Effects of Tigecycline Combined with Azithromycin Against Biofilms of Multidrug-Resistant *Stenotrophomonas maltophilia* Isolates from a Patient in China

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Purpose: Our aim was to investigate in vitro biofilm formation by *S. maltophilia* and the effects of antibacterial agents used to prevent biofilm formation.

Methods: Two trimethoprim/sulfamethoxazole-resistant *S. maltophilia* strains were isolated from the pleural effusion of a patient with cancer. The minimum inhibitory concentrations (MICs) of amikacin, azithromycin, cefoperazone/sulbactam, and tigecycline were determined. The checkerboard method was used to determine the fractional inhibitory concentration indices (FICIs). A crystal violet biofilm assay and confocal laser scanning microscopy (CLSM) were used to observe biofilm formation. In vitro effects of azithromycin combined with tigecycline on biofilms of *S. maltophilia* strains were tested.

Results: The two *S. maltophilia* isolates were confirmed to produce strong biofilms. Crystal violet biofilm assay and CLSM analysis of *S. maltophilia* biofilm were in the initial adhesive stage after 2 h incubation. Biofilm was in the exponential phase of growth at 12 h and reached maximal growth at 36–48 h. Compared with tigecycline or azithromycin alone, the combination of tigecycline and azithromycin increased the inhibiting effect *S. maltophilia* biofilm biomass after incubation for 12 h. Compared with the control group, in almost all strains treated with tigecycline and azithromycin, the biofilm was significantly suppressed significance ($P < 0.001$). We found that 2x MIC azithromycin combined with 1x MIC tigecycline had the best inhibiting effect against the biofilm, the biofilm inhibition rates of three strains were all over 60%, the biofilm thickness was inhibited from $36.00 \pm 4.00 \mu\text{m}$ to $8.00 \mu\text{m}$, from $40.00 \mu\text{m}$ to $6.67 \pm 2.31 \mu\text{m}$, and from $32.00 \mu\text{m}$ to $13.33 \pm 2.31 \mu\text{m}$ in SMA1, SMA2 and ATCC17666, respectively.

Conclusion: Azithromycin combined with tigecycline inhibited biofilm formation by *S. maltophilia*. Our study provides an experimental basis for a possible optimal treatment strategy for *S. maltophilia* biofilm-related infections.

Keywords: *Stenotrophomonas maltophilia*, azithromycin, tigecycline, biofilm, trimethoprim/sulfamethoxazole

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Introduction

Stenotrophomonas maltophilia (SMA) is recognized as a superbug that threatens human health by virtue of its natural and acquired resistances to a variety of antimicrobial agents.^{1,2} SMA causes healthcare-associated infections, and the infection rate has shown a substantial upward trend.^{3,4}

SMA infection often occurs in elderly patients, in patients with tumors, in patients with cystic fibrosis, in immunocompromised patients, in patients with prolonged hospitalization, and as a result of misuse of antimicrobial agents, especially carbapenems^{1,5,6} Persistent infections caused by SMA create substantial challenges for clinicians. Multiple drug resistance contributes to persistent infections and subsequent mortality. Biofilm formation has been proven to enhance bacterial resistance. Nevertheless, the relationship between biofilm formation and intractable SMA infections remains unclear.

Biofilm formation is becoming a predominant feature in nosocomial infections. Because biofilms are increasingly resistant to antibiotics, making monotherapy ineffective, combination therapy seem to be important for their eradication.⁷ Macrolides in combination with other antibiotics have shown conflicting results with respect to antibiofilm properties on *S. maltophilia*. Azithromycin in combination with fluoroquinolones, particularly moxifloxacin, significantly reduced the biofilm-inhibiting effect on *S. maltophilia* biofilms.⁸ One possible mechanism is that azithromycin may interfere with protein synthesis and consequently with the bactericidal activity of fluoroquinolones.⁸ By contrast, erythromycin acts synergistically with levofloxacin, cefoperazone/sulbactam, and piperacillin, enhancing susceptibility against biofilms. These findings suggest that specific combined macrolide could be an effective treatment for *S. maltophilia* infection.⁹ Several studies have indicated that a combination of fluoroquinolones and azithromycin has a synergetic effect on *Pseudomonas aeruginosa* biofilms.¹⁰ Bacterial biofilm structure and composition confer inherent resistance to antimicrobial agents. The biofilm matrix serves as an obstacle to antimicrobial agents, which can be bound to matrix components or be consumed by them.¹¹ Nevertheless, this trait varies by matrix composition, biofilm age, and specific antimicrobial agent.^{12,13} The aim of antibiofilm strategies was to prevent initial bacterial adhesion, inhibit biofilm maturation inhibition, and eradicate biofilm production. Premature biofilms are more susceptible to antimicrobial agents than are mature biofilms.¹⁴

In the present study, we measured the influence of biofilm production on refractory infection, and the in vitro effects of antimicrobial agents against biofilms produced by this organism.

