**Review comments:** 3/19 questions

**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?

**[Our response]**

In the methods and supplementary data (See main manuscript: page ? and line ?; Online supplement: page ? and line ?), we include the text that a macrolide non-recipient was defined as someone who had not received any macrolide therapy in the 6 months prior to enrolment in the study.

We chose to set 6 months as a cut-off due to reports from previous literature. For example, Malhotra *et al.*, showed that macrolide resistance genes returned to baseline levels at 6 months (Malhotra-Kumar *et al.* 2007). This is further supported by a meta-analysis (Costelloe et al. 2010).

Reference: PMID: 17292768, 2010PMID: 20483949

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

**[Our response]**

We have confirmed that all participants including patients and close contacts did not receive any antibiotic during the 4-week wash-out period. This includes clindamycin.

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?

**[Our response]**

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 and amended any discrepancies (See main manuscript: page ? and line ?; Online supplement: page ? and line ?).

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?)

**[Incomplete note]:**

The majority of the close contacts are cohabitants <- major determinant of transmission (Personally, I do not recommend explain this, as it will be pointed to our sample size issue)

**[Our response]**

We provide a breakdown of the relationship between patients and close contacts in Table R1, below. We categorise the relationships according to both level of contact and type of relationship as both may influence likelihood of transmission. As these tables illustrate, 92% (86/93) of close contacts are cohabitant to patients. Of these, 33% are family members and 67% are partners. We agree that this detail is an important consideration and have included this table in online supplement (e-Table ?).

We also agree that both the type of relationship and level of contact may influence the degree of transmission. Our study set out to address whether there was any evidence of transmission of macrolide resistance genes between patients and close contacts and whether macrolide use was associated with transmission risk. While the different degree of contact is an important consideration for the propensity of AMR transmission, such subgroup analysis is beyond the scope of this study.

To emphasize the importance of different degree of contact on propensity of AMR transmission, a small discussion has been included in the revised manuscript (See main manuscript: page ? and line ?)

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

|  |  |  |  |
| --- | --- | --- | --- |
| **Contact levels** | **Sample size** | **Relationship types** | **Percentage (%)** |
| Cohabitant  (>6 months) | 86 | Family members  (Parent, siblings and children) | 33  (28/86) |
| Partner, spouse and de facto | 67  (58/86) |
| Friend | 0  (0/86) |
| Regular contact  (>2 times per week in the last 2 years) | 7 | Family members  (Parent, siblings and children) | 43  (3/7) |
| Partner, spouse and de facto | 14  (1/7) |
| Friend | 43  (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.

**[Incomplete note]:**

We opted to assess a wide range of AMR genes to comprehensively assess the effect of macrolide treatment on gene carriage and transmission.

**[Our response]:**

We agree with the reviewer that adjusting for multiple testing is an important consideration and this is also how we would normally approach this type of analysis. Our initial analysis was performed without adjustment as we were concerned about inflating the risk of Type II error (Sarkar 2004, Lehmann *et al.* 2005).

Below we provide the outcome tables without and with false discovery rate (FDR) adjustment (Benjamini and Hochberg method) using the “stats” package in R (Jafari *et al.* 2019). As Table R4 shows, difference in *erm*(B) levels between MR and MNR group is not significant after FDR adjustment. In response to the reviewer’s comment, we have amended the manuscript to reflect this outcome (See main manuscript: page ? and line ?).

Besides, 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. Despite the loss of *erm*(B) significance between MR and MNR group after FDR correct, the adjustment does not affect our main outcomes.

Reference: PMID: 30124010

**Table R2.** Resistance gene detection frequency in patients stratified by macrolide use.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Resistance gene** | **MR** | **MNR** | ***P* values** | ***P* values**  **(post-FDR)** |
| *erm*(A) | 3.8%  (2/53) | 5.0%  (2/40) | 0.99 | 0.99 |
| *erm*(B) | 89%  (47/53) | 95%  (38/40) | 0.46 | 0.66 |
| *erm*(C) | 19%  (10/53) | 13%  (5/40) | 0.57 | 0.71 |
| *erm*(F) | 68%  (36/53) | 78%  (31/40) | 0.36 | 0.62 |
| *mef* | 74%  (39/53) | 83%  (33/40) | 0.33 | 0.62 |
| *msr*(A) | 36%  (19/53) | 25%  (10/40) | 0.37 | 0.62 |
| *msr*(E) | 66%  (35/53) | 73%  (29/40) | 0.65 | 0.72 |
| *tetM* | 94%  (50/53) | 100%  (40/40) | 0.26 | 0.62 |
| *tetO* | 64%  (34/53) | 78%  (31/40) | 0.18 | 0.62 |
| *tetW* | 85%  (45/53) | 98%  (39/40) | 0.07 | 0.62 |

