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Carriage and transmission of macrolide resistance genes in patients with chronic respiratory conditions and their close contacts

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SUMMARY CONFLICT OF INTEREST STATEMENTS

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ABSTRACT

Background: Long-term macrolide therapy has been shown to provide benefit to those with a range of chronic respiratory conditions. However, there remain concerns about the impact of macrolide exposure on the carriage and abundance of antibiotic resistance genes within the oropharynx. The potential for onward transmission of resistance from macrolide recipients to their close contacts is also poorly understood.

Research Question: Does long-term macrolide use impact carriage of resistance within the oropharyngeal microbiota in people with chronic respiratory conditions and risk of onward transmission to their close contacts?

Study Design and Methods: Oropharyngeal swabs were collected from 93 individuals with chronic respiratory conditions, of whom 53 were receiving long-term macrolide therapy. An oropharyngeal swab was also collected from a close co-habiting contact of each subject. Detection and abundance of ten macrolide-associated resistance genes with the potential to disseminate via horizontal gene transfer were assessed by quantitative PCR.

Results: Detection of resistance genes in macrolide recipients was comparable to that in non-recipients. However, the normalised gene abundance of *erm*(B) was significantly higher in the macrolide recipient group (p=0.045). In the close contacts, no between-group differences in resistance gene detection or abundance were identified. Within-group analysis showed that the detection of *erm*(F) and *mef* in macrolide recipients, but not non-recipients, was significantly associated with detection in close contacts (p=0.003 and p=0.004 respectively). However, between-group analysis showed that treatment group did not predict co-carriage between patients and their close contacts (p>0.05 for each gene).

Interpretation: While levels of *erm*(B) were higher in those receiving long-term macrolide therapy and there was evidence of gene co-carriage with close contacts, there was no evidence that macrolide use increased the onward transmission risk to their close contacts. This study

therefore addresses concerns that long-term macrolide therapy could promote the dissemination of transmissible macrolide resistance.

KEY WORDS LIST

Antibiotic resistance; Asthma; Azithromycin; Cystic fibrosis; Erythromycin; Macrolides; Non-CF bronchiectasis

ABBREVIATION LIST

CF: cystic fibrosis;

MNR: macrolide non-recipients;

MNRCC: close contact of a macrolide non-recipient;

MR: macrolide-recipients;

MRCC: close contact of a macrolide recipient;

qPCR: quantitative Polymerase Chain Reaction

INTRODUCTION

Macrolide antibiotics have a range of clinical uses. Antibiotics such as azithromycin, erythromycin, clarithromycin, and roxithromycin, are used widely in the treatment of specific respiratory[1,2] and soft-tissue[3] infections. Increasingly, macrolide antibiotics, are also being used in the long-term management of chronic respiratory conditions, including cystic fibrosis,[4-6] asthma,[7-10] chronic obstructive pulmonary disease,[11,12] and bronchiectasis.[13-17] Using macrolides in this way appears to be both safe and effective.[6,7,14] However, the potential for increased carriage and dissemination of macrolide resistance is a concern both for treatment recipients and for the wider community.[17-20]

We hypothesised that the carriage of macrolide resistance genes, and non-macrolide resistance genes under co-selection, would be more frequent within the oropharyngeal microbiota of those receiving long-term macrolide therapy for chronic respiratory conditions compared to macrolide-naïve patients. We further hypothesised that any differences in resistance carriage between recipient and non-recipient patients would be reflected in resistance carriage rates in co-habiting close contacts of patients, consistent with person-to-person transmission.

We report a quantitative PCR (qPCR)-based assessment of oropharyngeal detection and abundance of 10 macrolide-associated antibiotic resistance genes in 93 individuals with chronic respiratory conditions, of whom 53 were receiving long-term macrolide, and their close contacts.

METHODS

Study design and setting

This is a cross-sectional, single time point cohort study. The study was designed to test whether the detection and abundance of macrolide-associated antibiotic resistance gene in the oropharyngeal microbiota differed between individuals with chronic respiratory conditions

who were receiving either long-term macrolide therapy or not (Online Supplement e-Figure 1). In addition, the study was designed to test whether resistance genes detected in individuals with chronic respiratory conditions were detected in close-contacts, and whether long-term macrolide therapy influenced detection in close-contacts. The study was approved by the Mater Health Services Human Resource Ethics Committee (HREC/14/MHS/68) and the Metro South Human Resource Ethics Committee (HREC/15/QPAH/245) in Queensland, Australia. All study participants provided written informed consent. Participants were approached for recruitment at one of three centres: 1) Mater Hospital South Brisbane, Queensland, Queensland, 2) Greenslopes Private Hospital, Queensland, or 3) Concord Repatriation General Hospital New South Wales.

Criteria for participant and close contact recruitment

Patients were considered to be macrolide-recipients (MR) if they were receiving macrolide maintenance therapy (either erythromycin or azithromycin at standard doses for the specific condition) for a chronic respiratory condition for at least 6 months but had not received any other antibiotic class in the previous 4 weeks. Patients were considered to be macrolide non-recipients (MNR) if they had a chronic respiratory condition but had not received macrolide maintenance therapy or any macrolide antibiotic in the prior 6 months and had not received any other antibiotic class in the previous 4 weeks.

Close contacts were defined as someone who was either a close household contact (spouse, de facto, or family members who has lived with the patient for the preceding 6 months) or was a close family member (parent or sibling or partner) or friend who has had close contact with the patient at least twice per week over the preceding 2 years. Full details of the relationship between close contact and patient is provided in Online Supplement e-Table 1. Close contacts were grouped according to the macrolide usage of the patient: a close contact of a macrolide

recipient (MRCC); or a close contact of a macrolide non-recipient (MNRCC). Close contacts were excluded if they had received macrolides in the previous 6 months or had received antibiotics (any class) or hospital treatment in the prior four weeks. Further details of subject inclusion and exclusion criteria are provided in the Online Supplementary Methods.

Sample collection

Collection of oropharyngeal microbiota samples was performed using Copan Amies Transport swabs without charcoal (Interpath Services, Heidelberg West, VIC, AU). Swabs were passed over the tonsils and posterior pharyngeal wall, while avoiding contact with jaws, teeth and gingiva, as described previously by Malhotra-Kumar *et al*.[19] Swabs were stored at -80 °C prior to processing for analysis. Close contact swabs were collected within one week of the index subject (samples were usually collected on the same day).

Sample preparation and DNA extraction

Swabs were heated at 95 °C for 3 min, vortexed in the collection medium for 30 s, and centrifuged at 13,000 × *g* for 5 min at 30 °C. Pellets were resuspended in 400 µL Tris-EDTA buffer and nucleic acid extraction was performed by using ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Irvine, CA, USA), including beat beating at 6.5 m/s on a FastPrep (MP Biomedicals, Irvine, CA, USA) for 5 min, in accordance with the manufacturer's instructions. DNA yield was quantified using a Qubit 2.0 (Invitrogen, Carlsbad, CA, USA).

Resistance gene detection and quantification

Ten antibiotic resistance genes were measured comprising of seven macrolide resistance genes: *erm*(A), *erm*(B), *erm*(C), *erm*(F), *msr*(A), *msr*(E), and *mef,* and three tetracycline resistance genes: *tetM*, *tetO* and *tetW*. Genes were assessed according to two measures: detection and

abundance. Detection was defined as the presence or absence of a gene, within the sensitivity limits of the assay used. Abundance was defined as the amount of a gene detected, relative to the total amount of bacteria within the sample.

SYBR Green-based assays were used to quantify nine genes (*erm*(A), *erm*(B), *erm*(C), *erm*(F), *msr*(A), *msr*(E), *tetM*, *tetO* and *tetW*) and a TaqMan probe-based assay was used to quantify *mef*. The latter TaqMan assay measures has specificity for both *mef*(A) and *mef*(E), which are referred to as *mef* throughout this manuscript. Oligonucleotides used for each reaction, and amplicon sizes are provided in Online Supplement e-Table 2. Detection of amplification signal greater than the lowest positive serial dilution was considered positive. Resistance gene abundance was normalised to the 16S Ct value (Online Supplement e-Table 2) of each sample: ΔCt ($\Delta Ct=Ct_{target\ gene}-Ct_{16S}$) and transformed so a higher value represents higher gene abundance, as described previously.[20] A detailed description of qPCR methods is provided in Online Supplementary Methods.

Statistical analysis

All data analyses were performed using either R software (version 4.1.0) or GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA). Student's *t* test was used to analyse unpaired parametric data. Mann-Whitney U test was utilized to analyse unpaired non-parametric data. Fisher's exact test was employed to analyse proportional data. Correction for multiple comparison testing was performed using the Benjamini-Hochberg method.

Two different binary logistic regression models were employed to assess the propensity for macrolide use to result in transmission of macrolide resistance genes between patients and close contacts. The first, within group comparison, tested the odds of macrolide resistance genes being co-carried between patients and close contacts. The presence or absence of each resistance gene in the patient was the independent variable, while presence or absence in the

close contact was the dependent variable. The outcome of this model provided information on the likelihood of macrolide resistance gene co-carriage between macrolide recipients and their close contacts. The second, between group comparison, assessed whether evidence of potential transmission between patients and close contacts (dependent variable) was associated with treatment group (independent variable). Potential transmission was defined as a resistance gene being detected in both patients and their close contacts (1-1), while no evidence of transmission was defined as gene detection in only patients or close (1-0 or 0-1). Instances where neither patients nor their close contacts carried the resistance gene were excluded from the second model as these cases cannot provide evidence of either transmission or no transmission.

RESULTS

Cohort overview

A total of 149 individuals with chronic respiratory conditions were recruited. Of these, 54 were excluded due to failure to recruit a close contact or to collect all necessary clinical samples and data. A further two patients were excluded due to having received clarithromycin treatment in the 6 months prior (Online Supplement e-Figure 1). Of the close contacts, 92% (86/93) were household contacts and were either family members or partners, with the remaining 7/93 having regular contact of >2 times per week for the prior 2 years (Online Supplement e-Table 1). Baseline demographic and disease characteristics are shown for both patient and close contact groups in Table 1.

Macrolide recipients (MR) compared to macrolide non-recipients (MNR)

The detection (presence/absence) of 10 resistance genes was assessed. Detection rates varied substantially, from 97% detection for *tetM* to 4.3% for *erm*(A) across both MR and MNR

groups. No significant differences were identified in the detection frequency of any resistance gene between MR and MNR (Table 2).

