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Effects and time-kill assessment of amoxicillin used in combination with chloramphenicol against bacteria of clinical importance

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With the emergence of multidrug-resistant organisms in an era when drug development faces challenges causing pharmaceutical companies to curtail or abandon research on anti-infective agents, the use of combined existing antimicrobial agents may be an alternative. This study evaluated the effects of combining amoxicillin and chloramphenicol, to which many bacteria have become resistant, *in vitro* against Gram positive and Gram negative bacteria by agar diffusion, checkerboard and time-kill assays. The test isolates were susceptible to amoxicillin with minimum inhibitory concentrations (MICs) ranging between 0.448 and 500 µg/ml and between 1.953 and 31.25 µg/ml for chloramphenicol. Upon combining these agents, there was a drastic reduction in their MICs indicating an increased antibacterial activity that showed synergistic interaction against all the bacteria. At the highest concentrations, the inhibition zones ranges were 20.33–38.33±0.58 µg/ml for amoxicillin, 27.67–37.67±0.58 µg/ml for chloramphenicol and 31.67–39.33±0.58 µg/ml for the combined agents. The fractional inhibitory concentration indices (FICIs) showed synergy ranging from 0.129 to 0.312 while FICIs for additive interaction were between 0.688 and 1.0. There was no antagonistic interaction. At the $^1/_2$MICs of the combined antibiotics, all the tested bacteria, except for *Klebsiella pneumoniae* ATCC 4352, *Proteus vulgaris* CSIR 0030 and *Enterococcus cloacae* ATCC 13047 were eliminated before 24 h. At the MICs, all the tested bacteria were eliminated except *Enterococcus cloacae* ATCC 13047 which was almost totally eliminated. Post-antibiotic assessment after 48 h showed that all the cultures were sterile except for that of *Enterococcus cloacae* ATCC 13047. The lack of antagonism between these antibacterial agents in checkerboard and time-kill assays suggested that combining amoxicillin with chloramphenicol can provide an improved therapy in comparison to the use of each antibiotic individually. The study indicates the potential beneficial value of combining amoxicillin and chloramphenicol in the treatment of microbial infections in clinical settings.

Keywords: drug-drug interactions; fractional inhibitory concentrations; multidrug resistance; time-kill assessment

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Abbreviations: FICIs, fractional inhibitory concentration indices; MDR, multidrug resistance; MICs, minimum inhibitory concentrations

INTRODUCTION

In the late twentieth century the availability and success of antibiotics and vaccinations resulted in a confidence that technology and modern medicine would be victorious against infectious diseases. During this early period of the antibiotic usage bacterial infections were considered tamed as potentially lethal infections were being cured with antibiotics. However, while the introduction of antimicrobial agents was accompanied by negative rather than positive impact on the patients, the number of individuals to be treated with antibiotics increased with enhanced pathogenicity and invasiveness. The widespread use of antibiotic, therefore, resulted in the emergence of outbreaks and epidemics of antibiotic-resistant pathogens including multidrug resistant strains (Normak & Normak, 2002). Today, resistant pathogens, an underappreciated threat to public health throughout the globe (Zhang *et al.,* 2006), are rapidly growing problems leading to an urgent need for novel antimicrobial agents (Kumar & Schweizer, 2005; Edgar *et al.,* 2012).

Although there is a continued effort into seeking new therapies in response to the consequences of the pressure on the widespread use of antibiotics or problems associated with increasing drug resistance (Cameron *et al.,* 2004), bacteria have, also, continued to develop different resistance mechanisms to virtually all antibiotics in general clinical practices (Clatworthy *et al.,* 2007). These resistance mechanisms may include altered penicillin-binding proteins, presence of various β-lactamases and loss of porins (Bou & Martïnez-Beltran, 2000). While active efflux and enzymatic inactivation are the mechanisms responsible for resistance to aminoglycosides (Smith *et al.,* 2007), the most common mechanisms of resistance to chloramphenicol are decreased outer membrane permeability (Burns *et al.,* 1989), enzymatic inactivation by acetylation essentially by acetyltransferase or by chloramphenicol phosphotransferases (Schwartz *et al.,* 2004; Aakra *et al.,* 2010), target site modulation (Montero *et al.,* 2007) and presence of efflux pump (Daniels & Ramos, 2009). To overcome various resistance mechanisms and dissemination of antibiotic resistance genes, exploring the possible synergy between conventional antibiotics becomes necessary. This study, therefore, aimed at investigating the combinatory effects and time-kill assessment of amoxicillin and chloramphenicol against bacteria of clinical importance. These antibiotics have long been used for the treatment of enteric fever. However, most enteric organisms including *Salmonella typhi* causing typhoid fever have become highly resistant to them (Kabra, 2000; Das & Bhattacharya, 2000).

