Resveratrol Improves The Antimicrobial Properties Of Polymyxin B Against Multidrug-Resistant Pseudomonas Aeruginosa

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Research Article

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Abstract

Background: Resveratrol has been reported to have synergistic effect with different antibiotics against various kinds of bacteria species. In this study, we investigated if resveratrol can increase the antimicrobial efficacy of polymyxin B against multidrug resistant (MDR) Pseudomonas aeruginosa.7 different MDR Pseudomonas aeruginosa isolates obtained from clinical specimen were tested with broth microdilution method to determine their sensitivity to polymyxin B and resveratrol respectively. Checkerboard analysis was performed to analyze the biological interaction between resveratrol and polymyxin B. Time-kill assays were carried out to monitor the bactericidal activity against Pseudomonas aeruginosa cells. And the biofilm-forming bacteria were selected for biofilm formation analysis.

Results: Though resveratrol showed no intrinsic bactericidal activity against Pseudomonas aeruginosa (MIC >512μg/ml for all isolates), it could effectively increase the sensitivity of those isolates to polymyxin B, with the MIC of polymyxin B reduced by 4-8 fold with 64μg/ml resveratrol. The synergistic effect between resveratrol and polymyxin B on bactericidal activity was also observed with all tested isolates (FICI <0.5). Biofilm formation assay showed that resveratrol in combination with polymyxin B significantly inhibited the biofilm formation of Pseudomonas aeruginosa (P <0.01). With established biofilm, the sensitivity of the tested isolate to polymyxin B was sharply reduced, leading to high concentration polymyxin B resistance (MIC >32μg/ml). The bactericidal activity of polymyxin B against those cells protected by established biofilm could still be promoted by resveratrol (P <0.01).

Conclusions: Resveratrol was able to improve the antimicrobial properties of polymyxin B from various aspects, it may be an ideal partner for the combination therapy of polymyxin B against MDR Pseudomonas aeruginosa.

Introduction

Pseudomonas aeruginosa is a type of Gram-negative bacterium that widely exists in various ecological environments, and also one of the most common opportunistic pathogens that cause clinical infection[1–4]. In 2017, it has been recognized as one of the most life-threatening bacteria and listed as a priority pathogen for Research and Development of new antibiotics by the World Health Organization[5]. In the past few years, with the heavy application of antibiotics in clinical practice, antibiotic resistance became more and more serious for Pseudomonas aeruginosa[6–7]. Especially in recent few years, with the frequent emergence of MDR Pseudomonas aeruginosa, the treatment of Pseudomonas aeruginosa infection has become a big challenge facing by the clinicians[8–10]. Though having not yet been confirmed by clinic survey, combination therapy of antimicrobial agents is considered to be a better strategy than monotherapy for the treatment of Pseudomonas aeruginosa infection, since it can be expected to increase clinical efficacy, and prevent the emergence of antibiotic-resistant strains [11–14].

Polymyxin B, a type of traditional antibiotic that has been neglected for several decades due to its relatively poor clinical efficacy and high risk of nephrotoxicity, was reemployed in clinical practice in recent few years for the emergence and high prevalence of MDR bacteria[15–17]. It even be regarded as the last resorted

agent for the treatment of MDR bacteria infections, even though this special antibiotic is far from being an ideal antimicrobial agent[18–19]. Unfortunately, polymyxin B resistance has emerged in various bacterial species and has been frequently reported in recent few years[20–21]. Given the increasingly serious antibiotic resistance of pathogenic bacteria and the current situation of polymyxin B application, polymyxin B was suggested to be used in combination with other active antimicrobial agents when it is possible, aiming to improve the antimicrobial effect and prevent the emergence of polymyxin B resistance[22–23].

Resveratrol is a naturally occurring polyphenolic compound that has received massive attention for its health benefits, including anti-carcinogenesis and anti-inflammation[24–25]. This compound has also shown its potential value in antibacterial therapy. For example, it has been reported to enhance the antimicrobial efficacy of aminoglycosides against Staphylococcus aureus, to rescue the antibacterial activity of chlorhexidine against carbapenem-resistant Acinetobacter baumannii, and even to increase the bactericidal effect of polymyxin B against Klebsiella pneumonia and Escherichia coli[26–28]. However, the potential biological function of resveratrol on the antibacterial property of polymyxin B against Pseudomonas aeruginosa was still unknown, which deserves further investigation.

Here we investigate the influence of resveratrol on bacterial sensitivity to polymyxin B, the inhibitory function of polymyxin B on biofilm formation, and the bactericidal effect of polymyxin B against those cells protected by established biofilm, to study whether resveratrol and polymyxin B have synergistic effect on antibacterial activity against MDR Pseudomonas aeruginosa, intending to establish an ideal partner for the combination therapy of polymyxin B for the treatment of MDR Pseudomonas aeruginosa infection.