**Table R3.** Normalised resistance gene abundance in patients stratified by macrolide use.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Resistance gene** | **MR** | **MNR** | ***P* values** | ***P* values**  **(post-FDR)** |
| *erm*(A) | 0  (0.0-10.5) | 0  (0.0-7.1) | 0.39 | 0.99 |
| *erm*(B) | 7.5  (0.0-12.4) | 6.9  (0.0-10.8) | 0.045\* | 0.66 |
| *erm*(C) | 0  (0.0-13.2) | 0  (0.0-8.0) | 0.14 | 0.71 |
| *erm*(F) | 7.6  (0.0-12.4) | 6.2  (0.0-11.9) | 0.22 | 0.62 |
| *mef* | 4.4  (0.0-6.7) | 3.9  (0.0-7.5) | 0.20 | 0.62 |
| *msr*(A) | 0  (0.0-13.2) | 0  (0.0-9.1) | 0.15 | 0.62 |
| *msr*(E) | 7.3  (0.0-13.0) | 5.7  (0.0-15.9) | 0.07 | 0.72 |
| *tetM* | 5.9  (0.0-8.9) | 5.5  (2.1-7.6) | 0.42 | 0.62 |
| *tetO* | 6.4  (0.0-10.5) | 6.3  (0.0-12.3) | 0.39 | 0.62 |
| *tetW* | 5.2  (2.7) | 4.8  (2.2) | 0.46 | 0.62 |

\* indicates the significance of this comparison lost after FDR correction

**Table R4.** 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** | ***P* values**  **(post-FDR)** | **Odds ratio**  **(95% CI)** | ***P* value** | ***P* values**  **(post-FDR)** |
| *erm*(B) | 3.4  (0.5-22.9) | 0.21 | 0.38 | 1.3×10-7  (0-Inf) | >0.99 | 0.99 |
| *erm*(C) | 5.1  (0.6-41.9) | 0.13 | 0.29 | 8.5  (0.4-163.9) | 0.16 | 0.99 |
| *erm*(F) | 11.8  (2.3-59.6) | 0.0029† | 0.020 | 1.7  (0.4-7.6) | 0.50 | 0.99 |
| *mef* | 7.3  (1.9-28.4) | 0.0044† | 0.020 | 1.3  (0.3-6.9) | 0.75 | 0.99 |
| *msr*(A) | 1.5  (0.5-4.9) | 0.48 | 0.62 | 1.8  (0.4-8.2) | 0.43 | 0.99 |
| *msr*(E) | 0.8  (0.3-2.7) | 0.74 | 0.83 | 1.1  (0.3-4.5) | 0.87 | 0.99 |
| *tetM* | 2.1×10-7  (0-Inf) | >0.99 | 0.99 | N/A | N/A | N/A |
| *tetO* | 2.7  (0.8-8.5) | 0.099 | 0.29 | 1.7  (0.4-7.6) | 0.50 | 0.99 |
| *tetW* | 2.4  (0.5-12.0) | 0.29 | 0.44 | 2.5×10-7  (0-Inf) | >0.99 | 0.99 |

† indicates the significance of this comparison remained after FDR correction

**Table R5.** 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** | ***P* values**  **(post-FDR)** |
| *erm*(A) | 1.0  (0-Inf) | >0.99 | 0.99 |
| *erm*(B) | 1.0  (0.4-2.9) | 0.96 | 0.99 |
| *erm*(C) | 1.0  (0.07-13.9) | >0.99 | 0.99 |
| *erm*(F) | 1.0  (0.4-2.5) | 0.97 | 0.99 |
| *mef* | 1.6  (0.6-3.9) | 0.33 | 0.99 |
| *msr*(A) | 1.3  (0.3-5.0) | 0.73 | 0.99 |
| *msr*(E) | 0.6  (0.2-1.5) | 0.25 | 0.99 |
| *tetM* | 0.5  (0.09-2.7) | 0.43 | 0.99 |
| *tetO* | 0.9  (0.4-2.2) | 0.82 | 0.99 |
| *tetW* | 0.7  (0.3-2.0) | 0.55 | 0.99 |

(1)“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.