MATERIALS AND METHODS

Bacterial culture and preparation of antibiotic solutions. The bacteria used in this study included *Shigella sonnei* ATCC 29930, *Salmonella typhi* ATCC 13311, *Staphylococcus aureus* OK_{2a}, *Acinetobacter calcoaceuticus* UP, *Enterococcus cloacae* ATCC 13047, *Proteus vulgaris* CSIR 0030, *Pseudomonas aeruginosa* ATCC 19582, *Bacillus cereus* ATCC 10702, *Klebsiella pneumoniae* ATCC 4352 and *Staphylococcus aureus* ATCC 6538. Antibiotic powders of Amoxicillin (Duchefa) and Chloramphenicol (Duchefa) were used. Stock antibiotic solutions were prepared and dilutions made according to the manufacturer's recommendations.

Antibiotic susceptibility testing-Agar diffusion method. Each bacterial strain's colony suspension was matched with 0.5 McFarland standards to give a resultant concentration of 1.5×10^7 cfu/ml. The antibiotic susceptibility was determined by swabbing the Mueller-Hinton agar (MHA) (Oxoids UK) plates with the adjusted bacterial strains. Agar wells were made with heat sterilized 6 mm cork borer before being filled with 100 µl of different solutions (62.5 µg/ml, 125 µg/ml and 250 µg/ml) of each of the antibiotics. These concentration ranges were chosen to cover the maximum serum therapeutic range that could be reached *in vivo* when 100 µl of each antibiotic was dispensed. Different solutions containing the same concentrations of amoxicillin and chloramphenicol were prepared and used to determine the effect of combining the antibiotics with care taken to prevent spillage of the solutions onto the agar surface. The plates, in triplicate, were allowed to stand for 1 h before being incubated at 37°C for 24 h. After incubation, the diameter of the inhibition zones produced by each antibiotic alone and their combinations were measured with a transparent ruler. Synergism was stated when inhibition zones of combination treatment were at least 0.5 mm larger than those produced by the individual antibiotics.

Determination of minimal inhibitory concentration (MIC). To determine the MICs of each antibiotic, 100 µl of each bacterium was added to different concentrations (0.0019–500 µg/ml) of each of the antibiotics prepared by serial dilution in double strength Mueller Hinton broth. To determine the effects of combining these antibiotics, the same concentrations of each antibiotics used for determining their MICs were combined before the solutions were inoculated with 100 µl of each of the bacterial strains and incubated at 37°C for 24 h. Blank Mueller Hinton broth was used as negative control. 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 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 incubating for 24 h at 37°C. FIC indices were calculated using the formula FIC index = (MIC of amoxicillin in combination/MIC of amoxicillin alone) + (MIC of chloramphenicol in combination/MIC of chloramphenicol alone). In this study, synergy was defined as $\sum FIC \leq 0.5$, additivity as $0.5 < \sum FIC \leq 1$, indifference as $1 < \sum FIC \leq 4$ and antagonism as $\sum FIC > 4$ determined by the checkerboard method according to Petersen and coworkers (2006).