The relative abundance (gene levels normalised to the bacterial 16S gene) of the same 10 genes were also assessed. The relative abundance of *erm*(B), a gene what was detected frequently in both groups (MR 89% and MNR 95%), was significantly higher in MR than MNR (p=0.045; Figure 1). However, this finding was not significant after correction for multiple comparisons and no significant differences were observed in the relative abundance of any other assessed genes (Table 3).

Onward transmission: Macrolide recipient close contacts (MRCC) compared to macrolide non-recipients close contacts (MNRCC)

As a means of investigating onward transmission of macrolide resistance genes, we first compared the detection and abundance of resistance genes between the macrolide recipient close contact (MRCC) and macrolide non-recipients close contacts (MNRCC) (Online Supplement e-Table 3). Similar to MR and MNR groups, detection frequency of macrolide resistance genes in close contacts was variable, ranging from 96% prevalence for *tetM* to 0% for *erm*(A). However, no significant between-group differences in the detection frequency were identified (Online Supplement e-Table 3). The relative abundance of these genes was also compared and were not significantly different between MRCC and MNRCC (Online Supplement e-Table 3).

Onward transmission: Resistance gene detection between patients and their close contacts

To assess whether macrolide resistance gene carriage in close contacts was dependent on carriage in their paired patients, within-group, paired analysis was performed. In the macrolide recipient group, detection of *erm*(F) and *mef* in MRCCs was significantly associated with the

detection of these genes in their matched MR patient (p=0.003 and p=0.004 respectively, Table 4). This association was not observed for any other genes in the macrolide recipient group, nor for any genes in the macrolide non-recipient group (Table 4). Detection of resistance genes in patients and their close contacts is shown in Online Supplement e-Figure 2.

Despite the association of *erm*(F) and *mef* detection between MRCC and MR patients, the relative abundances of these genes were significantly lower in close contacts compared to corresponding MR patients (Figure 2).

Onward transmission: Paired resistance gene detection between macrolide recipient and macrolide non-recipient groups

To further investigate the impact of long-term macrolide therapy on macrolide resistance gene transmission, we compared the risks of patient-close contact macrolide resistance gene transmission between groups (macrolide recipients vs macrolide non-recipients) using our second binary logistic regression model (details see statistical analysis section). The risk of a patient and close contact both carrying a macrolide resistance gene was not significantly higher in macrolide recipients and macrolide non-recipients ($p>0.05$, Table 5). In other words, none of the resistance genes assessed were more likely to be detected in the close contacts of the macrolide recipient group compared to the close contacts of the macrolide non-recipient group.

DISCUSSION

There is a concern that an increased use of macrolide antibiotics in the clinical management of common chronic respiratory diseases could contribute to the growing population-level burden of antibiotic resistance carriage. Our aim was therefore to investigate the impact of long-term macrolide treatment on the carriage of resistance determinants within the oropharyngeal microbiota in patients with chronic respiratory conditions, and potential onward transmission

to close contacts. We found that patients who receive long-term macrolide therapy had higher levels of *erm*(B) compared to patients who were macrolide naïve for at least 6 months. However, analysis of the close contacts revealed macrolide use was not associated with co-carriage of resistance genes in the close contacts. Our cohort consisted of adults with asthma, CF and bronchiectasis, conditions in which long-term macrolide therapy has been shown to confer substantial benefit.[4-7,13-15]

The resistance genes assessed here have been demonstrated to be common determinants of macrolide susceptibility in respiratory bacterial pathogens or to increase in oropharyngeal prevalence with long-term macrolide exposure.[19,21] Seven of the assessed genes confer macrolide resistance via modification of the ribosomal target site (*erm*(A), *erm*(B), *erm*(C), and *erm*(F)), protection of the target site (*msr*(A) and *msr*(E)), or a macrolide efflux pump (*mef*, *msr*(A) and *msr*(E)), and are all associated with mobile genetic elements.[22-27] Three tetracycline resistance genes: *tetM*, *tetO*, and *tetW* that are associated with macrolide exposure,[19,20] and which can be co-located on mobile genetic elements with macrolide resistance genes,[20,22,28,29] were also assessed.

Our investigation highlighted how frequently many of the assessed determinants are carried, both in patients being treated for chronic respiratory conditions and in non-recipients of macrolides. A previous study by Malhotra-Kumar *et al.* reported that ~80% of oropharyngeal streptococci harbour the *mef* gene, despite subjects being healthy and having no macrolide exposure.[19] Indeed, we detected *mef* in 63% of macrolide non-recipient close contacts. We also detected *erm*(B) and *tetM* in a high proportion of macrolide non-recipient close contacts (85%, and 95%, respectively), again, in keeping with the high relative prevalence of these genes in oropharyngeal streptococci, reported previously to be 30% and 40%, respectively.[19] Such high frequency of detection might reflect their common presence on mobile genetic elements,

including conjugative transposons and conjugative plasmids,[30,31] that can move readily between bacterial species.[30,31]

Interestingly, we did identify a significant increase in the relative abundance of *erm*(B) in macrolide recipients compared to non-recipients, supporting the previous data with the increased abundance of *erm*(B) found in the oropharynx of patients with bronchiectasis after 48 weeks of erythromycin therapy.[21] This finding was not significant after correction for multiple comparisons. Importantly, the absence of substantial differences in gene carriage frequency, either between macrolide recipient and non-recipient groups, or between patient groups and their respective close contacts, suggests that, despite the difference in the relative abundance of *erm*(B), chronic macrolide exposure is not associated with noticeably increased carriage frequency of macrolide-associated resistance genes. Several common oropharyngeal bacterial genus have been found to carry *erm*(B), including *Streptococcus*,[32,33] *Haemophilus*,[34] *Gemella*,[35,36] *Staphylococcus*,[35,37] *Enterococcus*,[35] *Neisseria*,[38] and *Campylobacter*.[39] Higher levels of these bacteria that carried *erm*(B) may potentially increase the likelihood of onward transmission, with the risk of such transmission greatest for those living in close physical proximity. However, our comparison of resistance genes frequency between the two close contact groups did not show any evidence of increased resistance gene carriage in macrolide recipient close contacts and is therefore not likely to be of importance.

Nonetheless, to explore the potential risk for onward transmission, we undertook three analyses. Firstly, by comparing close contacts of macrolide recipients and non-recipients, we found that both groups had comparable levels of resistance gene carriage, indicating no increased resistance gene carriage in close contacts of patients receiving macrolides. Secondly, by comparing detection rates within groups, we identified that carrying *erm*(F) or *mef* (not *erm*B as above) by close contacts of macrolide recipients was significantly associated with carriage in their paired patient. Such an association was not observed in macrolide non-recipients,

indicating potential onward transmission. The *erm*(F) and *mef* genes are located on transmissible elements found in *Prevotella* spp. and *Streptococcus* spp., respectively.[40-42] Along with *erm*(B), *mef* is the most common determinant of macrolide resistance in *S. pneumoniae* representing a clinical concern if disseminated within the community. However, subsequent analysis comparing transmission risk between macrolide recipient and non-recipient groups found that macrolide use was not predictive of co-carriage. For example, macrolide use was associated with co-detection of *mef* in 29/53 pairs, while no macrolide use was associated with co-detection in 21/40 pairs. This led to only a 2.2% increase in co-detection events in the macrolide group, which was not significant. Taking all three analyses together, our findings indicate that, while *erm*(F) and *mef* had a significant likelihood of co-carriage between macrolide recipients and their close contacts, there was no indication that close contacts of macrolide recipients had a higher carriage compared to close contacts of macrolide non-recipients, or that macrolide use was significantly associated with this effect. Had we identified evidence that macrolide use was associated with co-detection between patients and close contacts, further studies would be needed to support any conclusion that onward transmission occurred. This is because co-detection is circumstantial, but not definitive evidence of transmission. Studies to provide stronger evidence of transmission would necessitate longitudinally collected samples, with precise methods to detect signatures of transmission, such as strain typing from cultured isolates. However, a lack of co-detection does strongly reflect a lack of onward transmission and support the conclusions of this study.

Our study did have limitations and considerations that should be noted. Macrolide recipient and non-recipient groups differed in their clinical characteristics and treatment exposures, including the former having a higher frequency of hospital admission and a greater burden of acute non-macrolide antibiotics. However, these disparities were not found to influence oropharyngeal carriage of macrolide resistance determinants directly in our study, and patients

had not received non-macrolide antibiotics in the 4 weeks prior to the study. Second, close contacts did not have a chronic lung disease and both patients and close contacts were stable at the time of sample collection. This study therefore does not reflect findings of transmission of antimicrobial resistance between patients during periods of exacerbation, where dissemination of potentially resistant bacteria through the production of cough aerosols has been identified.[43,44] Further, during periods of stability, intact commensal microbial systems, including those in the oropharynx, resist colonisation by external bacterial populations.[45] This protection is greatly reduced when microbiota are disrupted, such as during respiratory viral infection.[46]

Analysis was based on carriage of the main resistance genes associated with macrolide use in the oropharynx; it did not include assessment of phenotypic resistance, resistance in other sites such as the gut, or apply a metagenome-wide assessment of resistance (i.e. resistome analysis). Phenotypic resistance in bacteria of clinical concern, such as streptococci, is strongly associated with the presence of genes included in this study.[47] However, macrolide resistance in other bacteria of clinical concern, such as *Mycoplasma pneumoniae, Legionella pneumophila* and nontuberculous mycobacterium, are attributed to point mutations in ribosomal binding sites, which were not included in this study. While these bacteria are rarely carried asymptomatically in the oropharynx, the findings from this study cannot be extended to resistance attributed to point mutations. Our analysis did not include determination of shifts in microbiota composition and did not attempt to determine whether increases in the relative abundance of individual resistance genes resulted from increases in the size of pre-existing resistant bacterial populations or through the horizontal transfer of resistance determinants to new populations. Resistance conferred by point mutations or amino acid alteration in ribosomal proteins, two common macrolide resistance mechanisms for certain pathogens,[48,49] was not assessed. Finally, it should be noted that this study was performed in Australia, where

community use of macrolides is more restricted compared to other countries such as the U.S.A. While detection of macrolide resistance genes in this cohort were comparable to other studies, these findings should be considered within the context of the rates of macrolide usage and resistance of the region.

In summary, we report that detection of macrolide and macrolide-associated resistance genes is common in the oropharyngeal microbiota, irrespective of long-term macrolide use. In keeping with previous studies, increased abundance of the *erm*(B) gene in patients was associated with long-term macrolide use. Finally, while some resistance genes were significantly co-carried between patients and close contacts in the macrolide recipient group, macrolide use was not associated with increased risk of carriage in close contacts.