(3) 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).

**[Our response]:**

The authors are very aware that determining evidence of transmission is incredibly difficult to demonstrate in a cross-sectional cohort study.

In our previous open-plan hospital ward-related AMR transmission paper by Ashokan *et al.*, a diverse range of AMR bacteria and genes were found in rectal swabs collected from 59 resident inpatients. Despite no association between microbial composition and the distance between patients, a substantial number of AMR genes, including clinical important ones (OXA-1 and NDM-7), contribute to a significant location-resistome relationship and such a relationship is consistent with patient-to-patient AMR transmission (Ashokan *et al.* 2021). These results suggest the clinical importance of AMR genes as a biomarker for transmission.

Current studies on AMR transmission between household contacts mainly focus on AMR bacteria (Lietzau *et al.* 2007, Valverde *et al.* 2008, Mollema *et al.* 2010) ; therefore, findings in our study provide useful early evidence on AMR gene transmission between patients and healthy close contacts. In our revised manuscript, we have included a detailed discussion of the need for large, longitudinal studies to assess the carriage and transmission of AMR (both gene and specific isolates) as well as the contribution of antibiotics to this transmission risk.

Reference:

1. Ashokan et al. 2021: PMID - 33736699
2. Lietzau et al. 2007: PMID - 17938057
3. Valverde et al. 2008: PMID - 18562591
4. Mollema et al. 2010: PMID - 19923490

**[Note]**

1. The authors are very aware that determining evidence of transmission is incredibly difficult to demonstrate in a cross-sectional cohort study.
2. We have published in this space [Anushia’s paper]
   1. Short description of Anushia’s paper
3. However, AMR genes are clinically important
4. We use detection of AMR genes as a marker for transmission
5. There are no studies of this sort
   1. Mughini-Gras 2019- PMID 31439317: Humans to human transmission are the main source of community acquired antibiotic-resistant bacteria
   2. Examples of antibiotic resistance (bacteria) transmission (MRSA):
      1. PMID11101914: household contacts (27/87, 31%) vs the community (14/77, 18.1%)
      2. PMID19923490: two-thirds (67%; n = 56) of household contacts -> MRSA positive
6. Therefore these findings provide useful early evidence
7. We have included a detailed discussion of the need for large, longitudinal studies to assess the transmission of AMR and the contribution of antibiotics to this transmission risk.
   1. Need to measure not just AMR gene but carriage and transmission of specific isolates

**Interesting paper**

**Microbial diversity in individuals and their household contacts following typical antibiotic courses**

(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.

(2)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.

**[Our response]**

We agree that macrolide and tetracycline resistance genes are highly prevalent in both environment and within the human microbiome （PMID: 22846103.

We have contributed to the literature to reflect this in our revised manuscript.

**[Note]**

**Reference**

**[Note]**

1. Agree that macrolide and tet resistance genes are highly prevalent in both the environment and within the human microbiome
2. We have contributed to the literature to show this [papers]
   1. Why might this be the case? E.g. high amount of tetracycline in the environment endogenously
3. As the reviewer notes, even in the close contacts of macrolide non-recipients, carriage of macrolide and tetracycline resistance genes were high [stats]
4. Such prevalence does indeed confound the ability to demonstrate that macrolide use is a primary contributor to carriage
5. This is why it was necessary to have a high sample size
6. **Global macrolide/tetracycline resistance:** 31.0% (pneumococcal macrolide resistance, PMID: 15963272), 8.7% and 24.3% (Tetracycline for MRSA and S, pneumoniae, PMID: 26989065)
   1. Most of these resistance studies above collected samples from patients, not healthy individuals
7. **Macrolide use drives macrolide resistance:** Macrolide use is the most important driver of macrolide resistance (PMID: 17292768-Malhotra, PMID: 29669883, PMID: 23532241, PMID: 17195698, PMID: 16469851)
8. **Household transmission is important:** Humans to human transmission are the main source of community acquired antibiotic-resistant bacteria (PMID 31439317) and transmission risk of MRSA is higher among household contacts than among community members (PMID 11101914)
9. **In this study, we not only assessed the carriage but also assessed the abundance**

**In Australian population, the macrolide carriage rate is lower than many other countries:** In Australia, 32.4% carried ermB, this rate is not high as compared with other countries (e.g, Morocco 90.2%, Belgium 90.2%, France 90.0%, Poland 80.8%) (PMID: 27709102)

(3)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.

