Evolution of antimicrobial resistance in *Mycobacterium abscessus* isolated from drinking water

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Background

Mycobacterium abscessus (Mabs) is an opportunistic pathogen increasingly associated with pulmonary disease, particularly in people with structural lung disease. In Australia, Mabs has been isolated from drinking water distribution systems (DWDS) and found to be indistinguishable from patient isolates suggesting acquisition of infection from drinking water. Chlorine and chloramine are used as disinfectants in DWDS and Mabs susceptibility to these agents has not been previously quantified. Little is known about long-term exposure to disinfectants and the evolution of antimicrobial resistance in Mabs isolated from DWDS.

Aims

1. To investigate if the antimicrobial susceptibility of Mabs isolates from Brisbane's tap water has changed over time.
2. To investigate whether prolonged exposure to sub-inhibitory concentrations of chlorine and chloramine result in decreased susceptibility to the agents they were exposed to and/or other antimicrobial agents.
3. To investigate whether prolonged exposure to sub-inhibitory concentrations of imipenem results in imipenem resistance and/or other antimicrobial resistance.

Methods

Water samples from Brisbane's municipal water supply were collected and cultured for Mabs in 2007, 2017-18 and 2021-22 as part of ongoing studies. Isolates were selected from each time point and antimicrobial susceptibility testing (AST) performed by broth microdilution following the Clinical Laboratory Standards Institute (CLSI) guidelines.

A single 2007 *M. abscessus* subsp. *abscessus* water isolate was selected and passaged over six weeks exposure to chlorine, chloramine, imipenem, and a no-antimicrobial control to conduct an *in vitro* evolution experiment as shown in **Figure 1**. DNA was extracted from samples stored after passage 2,4 and 6 from each condition as well as the original 2007 isolate. DNA was sequenced using Illumina NextSeq (150 bp paired end) at a depth allowing >120 times coverage. The Breseq tool[1] was used to determine structural variants of passaged cultures using default parameters and the original isolate as the reference strain. Only assigned, predicted structural variants were reported.

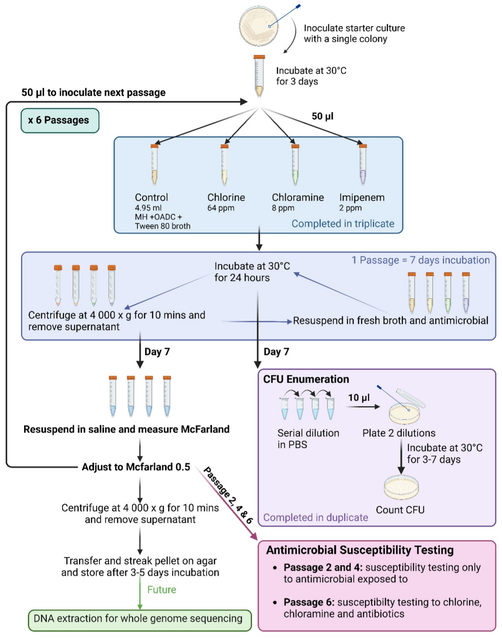

Figure 1: *in vitro* evolution experiment method.

References:
1. Deatherage DE, Barrick JE. Identification of mutations in laboratory-evolved microbes from next-generation sequencing data using breseq. *Methods in Molecular Biology* 2014; 1151:165-88.
2. Sayed ARM, Shah NR, Basso KB, Kamat M, Jiao YY, Moya B, et al. First penicillin-binding protein occupancy patterns for 15 beta-lactams and beta-lactamase inhibitors in *Mycobacterium abscessus*. *Antimicrobial Agents and Chemotherapy*. 2021;65(1):13.

In vitro evolution experiment

Minimum inhibitory concentrations (MICs) for the antimicrobial the passaged lines were exposed to was measured after passage 2,4 and 6. The chlorine passaged lines had a >16-fold increase in MIC at passage 6 and the imipenem passaged lines had a significant increase to 16-fold the initial MIC at passage 4 and 32-fold at passage 6. There was no change for the chloramine passaged lines or the control.