INTERPRETATION

This is the first cross-sectional cohort study that report the impact of long-term macrolide therapy on oropharyngeal macrolide resistance gene carriage in healthy close contacts of people with chronic lung diseases. Given long-term macrolide therapy was not associated with increased risk of acquiring macrolide resistance genes in the oropharynx of close contacts, our study addresses concerns that long-term macrolide therapy could promote the dissemination of transmissible macrolide resistance.

TAKE-HOME POINT

Study Question: Does long-term macrolide therapy impact carriage of macrolide resistance in patients with chronic respiratory conditions and does it increase the risk of onward transmission to close contacts?

Results: Macrolide resistance genes were higher in patients receiving long-term macrolide therapy; however, there was no evidence that long-term macrolide use increased the onward transmission risk to their close contacts.

Interpretation: This study provides new evidence to further support existing guidelines for the use of long-term macrolide therapy in people with chronic lung diseases.

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Author's Contributions: The study was conceived by GBR and LDB. LDB, LM, RT, RK, KH were responsible for subject recruitment and sample collection. YW was responsible for sample processing and data generation. YW, SLT, GBR undertook the data analysis. YW, SLT, GBR, drafted the manuscript, with the support of JMC and LEP. All authors approved the final version of the manuscript.

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Table 1. Study population characteristics

Characteristic	MR	MNR	*P* value	MRCC	MNRCC	*P* value
Sample size (N)	53	40		53	40	
Age, years*						
Median (95% CI)	41 (30-53)	60 (34-73)	0.25	53 (47-58)	62 (49-69)	0.54
Sex, n (%)†						
Male	27 (51)	18 (45)	0.68	22 (42)	18 (45)	0.83
Female	26 (49)	22 (55)		31 (58)	22 (55)	
Respiratory condition, n (%)						
Cystic fibrosis	35 (66)	10 (25)	0.0001	0	0	>0.99
Asthma	0	7 (18)	0.002	5 (9)	6 (15)	0.52
Non-CF Bronchiectasis	18 (34)	23 (58)	0.035	0	0	>0.99
Smoking status, n (%)†						
Non-smoker	44 (83)	32 (80)	0.79	35 (66)	21 (53)	0.21
Current smoker	0	0	>0.99	2 (4)	3 (7)	0.65
Ex-smoker	8 (15)	8 (20)	0.59	15 (28)	16 (40)	0.27
Not provided	1 (2)	0	>0.99	1 (2)	0	>0.99
Hospital admission in last 4 weeks, n (%)†	6 (11)	2 (5)	0.46	0	0	>0.99
Macrolide exposure, n (%)†						
Azithromycin	40 (75)	0	<0.0001	0	0	>0.99
Erythromycin	13 (25)	0	0.0004	0	0	>0.99

Abbreviations: MR: patients who were receiving long-term macrolide therapy; MNR: patients who were not receiving any macrolide therapy. MRCC: close contacts of patients who were receiving long-term macrolide therapy; MNRCC close contacts of patients who were not receiving any macrolide therapy. *Median (95% CI), Mann-Whitney U test; †n (%), Fisher's exact test. Respiratory condition categorized by primary diagnosis.

Table 2. Resistance gene detection frequency in patients stratified by macrolide use.

Resistance gene	MR	MNR	*P* values
erm(A)	3.8% (2/53)	5.0% (2/40)	0.99
erm(B)	89% (47/53)	95% (38/40)	0.46
erm(C)	19% (10/53)	13% (5/40)	0.57
erm(F)	68% (36/53)	78% (31/40)	0.36
mef	74% (39/53)	83% (33/40)	0.33
msr(A)	36% (19/53)	25% (10/40)	0.37
msr(E)	66% (35/53)	73% (29/40)	0.65
tetM	94% (50/53)	100% (40/40)	0.26
tetO	64% (34/53)	78% (31/40)	0.18
tetW	85% (45/53)	98% (39/40)	0.07

Abbreviations: MR: patients who were receiving long-term macrolide therapy; MNR patients who were not receiving any macrolide therapy. *P* value determined by Fisher's exact test.

Table 3. Normalised resistance gene abundance in patients stratified by macrolide use.

Resistance gene	MR	MNR	*P* values
erm(A)	0 (0.0-10.5)	0 (0.0-7.1)	0.39
erm(B)	7.5 (0.0-12.4)	6.9 (0.0-10.8)	0.045*
erm(C)	0 (0.0-13.2)	0 (0.0-8.0)	0.14
erm(F)	7.6 (0.0-12.4)	6.2 (0.0-11.9)	0.22
mef	4.4 (0.0-6.7)	3.9 (0.0-7.5)	0.20
msr(A)	0 (0.0-13.2)	0 (0.0-9.1)	0.15
msr(E)	7.3 (0.0-13.0)	5.7 (0.0-15.9)	0.07
tetM	5.9 (0.0-8.9)	5.5 (2.1-7.6)	0.42
tetO	6.4 (0.0-10.5)	6.3 (0.0-12.3)	0.39
tetW	5.2 (2.7)	4.8 (2.2)	0.46

Abbreviations: MR: patients who were receiving long-term macrolide therapy; MNR patients who were not receiving any macrolide therapy. Resistance gene abundance was normalised to the 16S Ct value of each sample. Data presented as median (min-max) or mean (SD) depending on data distribution. *P* values were determined by Mann-Whitney U test or Student's t test with Welch's correction. * P >0.05 after correction for multiple comparisons.

Table 4. Paired assessment of the resistance gene detection frequency between patients and close contacts stratified by macrolide use.

Resistance gene	MR vs MRCC		MNR vs MNRCC	
	Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)	*P* value
erm(B)	3.4 (0.5-22.9)	0.21	1.3×10^{-7} (0-Inf)	>0.99
erm(C)	5.1 (0.6-41.9)	0.13	8.5 (0.4-163.9)	0.16
erm(F)	11.8 (2.3-59.6)	0.0029*	1.7 (0.4-7.6)	0.50
mef	7.3 (1.9-28.4)	0.0044*	1.3 (0.3-6.9)	0.75
msr(A)	1.5 (0.5-4.9)	0.48	1.8 (0.4-8.2)	0.43
msr(E)	0.8 (0.3-2.7)	0.74	1.1 (0.3-4.5)	0.87
tetM	2.1×10^{-7} (0-Inf)	>0.99	N/A	N/A
tetO	2.7 (0.8-8.5)	0.099	1.7 (0.4-7.6)	0.50
tetW	2.4 (0.5-12.0)	0.29	2.5×10^{-7} (0-Inf)	>0.99

Abbreviations: MR: patients who were receiving long-term macrolide therapy; MNR patients who were not receiving any macrolide therapy. CC: close contacts. *P* value determined by binary logistic regression. Analyses could not be performed for *tetM* of the macrolide non-recipient group and for *erm*(A) (both groups) due to no variance between groups. * P value remained <0.05 after correction for multiple comparisons.

Table 5. Assessment of long-term macrolide use on onward transmission risk of macrolide resistance genes.

Resistance gene	Macrolide recipient group vs Macrolide non-recipient group	
	Odds ratio (95% CI)	***P* value**
erm(A)	1.0 (0-Inf)	>0.99
erm(B)	1.0 (0.4-2.9)	0.96
erm(C)	1.0 (0.07-13.9)	>0.99
erm(F)	1.0 (0.4-2.5)	0.97
mef	1.6 (0.6-3.9)	0.33
msr(A)	1.3 (0.3-5.0)	0.73
msr(E)	0.6 (0.2-1.5)	0.25
tetM	0.5 (0.09-2.7)	0.43
tetO	0.9 (0.4-2.2)	0.82
tetW	0.7 (0.3-2.0)	0.55

P value determined by binary logistic regression. Pairs with resistance genes being undetected in both patients and the close contacts are excluded from the analysis as the presence of those pairs in the analysis do not provide any clinical transmission information and will impact the accuracy of the results.

FIGURE LEGENDS

Figure 1. Comparison of *erm*(B) abundance in macrolide recipients and macrolide non-recipients.

erm(B) abundance normalised to bacterial 16S copies. Bars show mean and standard deviation; significance calculated by Mann-Whitney U test. *p<0.05

Figure 2. Comparison of *erm*(F) and *mef* abundance between patient/close contact pairs.

Gene levels normalised to bacterial 16S copies. Significance of gene abundance calculated by Wilcoxon matched-pairs signed rank test.