Materials and Methods

Clinical Observation of the Patient

On 22 May 2017, a 64-year-old man was admitted to the First Affiliated Hospital of Anhui Medical University with

a 1-month history of recurrent fever. The patient presented with high fever, accompanied by chest tightness. He had a history of surgery for esophageal cancer one month prior. Chest CT revealed right pleural effusion. Pleural tap revealed a white blood cell of 2986 cell/mm³. *K. pneumoniae* was isolated after admission; two weeks later, SMA1 was obtained from the pleural effusion. Twenty days later, SMA2 was obtained from pleural effusion as well. The patient was diagnosed with an infected pleural effusion.

(A) *K. pneumoniae* was resistant to cefepime, aztreonam, and cefoperazone-sulbactam. The patient was treated with IV imipenem (0.5 g, q8h, 17 days) from 22 May to 8 June. After imipenem treatment for 16 days, WBC in pleural effusion decreased to 1951 cell/mm³. However, the patient maintained a low-grade fever. SMA1 was isolated in the pleural effusion. The anti-bacterial therapy was changed to IV tigecycline (50 mg, q12h) for 14 days. However, the fever rebounded to 39°C on 16 June, and SMA2 was isolated from the pleural effusion. At this time, the thoracic drainage tube was removed. The anti-bacterial therapy was changed to IV tigecycline (2 g, q8h) combined with azithromycin (0.5 g, qd) for 17 days. Finally, the patient recovered. This study was conducted in accordance with the Declaration of Helsinki. We confirmed that written informed consent has been provided by the patient to publish the case details. This study was approved by the local Ethics Committee of Anhui Medical University.

S. maltophilia Isolates

SMA1 and SMA2 were isolated from the patient pleural fluid on 5 June and 19 June, respectively. The isolates were confirmed using the VITEK GNI system (bioMérieux Vitek Inc., Hazelwood, MO, USA) and Clin-ToF-II.

Antimicrobial Susceptibility Testing and Detection of Resistance Mechanisms

Antimicrobial susceptibility testing was determined using the microdilution method, according to the Clinical and Laboratory Standards Institute Performance Standards for Antimicrobial Susceptibility Testing, 23rd informational

supplement.¹⁵ The following antimicrobial agents were tested against *S. maltophilia* strains: trimethoprim/sulfamethoxazole (SXT), minocycline (MIN), levofloxacin (LVX), ticarcillin-clavulanate (TIC/CIA), chloramphenicol (CHL), ceftazidime (CAZ), amikacin (AMK), azithromycin (AZM), tigecycline (TGC), cefoperazone/sulbactam, aztreonam (ATM), imipenem (IPM), and meropenem (MEP). All antibiotics are from Sigma-Aldrich China, Inc. All experiments were repeated three times. The breakpoint of tigecycline for *S. maltophilia* was based on the standard of the US Food and Drug Administration. *S. maltophilia* 17,666, *Pseudomonas aeruginosa* ATCC 27,853, and *Escherichia coli* ATCC 25,922 were used as controls for antimicrobial susceptibility testing.

Polymerase chain reaction was used to detect β -lactamase genes (*LI* and *blaL2*), sulfamethoxazole resistance genes (*sul1*, *sul2*, and *sul3*), trimethoprim resistance genes (*dfrA1*, *dfrA5*, *dfrA12*, *dfrA13*, and *dfrA17*), quinolone resistance genes (*qnrX*, *qnrY*, and *qnrZ* gene), and class 1, 2, and 3 integrons. All experiments were repeated three times.

Checkerboard Assay

The checkerboard broth microdilution assay was performed in 96-well microtiter plates using 2-fold dilutions of two antibiotics diluted in cation-adjusted Mueller–Hinton broth (CAMHB). AZM ranging from $1/64 \times \text{MIC}$ to $2 \times \text{MIC}$ was dispensed in every row. TGC ranging between $1/64 \times \text{MIC}$ and $2 \times \text{MIC}$ was added in each column. An equal volume of standardized bacterial suspension of 1×10^6 CFU/mL was added and then all plates were incubated at 37°C in an aerobic atmosphere for 24 h. Fractional inhibitory concentration index (FICI) values were calculated as $\text{FICI} = \text{FIC A (MIC antibiotic A in combination)} / (\text{MIC antibiotic A} + \text{FIC B (MIC antibiotic B in combination)} / (\text{MIC antibiotic B}))$. To categorize the drug combination that consistently generated the lowest FICI after repeating the experiment in duplicate on two further occasions, the results were grouped as follows: FICIs of ≤ 0.5 were interpreted as synergistic; FICIs of >0.5 but ≤ 1 were considered additive; FICIs of >1 but ≤ 4 were considered indifferent; and FICIs >4 were interpreted as antagonistic.¹⁶ The measurements were performed on three different samples of each.