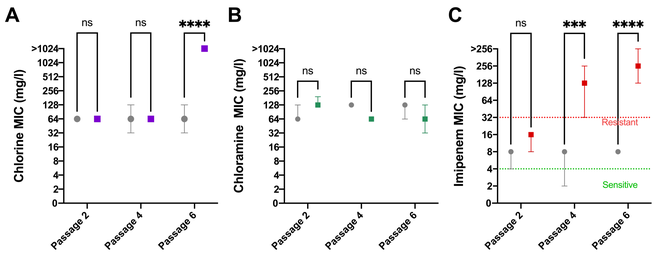

Figure 2: Chlorine, chloramine and imipenem susceptibility of *M. abscessus* passaged lines at subsequent passages. A Chlorine passaged lines. **B** Chloramine passaged lines. **C** Imipenem passaged lines. Grey represents AST of no-antimicrobial passaged lines. All data is represented as the median and range of three passaged lines under each condition. *** P<0.001, **** P<0.0001

After passage 6, further antimicrobial susceptibility testing was conducted to detect potential emergence of multi-drug resistance. The chloramine passaged lines had an increased imipenem MIC (p=0.033) however only one of the lines had an MIC outside the range of the original isolate. The chlorine passaged lines had a significantly decreased amikacin MIC (p=0.0093). The imipenem passaged lines had significantly increased MICs for linezolid and tigecycline. However, they required a longer incubation time due to a slower growth rate and when compared to the original isolate with the same incubation time there was no difference.

Table 1: Antimicrobial susceptibility of *M. abscessus* lines at the conclusion of passage 6. * p<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. Highlighted statistical significance markers are compared to control MIC read at day 3, where imipenem passaged lines were read at day 4.

	Minimum Inhibitory Concentration (mg/l)								
Passaged Line	**LZD**	**IMI**	**FOX**	**AMI**	**TGC**	**CLA (D3)**	**CLA (D14)**	**CL**	**CLM**
Original Isolate median (range) n=5	32 (8-32)	8 (4-16)	32 (32-64)	8 (4-16)	0.5 (0.25-0.5)	0.25 (0.12-0.25)	1 (1-2)	64	128 (64-128)
Control									
	32	8	32	8	0.5	0.12	1	64	64
	16	8	32	16	1	0.12	1	64	128
	16	8	32	4	0.5	0.25	1	128	128
Chlorine				**				****	
	16	8	32	4	0.25	0.12	1	>1024	128
	16	8	32	4	0.5	0.12	1	>1024	128
	32	16	32	4	0.5	0.12	1	>1024	128
Chloramine		*							
	16	32	16	4	0.5	0.12	1	64	128
	16	8	16	8	0.5	0.12	1	32	32
	32	16	32	8	0.5	0.12	1	32	64
Imipenem	***	****			*				
	>32	>64	32	8	1	0.25	1	64	128
	>32	>64	64	8	0.5	0.25	1	64	64
	>32	>64	64	16	1	0.25	1	128	128

LZD = Linezolid, IMI = Imipenem, FOX = Cefoxitin, AMI = Amikacin, DOX = Doxycycline, MIN = Minocycline, CLA (D3) = Clarithromycin at Day 3, CLA (D14) = Clarithromycin at Day 14 (inducible resistance), CL = Chlorine, CLM= Chloramine

Sensitive	Resistant
Intermediate	No interpretive criteria

A 10bp deletion present in the *ponA1* gene was identified in one of the imipenem passaged lines at passage 6. PonA1 is a penicillin binding protein that has been found to be inactivated by imipenem[2] suggesting that this deletion could be a genetic mechanism of imipenem resistance. No differences were found across the core genome for any other of the other sequenced samples compared with the original isolate. Future work will be conducted with the aim of identifying minority variants in the sequence data as DNA was extracted from sweeps of the bacterial population rather than single colonies.

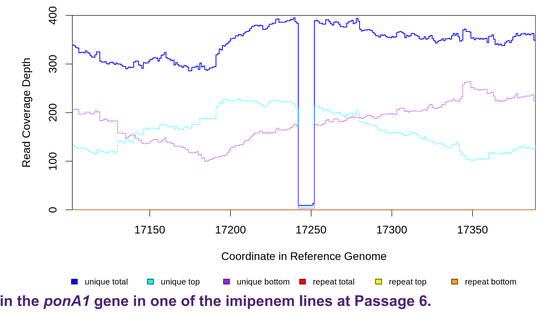

Figure 3: 10dp deletion in the *ponA1* gene in one of the imipenem lines at Passage 6.

Antimicrobial susceptibility testing of isolates

The Mabs isolates from 2021-22 did not have increased antibiotic resistance compared with the 2017-18 or 2007 isolates. There was no significant difference across the three time points except for four 2021-22 isolates that had low MICs for minocycline with three of the four also having low MICs for doxycycline.

Table 2: Antibiotic susceptibility of *M. abscessus* isolated from Brisbane's municipal water in 2007, 2017-2018 and 2021-2022.

