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Original Article

In vitro susceptibility of a penicillin-resistant and tolerable isolate of *Streptococcus pneumoniae* to combination therapy

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Abstract

Introduction: Preference for combination therapy to treat infection due to multidrug-resistant *S. pneumoniae* (MDRSP) has not been well elucidated in previous studies.

Methodology: In the present study, 19 antibiotics in combinations were tested against an MDRSP isolate. *In vitro* susceptibility studies including minimum inhibitory concentration (MIC), minimal bactericidal concentrations (MBC) and disk agar diffusion (DAD), tolerance to resistant antibiotics, checkerboard assay, time-kill curve, hemolytic assay, and autolysis assay were performed on the test strain to study its *in vitro* susceptibility to combination therapy.

Results: From the checkerboard assay and time-kill curve, it was observed that a combination of levofloxacin (MIC, 16 µg/mL) and ceftriaxone (MIC, 2 µg/mL), at sub-MIC concentration was synergistic and most effective against the MDRSP isolate (penicillin MIC, > 64 µg/mL). Hemolytic activities also increased significantly with combination therapy compared to monotherapy ($p < 0.05$). Moreover, the hemolytic activity of levofloxacin in combination with ceftriaxone was better than ciprofloxacin plus ceftriaxone or cefepime. The autolysis rate was also found to increase rapidly within one hour of exposure to levofloxacin plus ceftriaxone, and this was found to be significantly different from the other combinations at the fifth and sixth hour post incubation ($p < 0.05$).

Conclusions: This data suggests that this combination is bactericidal *in vitro*, and requires further studies in *in vivo* models for treatment against MDRSP infections.

Key words: *Streptococcus pneumonia;* multidrug-resistant; susceptibility; tolerance; combination therapy; antibiotics.

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Introduction

Streptococcus pneumoniae (SP), the most frequent isolate from clinical samples of respiratory tract infections and community acquired pneumonia (CAP) [1], is still characterized by high mortality and morbidity associated with significant health cost [2]. Over a few decades, the emergence of multidrug-resistant (MDR) strains of SP has become a global problem [3, 4] and demands innovative therapeutic modalities, particularly for treatment of MDR strains.

Treatment with a single antibiotic such as a beta-lactam or third-generation cephalosporin or a fluoroquinolone was considered to be effective previously [5], but with the emergence of multidrug-resistant *S. pneumoniae* (MDRSP) strains, use of a beta-lactam or macrolide as empirical therapy is of great concern. Though fluoroquinolones with increased activity against SP are now being recommended, there has been growing concern about the emergence of fluoroquinolone-resistant strains [6]. Emerging evidence suggests that dual therapy is superior to monotherapy, particularly for patients with severe CAP or bacteremic pneumococcal CAP [7, 8]. Combination therapy using antimicrobials with different mechanisms of action has been used to treat infections for decades, with the goal of producing a wider spectrum of action, preventing the emergence of multidrug-resistant (MDR) populations, reducing the dose of a single agent, or achieving a synergistic effect [9].

Autolytic response induced during stationary growth phase leads to excessive lysis of cultures *in vitro*. From a therapeutic perspective, autolysin (LytA) contributes to the penicillin- and vancomycin-induced lysis of pneumococci [10], which also enhances the release of pneumolysin [11], which plays an important role in pneumococcal pathogenicity. It has been reported that macrolide antibiotics, at therapeutically relevant concentrations, inhibit the production of these toxins by macrolide-resistant strains of SP, both *in*

vitro and *in vivo* [12, 13]. Hence, this study was conducted with the objective of determining the most effective antibacterial combination for treatment of multidrug-resistant pneumococcal infections.

Methodology

Strains and antimicrobial agents

The study drugs, which included ampicillin (AMP), azithromycin (AZM), amoxicillin/potassium clavulanate (AMC), oxacillin (OXA), ceftazidime (CAZ), cefotaxime (CTX), cefuroxime (CXM), ceftriaxone (CRO), clindamycin (CLI), imipenem (IPM), meropenem (MEM), levofloxacin (LVX), ciprofloxacin (CIP), rifampicin (RIF), vancomycin (VAN), trimethoprim/sulphamethoxazole (TMP-SXT), cefepime (FEP), and gentamicin (GEN) (HiMedia, Bombay, India), were used for all *in vitro* testing.

