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Intrinsic Acidity of N-Acetylcysteine Mediates Enhanced Inhibition of *Klebsiella pneumoniae* and Its Biofilms by Polymyxin B

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Abstract

This study investigated the combined antibacterial and anti-biofilm activity of polymyxin B (PB) with intrinsically acidic N-acetylcysteine (NAC) against *Klebsiella pneumoniae*. The minimum inhibitory concentrations (MICs) of PB, acidic NAC, and neutralized NAC against 34 *K. pneumoniae* strains were determined using the broth microdilution. Drug interactions were assessed by checkerboard assays and the fractional inhibitory concentration index (FICI), while biofilm inhibition was quantified using crystal violet staining. Polymyxin B resistance was identified in the reference multidrug-resistant strain *K. pneumoniae* ATCC BAA-1705. The PB–NAC combination showed an additive effect (FICI 0.53–0.63) against PB-resistant and PB-intermediately susceptible strains, whereas indifferent interactions were observed in PB-susceptible strains. Furthermore, sub-inhibitory concentrations of the combination produced significantly stronger biofilm inhibition than either agent alone. Neutralization of NAC markedly reduced its antibacterial and anti-biofilm activities, with substantial inhibition observed only at concentrations $\geq$ 32 mg/mL. These findings demonstrate that the combination of PB and acidic NAC exerts additive antibacterial effects, particularly against resistant *K. pneumoniae* strains, and enhances biofilm inhibition. Notably, the intrinsic acidity of NAC is essential for its antimicrobial and anti-biofilm activity.

Keywords: *Klebsiella pneumoniae*; drug combination; N-acetylcysteine; polymyxin B; biofilm

1. Introduction

Klebsiella pneumoniae is a major opportunistic pathogen within the Enterobacteriaceae family and is frequently responsible for both hospital- and community-acquired infections, particularly in immunocompromised individuals [1,2]. It colonizes mucosal sites such as the gastrointestinal tract and oropharynx and can subsequently cause severe invasive infections, including aspiration pneumonia, urinary tract infections, and bacteremia [3]. In recent years, the widespread use of antibiotics has driven an increasing prevalence of multidrug-resistant strains [4]. Beyond the human host, *K. pneumoniae* forms biofilms on environmental surfaces and medical devices [5]. These complex, dynamic microcolonies communities facilitate bacterial colonization and transmission, enhance tolerance to antibiotic and environmental adaptability, and contribute to chronic and recurrent infections [6,7]. Moreover, Biofilm-dispersed cells exhibit greater colonization capacity and immunogenicity than planktonic cells [8]. Together, antibiotic resistance and biofilm formation represent major contributors

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to the persistent clinical burden of *K. pneumoniae*, underscoring the urgent need for novel strategies that target both resistant strains and their biofilms.

Polymyxin B (PB) is a classic polypeptide antibiotic that exhibits rapid bactericidal activity against Gram-negative bacteria and is often regarded as a "last line of defense" for infections caused by multidrug-resistant Gram-negative pathogens [9]. Most clinical *K. pneumoniae* isolates remain susceptible to PB [10], and PB has also demonstrated inhibitory and eradication effects against bacterial biofilms [11]. However, increasing clinical use of PB-resistant strains, which poses a serious challenge to its continued clinical effectiveness.

N-acetylcysteine (NAC) is a pharmacological agent with mucolytic, antioxidant, and broad-spectrum antimicrobial activities and is widely used as an adjunctive therapy in clinical practice [12]. Previous studies have demonstrated that NAC, at concentrations exceeding the minimum inhibitory concentration (MIC), inhibits biofilm formation by *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus mutans*, although limited activity has been observed against *Pseudomonas aeruginosa* [13]. Furthermore, NAC has been proposed as a promising adjuvant for the treatment of multidrug-resistant (MDR) infections, as summarized in recent reviews [14]. NAC is a weak acid, and under conditions where the environmental pH < pKa, it can penetrate the extracellular matrix and effectively kill bacteria embedded within the biofilms of mucoid Pseudomonas aeruginosa strains [15]. However, it remains unclear whether the antimicrobial activity of NAC is intrinsically dependent on its acidic nature and what specific mechanistic role this property plays in its bactericidal and anti-biofilm effects.