Material And Methods

Bacterial Isolates and Chemicals

7 different isolates of MDR Pseudomonas aeruginosa, including 2 polymyxin B susceptible and 5 resistant isolates, were specially selected from a strain repository at the Department of Microbiology, Shiyan Renmin Hospital. All of those isolates were obtained from different inpatients and specimens and confirmed with the MALDI-TOF mass spectrometry (MS) method. Pseudomonas aeruginosa ATCC 27853 was used as the control strain. Polymyxin B MICs had been interpreted accordingly to the EUCAST clinical breakpoints, version 8.0 (www.eucast.org). Before each experiment, every isolate was cultured onto M-H agar plate and incubated at 37℃ for 24h, and then one colony was selected for further study. Both resveratrol and polymyxin B used in this study were purchased from Solarbio (Beijing, China) and dissolved with sterile water to prepare stock solution with the original concentration of 20mg/ml for polymyxin B and 100mg/ml for resveratrol. Tiny amount of dimethyl sulfoxide (DMSO) was used to assist the dissolution of resveratrol and well-designed DMSO control groups were always included for each experiment.

Susceptibility Testing and Genetic Analysis

MICs of resveratrol and polymyxin B were determined with the Broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guideline. Briefly, bacterial cell suspension prepared with cation-regulated Mueller-Hinton broth (CAMHB) was dispensed into wells of 96-well microtiter plate and made the final bacterial concentration of 5×10^5 CFU/ml for each well. Polymyxin B and resveratrol were separately added into those wells to get desired concentration, ranging from 32μg/ml to 0.125μg/ml with two-fold dilution for polymyxin B, and from 512μg/ml to 32μg/ml with two-fold dilution for resveratrol. After incubating at 37℃ for 24h, the result was then read, and the MIC was defined as the lowest concentration of drug required to visibly inhibit the growth of bacteria. PCR was performed on five polymyxin B resistant isolates with specially designed primers to detect five polymyxin B-resistance related genes including PmrA, PmrB, PhoP, PhoQ, and mcr-1, and the final products were sequenced and analyzed by Biomed (Beijing, China).

Checkerboard Studies

Basing on the susceptibility testing result, checkerboard assay was further conducted to determine the interaction between polymyxin and resveratrol on antibacterial activity. In this experiment, polymyxin B and resveratrol were prepared by two-fold serial dilutions and mixed to produce different concentration combinations. The result was read after incubated at 37℃ for 24 hours and interpreted as follows: $FICI \leq 0.5$, synergistic effects; $0.5 < FICI \leq 1.0$, additive effects; $1.0 < FICI \leq 2.0$, no interaction; and $FICI > 2.0$, antagonistic effects. The result was confirmed with at least three independent experiments.

Time-kill assays

Time-kill studies were performed as described previously with slight modification with all isolates[29]. Resveratrol used in this experiment was maintained at the concentration of 64μg/ml, while the concentration of Polymyxin B for each isolate was determined as the 1/4 MIC of polymyxin B. Bacteria suspension of 5×10^5 CFU/ml was freshly prepared and incubated with polymyxin B and resveratrol alone or in combination at 37℃. Cell counts were determined at the time points of 0, 1, 2, 4, 6, 8, 24 hours respectively, by plating 100μl culture abstracted at different time points on M-H agar plates with appropriate dilution. Synergistic activity was defined as ≥ 2log10 decrease in CFU/mL by two-drug combination compared with every single drug.

Biofilm formation

Bacterial cell suspension of approximately 5×10^5 CFU/ml was freshly prepared with LB broth and dispensed into wells of 96-well microtiter plate. After statically incubated at 37°C for 24h, the liquid phase was discarded and the wells were gently washed with PBS for five times. Biofilm was fixed by adding 99% methanol to each well for 30 minutes. The fixative was then removed and the plate was allowed to air dry before the addition of crystal violet for 15 minutes to stain the adherent biofilm. The well was washed five times with PBS to remove free cells and 100μl of 7% acetic acid was then added to solubilize the dye combined by biofilm. Absorbance, which reflects the established biofilm, was measured at 595nm on a reader. All of those isolates were ranked according to their OD value from large to small, and the top three isolates were selected for further study.

Biofilm formation inhibition analysis

Three isolates with high biofilm formation capability were selected and biofilm formation analysis was performed as described above. Bacterial cell suspension of approximately 5×10^5 CFU/ml was dispensed into wells of 96-well microtiter plate and mixed with polymyxin B and resveratrol alone or in combination. To ensure that the inhibitory function is mainly on biofilm formation rather than on bacteria growth, the subinhibitory concentration of drug that has less than 10% influence on bacteria growth was employed in this experiment. The influence on bacteria growth was measured by incubating the bacteria suspension of 5×10^5 CFU/ml with polymyxin B and resveratrol alone or in combination at 37°C for 24h and calculated as the decreased percentage of cell number compared with the control group. Resveratrol concentration of 8μg/ml was employed in this experiment, which has less than 10% influence on bacteria growth. While the concentration of polymyxin B for each isolate was determined, by incubating the bacterial suspension of 5×10^5 CFU/ml with a series of diluted polymyxin B in combination with 8μg/ml resveratrol, as the highest polymyxin B concentration of those combinations which has less than 10% influence on bacteria growth. With each newly prepared bacterial suspension, two experiments were performed simultaneously, one was for inhibitory function on biofilm formation analysis, the other for influence on bacteria growth analysis. After incubating at 37°C for 24h, OD value and cell count were measured respectively, and the result was confirmed with at last three independent experiments.