Determination of rate of kill. The Time-kill assay was performed using the broth macrodilution technique (Pankey *et al.,* 2005). The amoxicillin and chloramphenicol antibiotics were incorporated into 50 ml of Mueller Hinton broth at $^1/_2$MIC and MIC respectively. Controls consisting of Mueller Hinton broth with the respective antibiotic added alone at the test concentrations were included in each experiment. The experimental and control flasks were inoculated with each test organism to a final inoculum density of approximately 10^9 cfu/ml. Immediately after inoculation, aliquots (100 µl) of the negative control flasks were taken, serially diluted in sterile distilled water and plated on nutrient agar in order to determine the zero h counts. The test flasks were incubated at 37°C with shaking 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. The problem of antibiotics carryover was addressed by dilution. After incubation, emergent bacterial colonies were enumerated, the mean count (cfu/ml) for each test and controls was calculated and expressed as $\log_{10}$. The interactions were considered synergistic if there was a decrease of >2 $\log_{10}$ cfu/ml in colony counts after 24 h for the antibiotics combination compared to the activity of each antibiotic used alone. Additivity or indifference was described as a <2 $\log_{10}$ cfu/ml change in the average viable counts after 24 h for the combination, in comparison to the activity of each antibiotic used alone. Antagonism was defined as a >2 $\log_{10}$ cfu/ml increase in colony counts after 24 h for the combination compared to the activity of each antibiotic used alone (Pankey *et al.,* 2005).

Statistical analysis. All the data were subjected to one way analysis of variance (ANOVA) and the mean values were separated at $p<0.05$ using Duncan's Multiple Range Test. The one way ANOVA test was used to determine if there was any statistically significant difference in the size of inhibition zones for each bacterial isolate exposed to each antibiotic alone and the antibiotics combination. All statistical analyses were performed using SPSS software (2009).

RESULTS

According to our results, amoxicillin, chloramphenicol and their combinations exhibited significant antibacterial activity as shown in Table 1. The bacterial inhibition zones produced by amoxicillin ranged between 16.33±0.58 and 33.67±0.58 mm at the lowest concentration of 62.5 µg/ml while the inhibition zones ranged between 20.33±0.58 and 38.33±0.58 mm at the highest concentration of 250 µg/ml. The inhibition zones produced by chloramphenicol at 62.5 µg/ml ranged from 20.67±0.58 to 31.67±0.58 mm while those produced at the highest concentration of 250 µg/ml ranged between 27.67±0.58 and 37.67±0.58 mm. Although each antibiotic produced various inhibition zones when used alone, those of their combinations at the lowest concentration of 62.5 µg/ml ranged between 23.67±0.58 and 34.67±0.58 mm. At the highest concentration, 250 µg/ml, of the combined amoxicillin and chloramphenicol, the resultant inhibition zones ranged between 31.67±0.58 and 39.67±0.58 mm. Comparatively the inhibition zones produced by the combination of the two antibiotics were mostly wider than those produced when amoxicillin and chloramphenicol were used individually. At the highest concentration for amoxicillin, *Proteus vulgaris* CSIR 0030 had the least inhibition zone while *Shigella sonnei* ATCC 29930 had the widest inhibition zone. At the highest concentration of chloramphenicol, *Enterococcus cloacae* ATCC 13047 had the least inhibition zone while *Shigella sonnei* ATCC 29930 had the widest inhibi-

tion zone. For concentration of amoxicillin and chloramphenicol in combination, *Staphylococcus aureus* ATCC 6538 had the least inhibition zone while *Shigella sonnei* ATCC 29930 had the widest inhibition zone.

The antibacterial susceptibility testing was further performed by the broth macrodilution method following the recommendations of the Clinical and Laboratory Standard Institute (CLSI, 2009). The MIC breakpoints for the two antibiotics were considered and the susceptibility results were interpreted according to EUCAST (2013). While the MICs of the amoxicillin ranged between 0.488 and 500 µg/ml, that of chloramphenicol ranged between 1.953 and 31.25 µg/ml. *Shigella sonnei* ATCC 29930 had the least MIC of 0.488 µg/ml and *Salmonella typhi* ATCC 13311 had the highest MIC of 500 µg/ml for amoxicillin. While *Shigella sonnei* ATCC 29930, *Pseudomonas aeruginosa* ATCC 19582 and *Klebsiella pneumoniae* ATCC 4352 had the least MIC of 1.953 µg/ml for chloramphenicol, the highest MIC of this antibiotic for *Proteus vulgaris* CSIR 0030, *Enterococcus cloacae* ATCC 13047, *Staphylococcus aureus* OK_{2a} and *Salmonella typhi* ATCC 13311 equaled 7.813 µg/ml. A consideration for the MIC breakpoint showed that the isolates were susceptible to chloramphenicol with the exception of *Acinetobacter calcoaceuticus* UP, while they were mostly resistant to amoxicillin with the exception of *Shigella sonnei* ATCC 29930, *Acinetobacter calcoaceuticus* UP and *Pseudomonas aeruginosa* ATCC 19582. The *in vitro* antibacterial activity of these antibiotics and their combinations was further assessed on the basis of the FIC index representing the sum of the FICs (∑FICs) of each antibiotic tested. When the antibacterial combination was considered as synergistic for ∑FIC≤0.5, additive for 0.5<∑FIC≤1, indifferent for 1<∑FIC≤4 and antagonistic for ∑FIC>4, the antibacterial combinations showed synergistic interactions (70.0%) and additivity/indifference (30.0%), whereas antagonism was not recorded in the case of the test organisms. However, while the FICI for the synergistic interaction was between 0.129 and 0.312, the FICI for the additive interaction was between 0.688 and 1.0 (Table 2).