Drug synergy assays are widely used to screen treatment regimens for drug-resistant bacteria and have shown considerable promise in the management of extensively drug-resistant (XDR) Gram-negative infections [16]. Although existing reviews have summarized the resistance mechanisms of *K. pneumoniae* biofilms and explored emerging strategies for biofilm-associated infections [17], the combined effects of PB and NAC against *K. pneumoniae* and its biofilms have not yet been reported. In the present study, we employed broth microdilution assays, checkerboard analyses, and crystal violet staining to systematically evaluate the antibacterial and anti-biofilm effect of PB and NAC, both individually and in combination, against *K. pneumoniae*. By calculating the fractional inhibitory concentration index (FICI) and biofilm inhibition rate, we characterized the interaction between the two agents. Furthermore, by adjusting the pH of NAC, we elucidated the contribution of its intrinsic acidity to its antibacterial and anti-biofilm activities. These findings provide an experimental and theoretical basis for optimizing PB-based combination therapies and improving the prevention and treatment of biofilm-associated infections.

2. Materials and Methods

2.1. Bacterial Strains and Identification

A total of 34 *Klebsiella pneumoniae* strains were included in this study, comprising seven strains and 27 clinical isolates. The seven reference strains (designated as strains 1–7) included ATCC 43816, ATCC BAA-1705, ATCC 4352, ATCC 700603, ATCC 10031, ATCC 13884, and ATCC 13883. These were purchased from Shanghai Beijiguan Biotechnology Co., Ltd. (Shanghai, China) and stored at −80 °C.

Clinical isolates (27 strains, designated as strains 8–34): These were provided by the Hainan Clinical Microbiology Testing and Research Center, confirmed using the MicroScan WalkAway automated bacterial identification system, and subsequently stored at −80 °C.

After resuscitation, all strains were inoculated onto sheep blood agar plates and incubated at 37 °C for 18–24 h. The colonies exhibited typical *K. pneumoniae* colonial morphology, appearing circular, convex, grey-white, moist, and mucoid, with adjacent colonies tending to coalesce. A distinct stringing phenomenon was observed when colonies were

touched with an inoculation loop. Gram staining revealed short, stout Gram-negative bacilli arranged singly, in pairs, or in short chains. Capsule staining confirmed the presence of a clear capsule structure. Biochemical identification results were consistent with *K. pneumoniae* characteristics: oxidase-negative, catalase-positive, and fermentative in oxidation–fermentation (OF) medium.

2.2. Determination of Drug Susceptibility

The minimum inhibitory concentrations (MICs) of polymyxin B (PB), N-acetylcysteine (NAC), and pH-neutralized NAC (adjusted to pH 6.8–7.2 using NaOH) against the 34 *K. pneumoniae* strains were determined using the broth microdilution method, in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines [18]. Preconfigured 96-well microtiter plates (provided by Ningbo Xunjian Biotechnology Co., Ltd., Ningbo, China) were used:

NAC plates: Wells 1–11 contained a two-fold serial dilution of NAC ranging from 64 to 0.0625 mg/mL, with well 12 serving as the growth control.

Polymyxin B plates: Wells 1–11 contained PB concentrations ranging from 64 to 0.0625 μg/mL, with well 12 used as the growth control.

Each well was inoculated with a bacterial suspension adjusted to approximately 1.5×10^5 CFU/mL. The plates were incubated at 37 °C for 18–20 h. The MIC was defined as the lowest drug concentration that completely inhibited visible bacterial growth, as determined by visual inspection.

2.3. Evaluation of Combined Drug Efficacy

The combined effect of PB and NAC was evaluated using the checkerboard microdilution method [19]. In 96-well plates, NAC concentrations ranging from 1/16× MIC to 4× MIC were dispensed into columns B–H, while PB concentrations ranging from 1/128× MIC to 8× MIC were added to rows 2–12. Well A1 served as the blank control. Each well was inoculated with a bacterial suspension adjusted to a 0.5 McFarland standard (110 μL/well) and incubated at 37 °C with 100% humidity conditions for 20 h. The Fractional Inhibitory Concentration Index (FICI) was calculated as follows:

FICI = (MIC of drug A in combination/MIC of drug A alone) + (MIC of drug B in combination/MIC of drug B alone)

Drug interactions were interpreted as follows: synergy for FICI $\leq$ 0.5, additive effect for 0.5 < FICI $\leq$ 1, indifference for 1 < FICI $\leq$ 4, and antagonism for FICI > 4.

