**Task:**

1. **Revise easily addressed comments based on Geraint’s and Steven’s comments**
2. **Revise manuscript based on (1)**
3. **Draft and think more about how to answer the hard questions**

**Manuscript ID: CHEST-21-3596**

**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.

***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:*

*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 macrolide non-recipients as individuals who 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).

*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 wash-out period.

*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 (See main manuscript: page ? and line ?).

*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 definition of close contact. Whilst definition of CC varies widely depending on the context, we define CC as…

In the table below (Table R1), we provide information on the nature of relationships between patients and close contacts. For example, 92% (86/93) of close contacts were cohabitees. This table is now also included as Table SX in the revised manuscript), including the level of interaction.

**Table R1.** Summary of all participants’ relationship details in this study

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

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, we have included the information above in the revised manuscript and now highlight the potential significance of degrees of contact for AMR transmission in the discussion (See main manuscript: page ? and line ?)

***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.*

*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 manuscript to reflect this (See main manuscript: page ? and line ?).

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

*“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 co-detection versus evidence of transmission. We were careful to frame our assessments as looking at measures that are consistent with transmission and therefore supporting the potential for its occurrence, rather than saying definitively that 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.

As the reviewer notes, demonstrating transmission is extremely challenging, particularly when based on cross-sectional data. In our revision, we highlight the need for further longitudinal studies, where evidence of transmission is identified.

*“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. However, as we report, the detection in recipients, and close contacts of recipients, differed significantly with non-recipients, implicating direct exposure in carriage rates.

We now highlight these considerations in our revised manuscript.

*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) (See main manuscript: page ? and line ?). However, it did allow a more focused investigation, and led on from our metagenomic assessments of the AMR genes undertaken in macrolide recipients (PMID: 23532242; PMID: 28687413), we have already provided (PMID: 29669883; PMID: 30875247). Specifically, we comprehensively assessed the effect of long-term macrolides therapy (azithromycin and erythromycin) on respiratory microbiota composition and antibiotic resistance in patients with chronic respiratory diseases.

In the current study, we used the findings from our previously published papers to determine the selection of the 10 genes assessed in this study and aim to provide clinical evidences on two important clinical questions: 1) Does long-term macrolide use impact carriage of resistance determinants within the oropharyngeal microbiota in patients with chronic respiratory conditions 2) Does long-term macrolide use impact onward transmission risk of macrolide resistance genes between patients and their close contacts. The novelty of this study lies therefore in the transmission risk of macrolide resistance between patients and their close contacts, rather than representing a more wide-ranging assessment of resistome impact.

These genes were also selected for their importance, clinically. They are not found in opportunistic respiratory pathogens include *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae* and *Moraxella catarrhalis*, but are also found on mobile genetic elements that can transmit horizontally. For example,

* *erm*(A), *erm*(C) and *msr*(A) are found on *S. aureus* across three separate MGE: transposon Tn554 (PMID: 20668911), plasmid pUSA03 (PMID: 20668911) and pMS97 (PMID: 9809423).
  + *erm*(B) Transposons Tn2010 (PMID: 27709102)

*mef* Tn1207.1 (PMID: 15328112) on *S. pnuemoniae*  [single anatomical site]

*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 include discussion of this important point in lines XXX.

*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. For example, in our recent analysis of fecal resistomes in the context of individuals with cystic fibrosis, we found that aminoglycoside resistance was a primary contributor to the divergence in resistome composition between CF and healthy stool, reflecting the high tobramycin use in CF individuals (PMID: 33250435).

While we agree that the lung microbiome is more stable under selective pressure compared to the gut, the clinical importance of the oropharyngeal microbiome and AMR transmission is well demonstrated. For example, Man et al….

1. Important for upper and lower respiratory infections
   1. E.g. Man et al

In response to this comment, we expand on our discussion of the importance of the human microbiome (including the gut) as a potential transmission of resistance in the revised manuscript (See main manuscript: page ? and line ?)