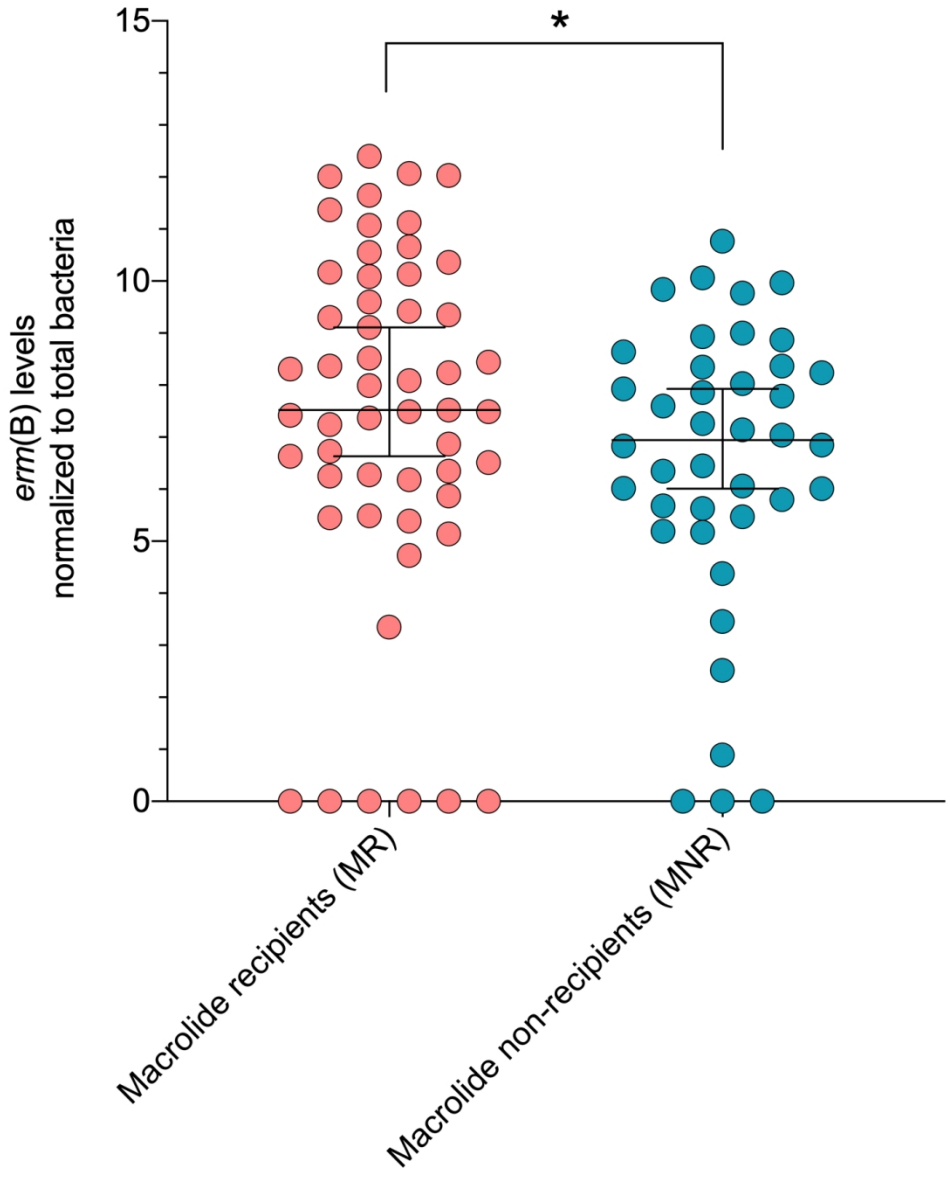

Figure 1. Comparison of erm(B) abundance in macrolide recipients and macrolide non-recipients.

125x154mm (300 x 300 DPI)

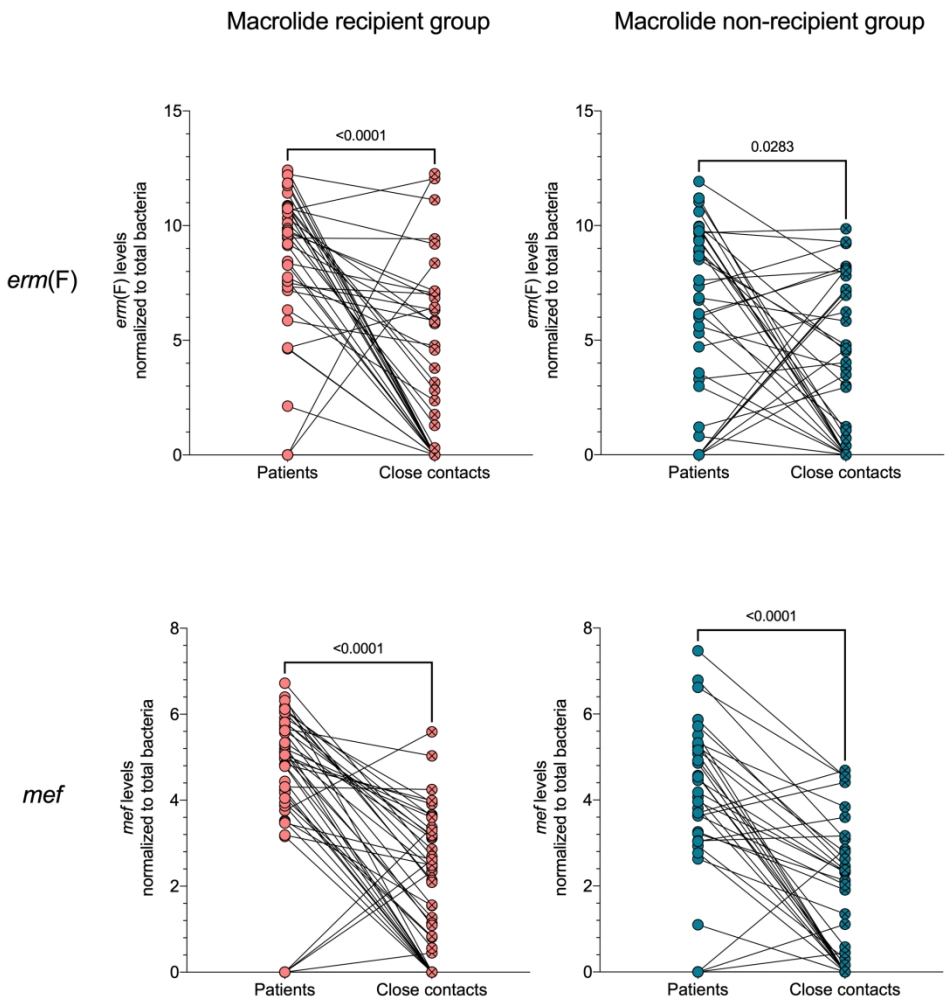

Figure 2. Comparison of erm(F) and mef abundance between patient/close contact pairs.

203x206mm (300 x 300 DPI)

ONLINE SUPPLEMENT

TITLE

Carriage and transmission of macrolide resistance genes in patients with chronic respiratory conditions and their close contacts

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SUPPLEMENTARY METHODS

Inclusion and exclusion criteria for subject recruitment

Macrolide recipient (MR)

- Inclusion criteria
    1. Age: 18 and above
    2. Must be able to provide written consent to participate
    3. Has at least one chronic lung disease including cystic fibrosis, asthma, or bronchiectasis
    4. Has been on azithromycin or erythromycin maintenance therapy for at least the preceding 6 months
- Exclusion criteria
    1. Unable to provide oropharyngeal swab sample for any reason
    2. Unable to accurately demonstrate / recall antibiotic exposure
    3. Prior non-macrolide antibiotic exposure (including clindamycin) in the prior 4 weeks

Macrolide non-recipient (MNR)

- Inclusion criteria
    1. Age: 18 and above
    2. Must be able to provide written consent to participate
    3. Has at least one chronic lung disease including cystic fibrosis, asthma, or bronchiectasis
    4. Is not currently on azithromycin/erythromycin maintenance therapy and has not received any macrolide antibiotics in the prior 6 months
- Exclusion criteria

1. Unable to provide oropharyngeal swab sample for any reason
2. Unable to accurately demonstrate / recall antibiotic exposure
3. Prior non-macrolide antibiotic exposure (including clindamycin) in the prior 4 weeks

Macrolide recipient close contacts (MRCC) and macrolide non-recipients close contacts (MNRCC)

- Inclusion criteria
  1. Must be able to provide written consent to participate
  2. Is either a close household contact (Spouse, *de facto* or family members) who has lived with the patient for the immediate proceeding period at least 6 months or was a close family member (parent or sibling or partner) or friend who has had close contact with the patient over the immediate proceeding period of 2 years, as defined by at least 2 times a week.
  3. Have not received any antibiotics (any class) in the prior 4 weeks
  4. Has not received any macrolide in the prior 6 months
  5. No chronic respiratory disease, excepting well-controlled asthma
  6. Has not been treated as a patient in hospital in the past 4 weeks
- Exclusion criteria
  1. Unable to provide oropharyngeal swab sample for any reason
  2. Unable to accurately demonstrate / recall antibiotic exposure
  3. Prior non-macrolide antibiotic exposure (including clindamycin) in the prior 4 weeks

Quantitation of total bacterial load, resistance gene carriage

Quantitative PCR (qPCR) assays were employed to quantify the total bacterial load (16S qPCR), detect resistance genes, and quantify abundance of resistance genes in each sample. The SYBR Green method was employed for measuring CT values of six macrolide resistance genes [*erm*(A), *erm*(B), *erm*(C), *erm*(F), *msr*(A), and *msr*(E)] and three tetracycline resistance genes [*tetM*, *tetO*, and *tetW*]. A Taqman assay was used for assessing CT values of the macrolide resistance gene *mef*. Each reaction using the SYBR Green method was prepared with 17.5 µL 2×PowerUp SYBGreen Master Mix (Applied Biosystems, Foster City, CA, United States), 15.1 µL of Nuclease-Free water, 0.7 µL of 10 µM forward primer, 0.7 µl of 10 µM reverse primer, mixed with 1 µl of DNA template. Each reaction using the Taqman method was prepared with 17.5 µL 2×Kappa Fast probe low rox (KAPA Biosystems, Woburn, MA, United States), 14.05 µL of Nuclease-Free water, 0.875 µL of 10 µM forward primer, 0.875 µl of 10 µM reverse primer, 0.7 µL of µM probe, mixed with 1 µl of DNA template. The annealing temperature of 16S rRNA gene, *erm*(A), *erm*(B), *erm*(C), *erm*(F), *msr*(A), *msr*(*E*), *tetM* and *tetW* gene was 60 °C. The annealing temperature of *mef* and *tetO* gene was 62 °C.

Amplification efficiency and detection limits were determined for each resistance gene using serial dilutions of a positive control (DNA from a bacterial isolate confirmed to harbour the resistance gene) as previously described.

e-Table 1. Relationship between patient and close contact

Contact levels	Proportion	Relationship types	Proportion
Household contact (>6 months)	86/93	Family members (Parent, siblings and children)	28/86
		Partner, spouse or *de facto*	58/86
		Friend	0/86
Regular contact (>2 times per week in the last 2 years)	7/93	Family members (Parent, siblings and children)	3/7
		Partner, spouse or *de facto*	1/7
		Friend	3/7

e-Table 2. Quantitative PCR primers and probes

Gene	Detection limit (Ct value)	Primer	Amplicon size (bp)	Reference
16S	27.50	F: 5'-TCCTACGGGAGGCAGCAGT-3' R: 5'-GGACTACCAGGGTATCTAATCCTGTT-3'	467	(E1)
***erm*(A)**	34.26	F: 5'-TCAGTTACTGCTATAGAAATTGATGGAG-3' R: 5'-ATACAGAGTCTACACTTGGCTTAGG-3'	358	(E2)
***erm*(B)**	34.64	F: 5'-GAAAGCCRTGCGTCTGACATC-3' R: 5'-CGAGACTTGAGTGTGCAAGAGC-3'	105	(E3)
***erm*(C)**	33.40	F: 5'-CTTGTTGATCACGATAATTTCC-3' R: 5'-ATCTTTTAGCAAACCCGTATTC-3'	190	(E4)
***erm*(F)**	32.59	F: 5'-CGGGTCAGCACTTTACTATTG-3' R: 5'-GGACCTACCTCATAGACAAG-3'	466	(E5, E6)
***msr*(A)**	34.04	F: 5'-TCCAATCATTGCACAAAATCTAAC-3' R: 5'-TCAATTCCCTCTATTTGGTGGT-3'	165	(E4)
***msr*(E)**	36.74	F: 5'-TCGATACGAAGAGGCGATGC-3' R: 5'-CTTCTGTTTGGTGCCGGTTG-3'	163	(E7)
tetM	33.82	F: 5'-CAGAATTAGGAAGCGTGGACAA-3' R: 5'-CCTCTCTGACGTTCTAAAAGCGTAT-3'	67	(E8)
tetO	31.96	F: 5'-AACTTAGGCATTCTGGCTCAC-3' R: 5'-TCCCACTGTTCCATATCGTCA-3'	515	(E3)
tetW	31.31	F: 5'-GAGAGCCTGCTATATGCCAGC-3' R: 5'-GGGCGTATCCACAATGTTAAC-3'	168	(E9)
mef	37.14	F: 5'-TATGGAGCTACCTGTCTGGA-3' R: 5'-GGTACTAAAAGTGGCGTAACC-3' Probe: HEX-CCGTAGCATTGGAACAGCTTTTC-BHQ1	85	(E10)

F, forward primer; R, reverse primer; cycle number,40;

e-Table 3. Resistance gene detection frequency and relative abundance in close contacts of macrolide recipient and macrolide non-recipients.

