

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

We would like to sincerely thank the Chest Editorial Team, the three external reviewers, and the statistical reviewer for the detailed evaluation of our manuscript. We provide a point-to-point response (black text) to each of the reviewer comments (*blue italic text*) below. Where changes have been made to the submitted manuscript, they have been tracked in red.

Reviewer 1:

With great interest I've read this really interesting paper on macrolide resistance genes. The authors have written a very good paper. The research has some important limitation, but the authors have all discussed them in the "discussion" session. Therefore, I have only a few less important remarks:

1. Do the authors have data or literature to support the statement in the Methods that a 6 month "wash-out" is enough to go back to a "baseline" situation?

We defined participants as macrolide naïve if they had not received any macrolide therapy in the 6 months prior to enrolment, a position that was based on the available literature. Specifically, Malhotra-Kumar *et al.* 2007, showed that levels of macrolide resistance genes had returned to their baseline values at 6 months post treatment (PMID: 17292768). This report was further supported by a meta-analysis undertaken by Costelloe *et al.*, 2010 (PMID: 20483949).

2. The authors have a 4-week wash-out for other antibiotics but how about e.g. clindamycin (Lincosamides) in this erm setting

We can confirm that no participants (patients or close contacts) were exposed to lincosamides during the 4-week period prior to sample collection. This has been clarified in the main text Methods section with the addition of the sub-heading "Criteria for participant and close contact recruitment" (see main manuscript: page 6-7, lines 115-132).

3. In the cohort overview results, the authors mention a 12-month interval used for macrolide as an exclusion but in the methods it's 6 months. Could the authors explain this discrepancy?

We apologise for this error. To clarify, all patients in the macrolide non-recipient group had not received any antibiotic in the 6 months prior to sample collection. We have now revised the manuscript to reflect this in the Method subheading "Criteria for participant and close contact recruitment."

4. Could the authors give more detail on the relationship between patients and controls? Are they siblings or spouses or others? This might influence contact. I imagine that transmission risk is different between a friend you see frequently or your sibling or your partner... Do the authors have details and could this have influenced the data (e.g. more similarity between partner vs friends?)

The reviewer raises an important issue on the relationship between the index patient and their close contact. We defined a close contact as either 1) a household contact (cohabitant) who lived with the patient for the proceeding 6 months or 2) a regular contact, being a family member (parent or children or sibling or partner) or friend who has had close contact with the patient more than 2 times per week over proceeding 2 years. Given that there was no existing standard definition, we set out our working definition in the study protocol.

In the table below (Table R1), we provide information on the nature of relationships between patients and close contacts. This table is now included as Online Supplement e-Table 1 in the revised manuscript. Of note, 92% (86/93) of close contacts were household contacts and were either family members or partners.

While the level of interaction between individuals is likely to influence AMR transmission risk, our study was not designed to investigate these relationships in detail. However, for the reviewer's interest, we have performed exploratory sub-analysis, only including household contacts (e.g. excluding the 7 contacts that do not share the same household. In summary, the results remained unchanged, with macrolide therapy associated with significantly higher *erm*(B) in patients, and evidence of co-carriage within the macrolide arm for *erm*(F) and *mef*.

In acknowledgement of the importance of contact between participant pairs, we have now included the relationship between pairs in the Online Supplement e-Table 1, as well as highlight these relationships in the Methods and Results sections (see main manuscript: page 6, line 127 and page 9, lines 194-197).

Table R1. 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 <i>de facto</i>	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 <i>de facto</i>	1/7
		Friend	3/7

Reviewer 2:

Wang and colleagues seek to assess whether long-term macrolide therapy poses a risk for onward transmission of resistance genes in patients with chronic respiratory disease and their co-inhabitants. Though limited in scope (focusing on a select number of macrolides + tetracycline genes by qPCR), I can't fault the technical execution of the molecular methods presented. I have some concerns about the experimental design, analysis, and conclusions.

1. That the relative abundance of ermB is higher in MR and MNR group. However, they also tested 9 additional resistance genes (multiple hypothesis testing) and I don't see where this has been adjusted for. With adjustment, significance will probably be lost.

Our initial analysis was performed without adjustment. However, in Table R2, below, we provide revised outcome tables that included corrections for false discovery (Benjamini and Hochberg method). As these data show, differences in *erm*(B) levels between MR and MNR group was not significant following FDR adjustment. We have amended the Methods, Results, and Discussion to reflect this (page 8, line 172-173; page 10, line 208; page 13, lines 282-283).