The clinical isolates of *S. pneumoniae* (AMRI SP-1) used for the experiments were obtained from a patient admitted to a hospital with severe pneumonia in Kolkata, West Bengal, India. A quality control strain of *S. pneumoniae* (ATCC 49619) was used for all *in vitro* susceptibility testing. Strains were stored in skimmed milk tryptone glucose glycerol medium (HiMedia) at -80°C and subcultured twice onto Columbia blood agar plates (BAP) supplemented with 5% sheep blood (BioMerieux, Lyon, France) overnight at 37°C in a 10% CO_2 air incubator before being used for all *in vitro* experiments. All *in vitro* experiments were carried out in Mueller-Hinton broth (MHB) (HiMedia).

In vitro susceptibility tests

In vitro susceptibilities of the isolates were compared with *S. pneumoniae* ATCC 49619 as per Clinical and Laboratory Standards Institute (CLSI) guidelines. Minimum inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) were determined by the tube dilution method in MHB supplemented with 5% sheep blood, and disk agar diffusion (DAD) test was performed using Mueller-Hinton agar supplemented with 5% sheep blood, following CLSI guidelines [14].

Tolerance study

For testing tolerance, samples were diluted in MHB broth containing 5% horse serum. When the culture (at 37°C) reached an optical density at 400 nm of about 0.1 to 0.2 (corresponding to 10^8 colony-forming unit [CFUs]/mL), antibiotics resistant to the clinical isolates were added at variable concentrations ranging from 2 to 50 times the MIC for the strain [15]. Viable number of bacteria were determined by plating appropriately diluted cultures on Columbia agar supplemented with 5% sterile sheep blood, six hours after initiation of the cultures.

Test for synergism

A checkerboard assay was performed according to the method described earlier [16]. For each combination, a synergy test was performed in a 96-well microtiter plate containing two antimicrobial agents in twofold dilutions (4 x MIC to 1/32 x MIC) dispensed in a checkerboard fashion on the day of the assay. The sum of fractional inhibitory concentrations (FICs) were calculated and were used to classify the effect of combinations of antimicrobial agents as synergistic, for FIC indexes ≤ 0.5; no interaction, for FIC indexes > 0.5–4; and antagonistic, for FIC indexes > 4 [17].

Time-kill assay

A time-kill assay was performed according to the method described earlier [18] with little modification. Each antibiotic was tested alone and in combination at concentrations to which they showed synergy in the checkerboard assay. Bacterial counts were performed at 0, 1, 2, 4, 6, 8, 12, and 24 hours of incubation at 37°C by plating aliquots of 10 and 100 µL after dilution in sterile saline (0.9%) onto Columbia BAP supplemented with 5% sheep blood. Synergy, indifference, and antagonism between the combined antibiotics were concluded from methods described earlier [18].

Hemolytic assay

AMRI SP-1 and ATCC 49619 were grown in MHB supplemented with 5% sterile horse serum in the presence as well as in the absence of antibiotics, either alone or in combination, to which the isolate showed synergistic interaction in the checkerboard assay. Subsequently, cells from growing culture were sampled at 0, 2, 4, 8, 12, and 24 hours of incubation at 37°C. Percent lysis of red blood cells (RBCs) was measured by methods described previously. Saponin (0.5%) was used as the positive control that represented 100% hemolysis [19].