Gene	Detection limit (Ct value)	% of close contact carried the gene		*P* values	Normalised gene levels		*P* values
		MRCC	MNRCC		MRCC	MNRCC	
***erm*(A)**	34.26	0% (0/53)	0% (0/40)	0.99	0 (0.0-0.0)	0 (0.0-0.0)	0.50
***erm*(B)**	34.64	85% (45/53)	85% (34/40)	0.99	3.4 (0.0-10.8)	4.2 (0.0-10.1)	0.48
***erm*(C)**	33.40	7.5% (4/53)	5.0% (2/40)	0.70	0 (0.0-11.1)	0 (0.0-9.9)	0.42
***erm*(F)**	32.59	45% (24/53)	65% (26/40)	0.09	0 (0.0-12.3)	3.3 (0.0-9.9)	0.065
mef	37.14	62% (33/53)	63% (25/40)	0.58	1.3 (0.0-5.6)	1.2 (0.0-4.7)	0.38
***msr*(A)**	34.04	36% (19/53)	30% (12/40)	0.66	0 (0.0-12.7)	0 (0.0-9.1)	0.26
***msr*(E)**	36.74	36% (19/53)	48% (19/40)	0.29	0 (0.0-13.5)	0 (0.0-19.7)	0.25
tetM	33.82	96% (51/53)	95% (38/40)	0.99	4.9 (2.3)	4.4 (1.9)	0.12
tetO	31.96	62% (33/53)	65% (26/40)	0.83	4.0 (0.0-10.0)	4.9 (0.0-10.1)	0.41
tetW	31.31	77% (41/53)	80% (32/40)	0.80	4.7 (0.0-11.3)	5.0 (0.0-8.9)	0.47

Abbreviations: MRCC: close contacts of patients who were receiving long-term macrolide therapy; MNRCC close contacts of patients who were not receiving any macrolide therapy. *P* value for detection frequency was determined by Fisher's exact test; *P* values for gene levels comparisons were determined by Mann-Whitney U test (non-parametric data, one-tailed test) and unpaired Student's t test with Welch's correction (parametric data, one-tailed test). Close contact defined as either a close household contact (Spouse, defacto or family members) who has lived with the patient for the immediate proceeding period at least 6 months or is a close family member (parent or sibling or partner) or friend who has had close contact with the patient over the immediate proceeding period of 2 years, as defined by at least 2 times a week.

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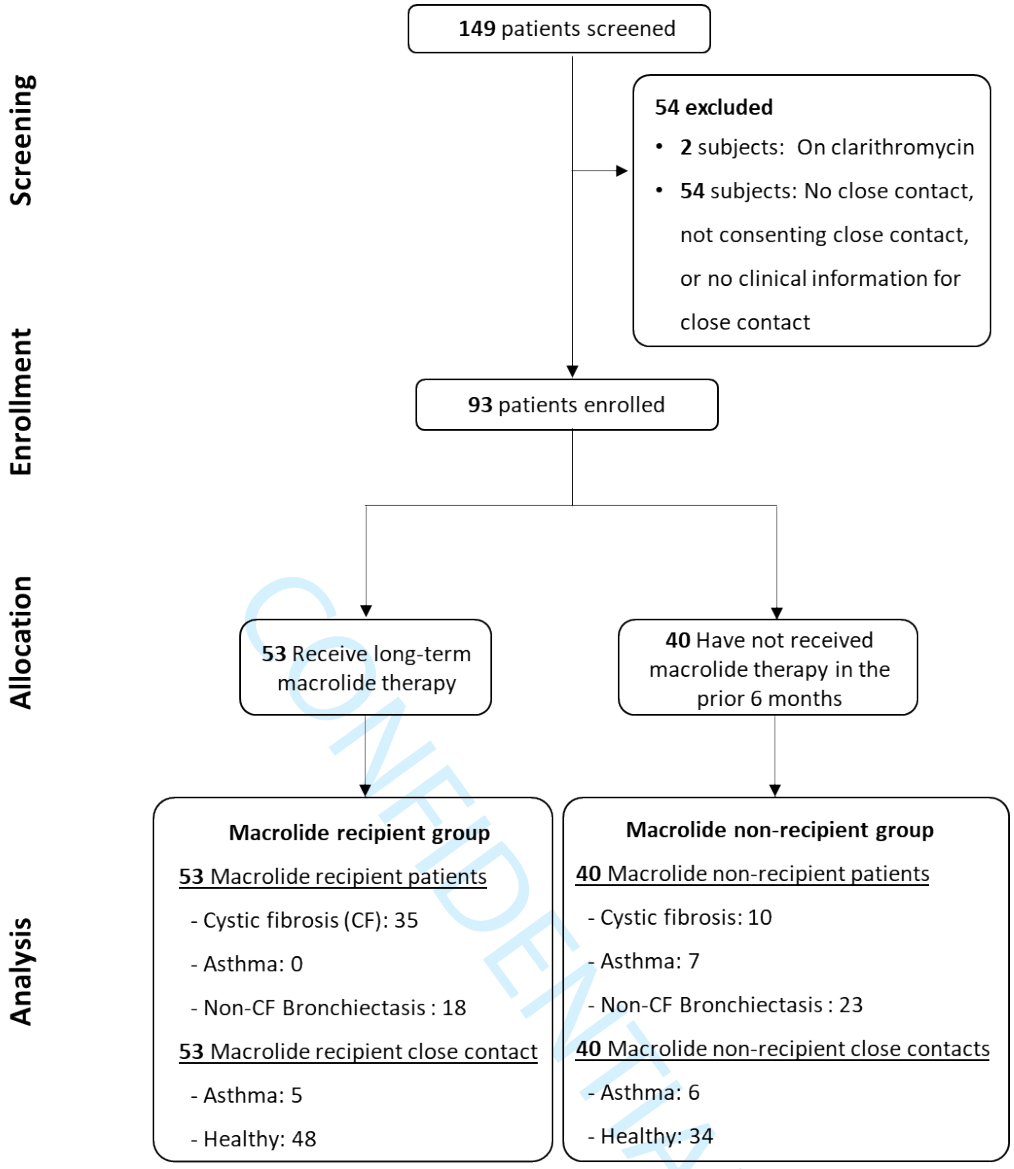
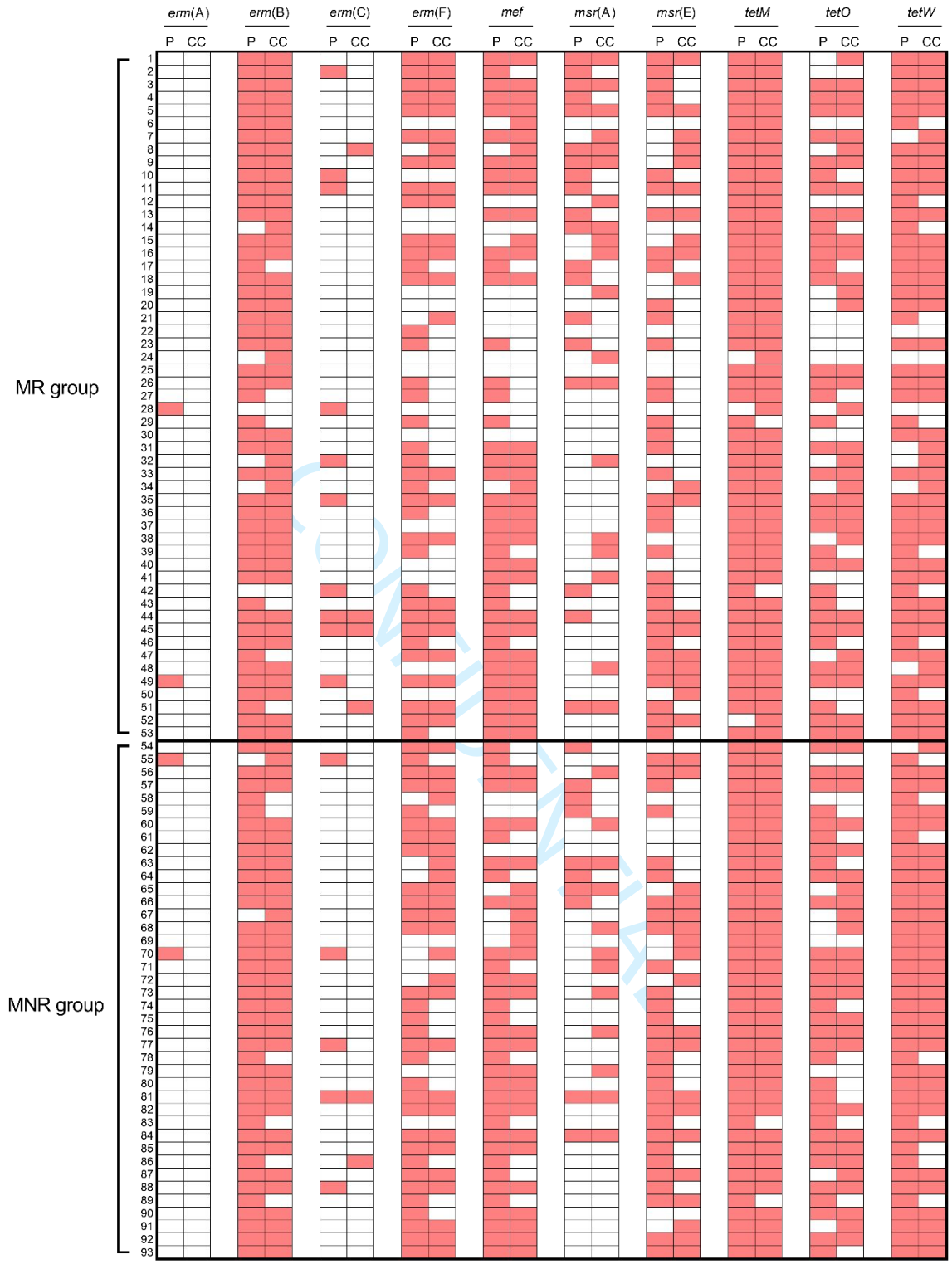