*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 concussively 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 comments and each point has been addressed 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.*

*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. However, the reviewer raises an important issue and we absolutely recognise the need for clarity in regards to the measures used. To address this, we now include expanded definitions of these terms at their first use within the text (See main manuscript: page ? and line ?).

Specifically….

*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.*

\* For discussion with all authors

*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.*

All close contacts had not 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; PMID: 20483949).

We have revised the methods section to make this clearer. Please see below

“…”

*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.*

[GR, ST, YW – work through together]

\* Incidence where there was evidence of transmission (e.g. 2/53 times).:

\* HOW MANY TIMES DID “TRANMISSION” OCCUR

The gene with the strongest evidence that macrolide use contributed to transmission is mef (p=0.35). Here, the odds ratio of 1.6 indicates that macrolide use results in a 60% increased risk of transmission. In this cohort, where 53 pairs were in the macrolide recipient group compared to 40 in the macrolide non-recipient, this means that macrolide use contributed to 2 additional co-carriage event. [effect size] Based on using this example of mef, we can postulate a sample size for this effect to be significant. Using XXXXXXXXXX, we would need XX people per group to demonstrate significant evidence of co-carriage.

1. Definition of co-carriage and transmission
   1. Co-carriage/dependency: incidence of 1-1 and 0-0 vs incidence of 0-1 and 1-0
   2. Transmission: incidence of 1-1 vs incidence of 0-1 and 1-0
2. How many times did transmission occur?

**[Preparation]**

The reviewer raises a very important point that we have discussed at length.

The clinical implications from this study are difficult to define. Macrolide resistance is a clinical concern in [settings].

In our study, we carefully selected seven clinically important macrolide resistance genes.

Direction 1

We have summarised the incidence of 1-1 pairs (indicating potential transmission) and incidence of 0-1/1-0 pairs (indicating no transmission) in each group and compared them in the table below.

From the results, we found that:

1. In macrolide group: A % of pairs have at least 1 macrolide resistance gene transmission case
2. In non-macrolide group: B % of pairs have at least 1 macrolide resistance gene transmission case
3. Considering A and B is comparable (chi-square), this result indicates that the overall macrolide transmission risk between two groups are comparable

Direction 2

Of all resistance genes tested in this study, ermB and mef are the primary resistance genes (most clinically important, how?). We have summarised the carriage of these two genes in pairs

From the results, we found that:

1. In macrolide group: A % of pairs have ermB; B% of pairs have mef
2. In non-macrolide group: C % of pairs have ermB; D% of pairs have mef
3. Considering A and C, B and D is comparable (chi-square), this result indicates that the overall macrolide transmission risk between two groups are comparable

**[Our responses]**

**[Tables]**

**Table R7.** Incidence of transmission (1-1 pair number in each group)

|  |  |  |
| --- | --- | --- |
| **Resistance gene** | **Macrolide group**  **(Percentage, %)** | **Non-macrolide group**  **(Percentage, %)** |
| *erm*(A) | 0  (0/53) | 0  (0/40) |
| *erm*(B) | 77  (41/53) | 80  (32/40) |
| *erm*(C) | 4  (2/53) | 3  (1/40) |
| *erm*(F) | 42  (22/53) | 53  (21/40) |
| *mef* | 55  (29/53) | 53  (21/40) |
| *msr*(A) | 15  (8/53) | 10  (4/40) |
| *msr*(E) | 23  (12/53) | 35  (14/40) |
| *tet*(M) | 91  (48/53) | 95  (38/40) |
| *tet*(O) | 45  (24/53) | 53  (21/40) |
| *tet*(W) | 68  (36/53) | 78  (31/40) |

*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 trasnmissable 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 the reviewer, 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 logistic regression model we performed. In this model, treatment group was included as an independent variable, and gene co-occurrence was included as the dependent variable. 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.

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. (See main manuscript: page ? and line ?)

*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 reviewer raises some important points here. In the discussion section of our revised manuscript, we highlight differences in macrolide use between regions and the implications of these variations for the interpretation and clinical significance of our findings. Specifically….