**[Our response]**

Our study has a specific positioning. Instead of assessing antibiotic effects on microbiome within household contacts as previously published by Abeles *et al.* 2016, our study mainly focusses on the long-term macrolide exposure on carriage and transmission risk of macrolide resistance genes in patients with chronic respiratory conditions and their close contacts.

To comprehensively evaluate the macrolide effects on carriage and transmission risk of AMR gene, we carefully selected six macrolide resistance genes, including *erm*(A), *erm*(B), *erm*(C), *erm*(F), *mef*, *msr*(A) and *msr*(E).

These genes are clinically important not only because these genes are known to be carried by bacteria commonly found in the human oropharynx (e.g. *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae* and *Moraxella catarrhalis*) and are able to confer resistance to macrolides via different mechanisms (Schroeder *et al.* 2016, Roberts *et al.* 2011, Schmitz *et al.* 2000, Choo *et al.*2018), but also because these genes are transmissible using mobile genetic elements such as plasmids or transposons. In details, *erm*(A), *erm*(C) and *msr*(A) were found on *S. aureus* transposon Tn554 (Malachowa *et al.* 2010), plasmid pUSA03 (Malachowa *et al.* 2010) and pMS97 (Matsuoka *et al.* 1998) respectively; Transposons Tn2010 (Schroeder *et al.* 2016) and Tn1207.1 (Cerdá Zolezzi *et al.* 2004) on *S. pnuemoniae* carried *erm*(B) and *mef* gene separately; *erm*(F) was found on *Bacteroides* transposons Tn4351 and Tn4400 (Chung *et al.* 1999); plasmid pS30-1 from *Acinetobacter* carried *msr*(E).

In addition to that, we also included three tetracycline resistance genes (*tetM*, *tetO* and *tetW*) as assessment targets, this is because these genes were either found on the same mobile genetic elements as macrolide resistance gene (*tetM* and *tetO*) or found to be strongly associated with long-term macrolide exposure (*tetW*). In details, Tn916 family both encode the streptococcal *erm*(B) gene and *tetM* gene (Cerdá Zolezzi *et al.* 2004); *tetO* and *mef* were both identified in a Tn1207.1-like transposon (Brenciani *et al.* 2004, Roberts 2005); *tetW* gene level was significantly higher in the airway after azithromycin treatment (compared with baseline) but not higher in placebo group (Taylor *et al.* 2019).

Taken together, we believe by assessing these clinically important macrolide resistance genes in our clinical cohorts, we are able to provide early evidence on carriage and transmission risk of macrolide resistance between patients with chronic respiratory disease and their healthy close contacts.

In response to this comment, we have provided a detailed overview of the clinical relevance of these macrolide resistance genes in the discussion (See main manuscript: page ? and line ?).

**Reference**

1. Abeles et al. 2016: PMID - 27473422
2. Schroeder et al. 2016: PMID - 27709102
3. Roberts et al. 2011: PMID - 21081549
4. Schmitz et al. 2000: PMID - 10837446
5. Choo et al. 2018: PMID - 29669883
6. Malachowa et al. 2010: PMID - 20668911
7. Matsuoka et al. 1998: PMID - 9809423
8. Cerdá Zolezzi et al. 2004: PMID - 15328112
9. Chung et al. 1999: PMID - 10511399
10. Brenciani et al. 2004: PMID - 15563518
11. Roberts 2005: PMID - 15837373
12. Taylor et al. 2019: PMID - 30875247

**[Note]**

* *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) is found on *S. pneumoniae* across two MGE: Transposons Tn917 and Tn2010 (PMID: 27709102; PMID: [25709602](https://www.ncbi.nlm.nih.gov/pubmed/25709602))
* *mef*(A/E)is found on *S. pnuemoniae* across two MGE: MEGA (*mef*(E)) and Tn1207.1 (*mef*(A)) (PMID: 11398110; PMID: 10952626)

