**Review comments**

**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?

\*Search literature – paste 1-3 references

**Interpretation of this question:** For NMR and all close contacts, they had not received any macrolide antibiotics in the prior 6 months. If they had macrolides before the 6 months and developed resistance already, whether the 6 months is enough for them to reduce the resistance level to the level before they receive any macrolide resistance.

**Tasks:**

1. Search the literature, study protocol or check our previous resistance paper
2. If unluckily, no findings, check with Lucy why they recruit NMR and CC using 6-month as recruitment criteria

**My response:**

1. If I found references to support this, my answer would be like:

Thank you for bringing this question. Yes, previously studies have reported that a 6-month wash-out would significantly reduce the resistance levels…, therefore, those individuals who had not received any macrolide antibiotics in the prior 6 months, were recruited in this study.

1. If I did not find any references to support this, my answer would be like: ?

**Steven’s /Geraint’s suggestions:**

**Revisions:**

**Final response:**

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

\*contact Lucy

**Interpretation of this question:** In the online supplements, it was written that close contacts have not received any antibiotics in the prior 4 weeks. I suppose the reviewer want to check the antibiotic history of close contacts, whether they have received clindamycin ?

**Tasks:**

1. Generate a master sheet and pull out all antibiotic usage data
2. Double confirm this in this clinical manifest and if any data missing, check with LucyB/LucyM

**My response:**

We have confirmed that all close contacts in this study did not receive any other antibiotics in the prior 4 weeks, which includes the clindamycin that might concerns the reviewer.

**Steven’s /Geraint’s suggestions:**

**Revisions:**

**Final response:**

1. 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?

\*Were any patients excluded because they received claritho between 6-12 months

\*Were any patients included because they received claritho between 6-12 months

**Interpretation of this question:** In the cohort overview results, we wrote “A further two patients were excluded due to having received clarithromycin treatment in the 12 months prior”**.** In our method, we define “NMR as those with a chronic respiratory condition who were not receiving macrolide maintenance therapy, and who had not received any macrolide antibiotic in the prior 6 months”, which confused the reviewer.

**Tasks:**

1. In AZM manifest, there are 2 MR, they have received clarithromycin treatment in the 12 months prior and were excluded by Lucy; however, Lucy did not specify the reason why clarithromycin is so important and deserve to use 12 months criteria not 6 months as other people. Need to confirm with Lucy about:
   1. The criteria here is because clarithromycin is more important?
   2. This manuscript combined AZM and SERPAT, the recruitment protocols are different, I united the recruitment cut-off as 6 moth. **NMR in AZM (cut-off is 12 month):** not receiving azithromycin in the last 2 years, not receive other macrolide in the 12 months prior. **NMR in SERPAT (cut-off is 6 month):** No macrolide use in the prior 6 months.

**My response:**

1. Our apologies on not bringing the message clearly. A revision on this have been made (See page ?, line ?)
2. In the cohort overview results, 2 MR were excluded as they have received clarithromycin treatment in the 12 months prior; however, the most important reason for excluding them is the clarithromycin rather than 12 months. This is because …(Lucy comments)?/ In the cohort overview results, 2 MR were excluded as they have received clarithromycin treatment in the 12 months prior; the reason why 12-month interval here is because ...?
3. Whether “receiving macrolide antibiotics in the prior 6 months” is our cut-off criteria for recruiting individuals

**Steven’s /Geraint’s suggestions:**

**Revisions:**

**Final response:**

1. 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?)

\*Contact Lucy

**Interpretation of this question:** Whether the transmission risk is different between friends, or sibling or partner?

**Tasks:**

1. Yes, looking through the details of relationship between patients and close contacts would be a good idea
2. Tear apart the clinical data and run a simple analysis on this (I think most of them are partner)

**My response:**

1. Thank you for bringing this question. Yes, we do have the relationship details. The majority of the close contacts (?%) are partner to the patients. ? analysis was conducted to assess the influence of relationship details on transmission risk
   1. Our results showed that the transmission risk was not affected by relationship details (P=?, See table below)
   2. Or, due to the fact that the majority of the close contacts are partners to the patients, we were unable to assess the influences of relationship details on transmission risk.

**Steven’s /Geraint’s suggestions:**

**Revisions:**

**Final response:**

**Questions:** Do we need to include this into our results considering this might adds up the significance ? I prefer not, it will mess up the current results and make it more complicated to explain to the audience, and I am concerned the sample size of other relationship other than partners is too small to test that

**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.

The authors demonstrate:

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.