2.4. Quantitative Analysis of Biofilm Formation

The inhibitory effects of the drugs on biofilm formation were evaluated using the crystal violet staining method [20]. After incubation, the culture medium from the susceptibility plates was discarded, and the wells were gently rinsed three times with phosphate-buffered saline (PBS) to eliminate planktonic bacteria. The plates were air-dried, and 110 μL of 99% methanol was added to each well for fixation. After a 15 min incubation, the methanol was removed. Each well was then stained with 110 μL of 0.1% crystal violet for 15 min at room temperature. Excess dye was rinsed with distilled water. The bound crystal violet was subsequently solubilized with 95% ethanol, and the optical density (OD) at 595 nm was measured using a microplate reader. Biofilm inhibition rates were calculated based on the OD values obtained.

2.5. Statistical Analysis

All experiments were performed independently in triplicate. Data were compiled using Microsoft Excel 2020, and statistical analyses were conducted using GraphPad Prism (version 9.5). Measurement data are presented as the mean ± standard devia-

tion (mean ± SD). Comparisons among groups were conducted using one-way analysis of variance (one-way ANOVA), with statistical significance defined as $\alpha = 0.05$.

3. Results

3.1. Analysis of Antimicrobial Activity

The minimum inhibitory concentrations (MICs) of polymyxin B (PB) and both intrinsically acidic and pH-neutralized (pH 6.8–7.2) N-acetylcysteine (NAC) against 34 *Klebsiella pneumoniae* strains (7 reference strains and 27 clinical isolates) were determined using the broth microdilution method. The results are summarized in Table 1.

Table 1. Minimum Inhibitory Concentrations (MICs) of Polymyxin B (PB), NAC, and Neutralized NAC against *K. pneumoniae* Strains.

Strain Designation	Original Designation	PB (μg/mL)	NAC (mg/mL)	Neutralized-NAC (mg/mL)
1	ATCC 43816	0.0625	4	>64
2	ATCCBAA-1705	4	4	>64
3	ATCC 4352	0.5	4	>64
4	ATCC 700603	0.5	4	>64
5	ATCC 10031	0.5	4	32
6	ATCC 13884	1	4	32
7	ATCC 13883	1	4	>64
8	OB30-3	0.0625	4	>64
9	OB35-55	0.0625	4	>64
10	OB30-45	0.25	4	>64
11	OB30-43	0.0625	4	32
12	OB30-19	0.0625	4	32
13	OB30-17	0.0625	4	>64
14	OB35-71	1	4	32
15	OB35-61	0.0625	4	>64
16	OB35-69	0.125	4	>64
17	OB35-68	0.125	4	>64
18	OB35-77	0.125	4	>64
19	OB30-79	0.125	4	>64
20	OB30-5	2	4	>64
21	OB30-7	0.0625	4	32
22	OB30-24	0.125	4	>64
23	OB30-22	0.125	4	>64
24	OB30-63	0.25	4	>64
25	OB35-70	0.25	4	>64
26	OB30-81	2	4	32
27	OB30-61	0.25	4	>64
28	OB30-37	0.125	4	>64
29	OB30-27	0.125	4	>64
30	OB35-58	0.25	4	>64
31	OB30-51	0.25	4	>64
32	OB30-39	0.125	4	>64
33	OB30-41	0.125	4	>64
34	OB30-75	0.25	4	>64

Note: PB, polymyxin B; NAC, N-acetylcysteine; Neutralized-NAC, Neutralized-N-acetylcysteine.

Polymyxin B: Susceptibility of *K. pneumoniae* to PB was interpreted according to EUCAST colistin breakpoints (susceptible, ≤2 mg/L; resistant, >2 mg/L) [21]. Among the reference strains, the MIC of ATCC BAA-1705 was 4 μg/mL, and was classified as resistant; the remaining six reference strains were susceptible. Among the 27 clinical isolates, all MIC values were ≤2 μg/mL, and all were classified as susceptible.

Antimicrobial activity of NAC: The MIC of intrinsically acidic NAC was consistently 4 mg/mL for all tested strains. In contrast, the antimicrobial activity of pH-neutralized NAC was markedly attenuated. Only 7 strains exhibited an MIC of 32 mg/mL, whereas for the remaining 27 strains, the MIC exceeded 64 mg/mL.