Autolysis assay

Autolysis rates of the clinical isolate AMRI SP-1 and the reference strain ATCC 49619 was determined by the use of a 10 mL culture of *S. pneumoniae* sampled from the mid-logarithmic growth phase (optical density $[OD]_{580}$ of 0.6 = 1–5 × 10^8 CFUs/mL)

and exposed to antibiotics LVX, 0.5 µg/mL (1/32 times MIC); CIP, 0.06 µg/mL (1/16 times MIC); CRO, 0.25 µg/mL (1/8 times MIC); and FEP, 0.06 µg/mL (1/8 times MIC) either alone or in combination when OD at 620 nm reached 0.25–0.3 (5×10^7 CFUs). Turbidity was measured hourly for six hours. The autolysis rate in terms of percent lysis was calculated by dividing OD by initial OD x 100 and area under the concentration versus time was calculated [20].

Statistical methods

The observers involved in data collection and analysis were not completely blind to treatment conditions. However, the methodology used for sample identification prevented subjective bias in the experiments. Data were expressed as mean ± standard deviation (SD) Means were compared between groups by one-way variance analysis followed by Scheffe's post-hoc test. $P < 0.05$ was considered significant.

Results

Determination of MICs, MBCs and DAD for different antibiotics tested against S. pneumoniae

Median MIC values for different antibiotics against the test isolates AMRISP-1 and ATCC-49619 were determined in triplicate according to the CLSI broth microdilution technique. The results obtained from MIC, MBC, and DAD of the pneumococcal isolate; the reference strain is listed in Table 1.

Tolerance study

The tested isolates' and control strain's level of tolerance to the antibiotics are represented in Table 2. Viable counts of bacteria were determined by plating appropriately diluted cultures. A decrease in CFUs of 3 to 4 $\log_{10}$ units of the test isolates was considered nontolerant, whereas killing by about 1 $\log_{10}$ CFUs at 50-fold concentration was considered tolerant following the definition of Moreillon *et al*. [21]. There was a significant difference between the group of antibiotics to which the strains were tolerant and between the non-tolerant ones ($p<0.05$).

Test for synergy

Checkerboard method was performed in duplicate for the MDR isolates for all possible drug combinations that would be of therapeutic interest. Out of the listed antibiotics tested for *in vitro* susceptibility (listed in Table 1) to determine MICs, MBCs, and zone of inhibition, combination studies using checkerboard assay were performed and are represented in Table 3.

Table 1. *In vitro* susceptibilities of *Streptococcus pneumonia* strains to different antimicrobial agents for all test isolates

Antibiotic	MIC (µg/mL)		MBC (µg/mL)		Zone diameter (mm)	
	ATCC 49619	AMRI SP-1	ATCC 49619	AMRI SP-1	ATCC 49619	AMRI SP-1
PEN	0.06[a]	> 64[b]	0.06	-	26	#
AMP	0.25[a]	> 32[b]	0.5	-	20	#
AMC	0.06[a]	2[a]	0.12	8	24	22
OXA	0.06[a]	0.25[a]	0.25	0.5	23	22
CAZ	8[c]	16[b]	16	>64	23	15
CRO	1[a]	2[b]	4	4	22	16
CTX	4[a]	0.5[a]	8	1	23	22
CXM	0.5[a]	2[a]	1	64	25	23
FEP	0.06[a]	0.5[a]	0.06	1	25	24
IPM	0.06[a]	0.12[a]	0.12	1	23	25
MEM	0.06[a]	0.06[a]	0.12	1	24	24
AZM	0.12[a]	> 8[b]	2	16	23	10
CLI	0.5[a]	4[b]	0.5	16	17	13
LVX	0.12[a]	16[b]	0.12	32	19	12
CIP	0.06[a]	1[a]	0.12	2	23	25
RIF	0.06[a]	0.12[a]	0.12	1	20	21
VAN	1[a]	> 64[b]	4	-	19	#
GEN	0.5[a]	2	1	4	23	20
TMP-SXT	1[a]	> 64[b]	2	-	18	#