Bactericidal activity analysis with established biofilm

The isolate with the highest biofilm formation capability was selected for this experiment. Biofilm was cultivated as described above and the cells protected by established biofilm were obtained by discarding the content and gently washing the wells with sterilized PBS solution for five times. Change in bacteria sensitivity of this isolate to polymyxin B was confirmed by comparing the influence of polymyxin B on the growth of cells with or without established biofilm. Briefly, 24 wells with established biofilm were divided into two groups. One group was for calculating the average quantity of cells attached to the bottom of well. 100ul sterilized PBS solution was added into each well, and a sterile pipette tip was used to scrape and blow the bottom of the well to release the adherent cells and mix the suspension. The cell number was counted by plating the suspension onto M-H ager plates with appropriate dilution. The other group was for analyzing the sensitivity of those cells protected by established biofilm. 100ul cation-regulated Mueller-Hinton broth with 8μg/mL polymyxin B was added into each well and incubated at 37°C for 24h. Then total cell number in each well, including both the adherent and planktonic cell, was counted by plating onto M-H ager plates with appropriate dilution. While when the mean number of cells attached to the bottom of well with established biofilm had been obtained with the former group, 100ul freshly prepared bacterial suspension, which could provide approximately similar(equal or small above but no more than 10%)amount of cells as the calculated result, was mixed with 8μg/mL polymyxin B and added into wells of 96-well microtiter plate. After incubated at 37°C for 24h, the total cell number was counted and compared with that of the group with established biofilm.

As for bactericidal activity analysis on cells protected by biofilm, every 12 wells with established biofilm from the same batch were divided into a group. 100ul cation-regulated Mueller-Hinton broth, mixed with

polymyxin B and resveratrol alone or in combination, was added into each well. Given the decreased sensitivity of cells with established biofilm, relatively high concentration of drugs, 32μg/mL for polymyxin B and 128μg/ml for resveratrol, were employed in this experiment. After incubating at 37°C for 24h, the total cell number was counted and statistical analysis was performed. The result of this experiment was confirmed with three independent experiments.

Statistical Analysis

Every performance was done in triplicate at least. Statistical analysis was carried out with Student's t-test and $P < 0.01$ was considered as significant.

Results

Bacterial isolates

Among 7 MDR Pseudomonas aeruginosa isolates selected for this study, as well as one control isolate ATCC27853, 3 isolates (PA4, PA5, ATCC27853) were sensitive to polymyxin B and 5 isolates (PA1, PA2, PA3, PA6, PA7) were resistant (Table 1). PmrA, PmrB, PhoP, PhoQ, and mcr-1 are five genes that have been reported to be closely associated with polymyxin B resistance. PCR and gene sequencing were performed with all polymyxin B resistant isolates for those five genes. PmrB mutations (S2P, V15I, G68S, Y345H) were found in the isolates PA1, PA2, and PA7, while PmrA mutation (L71R) in PA1, PA3, PA6, and PhoQ mutations (V260G, K123Q) in PA1 and PA6 (Table 2). No mutation in PhoP was found in any of those five isolates. The presence of mcr-1 in bacterial cell has been proved to lead to polymyxin B resistance. However, it could not be detected in any of those tested isolates, which suggested that polymyxin B resistance of those isolates may be unrelated to this gene. Biofilm formation is another character of Pseudomonas aeruginosa, which can lead to antibiotic resistance, but not all Pseudomonas aeruginosa isolates have the same biofilm formation capability. In this study, biofilm formation capability was measured as the OD value of the crystal violet adhered by established biofilm, and the top three isolates were PA2, PA1, and PA7, with the OD_{595} of 0.732, 0.674, and 0.485 respectively, compared with the OD_{595} of 0.023 for the negative control strain ATCC27853.

Table 1
Results of checkerboard assays of polymyxin B in combination with resveratrol.

Isolates[a]	Source	Biofilm formation (OD595)[b]	MIC (mg/L)				
			Polymyxin B	resveratrol	PB + 128μg/ml resveratrol	PB + 64μg/ml resveratrol	PB + 32μg/ml resveratrol
PA1	Sputum	0.674	16	≥512	4*	4*	8
PA2	Urine	0.732	4	≥512	0.5*	1*	1*
PA3	Sputum	0.287	8	≥512	1*	2*	2*
PA4	Wound	0.021	2	≥512	0.5*	0.5*	1
PA5	Wound	0.093	2	≥512	0.25*	0.25*	0.5*
PA6	Wound	0.159	4	≥512	1*	1*	2
PA7	Sputum	0.485	4	≥512	1*	1*	2
PA8	ATCC	0.023	1	≥512	0.25*	0.25*	0.5

[a]PA1-PA7 represent seven different clinical isolated MDR Pseudomonas aeruginosa and P8 the control starin of Pseudomonas aeruginosa ATCC 27853.