To validate the synergy detected in the checkerboard antibacterial assay, the time-kill analysis was performed and showed synergistic effects of the antibacterial combinations against all the test isolates. At the ½ MIC of the combined antibiotics, no growth was recorded for all the test bacteria except for *Klebsiella pneumoniae* ATCC 4352, *Proteus vulgaris* CSIR 0030 and *Enterococcus cloacae* ATCC 13047 that had their mean log cfu/ml drastically reduced to 2.614±0.025, 2.583±0.042 and 3.757±0.035 respectively after 24 h of incubation while the growth of *Enterococcus cloacae* ATCC 13047 alone was detected after 48 h. At the MIC of the combined antibiotics, no growth was recorded for all the tested bacteria except for *Enterococcus cloacae* ATCC 13047 alone. However, after 48 h of incubation all the tubes were sterile (Table 3). The synergy or additivity showed by the combinations of amoxicillin and chloramphenicol in checkerboard analysis was, therefore, affirmed by the degree of synergistic effects exerted on the bacteria as tested by the time-kill analysis.

DISCUSSION

Multidrug resistance (MDR) is defined as a resistance of an organism to ≥3 antibiotic classes (Lynch & Zhanel, 2005). In an era with the emergence of multidrug resistant organisms and lack of treatment options for infections with certain microorganisms, bacteria have be-

Table 1. Antimicrobial activity (average inhibition zone in mm) of amoxicillin, chloramphenicol and their combinations on bacterial isolates

	AMX			CHL			Amx-Chl combinations		
	62.5	125	250	62.5	125	250	62.5	125	250
Bacteria used	µg/ml								
Staphylococcus aureus ATCC 6538	18.33±0.58^{h}	23.67±0.58^{e}	27.33±0.58^{c}	20.67±0.58^{g}	24.33±0.58^{d}	29.67±0.58^{b}	23.67±0.58^{f}	27.33±0.58^{c}	31.67±0.58^{a}
Bacillus cereus ATCC 10702	21.00±1.00^{h}	24.33±1.15^{f}	27.67±0.58^{c}	23.67±0.58^{g}	26.67±0.58^{d}	29.67±0.58^{b}	24.67±0.58^{e}	27.67±0.58^{c}	32.67±0.58^{a}
Klebsiella pneumoniae ATCC 4352	16.33±0.58^{h}	17.33±1.15^{g}	20.67±0.58^{f}	24.67±0.58^{e}	29.67±0.58^{c}	31.67±0.58^{b}	24.67±0.58^{e}	27.67±0.58^{d}	32.67±0.58^{a}
Pseudomonas aeruginosa ATCC 19582	17.67±0.58^{i}	19.67±0.58^{h}	22.33±0.58^{g}	27.67±0.58^{e}	30.33±0.58^{d}	32.67±0.58^{b}	26.33±0.58^{f}	30.67±0.58^{c}	34.67±0.58^{a}
Proteus vulgaris CSIR 0030	15.33±0.58^{i}	17.67±0.58^{h}	20.33±0.58^{g}	21.33±0.58^{f}	27.67±0.58^{d}	30.67±0.58^{b}	24.67±0.58^{e}	29.67±0.58^{c}	33.67±0.58^{a}
Enterococcus cloacae ATCC 13047	19.67±0.58^{h}	21.67±0.58^{g}	24.67±0.58^{e}	22.33±0.58^{f}	26.33±0.58^{d}	27.67±0.58^{c}	24.67±0.58^{e}	28.33±1.15^{b}	32.33±1.15^{a}
Acinetobacter calcoaceuticus UP	27.67±0.58^{e}	29.67±0.58^{d}	31.33±0.58^{c}	24.67±0.58^{g}	27.67±0.58^{e}	29.67±0.58^{d}	26.67±0.58^{f}	32.33±1.15^{b}	38.33±1.53^{a}
Staphylococcus aureus OK_{2a}	20.33±0.58^{g}	22.67±0.58^{f}	24.67±0.58^{e}	24.67±0.58^{e}	26.67±1.53^{d}	32.67±0.58^{b}	27.33±1.15^{c}	32.67±0.58^{b}	34.67±0.58^{a}
Salmonella typhi ATCC 13311	16.67±0.58^{i}	18.67±0.58^{h}	22.67±0.58^{g}	23.67±0.58^{f}	27.67±0.58^{d}	31.67±0.58^{c}	27.67±0.58^{d}	32.67±0.58^{b}	34.67±0.58^{a}
Shigella sonnei ATCC 29930	33.67±0.58^{h}	36.33±0.58^{e}	38.33±0.58^{b}	31.67±0.58^{i}	35.67±0.58^{f}	37.67±0.58^{c}	34.67±0.58^{g}	36.67±0.58^{d}	39.67±0.58^{a}

