

Antimicrobial Activity of a Triple Antibiotic Combination Toward Ocular *Pseudomonas aeruginosa* Clinical Isolates

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Purpose: *Pseudomonas aeruginosa* is a leading cause of corneal infections. Recently, we discovered an antimicrobial drug combination, polymyxin B/trimethoprim (PT) + rifampin, that displayed impressive efficacy toward *P. aeruginosa* in both in vitro and in vivo studies. As such, this combination was further evaluated as a potential keratitis therapeutic through testing the combination's efficacy against a diverse set of *P. aeruginosa* clinical isolates.

Methods: Minimum inhibitory concentrations (MICs) of moxifloxacin, levofloxacin, erythromycin, tobramycin, PT, polymyxin B (alone), trimethoprim (alone), and rifampin were determined for 154 ocular clinical *P. aeruginosa* isolates, 90% of which were derived from corneal scrapings. Additionally, the efficacy of PT + rifampin was evaluated utilizing fractional inhibitory concentration (FIC) testing.

Results: While 100% of isolates were resistant to erythromycin (average MIC $224 \pm 110 \mu\text{g}\cdot\text{mL}^{-1}$) and trimethoprim (alone) ($206 \pm 67.3 \mu\text{g}\cdot\text{mL}^{-1}$), antibiotic resistance was generally found to be low: moxifloxacin (2% of isolates resistant; average MIC $1.08 \pm 1.61 \mu\text{g}\cdot\text{mL}^{-1}$), levofloxacin (3.9%; $1.02 \pm 2.96 \mu\text{g}\cdot\text{mL}^{-1}$), tobramycin (1%; $0.319 \pm 1.31 \mu\text{g}\cdot\text{mL}^{-1}$), polymyxin B (0%; $0.539 \pm 0.206 \mu\text{g}\cdot\text{mL}^{-1}$), PT (0%; $0.416 \pm 0.135 \mu\text{g}\cdot\text{mL}^{-1}$), and rifampin (0%; $23.4 \pm 6.86 \mu\text{g}\cdot\text{mL}^{-1}$). Additionally, FIC testing revealed that PT + rifampin eradicated 100% of isolates demonstrating additive or synergistic activity in 95% of isolates (average FIC index 0.701 ± 0.132).

Conclusions: The drug combination of PT + rifampin was effective against a large panel of clinically relevant *P. aeruginosa* strains and, as such, may represent a promising therapeutic for *P. aeruginosa* keratitis.

Translational Relevance: This work furthers the preclinical development of a novel antibiotic combination for the treatment of corneal infections (bacterial keratitis).

Introduction

Bacterial keratitis (corneal infection) is a serious disease requiring urgent topical antimicrobial treatment to mitigate ocular tissue damage and preserve vision. While a wide variety of micro-organisms have been implicated in bacterial keratitis, *Pseudomonas aeruginosa* stands out as the leading Gram-negative pathogen implicated in this disease, particularly in contact lens wearers.¹⁻³ In fact, some studies have indicated that among all organisms responsible for

contact lens-associated keratitis, the prevalence of *P. aeruginosa* can be as high as 70%.⁴⁻⁶ With a current estimated 38.5 million contact lens wearers in the United States⁷ and the rising popularity of multifocal, toric, and novelty contact lenses, *P. aeruginosa* keratitis has become a major health care and ophthalmic concern.

Currently, topical ophthalmic fluoroquinolones are widely used in the treatment of *P. aeruginosa* bacterial keratitis given their broad-spectrum activity, excellent tissue penetration, and patient tolerability. Fortunately, in the United States, circulating

antibiotic resistance among ocular *P. aeruginosa* isolates toward fluoroquinolones as well as other ophthalmic antibiotics has remained low.^{8,9} However, reports are now emerging describing significant *P. aeruginosa* resistance globally. For example, *P. aeruginosa* resistance to moxifloxacin, a commonly utilized fourth-generation fluoroquinolone as well as the aminoglycoside gentamicin, has been reported upward of 50% in large patient series based in India.^{10,11} Unfortunately, the clinical consequences of resistant infections are significant and include increased disease severity and worse visual outcomes.^{12,13} However, despite this worrisome trend of emerging antibiotic resistance, there is a paucity of commercial alternatives to fluoroquinolones in the treatment of keratitis.