It is important to again highlight that the novelty of our study lies in the detection and quantification of AMR genes in healthy close contacts and the macrolide effects on onward transmission risk. The non-significance of *erm*(B) differences between MR and MNR group after FDR correction therefore does not affect our principal outcomes substantially. Our main finding, that long-term macrolide use was not significantly associated with detection of macrolide resistance genes in close contacts, remains true after correction for multiple testing. We agree that indicating whether statistical findings were significant both before and after FDR correction provides the greatest clarity and have revised all results to reflect this.

Table R2. Normalised resistance gene abundance in patients stratified by macrolide use.

Resistance gene	MR	MNR	<i>P</i> value	Corrected <i>P</i> value
<i>erm</i> (A)	0 (0.0-10.5)	0 (0.0-7.1)	0.39	0.46
<i>erm</i> (B)	7.5 (0.0-12.4)	6.9 (0.0-10.8)	0.045	0.35
<i>erm</i> (C)	0 (0.0-13.2)	0 (0.0-8.0)	0.14	0.37
<i>erm</i> (F)	7.6 (0.0-12.4)	6.2 (0.0-11.9)	0.22	0.37
<i>mef</i>	4.4 (0.0-6.7)	3.9 (0.0-7.5)	0.20	0.37
<i>msr</i> (A)	0 (0.0-13.2)	0 (0.0-9.1)	0.15	0.37
<i>msr</i> (E)	7.3 (0.0-13.0)	5.7 (0.0-15.9)	0.07	0.35
<i>tetM</i>	5.9 (0.0-8.9)	5.5 (2.1-7.6)	0.42	0.46
<i>tetO</i>	6.4 (0.0-10.5)	6.3 (0.0-12.3)	0.39	0.46
<i>tetW</i>	5.2(2.7)	4.8(2.2)	0.46	0.46

2. *“Onward transmission” of resistance genes, i.e. (that is) increased relative risk of resistance gene co-detection in co-habitants of patients on long-term macrolide therapy. However, onward transmission is somewhat speculative since it is not really possible to establish “transmission” of a resistance gene by the methodology employed. A patient could have acquired the resistance gene from their co-inhabitant initially for example. We are really looking at the effect of “household antibiotic use” on risk of resistance detection in untreated individuals. The word transmission is misleading. One would need to isolate a resistant organism from both patient and co-inhabitant and test this by strain typing or WGS analysis (as a start).*

We agree with the reviewer regarding the challenge of distinguishing between co-detection and transmission. We were careful to frame our assessments as being based on measures that are consistent with transmission and therefore support the potential for its occurrence, rather than saying definitively whether transmission had occurred. It is important to note that, while co-detection does not necessarily indicate that transmission has occurred, the absence of co-detection strongly suggests that it has not. Given our findings show that macrolide use was not a significant predictor of resistance gene co-detection between patients and close contacts, we feel that our conclusion that macrolide therapy was not associated with evidence of onward transmission is accurate.

As the reviewer notes, demonstrating transmission is extremely challenging, particularly when based on cross-sectional data. In our revision, we highlight that the methodology of this study allows us to test whether onward transmission has not occurred. We further add that, had we identified transmission, further longitudinal studies would be needed to support evidence of transmission. This is included in page 14, lines 314-321 and pasted below.

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.

3. *“Carriage and transmission of resistance genes” For macrolides, this is an incredibly difficult question to address by the applied methodology given the apparently high prevalence of macrolide resistance in the environment/microbiome. Macrolide and Tetracycline resistance genes are frequently the most highly detected resistance genes reported in resistome studies. Thus, even healthy individuals may harbour these genes anyway, perhaps through maintenance via other, as yet unrecognised, selective pressures.*

We agree that macrolide and tetracycline resistance genes are highly prevalent in both environmental and human microbiome datasets. Certainly, had they been detected in all, or almost all, of the samples assessed here, our ability to draw meaningful conclusions would have been substantially reduced. Such high detection frequency was observed for *tetM* and *tetW*, detected in 179/186 and 157/186 samples, respectively, in line with that reported in other studies (PMID: 17292768, 30875247). Similarly, genes rarely detected would have necessitated a much higher sample size to detect evidence of transmission. However, the detection of key macrolide resistance genes were prevalent, but not ubiquitous, allowing us to test the effect of macrolide use on detection in patients, abundance in patients, and co-detection with close contacts. We now highlight these considerations in our revised manuscript (see main manuscript: page 12, lines 268-275 and copied below).