e-Figure 1. Study design

Subjects were recruited by respiratory physicians. Macrolide recipients: patients who receive macrolide maintenance therapy; Macrolide non-recipients: patients who have not received macrolide maintenance therapy in at least 6 months; Close contact defined as either a close household contact (Spouse, *de facto* or family members) who has lived with the patient for the immediate proceeding period at least 6 months or is a close family member (parent or sibling or partner) or friend who has had close contact with the patient over the immediate proceeding period of 2 years, as defined by at least 2 times a week. All patients were categorized based on their primary diagnosis.



e-Figure 2. Resistance gene presence/absence map.

Gene detection (red) determined by a qPCR amplification signal greater than the lowest positive serial dilution; P: patients; CC: paired close contact of patient; MR group: Macrolide recipient group; MNR group: Macrolide non-recipient group

Carriage and transmission of macrolide resistance genes in patients with chronic respiratory conditions and their close contacts

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SUMMARY CONFLICT OF INTEREST STATEMENTS

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ABSTRACT

Background: Long-term macrolide therapy has been shown to provide benefit to those with a range of chronic respiratory conditions. However, there remain concerns about the impact of macrolide exposure on the carriage and abundance of antibiotic resistance genes within the oropharynx. The potential for onward transmission of resistance from macrolide recipients to their close contacts is also poorly understood.

Research Question: Does long-term macrolide use impact carriage of resistance within the oropharyngeal microbiota in people with chronic respiratory conditions and risk of onward transmission to their close contacts?

Study Design and Methods: Oropharyngeal swabs were collected from 93 individuals with chronic respiratory conditions, of whom 53 were receiving long-term macrolide therapy. An oropharyngeal swab was also collected from a close co-habiting contact of each subject. Detection and abundance of ten macrolide-associated resistance genes with the potential to disseminate via horizontal gene transfer were assessed by quantitative PCR.

Results: Detection of resistance genes in macrolide recipients was comparable to that in non-recipients. However, the normalised gene abundance of *erm*(B) was significantly higher in the macrolide recipient group (p=0.045). In the close contacts, no between-group differences in resistance gene detection or abundance were identified. Within-group analysis showed that the detection of *erm*(F) and *mef* in macrolide recipients, but not non-recipients, was significantly associated with detection in close contacts (p=0.003 and p=0.004 respectively). However, between-group analysis showed that treatment group did not predict co-carriage between patients and their close contacts (p>0.05 for each gene).

Interpretation: While levels of *erm*(B) were higher in those receiving long-term macrolide therapy and there was evidence of gene co-carriage with close contacts, there was no evidence that macrolide use increased the onward transmission risk to their close contacts. This study

therefore addresses concerns that long-term macrolide therapy could promote the dissemination of transmissible macrolide resistance.

KEY WORDS LIST

Antibiotic resistance; Asthma; Azithromycin; Cystic fibrosis; Erythromycin; Macrolides; Non-CF bronchiectasis

ABBREVIATION LIST

CF: cystic fibrosis;

MNR: macrolide non-recipients;

MNRCC: close contact of a macrolide non-recipient;

MR: macrolide-recipients;

MRCC: close contact of a macrolide recipient;

qPCR: quantitative Polymerase Chain Reaction

INTRODUCTION

Macrolide antibiotics have a range of clinical uses. Antibiotics such as azithromycin, erythromycin, clarithromycin, and roxithromycin, are used widely in the treatment of specific respiratory[1,2] and soft-tissue[3] infections. Increasingly, macrolide antibiotics, are also being used in the long-term management of chronic respiratory conditions, including cystic fibrosis,[4-6] asthma,[7-10] chronic obstructive pulmonary disease,[11,12] and bronchiectasis.[13-17] Using macrolides in this way appears to be both safe and effective.[6,7,14] However, the potential for increased carriage and dissemination of macrolide resistance is a concern both for treatment recipients and for the wider community.[17-20]

We hypothesised that the carriage of macrolide resistance genes, and non-macrolide resistance genes under co-selection, would be more frequent within the oropharyngeal microbiota of those receiving long-term macrolide therapy for chronic respiratory conditions compared to macrolide-naïve patients. We further hypothesised that any differences in resistance carriage between recipient and non-recipient patients would be reflected in resistance carriage rates in co-habiting close contacts of patients, consistent with person-to-person transmission.

We report a quantitative PCR (qPCR)-based assessment of oropharyngeal detection and abundance of 10 macrolide-associated antibiotic resistance genes in 93 individuals with chronic respiratory conditions, of whom 53 were receiving long-term macrolide, and their close contacts.

METHODS

Study design and setting

This is a cross-sectional, single time point cohort study. The study was designed to test whether the detection and abundance of macrolide-associated antibiotic resistance gene in the oropharyngeal microbiota differed between individuals with chronic respiratory conditions

who were receiving either long-term macrolide therapy or not (Online Supplement e-Figure 1). In addition, the study was designed to test whether resistance genes detected in individuals with chronic respiratory conditions were detected in close-contacts, and whether long-term macrolide therapy influenced detection in close-contacts. The study was approved by the Mater Health Services Human Resource Ethics Committee (HREC/14/MHS/68) and the Metro South Human Resource Ethics Committee (HREC/15/QPAH/245) in Queensland, Australia. All study participants provided written informed consent. Participants were approached for recruitment at one of three centres: 1) Mater Hospital South Brisbane, Queensland, Queensland, 2) Greenslopes Private Hospital, Queensland, or 3) Concord Repatriation General Hospital New South Wales.

Criteria for participant and close contact recruitment

Patients were considered to be macrolide-recipients (MR) if they were receiving macrolide maintenance therapy (either erythromycin or azithromycin at standard doses for the specific condition) for a chronic respiratory condition for at least 6 months but had not received any other antibiotic class in the previous 4 weeks. Patients were considered to be macrolide non-recipients (MNR) if they had a chronic respiratory condition but had not received macrolide maintenance therapy or any macrolide antibiotic in the prior 6 months and had not received any other antibiotic class in the previous 4 weeks.

Close contacts were defined as someone who was either a close household contact (spouse, de facto, or family members who has lived with the patient for the preceding 6 months) or was a close family member (parent or sibling or partner) or friend who has had close contact with the patient at least twice per week over the preceding 2 years. Full details of the relationship between close contact and patient is provided in Online Supplement e-Table 1. Close contacts were grouped according to the macrolide usage of the patient: a close contact of a macrolide

recipient (MRCC); or a close contact of a macrolide non-recipient (MNRCC). Close contacts were excluded if they had received macrolides in the previous 6 months or had received antibiotics (any class) or hospital treatment in the prior four weeks. Further details of subject inclusion and exclusion criteria are provided in the Online Supplementary Methods.

Sample collection

Collection of oropharyngeal microbiota samples was performed using Copan Amies Transport swabs without charcoal (Interpath Services, Heidelberg West, VIC, AU). Swabs were passed over the tonsils and posterior pharyngeal wall, while avoiding contact with jaws, teeth and gingiva, as described previously by Malhotra-Kumar *et al*.[19] Swabs were stored at -80 °C prior to processing for analysis. Close contact swabs were collected within one week of the index subject (samples were usually collected on the same day).

Sample preparation and DNA extraction

Swabs were heated at 95 °C for 3 min, vortexed in the collection medium for 30 s, and centrifuged at 13,000 × *g* for 5 min at 30 °C. Pellets were resuspended in 400 µL Tris-EDTA buffer and nucleic acid extraction was performed by using ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Irvine, CA, USA), including beat beating at 6.5 m/s on a FastPrep (MP Biomedicals, Irvine, CA, USA) for 5 min, in accordance with the manufacturer's instructions. DNA yield was quantified using a Qubit 2.0 (Invitrogen, Carlsbad, CA, USA).

Resistance gene detection and quantification

Ten antibiotic resistance genes were measured comprising of seven macrolide resistance genes: *erm*(A), *erm*(B), *erm*(C), *erm*(F), *msr*(A), *msr*(E), and *mef,* and three tetracycline resistance genes: *tetM*, *tetO* and *tetW*. Genes were assessed according to two measures: detection and

abundance. Detection was defined as the presence or absence of a gene, within the sensitivity limits of the assay used. Abundance was defined as the amount of a gene detected, relative to the total amount of bacteria within the sample.

SYBR Green-based assays were used to quantify nine genes (*erm*(A), *erm*(B), *erm*(C), *erm*(F), *msr*(A), *msr*(E), *tetM*, *tetO* and *tetW*) and a TaqMan probe-based assay was used to quantify *mef*. The latter TaqMan assay measures has specificity for both *mef*(A) and *mef*(E), which are referred to as *mef* throughout this manuscript. Oligonucleotides used for each reaction, and amplicon sizes are provided in Online Supplement e-Table 2. Detection of amplification signal greater than the lowest positive serial dilution was considered positive. Resistance gene abundance was normalised to the 16S Ct value (Online Supplement e-Table 2) of each sample: ΔCt ($\Delta Ct=Ct_{target\ gene}-Ct_{16S}$) and transformed so a higher value represents higher gene abundance, as described previously.[20] A detailed description of qPCR methods is provided in Online Supplementary Methods.

Statistical analysis

All data analyses were performed using either R software (version 4.1.0) or GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA). Student's *t* test was used to analyse unpaired parametric data. Mann-Whitney U test was utilized to analyse unpaired non-parametric data. Fisher's exact test was employed to analyse proportional data. Correction for multiple comparison testing was performed using the Benjamini-Hochberg method.