MIC: minimum inhibitory concentration; MBC: minimal bactericidal concentrations; PEN: penicillin; AMP: ampicillin; AMC: amoxicillin/potassium clavulanate; OXA: oxacillin: CAZ: ceftazidime; CRO: ceftriaxone; CTX: cefotaxime; CXM: cefuroxime; FEP: cefepime; IPM: imipenem; MEM: meropenem; AZM: azithromycin; CLI: clindamycin; LVX: levofloxacin; CIP: ciprofloxacin; RIF: rifampin; VAN: vancomycin; GEN: gentamicin; TMP-SXT: trimethoprim/sulphamethoxazole; – Not within the detectable limit; # No zone of inhibition detected; [a] Sensitive; [b] resistant; [c] intermediate

Table 2. Tolerance study of the multidrug-resistant clinical isolates of *S. pneumoniae* (AMRI SP-1) to different resistant antibiotics

Drugs	AMRI SP 1		
	MIC (µg/mL)	Tolerance level µg/mL	Decrease in $\log_{10}$ CFU/mL[a]
AMP	32	30 x	3.2 ± 0.25
CAZ	8	2 x	3.5 ± 0.21
CLI	16	> 50 x	0.67 ± 0.15
LVX	4	50 x	0.43 ± 0.21
VAN	16	5 x	3.6 ± 0.21
TMP-SXT	64	2 x	3.6 ± 0.15

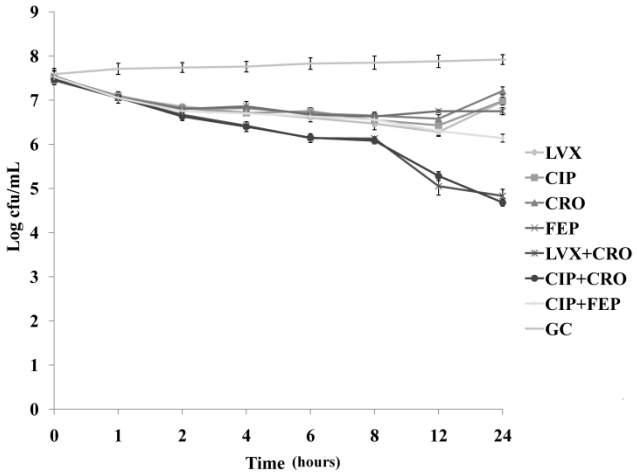
MIC: minimum inhibitory concentration; CFU: colony-forming unit; AMP: ampicillin; CAZ: ceftazidime; CLI: clindamycin; LVX: levofloxacin; VAN: vancomycin; TMP-SXT: trimethoprim-sulphamethoxazole; x: Number of times the MIC of an antibiotic (µg/mL) at which 99.9 % killing was observed. Decrease in $\log_{10}$ CFU/ml after antibiotic exposure was performed in triplicate and expressed as mean ± standard deviation; [a] Significant decrease in $\log_{10}$ CFU/mL ($p < 0.05$)

Table 3. Evaluation of synergy by checkerboard assay

Combination (A + B)		FIC A	FIC B	∑ FIC	Remarks
Antibiotic A	Antibiotic B				
Ampicillin	Azithromycin	0.093	0.037	0.468	Synergy
Ampicillin	Levofloxacin	0.281	0.562	0.843	Indifference
Ampicillin	Gentamicin	0.039	0.620	0.659	Indifference
Levofloxacin	Ceftriaxone	0.046	0.375	0.421	Synergy
Levofloxacin	Cefotaxime	$	$	$	$
Levofloxacin	Cefepime	0.035	1.12	1.159	Indifference
Levofloxacin	Azithromycin	$	$	$	$
Levofloxacin	Rifampicin	$	$	$	$
Vancomycin	Rifampicin	0.064	17.16	17.22	Antagonism
Vancomycin	Imipenem	0.251	134.33	134.58	Antagonism
Azithromycin	Ciprofloxacin	0.132	1.062	1.194	Indifference
Ciprofloxacin	Ceftriaxone	0.312	0.156	0.468	Synergy
Ciprofloxacin	Cefepime	0.124	0.248	0.372	Synergy
Amoxicillin/potassium clavulanate	Rifampicin	0.032	0.531	0.562	Indifference
Gentamicin	Ceftriaxone	0.187	0.187	0.374	Synergy