[b]OD value of the crystal violet adhered by established biofilm measured at the spectrum of 595nm.

*Combinations in which the selected polymyxin B and resveratrol concentration yielded a synergistic activity (FICI < 0.5).

MDR was defined as resistance to at least three of those antibiotic classes, including aminoglycosides, antipseudomonal penicillins, cephalosporins, carbapenems, and fluoroquinolones.

Table 2
Mutational analysis of the PmrAB and PhoPQ regulatory pathway

Isolates[a]	PmrA	PmrB				PhoP	PhoQ
		Unknown	TMI	PD	HA TPase_c		
PA1	L71R				Y345H		V260G
PA2		S2P	V15I	G68S			
PA3	L71R						
PA6	L71R						K123Q
PA7					Y345H		
[a]Five polymyxin B-resistant MDR Pseudomonas aeruginosa isolates.							
Obtained gene sequences were compared with that of the polymyxin B-susceptible Pseudomonas aeruginosa PAO1.							

Antibacterial activity of resveratrol and its synergistic effect with polymyxin B

To assess the combinatorial effect of resveratrol and polymyxin B against MDR Pseudomonas aeruginosa, we first determined the MIC of resveratrol and polymyxin B respectively with the broth microdilution method. The MIC values of resveratrol for all isolates were > 512μg/ml, indicating lack of intrinsic bactericidal activity. However, resveratrol could effectively increase the sensitivity of those isolates to polymyxin B, with the MIC of polymyxin B reduced by 4–8 fold with 64μg/ml resveratrol. The synergistic effect of resveratrol and polymyxin B could be observed with all isolates, with the FIC index keeping below 0.5 (Table 1).

Synergistic Activity of resveratrol and polymyxin B in Time-Kill Assays

To further confirm the combination effect of resveratrol and polymyxin B on bactericidal activity, time-kill assay was performed with the inoculums of 5×10^5 CFU/ml incubated with 1/4 MIC polymyxin B, alone or in combination with 64μg/ml resveratrol. According to the checkerboard assay results, the combination of 1/4 MIC polymyxin B and 64μg/ml resveratrol, with which the FICI is less than 0.5, can be expected to provide synergistic effect on bactericidal activity. That had been confirmed with all 8 isolates, as shown in Fig. 1. When compared with the control group, the single treatment group showed little or no time-dependent bactericidal activity, as bacterial load did not decrease even after 24h treatment. While in comparison, cells in all isolates except in ATCC27853 could be effectively eradicated by the combination treatment of 1/4 MIC polymyxin B and 64μg/ml resveratrol within 2-8h. In the isolate ATCC27853, though the combination of the chosen concentration of polymyxin B and resveratrol could not effectively eradicate bacterial cells, a decrease in cell number in the initial 8h and a wide gap in bacterial load after 24h could

still be observed compared with the single treatment group. For all isolates in this study, at least 2log10 decrease in CFU/mL can be obtained with the combination treatment compared with the single treatment after 24h, which supports the concept that resveratrol and polymyxin B has synergistic effect on bactericidal activity.

Combination effect of resveratrol and polymyxin B on inhibiting biofilm formation

Biofilm formation is a special character for many Pseudomonas aeruginosa isolates, which provide bacterial cells with high protection and lead to antibiotical treatment failure. To investigate if resveratrol has combination effect with polymyxin B on inhibiting biofilm formation, three isolates (PA1, PA2, PA7) with high biofilm formation capability were selected for this experiment. The subinhibitory concentration of resveratrol and polymyxin B, defined as the concentration that has less than 10% influence on bacteria growth compared with that of the untreated group, was employed for each group. Biofilm was cultivated with the bacterial suspension mixed with a subinhibitory concentration of resveratrol and polymyxin B alone or in combination and measured as the OD value of crystal violet adhered by established biofilm. At the same time, the influence of the chosen concentration of resveratrol and polymyxin B used alone or in combination on bacteria growth was monitored with the same bacteria suspension as used for biofilm formation. After incubated at 37°C for 24h, significant decrease in OD value ($P < 0.01$), but no more than 10% decrease in cell number, can be observed in all three isolates for the combination group compared with the polymyxin B or resveratrol single treatment group (Fig. 2). This result demonstrates that resveratrol and polymyxin B have combination effect on inhibiting biofilm formation.

Resveratrol increases the bactericidal activity of polymyxin B against the cells protected by established biofilm

Since it has been reported that biofilm can protect bacterial cells from antibiotic pressure, we intend to see if resveratrol can increase the bactericidal activity of polymyxin B against the cells with established biofilm[30–31]. In this experiment, biofilm was cultivated with the isolate PA2, which has the highest biofilm formation capability, and biofilm influence on the sensitivity of this isolate to polymyxin B was measured by comparing the growth of bacteria with or without established biofilm.