3.2. Synergistic Interactions Between Polymyxin B and N-Acetylcysteine

To evaluate potential synergistic interactions, the combination of polymyxin B (PB) and N-acetylcysteine (NAC) was tested against a selected panel of seven *K. pneumoniae* strains (Table 2). This panel included one PB-resistant strain (MIC = 4 μg/mL) and six susceptible strains with MICs ranging from 0.5 to 2 μg/mL, thereby allowing an assessment of the relationship between baseline PB susceptibility and combination efficacy.

Table 2. Synergy Testing Results for *K. pneumoniae* Strains with Different Polymyxin B Susceptibility.

Strain	MIC of PB Alone (μg/mL)	MIC of NAC Alone (mg/mL)	MIC of PB in Combination (μg/mL)	MIC of NAC in Combination (mg/mL)	FICI (NAC + PB)
2	4	4	0.125	2	0.53
20	2	4	0.25	2	0.63
26	2	2	0.0625	1	0.53
6	1	4	2	0.25	2.06
14	1	4	1	1	1.25
4	0.5	4	1	0.5	2.12
5	0.5	4	0.5	1	1.25

Note: PB, polymyxin B; NAC, N-acetylcysteine; MIC, minimum inhibitory concentration; FICI, fractional inhibitory concentration index. FICI = (MIC of PB in combination/MIC of PB alone) + (MIC of NAC in combination/MIC of NAC alone). Drug interactions were interpreted as follows: synergy, FICI $\leq$ 0.5; additive effect, 0.5 < FICI $\leq$ 1; indifference, 1 < FICI $\leq$ 4; antagonism, FICI > 4.

Analysis using the fractional inhibitory concentration index (FICI) revealed that the interaction outcome was closely associated with the PB MIC. An additive effect was specifically observed in strains with reduced PB susceptibility. This group included the PB-resistant strain 2 (FICI = 0.53) and the susceptible strains 20 and 26, which exhibited MICs at the susceptibility breakpoint (2 μg/mL), with FICI = 0.63 and 0.53, respectively.

In contrast, indifferent effects were consistently found among strains with lower PB MICs (0.5 to 1 μg/mL). Strains 6 and 14 (PB MIC = 1 μg/mL) yielded FICI values of 2.06 and 1.25, respectively, whereas strains 4 and 5 (PB MIC = 0.5 μg/mL) yielded FICI values of 2.12 and 1.25, all indicating an indifferent interaction.

Collectively, these results, summarized in Table 2, demonstrate a clear pattern: the additive effect of the PB-NAC combination is not universal but is selectively associated with strains exhibiting diminished sensitivity to PB alone.

3.3. Inhibitory Effects of Polymyxin B, Intrinsically Acidic NAC, and Neutralized NAC on Biofilm Formation in K. pneumoniae

The seven strains described above were selected for biofilm analysis, including one polymyxin B (PB)-resistant strain and six PB-susceptible strains with different susceptibility levels. The inhibitory effects of PB, intrinsically acidic N-acetylcysteine (NAC), and pH-neutralized NAC on biofilm formation were quantitatively evaluated using the crystal violet staining method. The results are presented in Figures 1 and 2.

