The Effects of Vancomycin and Ceftazidime on Biofilm Formation in *Elizabethkingia*

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Abstract: *Elizabethkingia* infections are associated with high mortality rates which is due in part to the antibiotic resistance expressed by all Gram-negative *Elizabethkingia* species. Biofilm formation by *Elizabethkingia* provides the bacteria with further defense against the action of antibiotics. Vancomycin is an unusual drug used to treat *Elizabethkingia* infections, but its use is controversial, since it is normally only used to treat Gram-positive infections. Our study tests the effect of vancomycin and ceftazidime on biofilm formation for all known species of *Elizabethkingia*. Biofilm formation was measured at 24, 48, and 72-hour timepoints using a crystal violet biofilm assay. Biofilm formation occurred in all positive control wells for all three timepoints for all species. The biofilms of *Elizabethkingia meningoseptica* in the presence of subinhibitory vancomycin concentrations, and *Elizabethkingia ursingii* in the presence of inhibitory ceftazidime concentrations, were reduced compared to the controls. This suggests that both drugs can inhibit biofilm formation in a species-specific manner.

Keywords: *Elizabethkingia*, Vancomycin, Ceftazidime, Biofilm Formation, Minimum Inhibitory Concentration (MIC)

Introduction

Elizabethkingia is a rod-shaped, Gram-negative, non-motile bacteria that causes infection in neonatal or immunocompromised populations. The infections are commonly serious (e.g. meningitis) and area associated with high mortality rates in part, due to the intrinsic multiple antibiotic resistance expressed by these organisms (Kämpfer *et al.* 2011; Lau *et al.* 2016). Several different antibiotics have been used in patients infected with *Elizabethkingia*, and vancomycin is one such antibiotic. The use of vancomycin for the treatment of *Elizabethkingia* infections can be effective, however this organism is often demonstrated to be resistant to this drug (Dias *et al.* 2010). Biofilms are formed when a matrix of extracellular polysaccharides is formed by bacterial cells. Biofilm formation is one of the key defense mechanisms by *Elizabethkingia* and supports increased antibiotic resistance, immune system avoidance, and attachment, which prevents the flushing of the organism (Jacobs and Chenia 2011; Donlan 2002). The effect of antibiotics on biofilm formation in *Elizabethkingia* has not been investigated. We hypothesize that biofilm formation in *Elizabethkingia* will be affected by vancomycin and ceftazidime at sub inhibitory concentrations.

Methods

Growth Conditions:

Bacterial colonies for each strain were grown on heart infusion agar (HIA) supplemented with 5% defibrinated rabbit blood. One colony from the HIA was used to inoculate a 3 ml Muller-Hinton broth (MHB) and incubated for 24 hours at 37° C (200rpm). Three overnight cultures per species were completed.

Minimum inhibitory and bactericidal (MIC/MBC) assays:

The CLSI microtiter method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for each type strain (Clinical and Laboratory Standards Institute 2018).

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Biofilm Growth Conditions:

Table 1: Elizabethkingia *type strain MIC and MBCs (µg/ml).*

		Vancomycin		Ceftazidime	
Strain	**Species**	**MIC**	**MBC**	**MIC**	**MBC**
R26	*anophelis*	8	16	>256	>256
ATCC	*bruuniana*	4	8	16	>256
KC1913	*meningoseptica*	64	64	>256	>256
G4071	*miricola*	4	8	>256	>256
G4070	*occulta*	8	16	256	>256
G4122	*ursingii*	2	4	4	16

Type strains for *Elizabethkingia anopholies, Elizabethkingia bruuniana, Elizabethkingia meningoseptica, Elizabethkingia microla, and Elizabethkingia ursingii* were plated in triplicate as biological replicates on a 96 well microtiter plate. Plates contained treatment wells with 100 µl drug containing MHB (½ MIC for vancomycin or 32 µg/ml for ceftazidime), while control wells contained 100 µl MHB. The overnight cultures were diluted to an OD_{600nm} of 0.01 and 100 µl was placed into all control and treatment wells. Two hundred µl MHB was inserted into all uninoculated control wells to prevent evaporation in control and treatment wells during incubation. The plates were then incubated for 24, 48, or 72 hours at 37° C.

Crystal Violet Biofilm Assay:

After the 24, 48, or 72 hours of incubation, plates were read at OD_{600nm} with a BioTek Synergy H1 plate reader and bacterial growth was recorded. All growth media was removed and 100 µl autoclaved diH_2O was used to rinse non-adherent cells from wells.