Strictly following the operating procedures described in the section of method, we got approximately 6.8×10^5 CFU of cells attached to the bottom of each well, which could be regarded as protected by established biofilm. While in contrast, the same number of free cells without established biofilm were obtained from freshly prepared bacteria suspension and added into wells of 96-well microtiter plate. After incubated with 8μg/ml polymyxin B for 24h, the total number of cells for the group with established biofilm reached up to approximately 1.8×10^9 CFU, but just 4.3×10^3 CFU for the control group. About 5log10 increase in CFU supports the concept that established biofilm leads to the decreased sensitivity of PA2 to polymyxin B. To

investigate if resveratrol can increase the sensitivity of those cells protected by biofilm to polymyxin, well-prepared cells with established biofilm were treated with 128μg/ml resveratrol and 32μg/ml polymyxin B alone or in combination for 24h and then the total cell number in each well was counted. Significantly, cell counts of the group treated with polymyxin B in combination with resveratrol were much smaller than that of the group treated with polymyxin B alone ($P < 0.01$), as well as the group treated with the same concentration of resveratrol alone ($P < 0.01$). Those results demonstrate that the bactericidal activity of polymyxin B against those cells protected by established biofilm can be increased by resveratrol (Fig. 3).

Discussion

Bacterial infection, especially MDR bacterial infection, has always been a tricky problem in clinical practice and also a big threat to people's health. Every year, people all over the world suffer a lot from bacterial infection and huge economic losses are associated with preventing and treating bacterial infection[32–34]. Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium that contributes a lot to clinical bacterial infection. And due to the multiple antibiotic resistance mechanisms of Pseudomonas aeruginosa, it is easy for this bacterium to acquired antibiotic resistance[35–36]. In recent few years, with the high prevalence of MDR Pseudomonas aeruginosa, and the limited number of antibacterial agents that can be available for MDR bacterial infection, the treatment of Pseudomonas aeruginosa infection is challenging[37].

Polymyxin B is one of the very few antibiotic agents that can be used for the treatment of MDR Gram-negative bacterial infection. However, in recent few years, polymyxin B resistance has been frequently reported in various kinds of bacteria including Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, as well as Pseudomonas aeruginosa[28,38–39]. Though the polymyxin B resistance mechanism of Pseudomonas aeruginosa is still not completely understand, a common appreciated one is the specific modification of the lipid A component of the outer membrane lipopolysaccharide (LPS) which has negative charge and is the initial target of polymyxin B[40]. It has been reported that some specific mutations in PhoP/PhoQ and PmrA/PmrB two-component system (TCS) can lead to the overexpression of LPS modifying genes, which in turn result in polymyxin B resistance in Pseudomonas aeruginosa[41–43]. Another well-known mechanism is associated with the presence of gene mcr-1. Gene mcr-1 encodes a lipid A phosphoethanolamine transferase that can catalyze the modification of lipid A moiety on bacterial lipopolysaccharide (LPS), so the plasmid carrying mcr-1 gene can also cause polymyxin resistance in Pseudomonas aeruginosa[44]. Mutations of PmrB, PmrA, and PhoQ can be detected in all polymyxin B resistant isolates in this study, suggesting that polymyxin B resistance of those isolates may be associated with the mutations of those genes, and the conclusion of this study is likely to be suitable for those types of Pseudomonas aeruginosa. However, gene mcr-1 could not be found in any of those isolates, that was one limitation of this study.

Even though having been regarded as the last resort agent for the treatment of MDR bacteria infection, the poor clinical efficacy and the high risk of nephrotoxicity of polymyxin have seriously restricted its clinical application[17, 45]. Undoubtedly, any drug that can improve the sensitivity of MDR Pseudomonas

aeruginosa to this special antibiotic can greatly benefit patients. Given this consideration, resveratrol is selected as a candidate, since it can be well tolerated by human body and has been reported to have synergistic effect with different antibiotics against various kinds of bacteria species[46–48]. In this study, both the checkerboard assay and time-kill assay have demonstrated that the combination of resveratrol and polymyxin B has synergistic effect against MDR Pseudomonas aeruginosa, which indicates that, with the assistance of resveratrol, a lower concentration of polymyxin B is required for the treatment of MDR Pseudomonas aeruginosa infection. While even though in clinical practice, polymyxin B was recommended to be given with the maximum doses that could be tolerated by patients, increased sensitivity indicates that better bactericidal activity and shorter treatment course can be expected with this defined concentration of polymyxin B [49]. Given being well tolerated by human body, as well as showing synergistic effect with polymyxin B against Pseudomonas aeruginosa, resveratrol is considered to be an ideal partner of polymyxin B for the combination therapy of MDR Pseudomonas aeruginosa infection. It can be expected to improve the clinical efficacy of polymyxin B by increasing the bactericidal activity and reducing the risk of nephrotoxicity by shortening the treatment course.