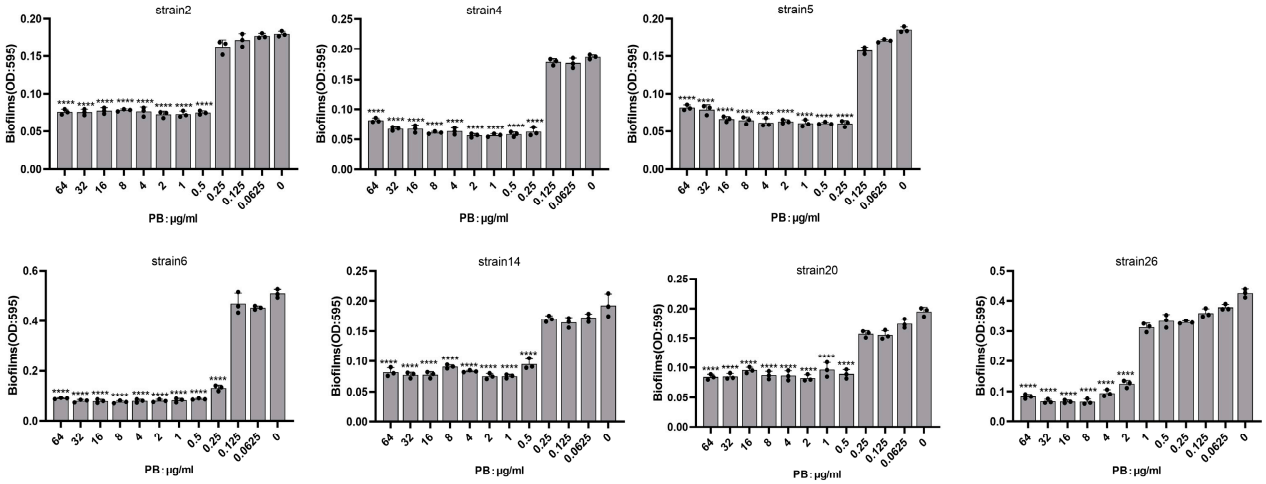


Figure 1. Polymyxin B (PB) inhibits biofilm formation of *Klebsiella pneumoniae* in a dose-dependent manner. Data are presented as mean ± SD ($n \geq 3$). **** $p < 0.0001$ versus the untreated control.

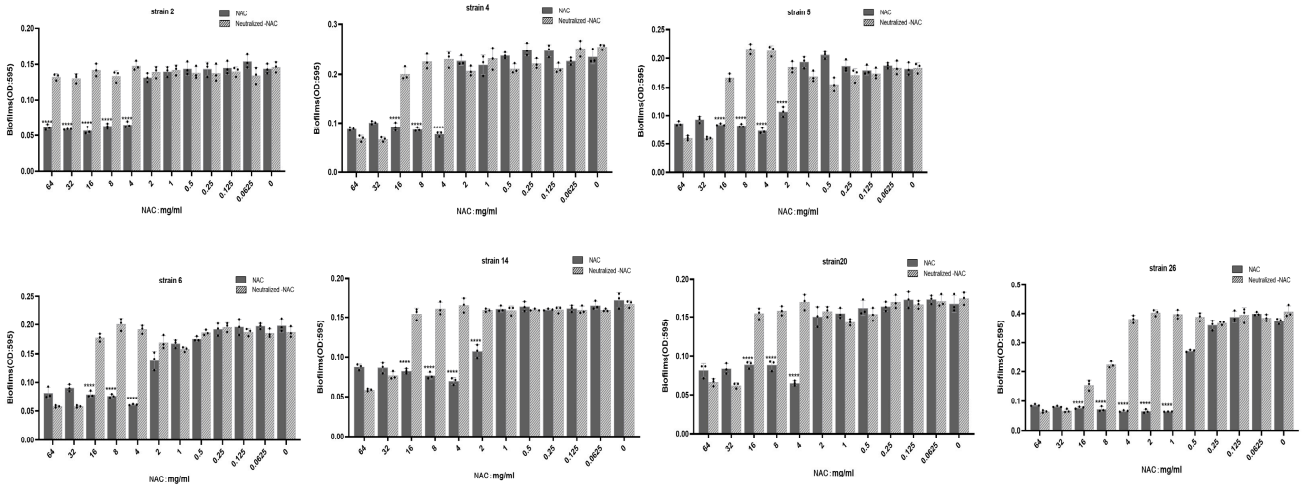


Figure 2. Inhibitory effects of intrinsically acidic and neutralized N-acetylcysteine (NAC) on *Klebsiella pneumoniae* biofilm formation. Data are presented as mean ± SD ($n \geq 3$). **** $p < 0.0001$ versus the untreated control.

3.3.1. Inhibitory Effect of Polymyxin B on Biofilms

Polymyxin B (PB) inhibited *K. pneumoniae* biofilm formation in a concentration-dependent manner (Figure 1). For most strains, biofilm biomass, as indicated by optical density values, decreased significantly at PB concentrations below the minimum inhibitory concentration (MIC). The minimum biofilm inhibitory concentration (MBIC) corresponded to 1/2 or 1/4 of the MIC for susceptible strains 4, 5, 6, 14, and 20. Notably, for the PB-resistant strain 2 (ATCC BAA-1705), biofilm inhibition occurred at 0.5 μg/mL (MBIC = 1/8 MIC), demonstrating that its resistance mechanism is independent of biofilm formation and that PB retains potent, anti-biofilm activity at sub-inhibitory concentrations. Only strain 26 exhibited an MBIC equivalent to its MIC. These findings confirm that PB effectively disrupts *K. pneumoniae* biofilms at sub-inhibitory concentrations, highlighting its potential to interfere with biofilm-associated infections even when bactericidal concentrations are not achieved.