Biofilm Formation Assay

Crystal Violet Biofilm Assay

To observe the biofilm formation and established biofilms, crystal-violet staining experiments were performed as

previously described with some changes.^{17–19} Briefly, overnight cultures of *S. maltophilia* were standardized to a 0.5 MacFarland standard (equivalent to 1.5×10^8 CFU/mL), and then diluted (1:100) with fresh Luria Bertani broth. Aliquots (200 μ L) of standardized inocula were added to the wells of sterile flat-bottom 96-well polystyrene tissue culture plates (Thermo Fisher Scientific, USA) and incubated at 37°C over a series of time-points (2, 4, 6, 8, 10, 12, 24, 36, and 48 h) in a closed and humidified plastic container. The medium was then discarded and non-adherent cells were removed by washing three times in sterilized ultrapure water. The cells were stained with 0.01% (w/v) crystal violet for 15 min. The excess stain was then removed by washing with water and the stained biofilms were dried for 30 min at ambient room temperature and extracted with 33% (v/v) glacial acetic acid. The amount of biofilm produced was quantified by measuring the optical density at 490 nm (OD490) using a plate reader (VarioskanFlash; Thermo Fisher Scientific, USA). Non-inoculated media were used as a control. The low cut-off point for biofilm production was chosen according to the criteria described by Di Bonaventura et al, the cut-off point was defined as three standard deviations above the mean optical density of the control (ODc) wells.¹⁷ Based on this cut-off, strains were classified into the following categories: no biofilm producer ($\text{OD} \leq \text{ODc}$), weak biofilm producer ($\text{ODc} < \text{OD} \leq 2 \times \text{ODc}$), moderate biofilm producer ($2 \times \text{ODc} < \text{OD} \leq 4 \times \text{ODc}$), and strong biofilm producer ($4 \times \text{ODc} < \text{OD}$).^{20,21} Each isolate was assayed three times and the results were presented as the average of the three assays.^{8,22}

Confocal Laser Scanning Microscopy (CLSM) of Biofilm Formation

Overnight bacterial culture of SMA1, SMA2 and ATCC17666 were standardized to a 0.5 MacFarland standard (equivalent to 1.5×10^8 CFU/mL) and then diluted (1:100) with fresh luria bertani broth. Then 400 μ L of the dilution were inoculated into tissue culture 24-well glass-bottom imaging plates and incubated at 37°C over a series of time-points (2, 6, 12, 24, 36 and 48 h) in a closed and humidified plastic container.²³ After washing out non-adherent cells with phosphate-buffered saline (PBS), biofilms were fixed with 3.7% (v/v) formaldehyde for 1 h, washed with sterile PBS, and then extracellular nucleic acids stained with fluorescent dyes 0.01% acridine orange (AO, Sigma) for 15 min at room temperature (RT). After staining, wells were washed with 1 mL of PBS to remove

unbound dye and dried for 15 min. All procedures were conducted in the dark. The excitation laser wavelength for AO was 488 nm, the emission wavelength is 512–654 nm. Confocal laser microscopy was used to observe the growth of biofilms, as well as structure and thickness at various times.²⁴ The biofilms were examined using a Zeiss LSM 510 META microscope (Zeiss, Germany).²⁰ CLSM was used to measure biofilm thickness using the ImageJ program.²⁵ Images were constructed using ZEN software (Carl Zeiss, Thornwood, NY, USA), as described previously.²⁶ The measurements were performed on three different samples of each.²⁷

Effect of Combined Tigecycline and Azithromycin on *S. maltophilia* Biofilms Using CLSM

CLSM was performed to visualize biomass and cells of biofilm affected by tigecycline and azithromycin using published procedures, with some modifications.²⁸ Briefly, overnight bacterial culture of *S. maltophilia* was adjusted and 200 µL dilution was inoculated into tissue culture-treated 24-well glass bottom imaging plates (Eppendorf AG, Hamburg, Germany, Cat. no. 0030741021). Sterile cell slides are placed at the bottom of the 24-well plates. After 12 h incubation, suspension medium was discarded and each well was washed three times with 1 mL of sterile PBS. Various concentrations of TGC (0.5x, 1x, and 2x of the MIC) were diluted by prepared fresh CAMHB in the wells of the plate. Various concentrations of azithromycin (0.5x, 1x, and 2x of the MIC) were added to wells on the same plate. Fresh CAMHB without antibiotics was added to the control wells. The plates were incubated at 37°C for 12 h. After washing out non-adherent cells with PBS, biofilms were fixed with 3.7% (v/v) formaldehyde for 1 h, washed with sterile PBS, and then extracellular nucleic acids stained with 0.01% AO for 15 min at RT. After staining, wells were washed with 1 mL of PBS to remove unbound dye and dried for 15 min. All procedures were conducted in the dark. The excitation laser wavelength for AO was 488 nm, the emission wavelength was 512–654 nm. Confocal laser microscopy was used to observe the growth of biofilms in different drug treatment groups, including structure and thickness. The biofilms were examined under Zeiss LSM 510 META microscope (Zeiss, Germany). CLSM was used to measure biofilm thickness using the ImageJ program.