For long-term consideration, the combination therapy of polymyxin B and resveratrol is likely to slow the evolution of polymyxin B resistance in Pseudomonas aeruginosa. According to the mutant selection window hypothesis, the development of antibiotic resistance in clinical practice is actually due to the accumulation of naturally occurring resistant mutants, and the combination strategy that can intervene in this enrichment step can be favorable for preserving the usefulness of antibiotics [50–51]. Though need to be confirmed with future long-term clinic data, the hypothesis that combination therapy of polymyxin B and resveratrol can slow the development of polymyxin B resistance in Pseudomonas aeruginosa has been partly proved in this study, with the fact that some polymyxin B resistant isolates can still be effectively eradicated by polymyxin B with the concentration of 2ug/ml or even lower when in combination with 64ug/ml resveratrol, which indicate that those resistant mutants are less likely to be accumulated during antibiotic combination therapy compared with the polymyxin B monotherapy.

Biofilm is a typical character for many Pseudomonas aeruginosa isolates, and it can protect bacteria from surrounding environmental pressure, confer bacterial cells with high antibiotic resistance, and even impede phagocytosis[30, 52]. So inhibiting biofilm formation will be an advantage for the treatment of Pseudomonas aeruginosa infection. Antibiofilm has been regarded as an important index for evaluating the antibacterial effect of antibiotics against those cells with high biofilm formation capability [53–54]. In this study, we have demonstrated that polymyxin B and resveratrol have combination effect on inhibiting the biofilm formation of Pseudomonas aeruginosa. This result indicates that, when used with resveratrol, a lower concentration of polymyxin B is required for inhibiting biofilm formation, and a better antibiofilm effect can be excepted. One concern of the combination antibiofilm strategy is for respiratory Pseudomonas aeruginosa infection where the cells are more likely to colonize and form biofilm and relative lower antibiotic concentration can be achieved with systemic administration. If the biofilm formation process can not be effectively curbed, the persistent respiratory Pseudomonas aeruginosa infection may result.

Established biofilm is frequently observed with chronic wound Pseudomonas aeruginosa infection and can result in poor wound healing[55–56]. According to previous reports, established biofilm can mediate high concentration antibiotic resistance, which means that those bacterial cells designated as susceptible to polymyxin B by traditional antibiotic susceptibility interpretation can not be effectively eliminated by polymyxin B with conventional recommended does strategy[57]. Even though the loading does of polymyxin B for systemic administration has been greatly limited for the high risk of nephrotoxicity, there is no such restriction for topical use, so high concentration of polymyxin B can still be used topically for the treatment of bacteria infection [58–59]. In this study, polymyxin B of 8ug/ml was employed for analyzing the sensitivity of Pseudomonas aeruginosa with established biofilm, and the increased resistance of Pseudomonas aeruginosa to polymyxin B was confirmed by comparing with those cells without established biofilm. To investigate if resveratrol can re-sensitize those cells protected by established biofilm to polymyxin B, polymyxin B and resveratrol were used together and the bacterial activity was monitored by cell counting. And the result has demonstrated that polymyxin B in combination with resveratrol has better bacterial activity against those cells with established biofilm compared with the polymyxin B monotherapy. This result suggests that if polymyxin B is used in combination with resveratrol in the treatment of Pseudomonas aeruginosa infection with established biofilm, a better clinical efficacy can be expected.

Conclusions

Though lacking intrinsic bactericidal activity, resveratrol may still be an ideal partner for the combination therapy of polymyxin B against MDR Pseudomonas aeruginosa, since it can be well tolerated by human body, and also greatly increase the sensitivity of MDR Pseudomonas aeruginosa to polymyxin B. Beside, it can improve the antimicrobial property of polymyxin B against MDR Pseudomonas aeruginosa from various aspects, such as inhibiting the biofilm formation, killing the cells protected by established biofilm. It can be forecasted that resveratrol has the potential to increase the clinical efficacy of polymyxin B for the treatment of MDR Pseudomonas aeruginosa infection and reduce the risk of the emergence of polymyxin resistance.

Abbreviations

MDR: multidrug resistant; MIC: Minimum inhibitory concentration; DMSO: Dimethyl sulfoxide; CLSI: Clinical and Laboratory Standards Institute; EUCAST: European Antimicrobial Susceptibility Testing Committee; FICI: Fractional inhibitory concentration index; M-H agar: Mueller-Hinton agar; LB broth: luria-bertani broth; CAMHB: cation-regulated Mueller-Hinton broth; PBS: Phosphate buffer solution; OD: Optical density; CFU: Colony-Forming Units; LPS: Lipopolysaccharide.

Declarations

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Authors' contributions

LL, JJ, LQ, and RX designed the study. LQ was in charge of this study and performed the majority of those experiments. RX drafted the manuscript and revised it critically for important intellectual content. SZ, HL, YJ and MA participated in the collection and identification of MDR Pseudomonas aeruginosa isolates and the genetic analysis. LL agrees to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated during the current study are available from the corresponding author upon reasonable request. Most of the data is included in this published article.