We recently described the synergistic antimicrobial activity of a novel drug combination, polymyxin B/trimethoprim (PT) + rifampin toward *P. aeruginosa* and *Staphylococcus aureus*, another leading cause of keratitis, in both in vitro and in vivo studies.^{14,15} While PT alone is commonly used for the treatment of mild bacterial conjunctivitis, its use in more serious corneal infections is limited due to weak antimicrobial potency and inadequate tissue penetration compared to fluoroquinolones.^{16–18} However, we have demonstrated that the combination of PT + rifampin overcomes these liabilities and displays antimicrobial efficacy in a murine model of bacterial keratitis that equals or exceeds that of fourth-generation fluoroquinolones.¹⁵ Importantly, PT + rifampin exhibits in vivo efficacy toward fluoroquinolone-resistant *S. aureus* and *P. aeruginosa* strains, suggesting that it may be an effective treatment option for infections that otherwise might fail currently available options.^{14,15}

To further investigate the incidence of antibiotic resistance and expand understanding of the potential therapeutic value of PT + rifampin for bacterial keratitis, we performed antimicrobial activity assays on a contemporary collection of 154 *P. aeruginosa* clinical ocular isolates. The entire strain set was first evaluated for antibiotic resistance to a panel of antibiotics that are commonly used for ocular treatment, including erythromycin, PT, levofloxacin, moxifloxacin, and tobramycin. Second, we evaluated the antimicrobial efficacy of PT + rifampin as well as rifampin (alone), trimethoprim (alone), and polymyxin B (alone) toward the entire isolate collection. Third, we evaluated whether the antimicrobial performance of PT + rifampin is a consequence of the combination's synergistic effects. Collectively, our results demonstrate that with the exception of erythromycin and trimethoprim, resistance levels remain low among US ocular

P. aeruginosa isolates. Additionally, we establish that the combination of PT + rifampin displays potent antimicrobial activity with 100% of the isolate collection susceptible to the combination, including strains resistant to fluoroquinolones. Further, we show that the antimicrobial effectiveness of PT + rifampin is associated with the combination's synergistic effects. Thus, PT + rifampin may represent a potential novel therapeutic to treat this blinding disease.

Materials and Methods

Bacterial Strains and Growth Conditions

A total of 154 *P. aeruginosa* clinical ocular isolates were commercially obtained from International Health Management Associates (IHMA) (Schaumburg, IL, USA). Individual isolates were grown overnight and subcultured in fresh Mueller–Hinton (MH) media shaking with aeration at 37°C to early exponential phase. A range of OD₆₀₀ = 0.2 to 0.4 was found to correspond to approximately 10⁸ colony-forming units (CFU)/mL, and subcultures were then diluted 1:100 in fresh media for subsequent minimum inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) testing. The pan-sensitive laboratory strain PAO1 was used as a control for this study.

MIC

Each clinical isolate was tested for susceptibility to a panel of eight clinically relevant antibiotics, including erythromycin, PT, rifampin, moxifloxacin, levofloxacin, trimethoprim, tobramycin, and polymyxin B, using the standard MIC testing guidelines.¹⁶ Individual wells of a 96-well microtiter plate were prepared by adding 88 μL of fresh MH broth, 2 μL of increasing concentrations of each antibiotic, and 10 μL of the appropriate bacterial culture to achieve a final concentration 10⁴ CFU/well and incubated for 16 hours at 37°C. The MIC value for each antibiotic was determined as the lowest concentration of antibiotic that inhibited bacterial growth as visualized by the naked eye. Resistance was characterized as having an MIC value of ≥4 μg/mL for levofloxacin, ≥16 μg/mL for tobramycin, and ≥4 μg/mL for polymyxin B per the 2021 Clinical and Laboratory Standards Institute (CLSI).¹⁹ Resistance for the remaining antibiotics (PT, rifampin, and moxifloxacin) was characterized as having MIC values ≥4× the MIC value for a susceptible laboratory strain, PAO1. Erythromycin and trimethoprim were

used as controls due to the known insusceptibility of *P. aeruginosa* to these antibiotics.²⁰