1. The positioning of this study focused on macrolide exposure on macrolide resistance
2. We chose 10 genes that reflect the most commonly carried genes associated with macrolide use.
3. ermA/B/C/F, msrA/E and mef are six common macrolide resistance genes that are carried on mobile genetic elements (ref)
   1. Clinically important
      1. ermA: is found in XX
      2. ermB – Haemophilus, strep..
   2. We provide a detailed overview of the clinical relevance of these genes in the discussion (lines XX).
4. Why tet included:
   1. tetM and ermB: Tn916-Tn1545 family (PMID: [15328112](https://www.ncbi.nlm.nih.gov/pubmed/15328112))
   2. tetM and tetO were found on the same mobile genetic elements with macrolide resistance gene (Tn5358, Tn1545 for tetM, Tn2009 for tetO, PMID: 12936983, PMID: 15837373-tetO, PMID: 7648031-tetM)
   3. tetW was recently found to be strongly associated with macrolide therapy (Steven’s paper, PMID: 30875247)
5. erm(A):
   1. S. pneumoniae (Syrogiannopoulos et al 2001, PMID: 11120994), Streptococcus pyogenes (Malhotra-Kumar 2018), S. aureus (Malachowa, PMID: 20668911), H, influenzae
   2. Tn554 on S.aureus (Malachowa, PMID: 20668911)
6. erm(B):
   1. Tn916 like elements: Tn6002 (S.pyogenes), Tn2009, Tn2010 (S. pneumoniae) (Schroeder et al. 2016: PMID – 27709102)
   2. Plasmid pI258 (PMID: 7726500)

Term(B) provides high-level resistance to macrolides (erythromycin MICs usually ≥256 μg/ml).

1. erm(C): Staphylococcus aureus
   1. H, influenzae(Roberts *et al.* 2011)
   2. S. aureus (Schmitz *et al.* 2000), plasmid pUSA03/ (Malachowa, PMID: 20668911), pE194(PMID: 7726500)
2. erm(F)
   1. H, influenzae(Roberts *et al.* 2011)
   2. ermF (GenBank accession No. M14730) is found on Bacteroides transposons Tn4351 and Tn4400, (PMID: 10511399)
3. mef
   1. Mef(E): Tn2010(Tn916 like elements:), MEGA(PMID: [15328112](https://www.ncbi.nlm.nih.gov/pubmed/15328112))-S.pneumoniae
   2. mefA; Tn1207.1 (PMID: 15328112)
4. msr(A)
   1. S. aureus (Schmitz *et al.* 2000)
   2. pMS97 on S, aureus (PMID: 9809423)
5. msr(E)
   1. Staphylococcus aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa, Streptococcus pyogenes, PMID: 34069640, PMID: 30875247(Steven)
   2. pS30-1 from Acinetobacter baumannii isolate PMID: 28533235

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.

**[Note]**

* We agree.
* We note this in the discussion.
* We have previous studies that have provided a metagenomic assessment of the AMR gene carriage.
* We used the findings from these papers to determine the selection of the 10 genes assessed here.

**[Our response]**

We agree that assessing macrolide resistance is a limitation of this study. We have already noted this and included this limitation in our discussion section initially (See main manuscript: page ? and line ?).

In our previously published randomized controlled trials (under BLESS and AMAZES study), we have already provided metagenomic assessments of the AMR genes (Choo *et al.* 2018; Taylor *et al.* 2018). 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. However, we did not assess the potential impact of long-term macrolides therapy on transmission risk of macrolide resistance between patients and their close contacts.

Therefore, in this 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.

In response, the novelty of this study lies in the transmission risk of macrolide resistance between patients and their close contacts given that metagenomic assessments of AMR genes have been already conducted in our previous studies.

**Reference**

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

**[Note]**

1. Tet genes are found on plasmids
2. Describe how you identified these genes

We provide an overview of the selection of resistance genes in response to the reviewer’s previous question. We would also highlight that these genes represent genes commonly associated with plasmids. For example plasmid 1 – ermB tetM. This plasmid is found in … Plasmid 2 – ermF tetW. This plasmid is found in …

**[Our response]**

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

We have provided an overview of the selection of resistance genes in response to the reviewer’s previous question (See our answers to question ?). We have also highlighted that these genes represent genes commonly associated with plasmids or other mobile genetic elements (e.g. transposons) (See our answers to question ?).

As we described, we did look at other resistance genes that are associated with macrolide exposure. For example, *tetM* was found on the same mobile genetic element Tn916 as *erm*(B) in erythromycin-resistant viridans group *streptococci* and *Gemella* spp, which is a well-characterized mobile genetic element (Clewell *et al.* 1995; Cerdá Zolezzi et al. 2004) and has been shown previously to increase streptococci after macrolide therapy. *tetO* and *mef* were both identified on a Tn1207.1-like transposon in *Streptococcus pyogenes* clinical isolates (Brenciani *et al.* 2004, Roberts 2005).

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.