3.3.2. Inhibitory Effect of Intrinsically Acidic and Neutralized N-Acetylcysteine on Biofilm

Intrinsically acidic N-acetylcysteine (NAC) inhibited *K. pneumoniae* biofilm formation in a strain-specific manner (Figure 2). For most strains, significant biofilm reduction

occurred at concentrations equivalent to the MIC. However, for strains 5 and 26, significant inhibition was observed at sub-MIC levels (1/2 and 1/4 MIC, respectively), indicating that an anti-biofilm activity of NAC can be partially decoupled from its direct bactericidal effects.

In contrast, neutralizing NAC's intrinsic acidity drastically reduced its potency. Against all strains except the PB-resistant strain 2, significant biofilm inhibition by neutralized NAC was observed only at a high concentration of 32 mg/mL. These results demonstrate that the intrinsic acidity of NAC is essential for effective biofilm inhibition at relevant concentrations.

3.4. Inhibitory Effects of N-Acetylcysteine Combined with Polymyxin B on Biofilm Formation

Based on the aforementioned results and synergy testing data, three strains (ATCC BAA-1705, strain 20, and strain 26) that exhibited additive interactions in the checkerboard assay were selected for further analysis. The biofilm inhibition rates were calculated for the selected concentration combinations and their corresponding single-drug treatments using the following formula: biofilm inhibition rate (%) = [1 − (OD of drug-treated well/OD of blank control well)] × 100%. The results were visualized using statistical software (Figure 3), as described below.

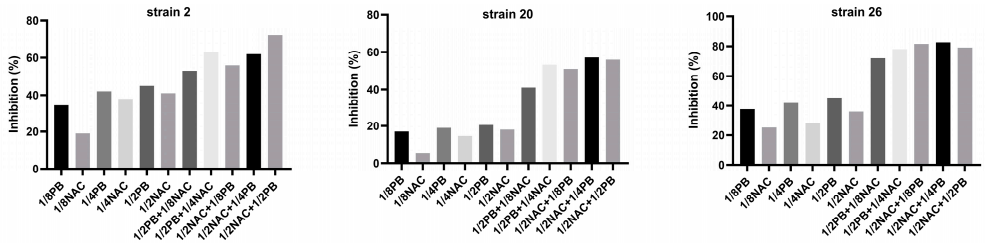


Figure 3. Comparison of the inhibitory effects of polymyxin B (PB) and N-acetylcysteine (NAC) at different sub-inhibitory concentrations (either as monotherapies or in combination) on biofilm formation by *Klebsiella pneumoniae* strains 2, 20, and 26.

For the reference strain ATCC BAA-1705 (strain 2), PB alone achieved a biofilm inhibition rate of approximately 40–50% at sub-inhibitory concentrations, whereas acidic NAC alone produced an inhibition rate of approximately 20%. Combination treatment markedly enhanced biofilm inhibition. Across the tested concentration combinations (1/2× PB + 1/8× NAC, 1/2× PB + 1/4× NAC, 1/2× NAC + 1/8× PB, 1/2× NAC + 1/4× PB, and 1/2× NAC + 1/2× PB), the inhibition rates were 53%, 63.3%, 56%, 62%, and 72%, respectively, all of which were significantly higher than those of the corresponding monotherapy groups.

For clinical strain 20, at sub-inhibitory concentrations (1/2× MIC, 1/4× MIC, 1/8× MIC), the inhibition rate of PB alone was approximately 20%. The inhibition rates of acidic NAC at the corresponding concentrations were 30%, 11%, and 5%, respectively, indicating that PB monotherapy was more effective than NAC monotherapy at these concentrations. Following combination treatment, the inhibition rates for the corresponding concentration pairs increased to 43.6–55%, which were significantly higher than those observed with sub-MIC PB or NAC alone.