FIC Testing of PT + Rifampin

A standard checkerboard assay was done to determine the efficacy of the drug combination (PT plus rifampin) on the clinical strain set according to CLSI guidelines.¹⁹ In total, 10 μ L of the indicated 10^6 CFU/mL bacterial culture was added to 88 μ L MH media and 2 μ L of antibiotics in individual wells of a 96-well microtiter plate with each column containing twofold increasing concentrations of PT (0.0–2.5 μ g/mL) and each row containing twofold increasing concentrations of rifampin (0.0–60 μ g/mL). PT + rifampin drug combinations were prepared separately in 200 μ L to minimize pipetting errors. The plates were then incubated for 16 hours at 37°C and visually inspected for growth. The fractional inhibitory concentration index (FICI) was calculated using the following formula: $FICI = (MIC \text{ of PT in combination} / MIC \text{ of PT alone}) + (MIC \text{ of rifampin in combination} / MIC \text{ of rifampin alone})$. The averaged FICI from three biological replicates was defined as either synergistic (FICI <0.5), additive (0.5–1), indifference (1–4), or antagonistic (FICI >4).²¹

Results

Strain Set Characteristics

In total, 154 ocular *P. aeruginosa* isolates were obtained from IHMA between 2016 and 2020. Table 1 provides the characteristics of the strain set. Forty-six percent ($n = 71$) of isolates were collected from male patients and 54% ($n = 83$) from female patients. Ages of patients at the time of isolate collection ranged from 1 to 104, with the majority of patients aged 40 to 59 (31%) and 60 to 79 years (27%). Ninety percent of the isolates were isolated from corneal scrapings, and 10% were broadly categorized as from eyes that could include corneal, conjunctival, intracamer, and/or intravitreal samples. The geographic representation included 139 (90%) of isolates from North America, 13 (8%) from Europe, 1 (1%) from Latin America, and 1 (1%) from Asia. Among isolates collected from the United States, 15 states were represented: Alabama ($n = 5$), California ($n = 46$), Colorado ($n = 3$), Florida ($n = 32$), Illinois ($n = 5$), Indiana ($n = 8$), Iowa ($n = 1$), Kentucky ($n = 4$), Michigan ($n = 3$), New Mexico ($n = 4$), New York ($n = 18$), North Carolina ($n = 1$), Texas ($n = 3$), Utah ($n = 5$), and Wisconsin ($n = 1$).

Table 1. Characteristics of 154 Ocular *Pseudomonas aeruginosa* Clinical Set

Characteristic	n (%)
Sex	
Male	71 (46)
Female	83 (54)
Age, y	
0–19	10 (6)
20–39	29 (19)
40–59	47 (31)
60–79	41 (27)
80–104	27 (18)
Source	
Cornea	138 (90)
Eye	16 (10)
Geography	
North America	139 (90)
Europe	13 (8)
Latin America	1 (1)
Asia	1 (1)
Year collected	
2016	22 (14)
2017	18 (12)
2019	45 (29)
2020	69 (45)

Antibiotic Resistance Profiles of Isolates toward Commercially Available Ophthalmic Antibiotics

MIC testing was performed on the entire strain set in triplicate to measure the effectiveness of five commonly used ophthalmic antibiotics: moxifloxacin, levofloxacin, erythromycin, tobramycin, and PT. Additionally, to further support the preclinical development of the novel drug combination PT + rifampin as a potential keratitis therapeutic, MIC testing was completed for the individual components: trimethoprim (alone), polymyxin B (alone), and rifampin. Resistance was characterized as having an MIC value above the 2021 CLSI¹⁹ break point when available or an MIC value $\geq 4 \times$ the MIC value for a susceptible laboratory strain, PAO1.

MIC testing revealed that overall resistance was very low among this set of clinical isolates, with only six strains (3.9%) resistant to one antibiotic and one strain that demonstrated multidrug resistance as defined by resistance to three or more classes of antibiotics²² (Table 2, Supplementary Table S1). More specifically, as expected, 100% of isolates were resistant to erythromycin (average MIC $224 \pm 110 \mu\text{g}\cdot\text{mL}^{-1}$) and trimethoprim ($206 \pm 67.3 \mu\text{g}\cdot\text{mL}^{-1}$) due to known