Note: The average inhibition zones with different superscript along the same row are significantly different (p<0.05)

Table 2. MICs and FICs values of amoxicillin, chloramphenicol and their combinations for bacterial isolates

	MICS (μg/ml)			FICs Indices			
Bacteria used	AMX	CHL	AMX-CHL	FICI Amx	FICI Chl	FICI	Remarks
Staphylococcus aureus ATCC 6538	3.906	3.906	1.953/1.953	0.5	0.5	1	Additive
Bacillus cereus ATCC 10702	31.25	3.906	1.953/0.976	0.062	0.249	0.312	Synergy
Klebsiella pneumoniae ATCC 4352	3.906	1.953	0.976/0.976	0.249	0.499	0.748	Additive
Pseudomonas aeruginosa ATCC 19582	0.977	1.953	0.448/0.448	0.459	0.229	0.688	Additive
Proteus vulgaris CSIR 0030	250	7.818	0.976/0.976	0.004	0.125	0.129	Synergy
Enterococcus cloacae ATCC 13047	250	7.818	0.976/0.976	0.004	0.125	0.129	Synergy
Acinetobacter calcoaceuticus UP	1.953	31.25	0.448/0.448	0.229	0.014	0.243	Synergy
Staphylococcus aureus OK_{2a}	125	7.818	0.976/0.976	0.008	0.125	0.133	Synergy
Salmonella typhi ATCC 13311	500	7.818	0.976/0.976	0.002	0.125	0.127	Synergy
Shigella sonnei ATCC 29930	0.488	1.953	0.06/0.06	0.123	0.031	0.154	Synergy

come resistant to 21 different antibiotics and each isolate is on average resistant to 7–8 antibiotics (D'Costa *et al.,* 2006). In this situation, combination therapy, where two or more antimicrobial agents are used simultaneously, is considered a potentially effective means of minimizing the emergence rate of bacterial resistance. Although there are a large number of antimicrobial agents for medical use and combination of two bactericidal drugs results in synergism, the combination of bactericidal and bacteriostatic agents often results in antagonism (Daschner, 1976) and there will always be a need to discover new agents. Consequently, since drug-drug combinations are convenient models that can give insight into the significance of synergistic and antagonistic interactions of dissimilar drugs (Hall *et al.,* 1983), amoxicillin and chloramphenicol were combined for their potential synergistic effects in view of the increasing resistance rate to these older antibacterial drugs.