**[Note]**

1. We agree that resistance in the gut is also important. The gut represents an important route of transmission.
2. E.g. Carbapenemase-producing enterobacteriaceae (CPE) <- ask lito about AMR infections transmitted via gut
3. Important reservoir of resistance.
4. We have published in this space
   1. Our recent paper already compared the fecal resistomes between adult CF and healthy individual (PMID: 33250435). 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 indivudals
   2. Our current study, however, focused on the airway resistance transmission; however, we agree that resistance in the gut is also important
5. The focus of this manuscript is the oropharyngeal AMR carriage.
6. Important for upper and lower respiratory infections
   1. E.g. Man et al
7. 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 ?)

**[Our response]**

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.

**[Note]**

# Point to the strength of the study (Cover letter could help)

**[Our responses]**

As we mentioned in our manuscript:

1. **Novelty and aims:** This study 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. It mainly focused on evaluating the impact of long-term macrolide therapy on airway macrolide resistance genes development and potential onward transmission risk of these genes to the close contacts
2. **Sound assessment methods:** To assess the impact of long-macrolide therapy on airway macrolide resistance genes development, a total of 93 people with chronic respiratory conditions (53 receiving long-term macrolides, 40 macrolide naïve) were included, and we analysed the carriage and abundance of 7 common macrolide resistance gene and 3 macrolide-related genes. To explore the potential risk for onward transmission, 93 paired samples from close contacts of subjects were collected and were subjected to three analyses: 1) by comparing resistance between close contacts of macrolide recipients and non-recipients 2) by comparing detection rates within groups 2) by comparing transmission risk between macrolide recipient and non-recipient groups
3. **Findings:** We found that long-term macrolide exposure is associated with increased macrolide resistance carriage within patients (before FDR), however, importantly, no increase in resistance carriage was observed in close contacts of patients.
4. **Limitation well-described:** Limitation of this study is also well-described in the discussion section
5. **Clinical significance:** Taken together, we think this study provide early evidences on macrolide resistance transmission between patients with chronic lung disease and their healthy close contacts.

**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.

**[Note]**

1. We recognise this issue
2. It is a complex issue where both contribute to our understanding
3. Within the author team, we have discussed this at length
4. We define these as … which we feel provides the clearest definition for the reader.
5. In response, we have [included definitions of each term] in attempt to further clarify.

**[Our responses]**

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.

**[Preparation]**

\* For discussion with Rachel and Lucy’s

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.

**[Preparation]**

\* we included in online supplement

\* This is an important feature of the study

\* we have now moved this to the main text

**[Our responses]**

We include information about duration of macrolide use and length of time without macrolide use in the Methods section under “study design and setting” (See main manuscript: page ? and line ?) and in the online supplement material.

For clarity, all close contacts have not received any macrolide in the prior 6 months. We chose to set 6 months as a cut-off due to reports from previous literature. For example, Malhotra *et al.*, showed that macrolide resistance genes returned to baseline levels at 6 months (PMID: 17292768). This is further supported by a meta-analysis by Costelloe *et al.* (PMID: 20483949).

In revising the manuscript, we have clarified the definitions of macrolide recipient (MR), macrolide non-recipient (MNR), close contact of macrolide recipient (MRCC) and close contact of macrolide non-recipient (MNRCC) (Also see main manuscript: page ? and line ?) and presented below.

* Macrolide recipient (MR): ?
* Macrolide non-recipient (MNR): ?
* Close contact of macrolide recipient (MRCC): ?
* Close contact of macrolide non-recipient (MNRCC): ?

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.

**[Note]**

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

\* HOW MANY TIMES DID “TRANMISSION” OCCUR

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.

**[Preparation]**

In our initial manuscript, the last sentence of the 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 is in reference to the “Macrolide resistance transmission model” in our method section, where the effect of macrolide use on transmission risk is tested. Here, there was no indication that macrolide use was associated with co-carriage of resistance genes. However, as the reviewer highlights, we do see evidence of co-detection of ermF and mef in the macrolide recipients. Independently, this was not found in the macrolide non-recipients. We feel that the higher co-detection of ermF and mef in the macrolide recipients is not evidence to suggest macrolide use is associated with co-detection. This is because:

1. The co-detection model includes the 0-0 pairs into the calculation(both patient and close contact are negative for the tested gene); however, 0-0 pairs does not provide any potential transmission information
2. Despite a significant higher co-detection of ermF and mef in the macrolide group, two treatment groups were subjected to the co-detection binary logistic model separately/individually (two sub-models). From the statistical side, P values can only be compared when they were generated at the same time in one model

To make it clear to the audience, we have included a new paragraph to clarify on how to interpret the results from two models. (See main manuscript: page ? and line ?)