Images were constructed using ZEN software (Carl Zeiss, Thornwood, NY, USA), as described previously.²⁶ The measurements were performed on three different samples of each.

In vitro Effects of Azithromycin, Tigecycline, Amikacin, Cefoperazone/Sulbactam Alone on *S. maltophilia* Biofilms

This test was performed on the *S. maltophilia* strains (SMA1, SMA2, and ATCC17666). After 12 h incubation periods at 37 °C, the supernatants from each well were gently discarded. Each well was then washed three times with sterile saline water without destroying the attached biofilm. Various concentrations of azithromycin, tigecycline, amikacin, and cefoperazone/sulbactam alone (1x, 2x, and 4x of the MIC) were prepared following dilution in 100 µL of fresh CAMHB in the wells of the micro-titer plate. Fresh CAMHB without antibiotics was added to the control wells. The plates were incubated at 37 °C for 12 h and the OD_{490 nm} was measured. All measurements were performed three times.

Effect of Tigecycline Combined with Azithromycin on *S. maltophilia* Biofilms

This test was performed on the *S. maltophilia* strains (SMA1, SMA2, and ATCC17666). After 12 h incubation periods at 37 °C, the supernatants from each well were gently discarded. Each well was then washed three times with sterile PBS without destroying the attached biofilm. Various concentrations of TGC (0.5x, 1x, and 2x of the MIC) were prepared following dilution in 100 µL of fresh CAMHB in the wells of the micro-titer plate. Various concentrations of azithromycin (0.5x, 1x, and 2x of the MIC) were added to wells on the same plate. Fresh CAMHB without antibiotics was added to the control wells. The plates were incubated at 37 °C for 12 h and the OD_{490 nm} was measured. The inhibition results were calculated using Eq (1) and were expressed as a percentage: % inhibition = 100 – (OD_{sample}/OD_{control}) x 100 (1).²⁹ All measurements were performed three times at least.

Statistical Analysis

All tests were performed in triplicate and repeated three times. Multiple comparisons of responses to antibiotics and inhibition of biofilm formation were performed using one-way analysis of variance. A P-value <0.05

was considered statistically significant. Statistical analysis was conducted using GraphPad Prism 6.

Results

Antimicrobial Susceptibility and Resistance Genes

Antimicrobial susceptibility testing showed that two strains displayed resistance to SXT, LVX, TIC/CIA, AZM, IPM, MEP, CAZ, and ATM, and were sensitive to SCF, MIN, CHL, and TGC. As shown in (Figure S1), the two isolates contained *sul1*, *qnrX*, *qnrY* and *qnrZ* genes, and *class 1* and *3* integrase of integrons.

In vitro Synergy Testing Using the Checkerboard Method

As shown in Table 1, the combination of azithromycin–tigecycline resulted in synergy for three strains, with FICIs ranging from 0.1875 to 0.375. The combination of cefoperazone/sulbactam–azithromycin resulted in additivity for three strains, with FICIs ranging from 0.625 to 1.0. The combination of amikacin–tigecycline resulted in no interaction for the three strains, with FICIs ranging from 1.5 to 3.0. The combination of amikacin–cefoperazone/sulbactam resulted in no interaction for three strains, with FICIs ranging from 1.25

to 2.25. The combination of tigecycline–cefoperazone/sulbactam resulted in no interaction for three strains, with FICIs ranging from 1.25 to 4.0.

In vitro Biofilm Formation by *S. maltophilia*

Crystal Violet Biofilm Assay

As shown in Figure 1, SMA1, SMA2 and ATCC17666 were in the initial adhesive stage after 2 h of incubation, and in the exponential phase of growth after 12 h of incubation. Maximum growth was achieved after about 48 h of incubation (plateau phase). As shown in Figure 2, the ability of the three strains to produce biofilm was strong.

CLSM Analysis of Biofilm Formation

CLSM analysis of biofilm formation in clinical isolates strains (SMA1, SMA2) and standard strain ATCC17666. CLSM analysis showed that the thicknesses of ATCC17666 biofilms were $3.99 \pm 0.01 \mu\text{m}$ at 2 h, $8.00 \mu\text{m}$ at 6 h, $16.00 \mu\text{m}$ at 12 h, $36.00 \mu\text{m}$ at 24 h, $34.67 \pm 2.31 \mu\text{m}$ at 36 h, and $42.67 \pm 2.31 \mu\text{m}$ at 48 h. Thicknesses of the SMA1 biofilms were $4.00 \mu\text{m}$ at 2 h, $8.00 \mu\text{m}$ at 6 h, $17.33 \pm 2.31 \mu\text{m}$ at 12 h, $36.00 \mu\text{m}$ at 24 h, $38.67 \pm 2.31 \mu\text{m}$ at 36 h, and $42.67 \pm 2.31 \mu\text{m}$ at 48 h. Thicknesses of the SMA2 biofilms were $4.00 \mu\text{m}$ at 2 h, $10.67 \pm 2.31 \mu\text{m}$ at 6 h, $18.67 \pm 2.31 \mu\text{m}$ at 12 h, $40.00 \pm 6.93 \mu\text{m}$ at 24 h, $44.00 \mu\text{m}$ at 36 h, and