For clinical strain 26, the inhibition rates of PB at 1/8×, 1/4×, and 1/2× MIC were 46%, 52%, and 55%, respectively, while the inhibition rates of acidic NAC at the corresponding concentrations were 25%, 27.6%, and 35.8%, respectively. After combination treatment, the inhibition rates for the tested concentration pairs (1/2× PB + 1/8× NAC, 1/2× PB + 1/4× NAC, 1/2× NAC + 1/8× PB, and 1/2× NAC + 1/4× PB) reached 70–85.5%, representing an improvement of approximately 20–30% compared with the respective monotherapy groups.

Collectively, these findings demonstrate that the combination of polymyxin B and intrinsically acidic N-acetylcysteine produces a substantially enhanced inhibitory effect on *K. pneumoniae* biofilm formation, including PB-resistant strains, and is consistently more effective than either agent alone at sub-inhibitory concentrations.

4. Discussion

Klebsiella pneumoniae is a common clinical opportunistic pathogen that causes a wide range of infectious diseases, and its antimicrobial resistance has increased steadily in recent years with the widespread use of antibiotics [22]. Polymyxin B (PB) is frequently employed as a critical therapeutic agent for infections caused by carbapenem-resistant *K. pneumoniae* (CRKP), and the emergence of PB resistance has attracted growing concern [23]. Previous combination therapy studies have shown that PB combined with tigecycline, fosfomycin, rifampicin, minocycline, doxycycline, and other antibiotics can exert synergistic antibacterial effects against multidrug-resistant (MDR) *K. pneumoniae* [24–27]. The combination of omadacycline and PB has also been reported to be synergistic against CRKP [28], and PB in combination with Reduning (a traditional Chinese medicine) enhances inhibition of CRKP [29]. N-acetylcysteine (NAC), a classic mucolytic agent with antioxidant and anti-inflammatory properties, has been confirmed to exhibit bactericidal, antibiofilm, and anti-virulence activities against *K. pneumoniae* [30,31]. NAC holds promise as an antibiotic adjuvant: it displays concentration-dependent bactericidal activity when used alone, and its combination with β-lactams can produce synergistic antibacterial effects against CRKP [32].

In the present study, we determined the minimum inhibitory concentrations (MICs) of PB and NAC against 34 *K. pneumoniae* strains (7 reference strains and 27 clinical isolates) using the broth microdilution method to evaluate in vitro antibacterial activity. All strains were susceptible to PB (MIC $\leq$ 2 μg/mL), except the reference strain ATCC BAA-1705, which was PB-resistant (MIC = 4 μg/mL). To our knowledge, this is the first study to identify and confirm *K. pneumoniae* ATCC BAA-1705 as a polymyxin B-resistant reference strain. This finding provides a valuable and well-characterized tool for future research aimed at elucidating PB resistance mechanisms and evaluating novel therapeutic strategies against resistant strains. The MIC of acidic NAC was 4 mg/mL for all strains. Checkerboard assays and fractional inhibitory concentration index (FICI) analysis showed that additive effects of PB and NAC occurred in PB-resistant strains (MIC = 4 μg/mL) or strains at the susceptibility breakpoint (MIC = 2 μg/mL), whereas indifferent effects were observed in PB-susceptible strains (MIC < 2 μg/mL). To our knowledge, this is the first report demonstrating that the combined effect of PB and NAC is dependent on the baseline MIC of PB.

Given the strong link between antibiotic resistance and biofilm formation and the limited availability of effective treatment options, increasing attention has been directed toward novel strategies such as antibiotic combinations, antimicrobial peptides, nanoparticles, natural products, and phage therapy. These advances have been summarized in recent reviews [33]. Current evidence indicates that PB has potent inhibitory and eradication effects on *K. pneumoniae* biofilms, which can be further enhanced by the addition of polysorbate 80 [34]. NAC has been shown to inhibit biofilm formation in *K. pneumoniae* KP1, *Staphylococcus aureus* 15981, and *Pseudomonas aeruginosa* DK1-NH57388A, and to target resistant bacteria and persister cells [35]. NAC also inhibits growth and interferes with biofilm formation in *Staphylococcus epidermidis* and *S. aureus*, and in combination with tigecycline, exerts a synergistic bacteriostatic effect against drug-resistant strains [36]. Furthermore, NAC enhances the bactericidal activity of fosfomycin and effectively reduces *Escherichia coli* biofilm formation [37].