Two different binary logistic regression models were employed to assess the propensity for macrolide use to result in transmission of macrolide resistance genes between patients and close contacts. The first, within group comparison, tested the odds of macrolide resistance genes being co-carried between patients and close contacts. The presence or absence of each resistance gene in the patient was the independent variable, while presence or absence in the

close contact was the dependent variable. The outcome of this model provided information on the likelihood of macrolide resistance gene co-carriage between macrolide recipients and their close contacts. The second, between group comparison, assessed whether evidence of potential transmission between patients and close contacts (dependent variable) was associated with treatment group (independent variable). Potential transmission was defined as a resistance gene being detected in both patients and their close contacts (1-1), while no evidence of transmission was defined as gene detection in only patients or close (1-0 or 0-1). Instances where neither patients nor their close contacts carried the resistance gene were excluded from the second model as these cases cannot provide evidence of either transmission or no transmission.

RESULTS

Cohort overview

A total of 149 individuals with chronic respiratory conditions were recruited. Of these, 54 were excluded due to failure to recruit a close contact or to collect all necessary clinical samples and data. A further two patients were excluded due to having received clarithromycin treatment in the 6 months prior (Online Supplement e-Figure 1). Of the close contacts, 92% (86/93) were household contacts and were either family members or partners, with the remaining 7/93 having regular contact of >2 times per week for the prior 2 years (Online Supplement e-Table 1). Baseline demographic and disease characteristics are shown for both patient and close contact groups in Table 1.

Macrolide recipients (MR) compared to macrolide non-recipients (MNR)

The detection (presence/absence) of 10 resistance genes was assessed. Detection rates varied substantially, from 97% detection for *tetM* to 4.3% for *erm*(A) across both MR and MNR

groups. No significant differences were identified in the detection frequency of any resistance gene between MR and MNR (Table 2).

The relative abundance (gene levels normalised to the bacterial 16S gene) of the same 10 genes were also assessed. The relative abundance of *erm*(B), a gene what was detected frequently in both groups (MR 89% and MNR 95%), was significantly higher in MR than MNR (p=0.045; Figure 1). However, this finding was not significant after correction for multiple comparisons and no significant differences were observed in the relative abundance of any other assessed genes (Table 3).

Onward transmission: Macrolide recipient close contacts (MRCC) compared to macrolide non-recipients close contacts (MNRCC)

As a means of investigating onward transmission of macrolide resistance genes, we first compared the detection and abundance of resistance genes between the macrolide recipient close contact (MRCC) and macrolide non-recipients close contacts (MNRCC) (Online Supplement e-Table 3). Similar to MR and MNR groups, detection frequency of macrolide resistance genes in close contacts was variable, ranging from 96% prevalence for *tetM* to 0% for *erm*(A). However, no significant between-group differences in the detection frequency were identified (Online Supplement e-Table 3). The relative abundance of these genes was also compared and were not significantly different between MRCC and MNRCC (Online Supplement e-Table 3).

Onward transmission: Resistance gene detection between patients and their close contacts

To assess whether macrolide resistance gene carriage in close contacts was dependent on carriage in their paired patients, within-group, paired analysis was performed. In the macrolide recipient group, detection of *erm*(F) and *mef* in MRCCs was significantly associated with the

detection of these genes in their matched MR patient (p=0.003 and p=0.004 respectively, Table 4). This association was not observed for any other genes in the macrolide recipient group, nor for any genes in the macrolide non-recipient group (Table 4). Detection of resistance genes in patients and their close contacts is shown in Online Supplement e-Figure 2.

Despite the association of *erm*(F) and *mef* detection between MRCC and MR patients, the relative abundances of these genes were significantly lower in close contacts compared to corresponding MR patients (Figure 2).

Onward transmission: Paired resistance gene detection between macrolide recipient and macrolide non-recipient groups

To further investigate the impact of long-term macrolide therapy on macrolide resistance gene transmission, we compared the risks of patient-close contact macrolide resistance gene transmission between groups (macrolide recipients vs macrolide non-recipients) using our second binary logistic regression model (details see statistical analysis section). The risk of a patient and close contact both carrying a macrolide resistance gene was not significantly higher in macrolide recipients and macrolide non-recipients (p>0.05, Table 5). In other words, none of the resistance genes assessed were more likely to be detected in the close contacts of the macrolide recipient group compared to the close contacts of the macrolide non-recipient group.

DISCUSSION

There is a concern that an increased use of macrolide antibiotics in the clinical management of common chronic respiratory diseases could contribute to the growing population-level burden of antibiotic resistance carriage. Our aim was therefore to investigate the impact of long-term macrolide treatment on the carriage of resistance determinants within the oropharyngeal microbiota in patients with chronic respiratory conditions, and potential onward transmission

to close contacts. We found that patients who receive long-term macrolide therapy had higher levels of *erm*(B) compared to patients who were macrolide naïve for at least 6 months. However, analysis of the close contacts revealed macrolide use was not associated with co-carriage of resistance genes in the close contacts. Our cohort consisted of adults with asthma, CF and bronchiectasis, conditions in which long-term macrolide therapy has been shown to confer substantial benefit.[4-7,13-15]

The resistance genes assessed here have been demonstrated to be common determinants of macrolide susceptibility in respiratory bacterial pathogens or to increase in oropharyngeal prevalence with long-term macrolide exposure.[19,21] Seven of the assessed genes confer macrolide resistance via modification of the ribosomal target site (*erm*(A), *erm*(B), *erm*(C), and *erm*(F)), protection of the target site (*msr*(A) and *msr*(E)), or a macrolide efflux pump (*mef*, *msr*(A) and *msr*(E)), and are all associated with mobile genetic elements.[22-27] Three tetracycline resistance genes: *tetM*, *tetO*, and *tetW* that are associated with macrolide exposure,[19,20] and which can be co-located on mobile genetic elements with macrolide resistance genes,[20,22,28,29] were also assessed.

Our investigation highlighted how frequently many of the assessed determinants are carried, both in patients being treated for chronic respiratory conditions and in non-recipients of macrolides. A previous study by Malhotra-Kumar *et al.* reported that ~80% of oropharyngeal streptococci harbour the *mef* gene, despite subjects being healthy and having no macrolide exposure.[19] Indeed, we detected *mef* in 63% of macrolide non-recipient close contacts. We also detected *erm*(B) and *tetM* in a high proportion of macrolide non-recipient close contacts (85%, and 95%, respectively), again, in keeping with the high relative prevalence of these genes in oropharyngeal streptococci, reported previously to be 30% and 40%, respectively.[19] Such high frequency of detection might reflect their common presence on mobile genetic elements,

including conjugative transposons and conjugative plasmids,[30,31] that can move readily between bacterial species.[30,31]

Interestingly, we did identify a significant increase in the relative abundance of *erm*(B) in macrolide recipients compared to non-recipients, supporting the previous data with the increased abundance of *erm*(B) found in the oropharynx of patients with bronchiectasis after 48 weeks of erythromycin therapy.[21] This finding was not significant after correction for multiple comparisons. Importantly, the absence of substantial differences in gene carriage frequency, either between macrolide recipient and non-recipient groups, or between patient groups and their respective close contacts, suggests that, despite the difference in the relative abundance of *erm*(B), chronic macrolide exposure is not associated with noticeably increased carriage frequency of macrolide-associated resistance genes. Several common oropharyngeal bacterial genus have been found to carry *erm*(B), including *Streptococcus*,[32,33] *Haemophilus*,[34] *Gemella*,[35,36] *Staphylococcus*,[35,37] *Enterococcus*,[35] *Neisseria*,[38] and *Campylobacter*.[39] Higher levels of these bacteria that carried *erm*(B) may potentially increase the likelihood of onward transmission, with the risk of such transmission greatest for those living in close physical proximity. However, our comparison of resistance genes frequency between the two close contact groups did not show any evidence of increased resistance gene carriage in macrolide recipient close contacts and is therefore not likely to be of importance.

Nonetheless, to explore the potential risk for onward transmission, we undertook three analyses. Firstly, by comparing close contacts of macrolide recipients and non-recipients, we found that both groups had comparable levels of resistance gene carriage, indicating no increased resistance gene carriage in close contacts of patients receiving macrolides. Secondly, by comparing detection rates within groups, we identified that carrying *erm*(F) or *mef* (not *erm*B as above) by close contacts of macrolide recipients was significantly associated with carriage in their paired patient. Such an association was not observed in macrolide non-recipients,

indicating potential onward transmission. The *erm*(F) and *mef* genes are located on transmissible elements found in *Prevotella* spp. and *Streptococcus* spp., respectively.[40-42] Along with *erm*(B), *mef* is the most common determinant of macrolide resistance in *S. pneumoniae* representing a clinical concern if disseminated within the community. However, subsequent analysis comparing transmission risk between macrolide recipient and non-recipient groups found that macrolide use was not predictive of co-carriage. For example, macrolide use was associated with co-detection of *mef* in 29/53 pairs, while no macrolide use was associated with co-detection in 21/40 pairs. This led to only a 2.2% increase in co-detection events in the macrolide group, which was not significant. Taking all three analyses together, our findings indicate that, while *erm*(F) and *mef* had a significant likelihood of co-carriage between macrolide recipients and their close contacts, there was no indication that close contacts of macrolide recipients had a higher carriage compared to close contacts of macrolide non-recipients, or that macrolide use was significantly associated with this effect. Had we identified evidence that macrolide use was associated with co-detection between patients and close contacts, further studies would be needed to support any conclusion that onward transmission occurred. This is because co-detection is circumstantial, but not definitive evidence of transmission. Studies to provide stronger evidence of transmission would necessitate longitudinally collected samples, with precise methods to detect signatures of transmission, such as strain typing from cultured isolates. However, a lack of co-detection does strongly reflect a lack of onward transmission and support the conclusions of this study.