Table 2. Antibiotic Resistance among the 154-Member Clinical Strain Set

Antibiotic	Overall Resistance, <i>n</i> (%)	MIC, Mean \pm SD, $\mu\text{g}\cdot\text{mL}^{-1}$
Moxifloxacin	3 (1.95)	1.08 \pm 1.61
Levofloxacin	6 (3.90)	1.02 \pm 2.96
Tobramycin	1 (0.65)	0.319 \pm 1.31
Erythromycin	154 (100)	224 \pm 110
Polymyxin B/trimethoprim	0 (0)	0.416 \pm 0.135
Polymyxin B	0 (0)	0.539 \pm 0.206
Trimethoprim	154 (100)	206 \pm 67.3
Rifampin	0 (0)	23.4 \pm 6.86
Resistant to ≥ 1 antibiotic	6 (3.90)	—
Resistance to ≥ 3 antibiotics	1 (0.65)	—

P. aeruginosa insusceptibility to these antibiotics. In contrast, resistance toward fluoroquinolones was low, with only three strains resistant to moxifloxacin ($1.08 \pm 1.61 \mu\text{g}\cdot\text{mL}^{-1}$) and six strains resistant to levofloxacin ($1.02 \pm 2.96 \mu\text{g}\cdot\text{mL}^{-1}$). Only one strain was found to be resistant to tobramycin ($0.319 \pm 1.31 \mu\text{g}\cdot\text{mL}^{-1}$), and 100% of isolates were sensitive to PT ($0.416 \pm 0.135 \mu\text{g}\cdot\text{mL}^{-1}$), polymyxin B (alone) ($0.539 \pm 0.206 \mu\text{g}\cdot\text{mL}^{-1}$), and rifampin ($23.4 \pm 6.86 \mu\text{g}\cdot\text{mL}^{-1}$).

Potent Antimicrobial Activity of PT + Rifampin

Previous studies have demonstrated that the combination of PT + rifampin is a synergistic, broad-spectrum antimicrobial. Moreover, PT + rifampin has also been shown to effectively eradicate both *P. aeruginosa* and *S. aureus* murine keratitis infections caused by antibiotic-resistant strains.^{14,15} Thus, in order to further investigate the therapeutic promise of PT + rifampin, we tested the antimicrobial susceptibility of the entire strain set to the combination.

The triple antibiotic combination, PT + rifampin, demonstrated synergistic or additive antimicrobial activity in 146 isolates (95%) with FICI values ranging from 0.446 to 1.005 (average $0.701 \pm .132$) (Table 3, Supplementary Table S2). Specifically, PT + rifampin displayed synergistic activity toward three isolates

(average FICI 0.457 ± 0.012) and additive toward 143 isolates (FICI 0.690 ± 0.111), and the combination had a neutral effect toward the remaining eight isolates (FICI = 1 ± 0.002). In line with these findings, we found a corresponding reduction in the MICs for both PT and rifampin when the drugs were tested against the clinical strain set in combination versus alone. For example, on average, there was a twofold reduction in the MIC for PT when measured alone versus in combination with rifampin ($0.416 \pm 0.135 \mu\text{g}/\text{mL}$ vs. $0.180 \pm 0.045 \mu\text{g}/\text{mL}$) and a fourfold reduction in the MIC of rifampin when in combination with PT versus alone ($23.4 \pm 6.86 \mu\text{g}/\text{mL}$ vs. $5.41 \pm 3.11 \mu\text{g}/\text{mL}$) (Table 3). Importantly, PT + rifampin displayed this potent activity, even toward drug resistant isolates. For example, IHMA1564153, a multidrug-resistant strain with resistance to moxifloxacin, levofloxacin, and tobramycin, was found to be highly susceptible to PT + rifampin with a synergistic FICI value of 0.458 ± 0.072 . Moreover, the efficacy of PT + rifampin was equivalent when comparing fluoroquinolone-resistant versus fluoroquinolone-sensitive isolates. The average FICI value of all six levofloxacin-resistant isolates (three of which were also resistant to moxifloxacin) was found to be 0.681 ± 0.18 , compared to 0.702 ± 0.132 for all fluoroquinolone-sensitive strains.