**\* Pre post FDR**

**Interpretation of this question:**

1. No FDR correction for qPCR results
2. Significance will be lost if FDR correction was employed

**Tasks:**

**My response:**

1. We agree that FDR correction is important, because …; However, the limitation of FDR correction is … considering only 9 genes not 100 genes were tested, FDR correction might be not necessary
2. Besides, previous papers have reported this result, our manuscript is more focused on how macrolide therapy affect the resistance gene carriage and abundance in close contacts and whether it will affect transmission

**Steven’s /Geraint’s suggestions:**

**Revisions:**

**Final response:**

1. “Onward transmission” of resistance genes, i.e. 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).
   * + Steven to send through reference of AMR dissemination

**Interpretation of this question:**

1. It is misleading to use the word “transmission” in this manuscript
2. Isolation of a resistant organism from both patient and co-inhabitant and test this by strain typing or WGS analysis is needed

**Tasks:**

**My response:**

1. I partially agree that, Indeed, a patient could have acquired the resistance gene from their healthy close contacts. However, as compared with other factors who lead to carriage of resistance gene in close contact, the macrolide therapy on their patient is the main factor for resistance development and to spread the resistance genes to close contacts, our study aims to assess that effect. To assess this, we also included patients who did not receive macrolide therapy and their close contacts as control group
2. Secondly, we made it clear at the end of the discussion section that the manuscript is about whether macrolide use is associated with increased risk of resistance gene carriage in close contacts. We did not draw conclusions on whether there is or there is no onward transmission. The conclusion we draw is there was no evidence to support long-term macrolide would affect onward transmission.
3. Besides, if there is an onward transmission from patients to their healthy close contacts, then theoretically, you will find associations of resistance genes between patients and close contacts given that macrolide therapy will induce antibiotic resistance which have been reported by many journals
4. I do not think it is necessary to isolate resistant organisms and test this as our manuscript focus on carriage and abundance of the resistant genes instead of tracing down to the transmission route.

**Steven’s /Geraint’s suggestions:**

**Revisions:**

**Final response:**

1. “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. 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.

\* Find 2-3 papers that describe the frequency of macrolide and tet resistance genes in humans/environment

Steven, can you help with this? Not sure his/her focuses? It seems to me that he/she think we did not do enough, which is lack of clinical significance.

**Interpretation of this question:**

**Tasks:**

**My response:**

**Steven’s /Geraint’s suggestions:**

**Revisions:**

**Final response:**

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

Tet genes are found on plasmids

Describe how you identified these genes

**Interpretation of this question:**

1. Only macrolide/tetracycline resistance was assessed. Macrolide exposure can co-select for other resistance genes which may be an even greater concern (thinking of MDR plasmids etc.).
2. The impact of macrolides on the gut is more relevant in terms of potential transmission of resistance.

**Tasks:**

**My response:**

1. We have listed the reasons why we only assessed resistance
2. This manuscript mainly focused on onward transmission of macrolide resistance gene
3. Gut microbiome is important (e.g. ref); however, the gut-lung axis connection is not strong as

**Steven’s /Geraint’s suggestions:**

**Revisions:**

**Final response:**

1. 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.

\* matergut

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.

Whether we also need to reply to this summary paragraph?

**Interpretation of this question:**

**Tasks:**

**My response:**

**Steven’s /Geraint’s suggestions:**

**Revisions:**

**Final response:**

**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.

* We recognise this issue
* It is a complex issue where both contribute to our understanding
* We define these as … Within the author team, we have discussed this at length
* In response, we have [included definitions of each term]

**Interpretation of this question:**

The terminology we used in the manuscript is difficult for readers to understand. Quantitative and presence/absence are suggested

**Tasks:**

**My response:**

Thank you for pointing this out, we agree. Detection and abundance have been replaced by **A and B** and the revision have been made throughout the whole manuscript.

**Steven’s /Geraint’s suggestions:**

**Revisions:**

**Final response:**

1. 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 Rachel and Lucy’s

**Interpretation of this question:**

Translate the resistant gene detection and abundance results into a clinical context, what does that mean in the clinical setting?

**Tasks:**

**Questions:** does this mean this clinical significance of this study should be discussed section?

It means if long-term macrolide therapy contributes to onward transmission of macrolide resistance genes from patients to their close contacts, then these genes carried by commensal bacteria in healthy close contact might spread to pathogenic bacteria developing macrolide resistance because of gene transfer. However, in our study we did not find any evidence to support macrolide therapy would contribute to increased transmission risk.

**My response:**

A discussion on clinical message from this study has been included in the revised manuscript (See page ? line ?)

**Steven’s /Geraint’s suggestions:**

**Revisions:**

**Final response:**

1. 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.

\* we included in online supplement

\* This is an important feature of the study

\* we have now moved this to the main text

**Interpretation of this question:** Does that mean whether close contacts have received macrolides in the prior 12 months? Weird I have made it really clear in online supplement that “Close contacts Has not received any macrolide in the prior 6 months and has not received any azithromycin in the prior 12 months”

**Tasks**: Confirm with lucy and check protocol

**My response:**

**Steven’s /Geraint’s suggestions:**

**Revisions:**

**Final response:**

1. 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.