Table 1 Checkerboards of the Various Antibiotics Used in This Study, with Their FICIs in *S. maltophilia* Strains

	SMA1			SMA2			ATCC17666		
	Single MIC (μg/mL)	Combination MIC (μg/mL)	FICI	Single MIC (μg/mL)	Combination MIC (μg/mL)	FICI	Single MIC (μg/mL)	Combination MIC (μg/mL)	FICI
AZM	256	32	0.375	256	32	0.25	256	32	0.188
TGC	0.5	0.125		1	0.125		0.5	0.031	
SCF	8	1	0.625	2	1	1	8	1	0.625
AZM	256	128		256	128		256	128	
AMK	2	2	3	8	4	1.5	64	8	2.125
TGC	0.5	1		1	1		0.5	1	
AMK	2	2	1.25	8	16	2.25	64	32	1.5
SCF	8	2		2	0.5		8	8	
TGC	0.5	0.125	1.25	1	2	4	0.5	0.063	2.125
SCF	8	8		2	4		8	16	

Notes: FICI was defined as $\text{FICI} = \text{FIC A (MIC antibiotic A in combination)} / (\text{MIC antibiotic A}) + \text{FIC B (MIC antibiotic B in combination)} / (\text{MIC antibiotic B})$. FICI index ≤ 0.5 , 0.5–1, 1–4, >4 were defined as synergistic, additive, indifferent or antagonism, respectively.

Abbreviations: MIC, the minimum inhibitory concentrations; AZM, azithromycin; TGC, tigecycline; SCF, cefoperazone/sulbactam; AMK, amikacin.

Figure 1 Average ODs at different time points (hours) of *S. maltophilia* strains. Results are expressed as means $\pm$ SDs.

Figure 2 OD values of *S. maltophilia* strains, showing their capacity to form biofilms. Results are expressed as means $\pm$ SDs. Compared with the control group, the difference is statistically significant, **** $P < 0.0001$. The ability of the three strains to produce biofilm was strong after 48 h of incubation.

Notes: No biofilm producer ($OD \leq OD_c$), weak biofilm producer ($OD_c < OD \leq 2 \times OD_c$), moderate biofilm producer ($2 \times OD_c < OD \leq 4 \times OD_c$), and strong biofilm producer ($4 \times OD_c < OD$). In our study, $OD_c = 0.05$.

Abbreviation: OD_c , optical density of the control.

$48.00 \pm 4.00 \mu m$ at 48 h (Figure 3). CLSM analysis showed bacterial biofilm beginning to form after 2 h incubation. The biological membrane structure is close, and the thickness increased over 12–24 h incubation. The three-dimensional space network structure gradually formed between 36–48 h incubation (Figure 4). The growth rates of the strains biofilms were similar between 0–12 h incubation, however between 12 h and 48 h incubation, the growth rates of SMA1 and SMA2 became faster than that of ATCC17666.

Figure 3 Average biofilm thickness (μm) at different time points of *S. maltophilia* strains by confocal laser scanning microscopy. Results are expressed as means $\pm$ SDs. The growth rate of the strain biofilm was similar before culture for 12 h, however between 12 h and 48 h incubation, the growth rate of SMA1 and SMA2 became faster, which was a little faster than that of ATCC17666.

In vitro Effects of Azithromycin, Tigecycline, Amikacin, and Cefoperazone/Sulbactam on *S. maltophilia* Preformed Biofilm

As shown in Figure 5, low concentrations of antibiotics had a weak inhibitory effect on biofilms and inhibited biofilm formation in the three *S. maltophilia* strains in a dose-dependent manner. Amikacin had a far greater inhibitory effect on the biofilm of the premature biofilms than did the other antibiotics. Compared with the control group, the inhibitory effect of amikacin on the biofilms of the three strains was statistically significant ($P < 0.01$). Both tigecycline and cefoperazone/sulbactam had inhibitory effects on the biofilms of SMA1 and SMA2 (biofilms formed after 12 h incubation). Compared with the control group, the inhibitory effects of tigecycline and cefoperazone/sulbactam on the biofilms of the two clinical isolates strains were statistically significant ($P < 0.01$); however, the inhibitory effect of tigecycline and cefoperazone/sulbactam on the biofilm of the ATCC17666 was not statistically. As shown in Figure 5, the inhibitory effects of azithromycin alone against preformed biofilms in SMA1, SMA2, and ATCC17666 occurring at various concentrations were poor. This may be because all three strains were resistant to azithromycin. Compared with the control group, the inhibitory effect of azithromycin *S. maltophilia* (SMA1, SMA2, ATCC17666) strains that have formed biofilms has almost not reached statistical significance.