$: Resistant to the combination; FIC: fractional inhibitory concentration; ∑ FIC (FIC A + FIC B) value < 0.5, synergy; 0.5–4, indifference; > 2, antagonism

Figure 1. Time-kill assay



Killing rates of levofloxacin (LVX) at a concentration of 0.5 µg/mL (1/32×MIC), ciprofloxacin (CIP) at a concentration of 0.06 µg/mL (1/16×MIC), ceftriaxone (CRO) at a concentration of 0.25 µg/mL (1/8×MIC), and cefepime (FEP) at a concentration of 0.06 µg/mL (1/8×MIC) either alone or in combination as LVX + CRO, CIP + CRO, and CIP + FEP for the MDRSP strain AMRI SP-1. GC indicates growth control. Experiments were performed in triplicate and the results expressed as mean ± standard deviation.

The combination that showed synergy with the MDR strain AMRI SP-1 was as follows: ciprofloxacin and cefepime> gentamicin and ceftriaxone > levofloxacin and ceftriaxone > ampicillin and azithromycin > ciprofloxacin and ceftriaxone. Antimicrobial combinations including ampicillin plus levofloxacin, ampicillin plus gentamicin, levofloxacin plus cefepime, azithromycin plus ciprofloxacin, and amoxicillin/potassium clavulanate plus rifampicin showed indifference. Combinations of vancomycin plus rifampicin and vancomycin plus imipenem were found to be antagonistic in action. Combinations of levofloxacin plus cefotaxime or azithromycin or rifampicin were ineffective in inhibiting the growth of the tested strain.

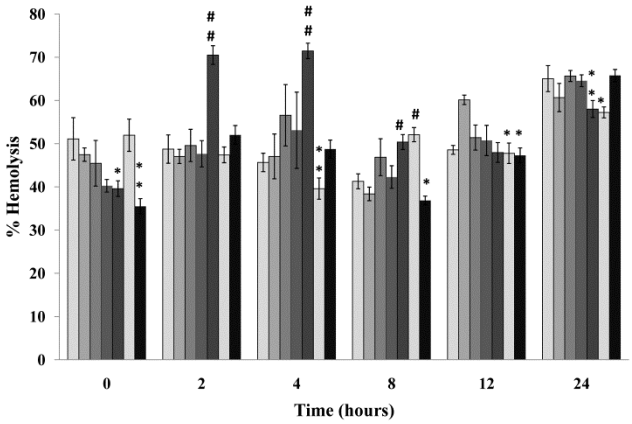
Time-kill assay

Combinations that showed high level of synergy in the checkerboard assay were examined to observe their killing activities using that particular antimicrobial agent either alone or in combination, at a concentration from which their sum of FIC was evaluated. Among the antimicrobials tested in combination, synergy was observed in the case of levofloxacin plus ceftriaxone and ciprofloxacin plus ceftriaxone. Representative time-kill curves that showed the most effective combination as determined from the viable cell count (CFUs) are demonstrated graphically for the isolate in Figure 1. The combination of fluoroquinolones (LVX or CIP) with a third-generation cephalosporin (CRO) showed synergy, as the combinations resulted in a greater than 2 $\log_{10}$ decrease in viable count at 24 hours. The time-kill curve also clearly showed a large decrease in viable titer at 12 hours of post-antibiotic exposure for these two combinations. A combination of CIP with FEP was able to decrease the viable titer by less than 1 $\log_{10}$ CFUs and was thus considered to be indifferent in action by definition. No antagonism was observed for the chosen combinations tested in the time-kill assay.