Ethics approval and consent to participate

The Ethics Committee of Shiyan Renmin Hospital, the Affiliated Hospital of Hubei University of Medicine, exempted this study from review because the present study focused on bacteria.

Consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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Figures

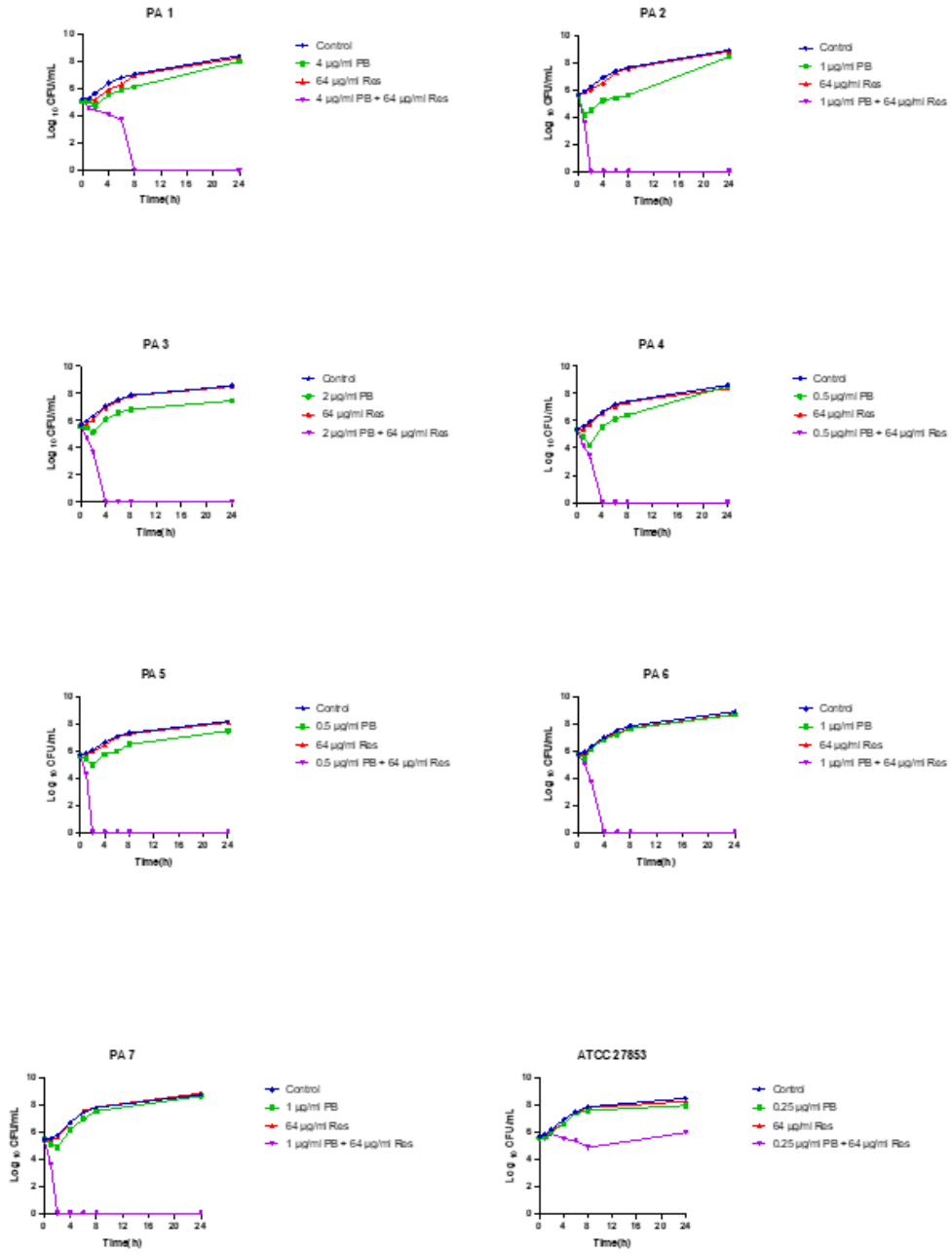

Figure 1

Time-killing curves of polymyxin B and resveratrol alone or in combination against 8 Pseudomonas aeruginosa isolates. PB = polymyxin B, Res = resveratrol.