In this study, we observed no antagonistic effects on any of the test isolates. The synergistic and additive effects of combining amoxicillin and chloramphenicol against the tested bacteria agree with some studies that demonstrated synergistic interactions between β-lactams and other therapeutic agents such as clavulanate (Abate & Miorner, 1998), ethanbutol (Getahun, 2000), vancomycin (Fox *et al.,* 2006) and aminoglycosides (Güzel & Gerçeker, 2008). However, while amoxicillin is still being considered a drug of choice within its class because it has better pharmacokinetics than other β-lactam antibiotics in case of treatment of infections caused by susceptible organisms (Shahhet *et al.,* 2011), chloramphenicol is being used sparingly in human medicine because of its bone marrow toxicity. However, due to the lack of new antibiotics and the global problem of advancing bacterial resistance caused by the indiscriminate use of the current antibiotics (Maviglia *et al.,* 2009), chloramphenicol is being reconsidered as an option for treatment of certain infections in critically ill patients (Nitzan *et al.,* 2010). Despite the potential renaissance of chloramphenicol as an effective antibiotic, there is a dearth of information on its interaction with other antibacterial agents, its co-administration or use in combination therapy.

Although the peptidyl transferase centre is the main target site for many antibiotics and substrate analogs (Spahn & Prescott, 1996), chloramphenicol binds to the 23S rRNA of the 50S ribosomal subunit and blocks the elongation of peptides during biosynthesis of proteins (Montero *et al.,* 2007). Chloramphenicol induces oxida-

Table 3. *In vitro* time-kill activity of Amoxicillin – Chloramphenicol combinations at ½ MIC and MIC against bacteria

	Mean ± S.D. Dev of Log cfu/ml at different concentrations					
	$^1/_2$ MIC			MIC		
Bacteria used	0 h	24 h	48 h	0 h	24 h	48 h
Staphylococcus aureus ATCC 6538	6.315±0.024	0	0	6.145±0.031	0	0
Bacillus cereus ATCC 10702	8.685±0.013	0	0	8.602±0.005	0	0
Klebsiella pneumoniae ATCC 4352	7.902±0.002	2.614±0.025	0	7.741±0.076	0	0
Pseudomonas aeruginosa ATCC 19582	9.677±0.059	0	0	9.7627±0.040	0	0
Proteus vulgaris CSIR 0030	8.659±0.035	2.583±0.042	0	8.9165±0.048	0	0
Enterococcus cloacae ATCC 13047	8.341±0.040	3.757±0.035	2.314±0.018	8.167±0.018	1.071±0.015	0
Acinetobacter calcoaceuticus UP	7.173±0.003	0	0	6.954±0.010	0	0
Staphylococcus aureus OK_{2a}	6.848±0.006	0	0	6.774±0.005	0	0
Salmonella typhi ATCC 13311	6.326±0.022	0	0	6.395±0.035	0	0
Shigella sonnei ATCC 29930	9.644±0.010	0	0	9.626±0.033	0	0

tive stress in sensitive bacteria (Aakra *et al.,* 2010), while β-lactams interfere with the production of peptidoglycan and break the cell of active dividing microorganisms in an iso-osmotic environment (Yellanki *et al.,* 2010). On this basis, the effectiveness of this antibacterial combination may not be overemphasized against resistant bacteria tested in this study. Combining these antibiotics may result in formation of a complex compound having different mechanisms of action and, possibly, acting on different target sites in addition to the target sites for which they were synthesized. Therefore, the synergy of amoxicillin and chloramphenicol may not only, prevent or suppress the emergence of resistant strains but decrease dose-related toxicity and attain a broad spectrum of activity while overcoming both intrinsic and genetic determinants conferring resistance to these antibiotics.

CONCLUSION

In conclusion, antimicrobial resistance is a significant global problem in the management of patients with infectious diseases. However, combining existing antibiotics may be an alternative means of combating bacterial resistance as the combined agents can exert their different antibacterial activities simultaneously. The lack of antagonism between amoxicillin and chloramphenicol *in vitro* in checkerboard and time-kill assays suggested that combining these two antibiotics can be an improved therapy in comparison to the use of each antibiotic individually. On the other hand, the observed synergy indicate the potential beneficial value of combining them in the treatment of microbial infections in clinical settings in the era of limited research on new drug development and discovery.

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