**[Note]**

1. Two models were employed to assess co-carriage (dependency) and transmission risk
   1. Revise discussion part as we confuse others about the first model?
2. The first model is within group comparison, the second model is between group comparison
   1. Our first analysis model focus on the co-carriage of resistance genes in each treatment group (incidence of 1-1 and 0-0 vs incidence of 0-1 and 1-0)
   2. Our second analysis model focus on the impact of macrolide therapy on transmission risk (Presence/absence of treatment (1/0) effect on transmission (1-1) and no transmission (0-1 and 1-0))
3. The significance of ermF and mef that found in the first model is not an indication of macrolide effects on transmission risk, this is because
   1. The first model aims to evaluate whether the resistance gene in close contacts is dependent/independent of that in patients in each treatment group, thus, 0-0 pairs were also included and considered as dependent pairs; however, 0-0 pairs can not be counted as transmission evidence
   2. Two treatment groups were subjected to the first model separately. Statistically speaking, P values cannot be compared directly
4. Our conclusion is:
   1. 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
   2. It is appropriate because
5. 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.

**[Note]**

* We agree that the community data you have is an Australian context, we had revised our manuscript and made it clear the conclusion drawn in this study was based on Australian population
* We provide the statistics for macrolide use between Australia, Europe and the USA.]
* Australia does use a lot of macrolide
* A discussion of macrolide usage especially Azithromycin is included in the discussion section

**[Our response]**

We agree that the community data you have is an Australian context, we had revised our manuscript and made it clear the conclusion drawn in this study was based on Australian population.

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

In Australia, the overall macrolide use dropped from **2.3** DDD/1 000 inhabitants/per day in 2017 to **~1.8** DDD/1 000 inhabitants/per day in 2019, which is lower than that in EU/EEA with an average use of **2.39** DDD/1 000 inhabitants/ per day and lower than that in US with an 3.62 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 ?)

**[Data]**

DDD: Defined daily dose

1. **Australia:**

Unit: DDD per 1,000 occupied bed days

***Source 1:*** *Australian Commission on Safety and Quality in Health Care. AURA 2021: fourth Australian report on antimicrobial use and resistance in human health. Sydney: ACSQHC; 2021.*

* 1. Macrolides: **~2.3** DDD/1 000 inhabitants/per day in 2017 drop to **~1.8** in 2019
  2. Azithromycin:
     1. the rate of inappropriate prescription of all azithromycin prescription is huge 26.5% (n=891)
     2. There was a huge rise in the proportion of private prescriptions for azithromycin throughout the 10-year period. 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. there was a slight increase in the overall rate of PBS prescribing of antimicrobials that have restricted benefits with azithromycin increasing from 0.84 to 1.1 per 100 prescriptions
     4. Top 10 most commonly prescribed antimicrobials in NAUSP (National Antimicrobial Utilisation Surveillance Program) contributor hospital

1. **Europe:**

Unit: DDD per 1 000 inhabitants per day

***Source:*** *European Centre for Disease Prevention and Control. Antimicrobial consumption in the EU/EEA (ESAC-Net) - Annual Epidemiological Report 2020. Stockholm: ECDC; 2021.*

J01F: Macrolides, lincosamides and streptogramins

* 1. Community usage:
     1. 2020: **2.39** DDD/1 000 inhabitants/ per day (compound annual growth rate -4%)
  2. Hospital usage:
     1. During 2011-2020: No significant EU/EEA trends were detected for consumption of macrolides, lincosamides and streptogramins (ATC group J01F).
     2. 2020: **0.17** DDD/1 000 inhabitants/ per day (compound annual growth rate 3.9%)

1. **USA: Although the WHO promotes the use of DDDs as metrics of drug use, but America do not**

Source: CDC antibiotic resistance & Patient Safety Portal

1. In 2020, 88 prescriptions of macrolides were dispensed in U.S. outpatient pharmacies for every 1,000 persons.
2. In 2020, 29 million prescriptions of macrolides were dispensed in U.S.outpatient pharmacies.

