellering (1996), antibiotic combinations that reduced the original inoculums by ≥ 2 $\log_{10}$ cfu/ml were considered synergy while antagonsism is a < 2 $\log_{10}$ change in cfu/ml when compared with the activity of the individual antibiotic after 24 h incubation period. The time-kill assay confirmed the synergy between kanamycin and metronidazole as indicated by the checherboard assay. These synergy that resulted in enhance antibacterial effects from antibiotics having different mechanisms of action could have resulted from the formation of a complex compound with enhanced antibacterial activity. Since kanamycin prevents bacteria from synthesizing proteins by binding to 16S rRNA of 30S subunit and metronidazole is reduced to cytotoxic polar compounds able to cause DNA strand breakage, DNA helix and nucleic acid destabilization in bacteria (Tocher and Edwards, 1992; 1994), the activity of the antibacterial combination could be complementary and resulted in the rapid death of bacterial colonies. This could be a means of achieving effective therapy at a reduced cost coupled with a drastic reduction or lost of vestibular and auditory toxicity often associated with the aminoglycosides. While the lack of antagonism between the antibiotics suggested that kanamycin or metronidazole may be effective in monotheryapy and combination therapy, the resultant synergy will reduce the dose of each drug in the combination and prevents the development of bacterial resistance (Barriere, 1992; Wu et al., 1999).

Although bactericidal drugs prevent the emergence of resistant mutants by killing the microorganism (Stratton, 2003) while synergy and bactericidal therapy could be achieved as long as the organism does not exhibit high-level resistance to aminoglycoside (Arias and Murray, 2008), it is evident that highly resistant bacteria with MIC ranging between 15.625 and >250 µg/ml for kanamycin were killed by its combination with metronidazole to which the MICs were between 15.625 and 125 µg/ml for the different isolates. However, while Mouton et al. (1997) and Jumbe et al. (2003) have conceptualized a microbial population as consisting of two distinct subpopulations with different susceptibility, the regrowth of *S. flexneri* KZN, *E. faecalis* KZN and *Staphylococcus aureus* OK_{2b} could be attributed to the preferential killing of the susceptible subpopulations allowing the selective increase of the resistant subpopulation of each of these resistant strains after 48 h incubation. Further treatment or subsequent doses of the antibacterial combinations would be sufficient to eliminate the resistant subpopulation.

Conclusion

In clinical settings, this study emphasizes the potential beneficial value of combining kanamycin and metronidazole for treating seriously ill patients with infections caused by the pathogens tested, especially in the absence of other therapeutic options. Future studies in *in vivo* infection models would provide a better understanding of the therapeutic potential and safety of kanamycin-metronidazole combinations.

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