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

\* HOW MANY TIMES DID “TRANMISSION” OCCUR

**Interpretation of this question:**

1. Sample size is big enough to draw the conclusion
2. Whether resistant gene transfer is clinically or even epidemiologically relevant.

**Tasks:**

**My response:**

1. If majority of patents have this gene but most of their close contacts do not have this gene, then yes, I think the gene transfer is clinically/ epidemiologically relevant; however, if both patients and close contacts have these gene, then probably is less clinically/ epidemiologically relevant

e.g. msrE in our results (overall patients: ~70%, CC: 40%)

1. Relative abundance of resistance genes also matters, to me, if the gene level is higher, it might indicate higher probability of inducing macrolide resistance as it means more commensal bacteria carried these gene, and the probability of spreading this gene to pathogens might be higher once infection occurs

**Steven’s /Geraint’s suggestions:**

**Revisions:**

**Final response:**

1. 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.
   1. Within group showed ….
   2. Between group showed …
   3. We conclude “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.”
   4. Justify

**Interpretation of this question:**

**Tasks:**

**My response:**

1. The reviewer misunderstood our analysis methods
2. Two logistic regression models were used in the manuscript: the first one (ermF and mef, the one he mentioned) is to assess the co-carriage of resistance gene in each group. The second one is to assess impact of macrolide therapy on transmission risk. He was confused and used the results from model 1 to interpret the transmission results, which is not what we actually did.
3. Secondly, to me, the reviewer compared two P values generated from the first model, which is not fair, From statistical point, it is not appropriate to compare two P values generated from two sub-models (underneath the first model) unless you put all data into one model
4. My thoughts:
   1. Two regression models are both paired analysis, but they address two different questions
   2. We firstly assessed the association between the presence of resistence genes in patients and the presence of these genes in their paired close contacts.
   3. In our association logistic mode, we found that whether patients have/don't the ermF or mef gene will significantly affect whether their close contacts have/don't have these genes in macrolide group. This results are not seen in non-macrolide group
   4. However, whether patients have genes affect their paired close contact have these genes does not answer transmission, it only reflect the associations as 0-0 pairs were also included in the model
   5. To further analyse whether the macrolide group is more likely to transmit resistence genes to the close contacts than non-macrolide group, we conducted another test using transmission comparison model
   6. In this logistic regression model, we found that as compared with non-macrolide group, the macrolide group is not more likely to transmit any resistence genes to their close contacts
   7. Therefore, long-term macrolide therapy won't affect transmission.

**Steven’s/Geraint’s suggestions:**

**Revisions:**

**Final response:**

1. In the discussion I think you have to note that the community data you have is an 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.
2. Get statistics

**Interpretation of this question:**

**Tasks:**

**My response:**

Thank you for bringing this up. Yes, we strongly agree with you on this point.

1. Australian and other developed countries’ usage of macrolide
2. Other developing countries data?
3. Onward transmission of resistance might have more clinical significance in other developing countries?

**Steven’s/Geraint’s suggestions:**

**Revisions:**

**Final response:**

1. 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.

**Interpretation of this question:**

1. Healthy people do not have disordered microbiome, which might protect them from macrolide resistance transfer
2. Should discuss in the discussion section

**Tasks:**

1. Track references that support the comments and discuss in last paragraph

**My response:**

Thank you for bringing this up. Yes, we agree with you. A revision has been made in the discussion section (See page ? Line ?)

In the discussion:

1. Respiratory and gut microbiome are important, our previous studies have found microbial communities are different between patients and healthy people…
2. Other studies have found e.g. CF patients can and do transfer multi-resistant organisms to each other, but not to healthy individuals.
3. Given that, an intact and normal respiratory/gut microbiome could play a protective role in macrolide resistance transfer.
4. Our study found the resistance level of MRCC and NMRCC is significantly lower than MR and MNR and no differences of resistance between MRCC and NMRCC, which could be partially explained by the protective role of intact/normal respiratory and gut microbiome

**Steven’s /Geraint’s suggestions:**

**Revisions:**

**Final response:**

**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.

**Interpretation of this question:** The logistic GEE model is suggested to compare within treatment pairs and between treatment groups without using two models

**Tasks:**

1. Learn more about this model and how do I use it to analyse my data?
2. Run this model on our data

**My response:**

1. Thank you so much for providing us the GEE model.
2. We employed this GEE model and re-analysed our data using this model and the outcomes are comparable (comparison results see below-a table)

**Steven’s/Geraint’s suggestions:**

**Revisions:**

**Final response:**