Figure 4 Confocal laser scanning microscopy images of *S. maltophilia* strain (ATCC17666) biofilms after 2, 6, 12, 24, 36, and 48 hours. Biofilm matrix components were stained with 0.01% AO. CLSM analysis showed bacterial biofilm beginning to form after 2 h incubation. The biological membrane structure is close, and the thickness increased over 12–24 h incubation. The three-dimensional space network structure gradually formed between 36–48 h incubation.

Figure 5 In vitro effects of azithromycin, tigecycline, amikacin, cefoperazone/sulbactam alone on *S. maltophilia* preformed biofilm after 12 h. Results are expressed as means $\pm$ SDs. Amikacin (all concentrations) had a far greater inhibitory effect on the biofilm of the premature biofilms (formed after 12 h incubation) than other antibiotics. Compared with the control group, the inhibitory effect of amikacin on the biofilm of the three strains was statistically significant ($P < 0.01$). Compared with the control group, the inhibitory effect of tigecycline and cefoperazone/sulbactam on the biofilm of the two clinical isolates strains was statistically significant ($P < 0.01$). Compared with the control group, the inhibitory effect of azithromycin *S. maltophilia* (SMA1, SMA2, and ATCC17666) strains that have formed biofilms had almost not reached statistical significance. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Abbreviations: MIC, the minimum inhibitory concentrations; AZM, azithromycin; TGC, tigecycline; AMK, amikacin; SCF, cefoperazone/sulbactam.

Effect of the Combination of Tigecycline and Azithromycin on Preformed Biofilm of *S. maltophilia* Using Crystal Violet Biofilm Assay

Based on the synergistic effect of azithromycin and tigecycline, the effects of various concentrations of azithromycin combined with tigecycline to inhibit the biofilms were tested.

Compared with tigecycline or azithromycin alone, the combination of tigecycline and azithromycin increased the inhibitory effect on *S. maltophilia* preformed biofilms. We tested several combinations of various drug concentrations, and all of the combinations increased the associated inhibition (Figure 6). Although this had the effect of synergistically inhibiting biofilm, it did not reach statistical significance at low concentrations; however, compared with the control

Figure 6 In vitro effects of tigecycline combined with azithromycin on biofilms of *S. maltophilia* strains after 12 h. Results are means $\pm$ SDs. *** $P < 0.001$. Compared with tigecycline or azithromycin alone, the combination of tigecycline and azithromycin increased the inhibitory effect on *S. maltophilia* preformed biofilms. The best inhibitory effect was observed when 2x MIC azithromycin was combined with 1x MIC tigecycline and when 2x MIC azithromycin was combined with 1x MIC tigecycline. The biofilm inhibition rates were 64% for SMA1, 76% for SMA2, and 67% for ATCC17666. The biofilm inhibition rates of three strains were all over 60%, suggesting good results. **Abbreviations:** MIC, the minimum inhibitory concentrations; TGC, tigecycline; AZM, azithromycin.

group, in almost all groups treated with antibiotics, biofilm formation was significantly suppressed ($P < 0.001$). The best inhibitory effect was observed when 2x MIC azithromycin was combined with 1x MIC tigecycline (Figure 6) and when 2x MIC azithromycin was combined with 1x MIC tigecycline. The biofilm inhibition rates were 64% for SMA1, 76% for SMA2, and 67% for ATCC17666. The biofilm inhibition rates of three strains were all over 60%, suggesting good results.

Effect of the Combination of Azithromycin and Tigecycline on *S. maltophilia* Preformed Biofilm Thickness and Structure Using CLSM Analysis

To further study the effects of antibiotics on biofilm formation by *S. maltophilia*, a combination of azithromycin and tigecycline was tested on the three *S. maltophilia* strains.

Interestingly, compared with *S. maltophilia* treated by azithromycin or tigecycline alone, the combination of azithromycin and tigecycline increased the inhibitory effect on *S. maltophilia* preformed biofilm. As shown in Figure 7, compared with the control group, the biofilms of almost all antibiotic treatment groups were significantly inhibited. Compared with tigecycline alone, the best inhibitory effect was observed with 2x MIC azithromycin combined with 1x MIC tigecycline ($P < 0.01$). When 2x MIC azithromycin combined with 1x MIC tigecycline was used, the biofilm thickness of SMA1 was inhibited from $36.00 \pm 4.00 \mu\text{m}$ to $8.00 \mu\text{m}$; that of SMA2 was inhibited from $40.00 \mu\text{m}$ to $6.67 \pm 2.31 \mu\text{m}$; and that of ATCC17666 was inhibited from $32.00 \mu\text{m}$ to $13.33 \pm 2.31 \mu\text{m}$.