Hemolysis assay

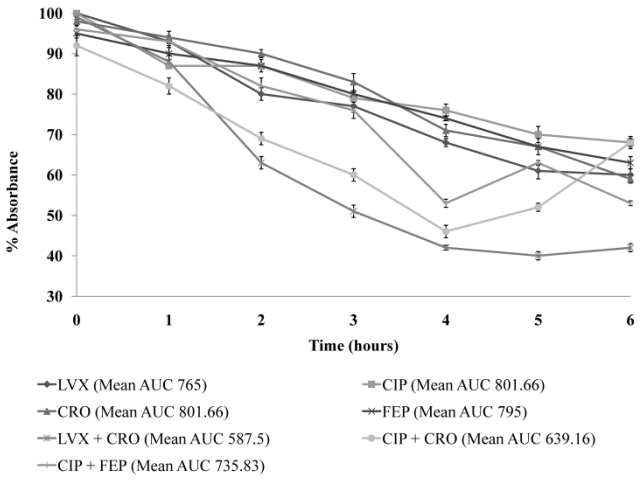
The effects of the sub-MIC (LVX, 0.5 mg/L [1/32×MIC]; CIP, 0.06 mg/L [1/16×MIC]; CRO, 0.25 mg/L [1/8×MIC]; and FEP, 0.06 mg/L [1/8×MIC]) level of antibiotics used for time-kill assays, either alone or in combination, were evaluated. After 0, 2, 4, 8, 12, and 24 hours of incubation post-antibiotic treatment, hemolytic activities were found to be significantly higher in the presence of LVX and CRO than in the presence of LVX or CRO alone at 4 hours,

Figure 2. Hemolysis assay



Pneumolytic activity of the supernatant obtained from MDRSP strain AMRI SP-1 culture, in the presence of antibiotics as LVX (levofloxacin 0.5 µg/mL, 1/32×MIC), CIP (ciprofloxacin 0.06 µg/mL, 1/16×MIC), CRO (ceftriaxone 0.25 µg/mL, 1/8×MIC) and FEP (cefepime 0.06 µg/mL, 1/8×MIC) either alone or in combination, were incubated to estimate the percent lysis of red blood cells. The absorbance of a solution of saponin at a concentration of 0.5% was considered to have undergone 100% lysis. Experiments were performed in triplicate and the percent hemolysis were expressed as mean with standard deviation. $P < 0.05$ was considered to be significant. *Indicates significant decrease, and # indicates significant increase in lysis owing to greater pneumolytic activity in combination therapy when compared to monotherapy (with a single antibiotic); **Indicates significant decrease, and ## indicates significant increase in hemolytic activity in combination therapy compared to both the individual antibiotics alone

Figure 3. Autolysis assay of the highly penicillin-resistant and -tolerant *S. pneumoniae* isolate AMRI SP-1 cells taken from the mid-exponential phase of growth and exposed to medium containing antibiotics at concentration predetermined by the checkerboard assay, either alone or in combination. Decrease in percent absorbance with time after antibiotic exposure was performed in triplicate and expressed as mean ± standard deviation. LVX (levofloxacin 0.5 µg/mL, 1/32×MIC), CIP (ciprofloxacin 0.06 µg/mL, 1/16×MIC), CRO (ceftriaxone 0.25 µg/mL, 1/8×MIC), and FEP (cefepime 0.06 µg/mL, 1/8×MIC).



whereas hemolytic activity was decreased significantly in the CIP and CRO combination when compared with CIP or CRO alone (Figure 2). The hemolytic activity of LVX with CRO was better than CIP with CRO or CIP with FEP. The MDRSP isolate had moderate hemolytic activity in different phases of growth, with the highest activity in the death phase, which was also significantly higher than in the control strain ATCC 49619 (data not shown), indicating this MDRSP strain to be highly pathogenic in nature.

Autolysis assay

Actively growing cultures from the mid-logarithmic phase of the reference strain and the clinical isolates were exposed to antibiotics, either alone or in combination, at an OD of 0.6 at 600 nm. The changes in OD were recorded to determine when the autolysin gene was activated maximally to induce autolysis. Combination therapy resulted in rapid lysis of the cells (decrease in OD from 99% to 88%) during zero to one hour, which decreased further with time than did monotherapy. Percent absorbance at 600 nm was plotted graphically with respect to time in hours (Figure 3). A significant difference in the rate of autolysis was observed with LVX plus CRO when compared with CIP plus CRO at the fifth and sixth hour.