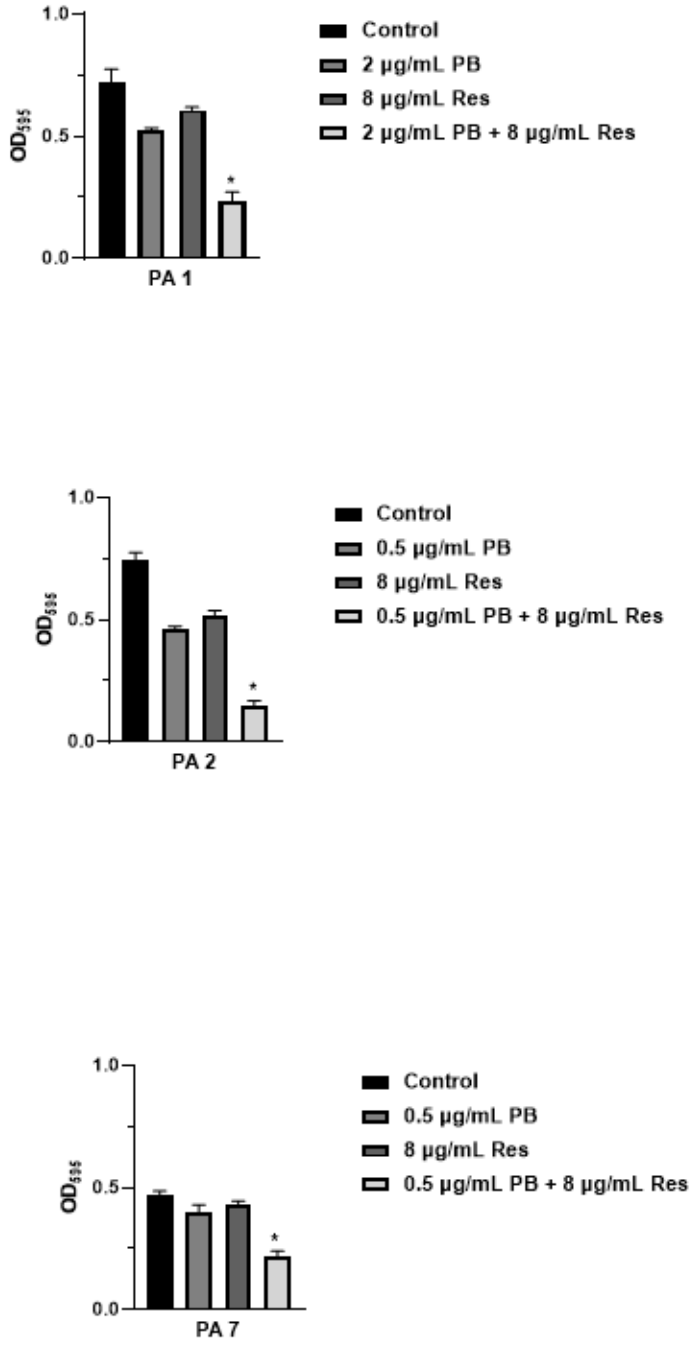

Figure 2

Biofilm inhibitory effects of polymyxin B combined with resveratrol on 3 Pseudomonas aeruginosa isolates. PB = polymyxin B, Res = resveratrol.

* There were significant differences for the combination group compared with every other group for each isolate (P <0.01).

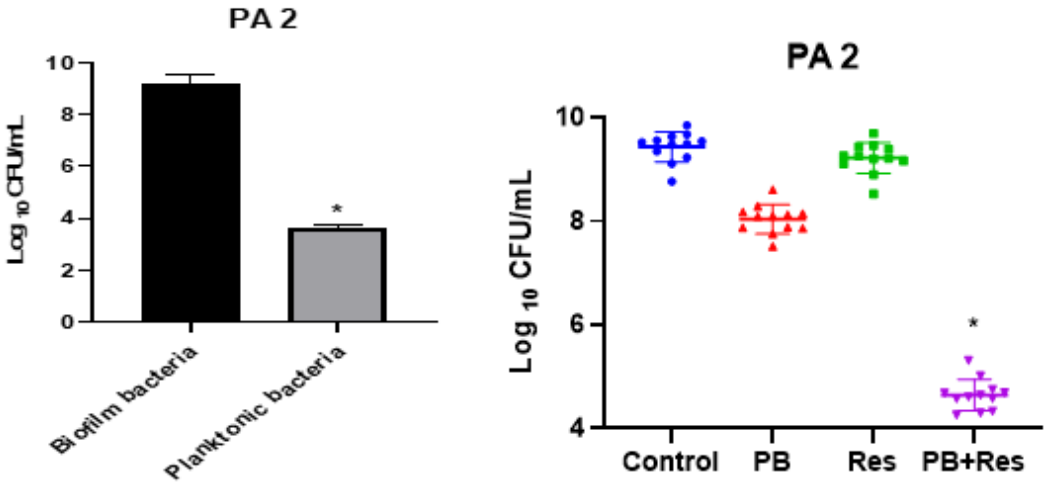

Figure 3

(A)Growth of PA2 cells with (Biofilm bacteria group) or without (Planktonic bacteria group) established biofilm after exposing to 8μg/mL polymyxin B for 24h.

(B)Combination effect of polymyxin B and resveratrol against the growth of PA2 cells with established biofilm. PB, 32 μg/mL polymyxin B; Res, 128μg/ml resveratrol; PB+Res, 32 μg/mL polymyxin B + 128μg/ml resveratrol.

* There were significant differences for this group compared with each other group (P <0.01).