[GR, ST, YW – work through together]

|  |  |  |  |
| --- | --- | --- | --- |
|  | Aus | US | Eur |
| CF | 36% |  |  |
| Bronchiectasis |  |  |  |
| Community use |  |  |  |

**Usage unit:** DDD/1000 inhabitants/day

**Macrolide usage:** AU & USA (macrolides only), EU/EAA (Macrolides, lincosamides and streptogramins)

**Data source:**

AU: *Australian Commission on Safety and Quality in Health Care. AURA 2019: fourth Australian report on antimicrobial use and resistance in human health. Sydney: ACSQHC; 2019.*

EU/EAA: *European Centre for Disease Prevention and Control. Antimicrobial consumption in the EU/EEA (ESAC-Net) - Annual Epidemiological Report 2019. Stockholm: ECDC; 2020.*

USA: *Centers for Disease Control and Prevention. Outpatient antibiotic prescriptions — United States, 2019*.

**Data**

Overall community usage in Australia dropped from ~2.3 to ~1.8

1. Community usage of macrolides in different regions at 2019
   1. AU: ~1.8
   2. EU/EAA : 2.8
   3. USA: 0.33
      1. Unit: antibiotic prescriptions / 1000 person, rate (121 in macrolides usage group)
      2. 121/365=0.33
2. A huge rise in the proportion of private prescriptions for azithromycin throughout the 10-year period in Australia (2010-2019)
   1. To support this view, average monthly private prescriptions of azithromycin were 423 in 2010 (0.07 per 100 GP visits), increasing to 1,424 in 2019 (0.16 per 100 GP visits)
3. The rate of inappropriate prescription of all azithromycin prescription is huge 26.5% (n=891) in Australia at 2019

We also provide the statistics for macrolide use between Australia, Europe and the USA.

In Australia, the overall macrolide use is **~1.8** DDD/1 000 inhabitants/per day, which is lower than that in EU/EEA with an average use of **2.8** DDD/1 000 inhabitants/ per day but higher than that in US with an 0.33 prescriptions/1 000 patients/per day.

However, there was a huge rise in the proportion of private prescriptions for azithromycin throughout the 10-year period in Australia. To support this view, average monthly private prescriptions of azithromycin were 423 in 2010 (0.07 per 100 GP visits), increasing to 1,424 in 2019 (0.16 per 100 GP visits). Besides, the rate of inappropriate prescription of all azithromycin prescription is huge 26.5% (n=891).

Given that, the macrolide use especially azithromycin in Australia is of great concern. A discussion of macrolide usage especially Azithromycin is included in the discussion section (See main manuscript: page ? and line ?)

*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.*

[GR, ST, YW – work through together]

We agree, 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.41, 42 In addition, altered mucus production and airway clearance are also likely to influence susceptibility.

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 some specific studies of CLD patients/CLD CCs]

***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):*

*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.*

**[Preparation]**

1. Thank you for the suggestion on GEE (generalized estimated equation) model
2. Advantage of this GEE model:
   1. What is GEE model
   2. This GEE model is extremely useful for …(Situation)
3. Agree that GEE could capture within and between group variance, under the condition that the DV is the same; however in our study, DVs are different (one is 0/1, the other is 1-1 pair/0-1 pair).
4. By using this GEE model, we could address three question:
   1. Whether patient have each resistance gene will affect whether its close contact have the gene
   2. Whether macrolide exposure will impact whether close contact have each resistance gene
   3. Whether the interaction of patient having the resistance gene and macrolide exposure will affect whether close contact have each resistance gene
5. However, our study focus on addressing two questions
   1. The co-carriage of each resistance gene (this question can be addressed by GEE model)
   2. Macrolide exposure effects on transmission risk
      * Transmission risk was defined as the incidence of 1-1 pair compared to incidence of 1-0/0-1 pair
      * Gee model could address this, but it requires re-dummy coding as DV are different, which is similar to our two models

Taken together, we appreciate your suggestions; GEE model is helpful but not more efficient than ours