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

4. There seem to be many unanswered questions that the study fails to address. Just looking at a few resistance genes at a single anatomical site is somewhat limited in scope considering the complex effects of antibiotics on the microbiome/resistome. That “long-term term antibiotic macrolide therapy was not associated with increased risk of acquiring macrolide resistance genes”. A major shortcoming is that only macrolide/tetracycline resistance was assessed.

We agree, the relationships between antibiotic exposure, commensal microbiota, and the dispersion of resistance determinants, is extremely complex and that limiting assessment to macrolide/tetracycline resistance determinants represents a limitation of this study (as noted in our discussion section). We selected these macrolide and tetracycline genes for a number of reasons: 1) All genes were derived from previous metagenomic assessment we (and others) have undertaken in the context of long-term macrolide use was used to select these genes (PMID: 30875247, 30151191, 32320621). 2) An *a priori* defined list of genes enabled a more focused investigation of the association of these genes with macrolide use. As each gene has been previously shown to be selected for with macrolide use, investigating onward transmission associated with macrolide use could be more targeted. 3) These genes are not only found in opportunistic respiratory pathogens such as *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae* and *Moraxella catarrhalis*, but are also located on

mobile genetic elements that can facilitate horizontal transmission. For example, *erm*(A), *erm*(C) and *msr*(A) are found in *S. aureus* across three separate mobile genetic elements (PMID: 20668911, 9809423), while *erm*(B) and *mef*(A) are found on Tn2010 and Tn1207.1 in *S. pneumoniae* (PMID: 27709102, 15328112).

With respect to our study's focus on a single anatomical site, the reviewer is correct in that assessment of multiple sites may provide a greater depth of analysis and potentially identify evidence that macrolide use influences gene co-detection between patients and close contacts. We selected oropharyngeal swabs as the oropharynx represents a site with high potential for transmission of antimicrobial resistance genes and respiratory pathogens. We, and others, have further shown that macrolide use influences carriage of resistance genes in the OP (PMID: 29669883, 17292768).

In response to the reviewer's comments, we have included discussion of the use of targeted assays to assess resistance gene carriage and the use of oropharyngeal swabs (page 15, lines 336-338).

5. Macrolide exposure can co-select for other resistance genes which may be an even greater concern (thinking of MDR plasmids etc.).

We agree that macrolide exposure can co-select for other resistance genes, including those carried on MDR plasmids. Indeed, this was the rationale for examining levels of tetracycline resistance genes. For example, *tetM* is found on the same mobile genetic element Tn916 as *erm*(B) in erythromycin-resistant viridans group *streptococci* (PMID: 7648031; PMID: 15328112). As another example, *tetO* and *mef* were both identified on a Tn1207.1-like transposon in *Streptococcus pyogenes* clinical isolates (PMID: 15563518, PMID:15837373). We discuss this important point in page 12 lines 259-267.

6. Further, the lung microbiome is relatively stable under antibiotic treatment and the gut is less so. The impact of macrolides on the gut may be much more relevant in terms of potential transmission of resistance. I understand that the focus is on the airway but what happens in the gut seldom stays in the gut, where resistance is concerned.

We agree with the reviewer's observations regarding the relative impact of antibiotic exposure on respiratory and gut microbiota, and recognise that the gut represents an important potential route of onward transmission. Indeed, a study by Dessein *et al.* showed that oral vancomycin had minimal effect on lung microbiota alpha diversity in mice, while the stool microbiota was substantially altered (PMID: 33076936). We have also shown that the faecal resistome of individuals with cystic fibrosis acts as a reservoir of aminoglycoside resistance, reflecting the high tobramycin use in CF individuals (PMID: 33250435). However, our decision to measure the impact of macrolide therapy on carriage and co-detection of resistance within the oropharynx was based on a number of considerations. Firstly, the

effect of macrolides on the oropharyngeal microbiota and AMR carriage is widely acknowledged (PMID: 29669883, 17292768, 20483949), providing a sound basis to extend this analysis and test whether co-detection within close contacts was also apparent. Secondly, the oropharynx represents a site with high potential for transmission between patients and close contact. For example, a study in army residents indicated that the number of members of a household was an independent risk factor for oropharyngeal carriage of *S. pneumoniae* (PMID: 21676361). Thirdly, alterations to the oropharyngeal microbiome have clinical implications for respiratory tract infections. This was nicely demonstrated by Man *et al.*, where disruption to the upper respiratory tract microbiome was predictive of subsequent lower respiratory tract infection (PMID: 30885620).