Crystal violet quantification demonstrated that both polymyxin B (PB) and N-acetylcysteine (NAC) monotherapy effectively inhibited *K. pneumoniae* biofilm formation. PB exhibited potent activity at sub-inhibitory concentrations, suggesting a mechanism distinct from its bactericidal action, potentially involving disruption of initial bacterial adhesion or intercellular aggregation. This is clinically significant, as it implies PB may impede the transition from acute to chronic biofilm-mediated infection even when tissue concentrations fall below the planktonic MIC. A pivotal finding was the potent anti-biofilm activity of PB against the resistant strain *K. pneumoniae* ATCC BAA-1705. The biofilm inhibitory concentration (0.5 μg/mL) was markedly lower than its planktonic MIC (4 μg/mL), clearly dissociating the phenotypic resistance mechanism from the biofilm formation process. This strain thus serves as a critical tool for delineating pathways of PB resistance that are independent of biofilm-mediated tolerance.

NAC also significantly reduced biofilm biomass at the sub-MIC levels in specific strains. This activity is likely mediated through mechanisms such as interference with quorum sensing or degradation of the extracellular polymeric matrix, rather than direct killing. This property positions NAC as a promising biofilm-disrupting adjuvant capable of enhancing antibiotic penetration and efficacy. Crucially, the combination of PB and NAC at sub-inhibitory concentrations yielded biofilm inhibition rates superior to any single agent. This synergy suggests complementary mechanisms of action—PB potentially targeting the bacterial cell surface and early adhesion, while NAC may destabilize the mature biofilm architecture. Together, these data advocate for the PB-NAC combination as a viable strategy to enhance biofilm eradication, meriting further investigation in complex in vivo infection models.

Previous work has reported that NAC, as a weak acid, exhibits pH-dependent antibiofilm and anti-persister activity, being effective only when the environmental pH is below its pKa. The proposed mechanism is that undissociated NAC penetrates the biofilm matrix and bacterial cells under acidic conditions, leading to cytoplasmic acidification and subsequent cellular damage [15]. In the present study, we neutralized NAC with NaOH to abolish its intrinsic acidity and systematically examined the impact on its activity. The MIC of neutralized NAC against *K. pneumoniae* increased markedly to 64 mg/mL, indicating a substantial reduction in antibacterial potency compared with intrinsically acidic NAC. Biofilm quantification further showed that a noticeable inhibitory effect on biofilms required concentrations $\geq$ 32 mg/mL.

Our findings are consistent with the fundamental mechanism reported previously, but they provide a complementary perspective. Whereas earlier studies focused on the external factor of whether the environmental pH is below the pKa, our direct comparison of intrinsically acidic versus neutralized NAC demonstrates that its inherent acidity is central to both its antibacterial and antibiofilm efficacy. Thus, NAC activity is not solely determined by the ambient pH but is critically dependent on its intrinsic acidic nature. This conclusion is mechanistically linked to the "molecular penetration–intracellular acidification" model under acidic conditions described in reference [15], while specifically identifying NAC's inherent acidity as the key intrinsic determinant of its functional activity.

This study has several limitations. First, the number of clinical isolates included was relatively limited, and larger strain collections are needed to further validate the generalizability of our conclusions. Second, in vivo experiments were not performed; therefore, animal models will be required to evaluate the efficacy and safety of PB–NAC combination therapy under physiological conditions. Future studies will incorporate more clinical isolates and in vivo models to further investigate the mechanisms of PB and NAC in combination and to provide stronger evidence for the clinical translation of this regimen.

5. Conclusions

This study demonstrates that the combination of polymyxin B (PB) and intrinsically acidic N-acetylcysteine (NAC) exerts additive antibacterial and anti-biofilm effects against *Klebsiella pneumoniae*, particularly against PB-non-susceptible strains. A pivotal finding was the potent anti-biofilm activity of PB against the resistant strain *K. pneumoniae* ATCC BAA-1705 at concentrations far below its planktonic MIC, effectively decoupling its resistance phenotype from biofilm formation. Furthermore, the intrinsic acidity of NAC was identified as an essential component for its activity.

Collectively, these results not only identify *K. pneumoniae* ATCC BAA-1705 as a valuable extensively drug-resistant (XDR) reference strain but also provide a novel combinatorial strategy for combating biofilm-associated infections caused by multidrug-resistant (MDR) and extensively drug-resistant (XDR) *K. pneumoniae*, laying a foundation for future research.

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