		Minimum Inhibitory Concentration (mg/l)								
Year	**Subspecies**	**LZD**	**IMI**	**FOX**	**AMI**	**DOX**	**MIN**	**TGC**	**CLA (D3)**	**CLA (D14)**
2007	*abscessus*	8	8	32	16	>16	>8	0.25	0.12	1
2007	*abscessus*	32	16	32	8	>16	>8	0.5	0.25	2
2007	*abscessus*	4	8	16	4	>16	>8	0.12	0.12	0.5
2017	*massiliense*	8	8	32	16	>16	>8	0.5	0.25	1
2018	*abscessus*	16	8	16	8	>16	>8	0.5	0.25	1
2017	*abscessus*	32	8	32	4	>16	>8	0.5	0.25	1
2017	*abscessus*	16	8	32	4	>16	>8	0.5	0.25	1
2021	*massiliense*	32	8	32	8	1	<1	0.12	0.12	0.5
2021	*massiliense*	16	8	32	8	0.5	<1	0.25	0.12	0.5
2021	*massiliense*	2	16	32	4	4	<1	0.5	0.25	0.25
2021	*abscessus*	32	16	64	8	>16	>8	0.25	0.25	1
2022	*massiliense*	32	16	64	16	16	2	0.5	0.25	1

LZD = Linezolid, IMI = Imipenem, FOX = Cefoxitin, AMI = Amikacin, DOX = Doxycycline, MIN = Minocycline, CLA (D3) = Clarithromycin at Day 3, CLA (D14) = Clarithromycin at Day 14 (inducible resistance)

Sensitive	Resistant
Intermediate	No interpretive criteria

The MICs for chlorine and chloramine did not increase across the three time points. There was no significant difference in chlorine MICs for *M. abscessus* subsp. *abscessus* (Maa) (p>0.9999) or subsp. *massiliense* (Mam) (p>0.9999). For Maa, the chloramine MICs were statistically significantly lower for the isolates from 2021-22 compared to the 2007 isolates (p=0.0012). This may be due to the effect of the single isolate in 2021-22 circled, which was a dominant circulating clone (DCC) 1 isolate compared to all other Maa isolates which were DCC5 isolates. If DCC5 isolates are more chloramine resistant than other Mabs isolates this may explain why DCC5 isolates have been the most commonly isolated from Brisbane's municipal water.

The chloramine MIC was significantly lower than the chlorine MIC for both Maa (p=0.0181) and Mam (p<0.0001). Mam had significantly lower MICs for chloramine than Maa (p=0.0325) where there was no subspecies difference for chlorine (p>0.9999).

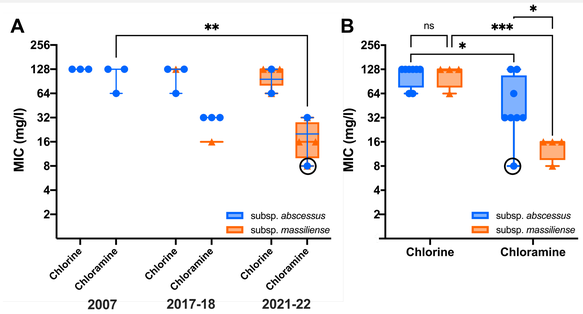

Figure 4: Chlorine and chloramine susceptibility of *M. abscessus* isolated from Brisbane's municipal water in 2007, 2017-2018 and 2021-2022. A Susceptibility over time. **B** Subspecies difference in susceptibility. Circled isolate is a DCC1, where all other Maa isolates were DCC5. * p<0.05, ** P<0.01, *** P<0.001, **** P<0.0001

Conclusions

- Antimicrobial susceptibility of Mabs water isolates did not differ between isolates obtained at the three time points suggesting that increased antimicrobial resistance is not responsible for the increased frequency of isolation.
- The MICs for chlorine and chloramine are much higher than the concentrations used in DWDS thus additional preventative measures should be used to protect against future nosocomial Mabs outbreaks. However, MICs measured in broth my not be reflective of Mabs disinfectant susceptibility in water.
- There are subspecies and potentially dominant circulating clone specific differences in chlorine and chloramine susceptibility that could explain why DCC5 isolates have been the most frequently isolated from Brisbane's water supply.
- Evolution of decreased chlorine and chloramine susceptibility is unlikely to occur in the DWDS due to the concentrations used in the evolution experiment being significantly greater than those found in DWDS.
- Laboratory evolution of imipenem resistance is possible under clinically relevant concentrations of imipenem and does not result in multi-drug resistance.
- A 10bp deletion in the *ponA1* gene may be a genetic mechanism of imipenem resistance.

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