In response to this comment, we note that this study does not reflect changes in AMR carriage within the entire human microbiome; in particular, it does not include assessment within the gut (page 15, lines 337-345).

7. In summary, the authors assess the impact of antibiotic therapy on macrolide/tetracycline resistance gene carriage/transmission in the airway and fail to detect a convincing signal that might suggest this is a real clinical problem. However, the study completely overlooks both other (non-macrolide/tetracycline) resistance mechanisms as well as the selective pressures placed on the intestinal microbiome and the risk of transmission of resistant gut pathogens. The authors have not sufficiently assessed this element and I'm concerned that it conveys a false message e.g. "macrolide selection is probably a non-issue". Antibiotic selection in the gut (initially on commensal species) likely preceded the global dissemination of cephalosporinase resistance genes (i.e. CTX-M-15), for example. The effect of antibiotic selection in the airway and corresponding impact on AMR dissemination has yet to be conclusively investigated. Thus, not looking at the gut, in the context of an AMR study is an oversight if onward transmission is the focus. Further the narrow range of analysis (10 genes) in the lung is a concern. Many other relevant resistance events could have been missed.

We thank the reviewer for their summary and have addressed each point in the respective sections above.

Reviewer 3:

The authors address the issue of whether long term macrolide therapy increases macrolide resistance and whether macrolide resistance increases in close contacts. There are a number of papers showing increased macrolide resistance and resistance genes with macrolide use, so the novelty lies in the issue of whether this spread.

1. I am not sure that clinical readers will understand the difference between gene detection and gene abundance, and perhaps some mention of qualitative vs quantitative or presence/absence vs total amount is appropriate for readers not familiar with microbiome work.

Assessment of both AMR gene “detection” and the “abundance” of AMR genes are important aspects of our analysis, and we feel that these are the most succinct and clear terms to use. However, we also recognise that not all readers will be familiar with this terminology and we therefore now provide these definitions in full at their first use within the text (see below) and include in page 7-8, lines 153-156.

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.

*2. For a clinical journal I think you need to put your data into a clinical context - what does all this mean in terms of clinically significant resistance in bacteria that would normally be treated by an oral macrolide (the real concern about community macrolide resistance). especially *S.pneumoniae* and other common streptococci.*

We agree with the reviewer that a clear clinical interpretation of these findings is essential and have considered this comment carefully. The concern that long-term macrolide therapy increases the population-level dissemination of macrolide resistance in clinically significant bacteria has been well documented (e.g. PMID: 24429132, 28889110) and was the rationale for this study. Our findings that macrolide use was not associated with co-detection of AMR in close contacts provides the first piece of evidence to refute the hypothesis that such resistance is in fact disseminated. Importantly, the genes investigated include *erm*(B) and *mef*, which together are the primary determinants of macrolide resistance in *S. pneumoniae* (PMID: 18178388). We would suggest that the absence of codetection of these genes is reassuring for clinicians weighing up the risks vs benefit of long term macrolide therapy for patients with recurrent exacerbations of chronic lung disease, especially within the context of macrolide resistant streptococci.

We acknowledge, however, that this study did not investigate point mutations, which is an important consideration and limits wider clinical interpretation. For example, point mutations that confer

macrolide resistance in *Mycoplasma pneumoniae*, *Legionella pneumophila* and nontuberculous mycobacterium (NTM) could not be measured in this study. This is because detection of AMR attributed to point mutations is incredibly methodologically challenging within a microbial community, such as the oropharynx. While these bacteria are rarely carried asymptotically in the oropharynx, macrolide resistance in these organisms is of important clinical concern. The risk of long-term macrolide use to select for, and disseminate, resistance within these organisms therefore remains unknown. We have expanded our discussion of the clinical interpretation of this study, including a sentence at the end of the abstract summarising our clinical interpretation of the findings (page 3-4, line 63-65), a section in the discussion regarding resistance in streptococci (page 14, line 304-305), and atypical bacteria and NTM (page 15, line 339-345).

3. There is no comment about whether the close contacts had administration of macrolides within a reasonable period (say 12 months) of sampling. With not vast numbers, this is a potential confounding variable.

No close contact had received any macrolide in the 6 months prior to the study, a period that was chosen based on the existing literature (e.g. PMID: 17292768, 20483949). Details of the inclusion and exclusion criteria for subject recruitment were initially included briefly in the main manuscript and provided in detail in the online supplement. Reviewer 1 also queried the length of time between macrolide exposure and sample collection for the macrolide naïve groups. In light of these comments, we have now amended manuscript to provide these details more clearly (see main manuscript: page 6-7, lines 116-132, and the relevant section included below). We have also clarified this in the online supplementary methods.