Discussion

Development of penicillin-resistant *Streptococcus pneumoniae* (PRSP) or MDRSP has necessitated researchers and pharmaceutical companies to develop new agents that are effective against these resistant strains. Approaches have been made to find new targets for antimicrobial activity through the use of combination agents that are effective against more than one target in the cell, but relevant clinical evidence with respect to combining agents and their dose of administration *in vitro* and *in vivo* has not been well elucidated for treatment of MDRSP strains.

We evaluated the susceptibility levels of the isolates to several major classes of antimicrobials (Table 1). Isolates were further exposed to increasing levels (times MIC) of those drugs found to be resistant for the test isolates to assess the tolerance level of the drug in action.

We used the checkerboard technique to investigate synergism of the combinations studied against the isolated SP. Combination of fluoroquinolones such as levofloxacin or ciprofloxacin plus ceftriaxone showed synergistic interaction *in vitro*, which was consistent with the studies previously conducted with other PRSP [22]. Thus, it was clear from our study that a combination of fluoroquinolone (levofloxacin or ciprofloxacin) with a third- or fourth-generation cephalosporin (ceftriaxone or cefepime) was effective in rendering damage to the MDRSP strain compared to a combination of beta-lactam plus macrolide, amino-penicillin plus fluoroquinolone, and vancomycin plus rifampicin or imipenem, which showed either indifference or antagonism.

Killing curves confirmed superior activity of levofloxacin plus ceftriaxone over ciprofloxacin plus cefepime or ceftriaxone in combination. Thus, newer-generation cephalosporin was not as effective as a primitive one when used in combination with levofloxacin against such MDRSP isolates. Interestingly, this finding confirms the effectiveness of combination therapy in empirical treatment against such severe infections.

We observed that the MDRSP isolates had moderate hemolytic activity in different phases of growth, with the highest activity in the death phase; however, the data is not shown. Interestingly, combination of levofloxacin and ceftriaxone was able to kill cells much more rapidly than the other combinations compared (Figure 2), showing it to be the most suitable combination for therapy in such severe cases of infections.

Cell wall degradation products released as a result of the action of enzyme autolysin are known to mediate inflammation and toxicity in several animal models. This major pneumococcal lysine can be activated to cause lysis of the bacteria in the stationary phase or upon antibiotic exposure [23]. In an exponentially growing culture, if a significant number of bacteria undergo autolysis, one would expect to find cytoplasmic proteins in the supernatant [24]. Here, we found that there was no significant difference in autolysis rates between the MDRSP and the control strain ATCC 49619 when the exponential phase culture were exposed to 0.6% sodium desoxycholate (data not shown). Thus, the combination of levofloxacin plus ceftriaxone was beneficial in killing the pathogen *in vitro*. As autolysin plays a fundamental role in cell division and separation, high activity and expression of autolysin may reduce the build up of peptidoglycan layers. Moreover, increased lysis indicates decreased production of protease that may be responsible for increased susceptibility to the combined antimicrobials [20].

Conclusions

Choosing an effective therapy for patients with MDRSP infections is becoming more challenging. Antimicrobial combination therapy may be used to extend spectrum coverage, prevent the emergence of resistant mutants, and gain synergy between antimicrobials [25]. A combination of levofloxacin and ceftriaxone may be used for therapy against treatment in severe cases of infection due to MDRSP strains, after the pharmacodynamic properties of this combination are studied *in vivo*. In our ongoing study, we are trying to use this combination for treatment in mice subjected to intranasal challenge with an MDRSP strain. An unpublished observation indicates a bactericidal effect of this combination in rendering damage to infections caused by such beta-lactam-, macrolide-, and fluoroquinolone-resistant strains.

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Authors' contributions

Authors AM and BB designed the study and designed protocol. AM, AN, RA, and SM performed all the experiments. AM, AN, and RA managed the literature searches and analyses. AM and AN undertook the statistical analysis; BB and AM wrote the manuscript. All authors contributed to and have approved the final manuscript.

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