Our study did have limitations and considerations that should be noted. Macrolide recipient and non-recipient groups differed in their clinical characteristics and treatment exposures, including the former having a higher frequency of hospital admission and a greater burden of acute non-macrolide antibiotics. However, these disparities were not found to influence oropharyngeal carriage of macrolide resistance determinants directly in our study, and patients

had not received non-macrolide antibiotics in the 4 weeks prior to the study. Second, close contacts did not have a chronic lung disease and both patients and close contacts were stable at the time of sample collection. This study therefore does not reflect findings of transmission of antimicrobial resistance between patients during periods of exacerbation, where dissemination of potentially resistant bacteria through the production of cough aerosols has been identified.[43,44] Further, during periods of stability, intact commensal microbial systems, including those in the oropharynx, resist colonisation by external bacterial populations.[45] This protection is greatly reduced when microbiota are disrupted, such as during respiratory viral infection.[46]

Analysis was based on carriage of the main resistance genes associated with macrolide use in the oropharynx; it did not include assessment of phenotypic resistance, resistance in other sites such as the gut, or apply a metagenome-wide assessment of resistance (i.e. resistome analysis). Phenotypic resistance in bacteria of clinical concern, such as streptococci, is strongly associated with the presence of genes included in this study.[47] However, macrolide resistance in other bacteria of clinical concern, such as *Mycoplasma pneumoniae, Legionella pneumophila* and nontuberculous mycobacterium, are attributed to point mutations in ribosomal binding sites, which were not included in this study. While these bacteria are rarely carried asymptomatically in the oropharynx, the findings from this study cannot be extended to resistance attributed to point mutations. Our analysis did not include determination of shifts in microbiota composition and did not attempt to determine whether increases in the relative abundance of individual resistance genes resulted from increases in the size of pre-existing resistant bacterial populations or through the horizontal transfer of resistance determinants to new populations. Resistance conferred by point mutations or amino acid alteration in ribosomal proteins, two common macrolide resistance mechanisms for certain pathogens,[48,49] was not assessed. Finally, it should be noted that this study was performed in Australia, where

community use of macrolides is more restricted compared to other countries such as the U.S.A. While detection of macrolide resistance genes in this cohort were comparable to other studies, these findings should be considered within the context of the rates of macrolide usage and resistance of the region.

In summary, we report that detection of macrolide and macrolide-associated resistance genes is common in the oropharyngeal microbiota, irrespective of long-term macrolide use. In keeping with previous studies, increased abundance of the *erm*(B) gene in patients was associated with long-term macrolide use. Finally, while some resistance genes were significantly co-carried between patients and close contacts in the macrolide recipient group, macrolide use was not associated with increased risk of carriage in close contacts.

INTERPRETATION

This is the first cross-sectional cohort study that report the impact of long-term macrolide therapy on oropharyngeal macrolide resistance gene carriage in healthy close contacts of people with chronic lung diseases. Given long-term macrolide therapy was not associated with increased risk of acquiring macrolide resistance genes in the oropharynx of close contacts, our study addresses concerns that long-term macrolide therapy could promote the dissemination of transmissible macrolide resistance.

TAKE-HOME POINT

Study Question: Does long-term macrolide therapy impact carriage of macrolide resistance in patients with chronic respiratory conditions and does it increase the risk of onward transmission to close contacts?

Results: Macrolide resistance genes were higher in patients receiving long-term macrolide therapy; however, there was no evidence that long-term macrolide use increased the onward transmission risk to their close contacts.

Interpretation: This study provides new evidence to further support existing guidelines for the use of long-term macrolide therapy in people with chronic lung diseases.

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Guarantor: Dr. Steven Taylor is the author responsible for the content of the manuscript.

Author's Contributions: The study was conceived by GBR and LDB. LDB, LM, RT, RK, KH were responsible for subject recruitment and sample collection. YW was responsible for sample processing and data generation. YW, SLT, GBR undertook the data analysis. YW, SLT, GBR, drafted the manuscript, with the support of JMC and LEP. All authors approved the final version of the manuscript.

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Table 1. Study population characteristics

Characteristic	MR	MNR	*P* value	MRCC	MNRCC	*P* value
Sample size (N)	53	40		53	40	
Age, years*						
Median (95% CI)	41 (30-53)	60 (34-73)	0.25	53 (47-58)	62 (49-69)	0.54
Sex, n (%)†						
Male	27 (51)	18 (45)	0.68	22 (42)	18 (45)	0.83
Female	26 (49)	22 (55)		31 (58)	22 (55)	
Respiratory condition, n (%)						
Cystic fibrosis	35 (66)	10 (25)	0.0001	0	0	>0.99
Asthma	0	7 (18)	0.002	5 (9)	6 (15)	0.52
Non-CF Bronchiectasis	18 (34)	23 (58)	0.035	0	0	>0.99
Smoking status, n (%)†						
Non-smoker	44 (83)	32 (80)	0.79	35 (66)	21 (53)	0.21
Current smoker	0	0	>0.99	2 (4)	3 (7)	0.65
Ex-smoker	8 (15)	8 (20)	0.59	15 (28)	16 (40)	0.27
Not provided	1 (2)	0	>0.99	1 (2)	0	>0.99
Hospital admission in last 4 weeks, n (%)†	6 (11)	2 (5)	0.46	0	0	>0.99
Macrolide exposure, n (%)†						
Azithromycin	40 (75)	0	<0.0001	0	0	>0.99
Erythromycin	13 (25)	0	0.0004	0	0	>0.99

Abbreviations: MR: patients who were receiving long-term macrolide therapy; MNR: patients who were not receiving any macrolide therapy. MRCC: close contacts of patients who were receiving long-term macrolide therapy; MNRCC close contacts of patients who were not receiving any macrolide therapy. *Median (95% CI), Mann-Whitney U test; †n (%), Fisher's exact test. Respiratory condition categorized by primary diagnosis.

Table 2. Resistance gene detection frequency in patients stratified by macrolide use.

Resistance gene	MR	MNR	*P* values
erm(A)	3.8% (2/53)	5.0% (2/40)	0.99
erm(B)	89% (47/53)	95% (38/40)	0.46
erm(C)	19% (10/53)	13% (5/40)	0.57
erm(F)	68% (36/53)	78% (31/40)	0.36
mef	74% (39/53)	83% (33/40)	0.33
msr(A)	36% (19/53)	25% (10/40)	0.37
msr(E)	66% (35/53)	73% (29/40)	0.65
tetM	94% (50/53)	100% (40/40)	0.26
tetO	64% (34/53)	78% (31/40)	0.18
tetW	85% (45/53)	98% (39/40)	0.07

Abbreviations: MR: patients who were receiving long-term macrolide therapy; MNR patients who were not receiving any macrolide therapy. *P* value determined by Fisher's exact test.

Table 3. Normalised resistance gene abundance in patients stratified by macrolide use.

Resistance gene	MR	MNR	*P* values
erm(A)	0 (0.0-10.5)	0 (0.0-7.1)	0.39
erm(B)	7.5 (0.0-12.4)	6.9 (0.0-10.8)	0.045*
erm(C)	0 (0.0-13.2)	0 (0.0-8.0)	0.14
erm(F)	7.6 (0.0-12.4)	6.2 (0.0-11.9)	0.22
mef	4.4 (0.0-6.7)	3.9 (0.0-7.5)	0.20
msr(A)	0 (0.0-13.2)	0 (0.0-9.1)	0.15
msr(E)	7.3 (0.0-13.0)	5.7 (0.0-15.9)	0.07
tetM	5.9 (0.0-8.9)	5.5 (2.1-7.6)	0.42
tetO	6.4 (0.0-10.5)	6.3 (0.0-12.3)	0.39
tetW	5.2 (2.7)	4.8 (2.2)	0.46

Abbreviations: MR: patients who were receiving long-term macrolide therapy; MNR patients who were not receiving any macrolide therapy. Resistance gene abundance was normalised to the 16S Ct value of each sample. Data presented as median (min-max) or mean (SD) depending on data distribution. *P* values were determined by Mann-Whitney U test or Student's t test with Welch's correction. * P >0.05 after correction for multiple comparisons.

Table 4. Paired assessment of the resistance gene detection frequency between patients and close contacts stratified by macrolide use.

Resistance gene	MR vs MRCC		MNR vs MNRCC	
	Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)	*P* value
erm(B)	3.4 (0.5-22.9)	0.21	1.3×10^{-7} (0-Inf)	>0.99
erm(C)	5.1 (0.6-41.9)	0.13	8.5 (0.4-163.9)	0.16
erm(F)	11.8 (2.3-59.6)	0.0029*	1.7 (0.4-7.6)	0.50
mef	7.3 (1.9-28.4)	0.0044*	1.3 (0.3-6.9)	0.75
msr(A)	1.5 (0.5-4.9)	0.48	1.8 (0.4-8.2)	0.43
msr(E)	0.8 (0.3-2.7)	0.74	1.1 (0.3-4.5)	0.87
tetM	2.1×10^{-7} (0-Inf)	>0.99	N/A	N/A
tetO	2.7 (0.8-8.5)	0.099	1.7 (0.4-7.6)	0.50
tetW	2.4 (0.5-12.0)	0.29	2.5×10^{-7} (0-Inf)	>0.99

Abbreviations: MR: patients who were receiving long-term macrolide therapy; MNR patients who were not receiving any macrolide therapy. CC: close contacts. *P* value determined by binary logistic regression. Analyses could not be performed for *tetM* of the macrolide non-recipient group and for *erm*(A) (both groups) due to no variance between groups. * P value remained <0.05 after correction for multiple comparisons.

Table 5. Assessment of long-term macrolide use on onward transmission risk of macrolide resistance genes.

Resistance gene	Macrolide recipient group vs Macrolide non-recipient group	
	Odds ratio (95% CI)	***P* value**
erm(A)	1.0 (0-Inf)	>0.99
erm(B)	1.0 (0.4-2.9)	0.96
erm(C)	1.0 (0.07-13.9)	>0.99
erm(F)	1.0 (0.4-2.5)	0.97
mef	1.6 (0.6-3.9)	0.33
msr(A)	1.3 (0.3-5.0)	0.73
msr(E)	0.6 (0.2-1.5)	0.25
tetM	0.5 (0.09-2.7)	0.43
tetO	0.9 (0.4-2.2)	0.82
tetW	0.7 (0.3-2.0)	0.55

P value determined by binary logistic regression. Pairs with resistance genes being undetected in both patients and the close contacts are excluded from the analysis as the presence of those pairs in the analysis do not provide any clinical transmission information and will impact the accuracy of the results.

FIGURE LEGENDS

Figure 1. Comparison of *erm*(B) abundance in macrolide recipients and macrolide non-recipients.

erm(B) abundance normalised to bacterial 16S copies. Bars show mean and standard deviation; significance calculated by Mann-Whitney U test. *p<0.05

Figure 2. Comparison of *erm*(F) and *mef* abundance between patient/close contact pairs.

Gene levels normalised to bacterial 16S copies. Significance of gene abundance calculated by Wilcoxon matched-pairs signed rank test.