As shown in the CLSM images, compared with *S. maltophilia* treated with azithromycin or tigecycline alone, the combination of azithromycin and tigecycline increased the inhibitory effect on *S. maltophilia*

Figure 7 In vitro effects of tigecycline combined with azithromycin on biofilm thickness of *S. maltophilia* strains after 12 h. Results are expressed as means $\pm$ SDs. Compared with the control group, the difference was statistically significant in clinical isolates SMA1 and SMA2. **** $P < 0.0001$, *** $P < 0.001$. Compared with the control group, the difference was statistically significant except 0.5x MIC tigecycline and 0.5x MIC azithromycin alone in standard strain ATCC17666. **** $P < 0.0001$, *** $P < 0.001$. **Abbreviations:** MIC, the minimum inhibitory concentrations; TGC, tigecycline; AZM, azithromycin.

preformed biofilms. As shown in Figure 7, 2x MIC azithromycin combined with 1x MIC tigecycline showed the best inhibitory effect, consistent with the results of the 96-well biofilm assays (Figure 6). Micrographs of no-antibiotic treatment groups showed spatial biomass distribution of the formed biofilms and relatively thick coating of the biofilms with well-organized architecture characterized by large clumps over a glass surface. However, after treatment with 1x MIC tigecycline and 2x MIC azithromycin alone, or 1x MIC tigecycline combined with 2x MIC azithromycin (Figure 8), the established biofilms were disrupted and scattered as microcolonies, and the biofilm cells remained in the form of flakes, evacuated, indicating that cells within the formed biofilms were probably challenged, killed, and detached from the established biofilms. Compared with *S. maltophilia* biofilm treated with 1x MIC tigecycline and 2x MIC azithromycin alone, biofilms of *S. maltophilia* were almost disrupted by 1x MIC tigecycline combined with 2x MIC azithromycin.

CLSM was used to visualize the biofilms formed on the glass bottom plates. Compared with *S. maltophilia* biofilm treated with tigecycline and azithromycin alone, microscopic examination showed that tigecycline combined with

azithromycin largely eradicated the biofilms formed on the glass bottom surface. This suggests that there were few biofilm cells.

Discussion

S. maltophilia is an opportunistic pathogen that is widely distributed in nature. The pathogen is the third most common infectious non-fermentative following *P. aeruginosa* and *Acinetobacter baumannii*.^{3,4} Biofilms lead to greater resistance to antimicrobial drugs than non-biofilm forming bacteria and are therefore more difficult to treat.^{30,31} The origin of various chronic infections is usually the formation of a biofilm by the infecting organism. Therefore, one of the important considerations for effective treatment of most chronic infections is to target the biofilm stage of the organism.^{32,33}

In the present study, a patient with esophageal cancer had an infected pleural effusion after surgery. After treatment of thoracic drainage tube drainage and imipenem for two weeks, SXT-resistant *S. maltophilia* was isolated from the pleural effusion. SXT-resistant *S. maltophilia* was still isolated after an interval of 20 days. Our result revealed the use of imipenem for two weeks selected the

Figure 8 In vitro effects of 1x MIC tigecycline combined with 2x MIC azithromycin on biofilms of *S. maltophilia* strains after 12 h. Green fluorescent staining (AO) represents extracellular DNA. CLSM images of panel of control groups (without antibiotics) *S. maltophilia* biofilms have spatial biomass distribution of mature biofilms and thick coating of biofilm with compact architecture characterized by large clumps. By contrast, see panels SMA1, SMA2, and ATCC17666. CLSM images of *S. maltophilia* biofilm treated with 1x MIC tigecycline and 2x MIC azithromycin alone, and 1x MIC tigecycline combined with 2x MIC azithromycin. Compared with the control group (without antibiotics), the established biofilms of the antibiotic treatment group were disrupted and biofilm cells were rare. Compared with *S. maltophilia* biofilm treated with 1x MIC tigecycline and 2x MIC azithromycin alone, when 1x MIC tigecycline was combined with 2x MIC azithromycin, biofilms of *S. maltophilia* were almost disrupted. As shown in the figure, the green fluorescence is very weak and the color is very dark when observed under a laser confocal microscope. This indicates that there are few biofilm cells and the structure was destroyed. Scale bar: 50 μ m.

Abbreviations: MIC, the minimum inhibitory concentrations; TGC, tigecycline; AZM, azithromycin.