Taken together, these data demonstrate the potent antimicrobial activity of the combination of PT + rifampin and its ability to effectively eradicate 100%

Table 3. FIC Testing of PT + Rifampin against 154-Member *P. aeruginosa* Ocular Strain Set

Strain	MIC ($\mu\text{g}\cdot\text{mL}^{-1}$)		MIC ($\mu\text{g}\cdot\text{mL}^{-1}$)		FICI
	Alone		Combination		
	PT	Rifampin	PT	Rifampin	
PAO1	0.41	15.6	0.21	1.9	0.634
Clinical isolate set average	0.416 ± 0.135	23.4 ± 6.86	0.180 ± 0.045	5.41 ± 3.11	0.701 ± 0.107

of this clinically relevant strain set. While overall resistance of *P. aeruginosa* keratitis isolates remains low, the PT + rifampin can successfully overcome existing antibiotic resistance with equal efficacy compared to antibiotic-sensitive strains, an important prerequisite for the development of any novel therapeutic agent.

Discussion

Antibiotic resistance is currently one of the most pressing concerns in modern medicine, resulting in significant clinical adverse outcomes due to treatment failures in addition to escalating health care costs.^{23–25} *P. aeruginosa*, one of the most common causes of ocular infections, has been designated by the Centers for Disease Control and Prevention as an ESKAPE pathogen, one of a group of six organisms of particular health care concern given their ability to “escape” traditional antimicrobial therapies as well as a “serious threat” in the recently published 2019 Antibiotic Resistant Threats in the United States.²⁶ Fortunately, however, our current data indicate very low circulating antibiotic resistance among *P. aeruginosa* ocular isolates, a finding that is supported by other large-scale surveillance studies. For example, the Antibiotic Resistance Monitoring in Ocular Microorganisms (ARMOR) study reported resistance rates of approximately 5% or lower to fluoroquinolones such as ofloxacin, ciprofloxacin, levofloxacin, and gatifloxacin in a set of nearly 700 clinical isolates collected from 2009 to 2018 in the United States.²⁷

Despite the current reassuring low rates of antibiotic resistance among *P. aeruginosa* ocular isolates in the United States, reports from India describe emerging antibiotic resistance particularly toward fluoroquinolones, the mainstay of treatment of corneal infections. Given that resistance will undoubtedly rise, the development of novel antimicrobial therapeutics is essential to stay ahead of the curve. In response to this growing need, we have recently described a novel drug combination containing PT + rifampin that displays impressive antimicrobial activity toward the two most common causes of keratitis, *S. aureus* and *P. aeruginosa*.^{14,15} Importantly, the potent activity of PT + rifampin extended to an in vivo keratitis model, where it was shown to successfully eradicate *P. aeruginosa* and *S. aureus* corneal infections, even those caused by fluoroquinolone-resistant clinical isolates.^{14,15} Thus, to further advance our understanding of the therapeutic potential of PT + rifampin, we evaluated the combination’s activity toward a diverse panel of clinically relevant ocular isolates.

Our results indicate that the combination of PT + rifampin is effective toward contemporary circulating *P. aeruginosa* strains, including those that are resistant to one or more currently available antibiotics. Importantly, the combination of PT + rifampin appears to be synergistic or additive toward 95% of these isolates, as reflected in the decreased concentrations of PT and rifampin required for efficacy when in combination compared to each agent individually.

Given that current standard of care necessitates intensive, frequent dosing of topical antibiotics in cases of severe keratitis, the potency of PT + rifampin may allow for reduced dosing schedules for patients and potentially decreased toxicity to the ocular surface. The effectiveness of this combination may be due, in part, to its multiple mechanisms of action. While polymyxin B acts as a detergent to disrupt bacterial cell membranes, trimethoprim inhibits bacterial DNA synthesis through the inhibition of dihydrofolate reductase, and rifampin inhibits DNA transcription through binding to bacterial RNA polymerase.^{28–30}

In summary, in an era of rising antibiotic resistance, the need for novel therapeutics is critical. This is particularly true for the treatment of keratitis, in which immediate empiric therapy that can successfully contend with circulating antibiotic resistance is necessary to prevent permanent ocular tissue damage. We have demonstrated the potency of a novel antibiotic combination, PT + rifampin, to successfully eradicate ocular clinical isolates of *P. aeruginosa* with varying resistance profiles, suggesting that the combination of PT + rifampin may represent a promising new therapeutic option to fill this critical need.

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