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.

4. With respect to the key issue of "does this resistance transfer to others, the key issue is whether the sample size is adequate to reach the conclusion of "no it doesn't". It is not defined what you would have considered sufficient gene transfer of resistance to be clinically or even epidemiologically relevant.

The reviewer raises a very important point that the authors have discussed at length. The clinical implications from this study are difficult to define. Macrolide resistance is a concern clinically in the management of sexually transmitted infections, non-tuberculous mycobacterium infections, and community acquired pneumonia where penicillin allergy is a concern. In these situations, we consider any increase in population level carriage of macrolide resistance a clinical concern.

In regards to our study design, we performed sample size calculations based on findings from the previous BLESS randomised controlled trial (PMID: 23532242), as there have not been studies investigating resistance gene co-carriage in close contacts previously. This trial reported a mean increase of 44% in oropharyngeal macrolide resistance after 1 month of erythromycin therapy and a baseline SD of 18%. We assumed transmission of macrolide resistance from index cases to cohabitants to be incomplete, occurring in an estimated 25% of cases. From this calculation, an estimated 44 pairs of patients and close contacts in each arm would provide 80% power at an alpha of 0.05 to demonstrate an 11% difference in proportional macrolide resistance rates between case-contacts and control-contacts.

We, however, did not see an 11% difference in co-carriage between groups, and hence our findings did not achieve statistical significance. Of the 10 resistance genes we assessed, we can use the example of *mef*, which is a gene associated with macrolide resistance in common respiratory pathogens. Here, 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 ($p=0.33$). While assigning a clinical or epidemiological weight to this is difficult, we now include in the discussion a detailed explanation of our results (page 12, lines 253-262; page 14, lines 308-310). We believe this discussion now presents the findings in a more comprehensible manner.

5. Given you did show erm and mef detection was more common in contacts of macrolide users, I am not clear at all how you can justify your conclusion that resistance is not transmissible into the community - in fact the opposite finding would appear to be supported by your data - as you acknowledge in your discussion. The last sentence of the abstract is therefore far too strong and not supported by your data.

As a reference for this comment, the last sentence our abstract states: “However, macrolide use was not associated with increased macrolide resistance gene detection rate and there was no evidence that long-term macrolide use increases the onward transmission risk to their close contacts.”

This conclusion was reached based on the regression model performed assessing the effect of treatment group on co-carriage. We found that treatment group did not significantly explain co-occurrence of any AMR gene assessed. We therefore conclude that macrolide use was not associated with evidence of transmission in this study.

The findings that the reviewer is referring to is the within group analysis. As the reviewer correctly identifies, here, we found that *erm*(F) and *mef* were significantly co-detected in the macrolide recipient group. However, this is not sufficient evidence that macrolide use is responsible for this co-selection, merely that genes were more likely to be co-detected in patients and close contacts. We felt it necessary

to present both analyses for transparency but opt to use the between-group assessment to draw our conclusions.

Throughout the manuscript, we attempt to provide these findings (and their interpretation) as clearly as possible. In the revised manuscript, we now include additional explanation to clarify our findings and our interpretation. For example, in page 12, lines 253-256 we summarise the findings at the start of the discussion. Further in page 14, lines 308-310, we provide detail of how macrolide use was not associated with co-detection of genes in the example of *mef*, as discussed above.

6. In the discussion I think you have to note that the community data you have is an Australian context. Australia has seen much less use of oral macrolides, and especially problematic ones like Azithromycin, than has, for example, the US and hence international surveillance data typically shows much less clinical macrolide resistance in your population.

The variation in macrolide use globally is an important consideration for this study and we thank the reviewer for this comment. As the reviewer notes, there are clear restrictions in Australia when it comes to the prescription of oral macrolide antibiotics. Despite the regulated use of macrolides, Australia has high rates of macrolide resistance. For example, antibiogram reports for Queensland, Australia, show that 30% of *Streptococcus pneumoniae* isolates were erythromycin resistant (www.snp.com.au/media/Multisite11068/community-antibiogram-report-2019.pdf), which is comparable to the 28.8% reported by the CDC for the US (www.cdc.gov/abcs/reports-findings/survreports/spneu18.pdf). Further comparisons can be drawn by comparing the findings from this study to previous studies. For example, 34/40 (85%) of macrolide naïve close contacts (MNRCC) carried either *erm*(B) or *mef*, which is comparable to the ~90% reported by the Malhotra-Kumar study conducted in Belgium. In light of this comment, we now highlight in the discussion that macrolide use and resistance is highly variable globally, which is a consideration for the generalisability of these findings (page 15-16, line 351-355) and copied below.