S. maltophilia as the dominant pathogenic bacteria. The two *S. maltophilia* isolates were confirmed to produce strong biofilms, which may have contributed to the refractory infection. The use of thoracic drainage tube drainage may contribute to biofilm formation. Interestingly, SMA2 had a higher ability to produce biofilm. There are many infectious bacteria, including *S. maltophilia*, *P. aeruginosa* and *Staphylococcus aureus*, which have the ability to form biofilms.³⁴ The ability of *S. maltophilia* to form biofilm is an important feature of its virulence.¹⁹ In our research, crystal violet biofilm assay and CLSM analysis of *S. maltophilia* biofilm were in the initial adhesive stage after 2 h incubation. Biofilm was in the exponential phase of growth at 12 h, and reached maximal growth at 36–48 h. Crystal violet biofilm assay and CLSM analysis showed these adherent cells are embedded within a self-produced matrix of an extracellular polymeric substance, and finally forming a complex three-dimensional architecture, which is consistent with the two previous investigations.^{19,20,25,35} The antibiotics used to treat *S. maltophilia* infections are usually selected based on the results of conventional antimicrobial susceptibility of plankton. However, it is known that organisms such as *S. maltophilia* actually grow in the form of biofilms that are more resistant to bacteria than bacteria floating on airway epithelial cells.^{8,36,37}

Previous research showed levofloxacin prevented biofilm formation of *S. maltophilia* isolates,³⁸ however, according to our study, a refractory infection in pleural effusion was caused by *S. maltophilia* with high biofilm production, characterized by resistance to SXT, LVX, and ceftazidime.¹¹ The patient was treated using TGC at first, however, the effect was not good, and biofilm production in the isolates may have contributed to the poor efficacy. The patient was recovered by the combination of azithromycin and tigecycline at last.

Macrolides in combination with other antibiotics have shown conflicting results regarding antibiofilm properties against *S. maltophilia*. Azithromycin in combination with fluoroquinolones significantly reduced the biofilm-inhibiting effect on *S. maltophilia* performed biofilms, especially combined with moxifloxacin.⁸ One possible mechanism is that azithromycin may interfere with the protein synthesis and consequently with the bactericidal activity of fluoroquinolones.⁸ In this research, we found that azithromycin–tigecycline showed synergistic effects against biofilm growth in the clinical *S. maltophilia* isolates, which contradicts to the previous research.⁸ By contrast, erythromycin acts synergistically with levofloxacin,

cefoperazone/sulbactam, and piperacillin, enhancing susceptibility in biofilms. For this reason, specific macrolides could be effective adjuncts for treatment of *S. maltophilia* infections.⁹ Several studies have indicated that a combination of fluoroquinolones and azithromycin has synergetic effects against *P. aeruginosa* biofilms.^{7,10} The results of these studies are consistent with our findings in SMA1, SMA2, and ATCC17666.

In our study, the clinical *S. maltophilia* isolates were resistant to SXT; however, the combination of azithromycin–tigecycline showed synergistic effect on the inhibition of clinical *S. maltophilia* isolates in vitro. Furthermore, both 0.5x MIC azithromycin and 0.5x MIC tigecycline had inhibitory effects on biofilms in the two clinical *S. maltophilia* isolates. The inhibitory effect was more substantial with the increase of drug concentration. Based on the synergistic effect of azithromycin and tigecycline, the effects of various concentrations of azithromycin combined with tigecycline to inhibit the biofilms were tested. For azithromycin, 0.5x MIC combined with 1x MIC tigecycline inhibited the biofilm to half the thickness. Inhibition of biofilms is critical to controlling recurrent infection. CLSM analysis showed that 2x MIC azithromycin combined with 1x MIC tigecycline almost inhibited biofilm growth in two clinical *S. maltophilia* strains. It penetrate the biofilm, eventually killing the bacteria penetrating the biofilm, and eventually killing the bacteria.³⁹

When mature biofilms are completely formed by biofilm-forming pathogens, conventional antibiotics usually fail to eradicate the infection because the multilayer architecture of the biofilm acts as a diffusion barrier. This can provide protection to biofilm-grown cells against antimicrobial agents or antibiotics. Therefore, high biofilm disrupting concentrations are required, and strategies focusing on destroying the formed biofilms are of particular importance against infection.⁴⁰ Compared with *S. maltophilia* biofilm treated with tigecycline and azithromycin alone, azithromycin combined with tigecycline increased the inhibitory effect on *S. maltophilia* biofilms more efficiently. Our results suggest that the combination of tigecycline and azithromycin induced synergistic disruption of the complex architecture of the biofilms, as measured both by microtiter plate assay and by CLSM analysis. We believe that antibiotics are likely to only slow down the progress of biofilm formation by eliminating unprotected planktonic bacteria and reducing even stopping the metabolic activity of bacteria on the biofilm surface, and our results agree with most studies.^{41–43}

Conclusion

Biofilm formation of *S. maltophilia* contributes to persistent infection. We found that azithromycin combined with tigecycline increased the inhibiting effect on *S. maltophilia* biofilms compared with tigecycline and azithromycin alone. The use of antibiotics can better inhibit the growth of bacterial at the early stage of biofilm formation, accordingly reducing the incidence of resistance. Our research provides an experimental basis for possible optimal treatment strategy for *S. maltophilia* biofilm-related infections; however, additional evidence is required to confirm these findings in future therapeutic studies.

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Disclosure

The authors report no conflicts of interest in this work.

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