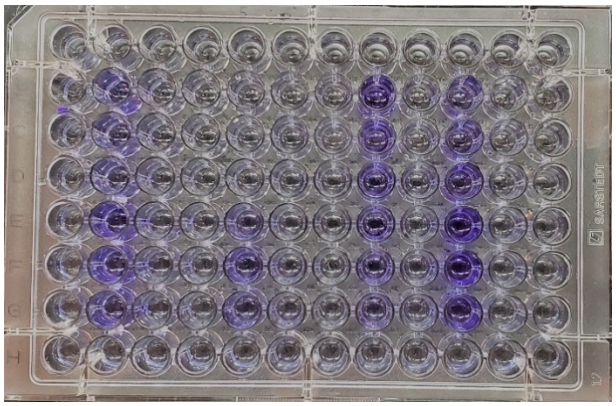

Figure 1: Crystal violet stained wells after rinsing with diH_20

One hundred µl 100% methanol was then placed into each well for 15 minutes to allow fixation of biofilm cells. The methanol was removed, and any excess was evaporated in a fume hood until all methanol was dissipated. One hundred µl of 0.2 % crystal violet was then added to all wells for 5 minutes. The wells were then rinsed with diH_2O to remove non-adherent crystal violet on biofilms (Figure 1). Plates were read at OD_{570nm} 1 minute after adding 100 µl 95% ethanol to dissolve crystal violet for measurement. Biofilm formation was determined positive if the well $OD_{570nm} \geq$ mean OD_{570nm} + 3 standard deviations of uninoculated control wells.

Results and Discussion

Each species of *Elizabethkingia* showed differences in MICs and MBCs for each drug (Table 1). All species formed biofilm in positive control wells for all timepoints, yet not not all controls wells formed biofilms (see ATCC, G4071, G4122, KC1913 in Tables 2 and 3). The control wells that did not from biofilms could be due to inappropriate dilution of overnight cultures (< 0.01) or harsh diH_2O rinsing which removed the biofilm. The crystal violet stain could have also been rinsed harshly from the well, causing the crystal violet bound to the biofilm to read lower than the actual amount of biofilm formation within the well. KC1913 in the presence of ½ MIC of vancomycin at 24-hour and 48-hour timepoints showed drug effect on biofilm formation (Table 2). G4122 in the presence of greater than MIC of ceftazidime also showed drug effect on biofilm formation (Table 3). For all other strains tested none or little effect was displayed between drug and biofilm

Table 2: Number of wells with biofilm formation in the presence of ½ MIC of vancomycin for all type strains of Elizabethkingia.

Isolate	Species	Vancomycin Concentration (µg/ml)	24 Hours		48 Hours		72 Hours	
			Control	Treatment	Control	Treatment	Control	Treatment
R26	*anophelis*	4	3	3	3	3	3	3
ATCC	*bruuniana*	2	2	3	3	3	3	3
KC1913	*meningoseptica*	32	3	0	3	1	3	3
G4071	*miricola*	2	1	3	3	3	3	3
G4070	*occulta*	4	3	3	3	3	3	3
G4122	*ursingii*	1	3	3	1	1	3	3

formation at any timepoint. However, biofilm formation occurred in KC1913 in the presence of ½ MIC of vancomycin at the 72-hour timepoint, proposing vancomycin resistant mutants formed (Table 2). The lack of biofilm formation for G4122 in the presence of ceftazidime could have been caused from using 32 µg/ml, which is higher than the MIC recorded for G4122 (Table 1 and 3). The exposure of greater than MIC likely caused no cell growth preventing biofilm formation.

The results display that *Elizabethkingia meningoseptica* in the presence of ½ MIC of vancomycin and *Elizabethkingia ursingii* in the presence of greater than MIC of ceftazidime had an impact on biofilm formation. Overall though, vancomycin and ceftazidime suggest no significant effect on stopping biofilm formation in all known species of *Elizabethkingia*. More trials should be conducted to further support these results and verify the impact of vancomycin and ceftazidime on biofilm formation in *Elizabethkingia.* Future studies will test biofilm formation for each type strain of *Elizabethkingia* in varied concentrations of vancomycin. Varied concentrations allow for results on what specific concentrations reduce or stop biofilm formation for vancomycin. Different drugs could also be used such as rifampin or clindamycin to view the way that they impact biofilm formation in *Elizabethkingia* as well as antibiotic synergy effect on biofilm formation. Future studies will also be conducted with Congo red stain to improve accuracy on measuring the actual amount of biofilm formation present in each well by staining the extracellular polysaccharide matrix.

Literature Cited

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Table 3: Number of wells with biofilm formation in the presence of 32 µg/ml ceftazidime for all type strains of Elizabethkingia.

Isolate	Species	24 Hours		48 Hours		72 Hours	
		Control	Treatment	Control	Treatment	Control	Treatment
R26	*anophelis*	3	3	3	3	3	3
ATCC	*bruuniana*	3	3	3	3	3	3
KC1913	*meningoseptica*	2	2	3	3	3	3
G4071	*miricola*	3	2	2	3	3	3
G4070	*occulta*	3	3	3	3	3	3
G4122	*ursingii*	3	0	3	1	3	0

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