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.

7. Very few of the close contact groups had any significant chronic lung disease. Do you think this may exert a protective effect on macrolide resistance transfer because they will not have as disordered a microbiome? We know that CF and bronchiectasis patients can and do transfer multi-resistant

organisms to each other, but not to healthy contacts. This should at least be reflected on in the discussion.

This is a really interesting area. It has certainly been shown previously that disruption of commensal microbiota increases the risk of acquisition of resistance when exposure occurs (PMID: 30383203). In addition, altered mucus production and airway clearance are also likely to influence susceptibility.

We agree with the reviewer that unperturbed gut microbiome in healthy close contacts provides colonisation resistance, which is able to prevent the expansion of potential pathogens (PMID: 29988120); however, it is unknown whether this effect applies to upper respiratory microbiomes which on the whole appear to be more resistant to perturbation even with antibiotic use (PMID: 33076936).

Better understanding AMR transmission between those with chronic lung disease is clearly extremely important, including between those attending the same respiratory clinic or between siblings with CF, for example. Investigating these issues would require a different study design to that employed here and therefore we feel it would be inappropriate for use to comment on these issues in detail in the manuscript. However, this issue should be investigated further in specific studies of where there is close contact between individuals with chronic lung diseases. In light of this comment, we have included discussion of the potential protective effects of healthy oropharyngeal microbiome on macrolide resistance transfer as well as the need for investigations of transmission risk among people with chronic lung diseases. This is included in the revised manuscript (page 15, lines 327-332) and copied below.

[...] 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

Statistical Review Comments:

The primary objective of this prospective study is to estimate and compare macrolide resistance gene detection rates and abundances between MR, MNR, MRCC, and MNRCC cohorts. Study design, data collection, primary endpoints, and statistical methods were clearly described. Logistic regression models were used to associate cohorts with detection and transmission status. Results were presented adequately and clearly. Conclusions were drawn appropriately. I have one minor suggestion (not a concern or question):

1. Seems authors could try logistic GEE model to estimate and compare 4 cohorts (MR, MNR, MRCC, and MNRCC) in one model with respect of resistant genes detection. This would be more efficient than first comparing within treatment pairs then between treatment groups.

We thank the reviewer for their positive comments of this work and their suggestion. We met with the statistical consultant at our institute to discuss the application of a logistic generalised estimating equation (GEE) model for this study. They agreed that GEE has the added strength of allowing for dependence within clusters. Upon discussion, we noted that our 2 aims had different assumptions and different dependent variables, precluding the ability to apply the one GEE model for both questions. The first, assessing whether gene presence/absence in the close contact is dependent on the patient can be modelled on all 4 possible conditions (present in both, absent in both, present only in close contact, present only in patient). The second however, assessing whether co-carriage was dependent on treatment group can only be accurately modelled in 3 of the 4 conditions (present in both, present only in close contact, present only in patient). Absence in groups both does not provide data either for or against transmission. While the statistical consultant agreed with the value of a GEE model, they also deemed the logistic models in the current manuscript sufficient.

As an additional means to compare the models in the current manuscript with that from a GEE, we performed a logistic GEE regression (output below). Here gene presence/absence in the close contact is the dependent variable, while patient gene presence/absence is an independent variable along with treatment group, and an interaction term for patient and treatment group. As the output interaction term shows, the findings from the GEE are comparable to those reported in the manuscript with treatment group not significantly predicting gene co-carriage in close contacts. For clarity and accessibility to the readers, we opt to keep the logistic regression in the manuscript and sincerely thank the statistician for their suggestion.

Table R3: Effect of the interaction between gene presence/absence in the patient and treatment group on gene presence/absence in the close contact

Resistance gene	Odds ratio (95% CI)	<i>P</i> value
<i>erm</i> (C)	0.6 (0.1-6.9)	0.78
<i>erm</i> (F)	7.0 (1.0-50.6)	0.085
<i>mef</i>	5.5 (0.8-36.7)	0.12
<i>msr</i> (A)	0.8 (0.3-2.4)	0.85
<i>msr</i> (E)	0.7 (0.2-3.3)	0.74
<i>tetO</i>	1.6 (0.3-7.8)	0.64

* Variance too small to perform logistic GEE model for *erm*(A), *erm*(B), *tetM*, *tetW*