Source: https://clincalc.com/DrugStats/Drugs/Azithromycin

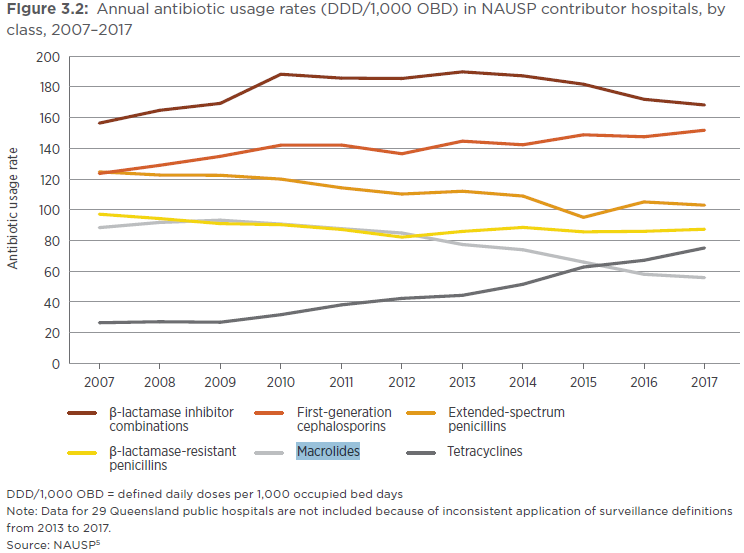
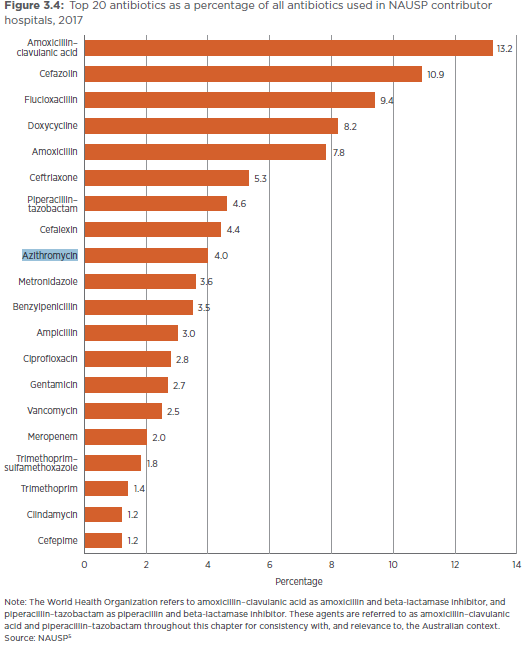
1. 2019: 3.62 prescriptions/1 000 patients/per day (calculated by myself)
2. **Other countries/region have higher macrolide usage (>15%)**

Unit: DDD per 1 000 inhabitants per day and proportion of total consumption

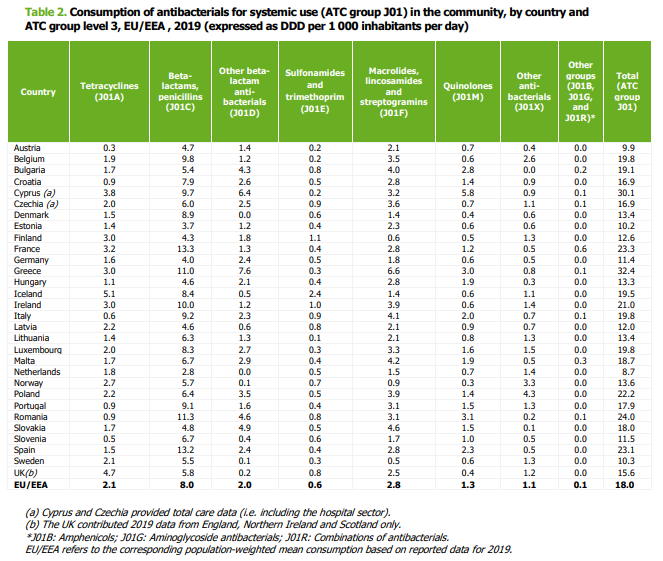
***Source:*** *WHO report on surveillance of antibiotic consumption: 2016-2018 early implementation. Geneva: World Health Organization; 2018. Licence: CC BY-NC-SA 3.0 IGO.*

1. Region of the Americas: Canada 3.29 (19.3%)
2. Region of Eastern Mediterranean Region: Jordan 4.66 (52.2%)
3. Region of Western Pacific Region: Japan 4.59 (32.3%), Republic of Korea 4.69 (17.0%)

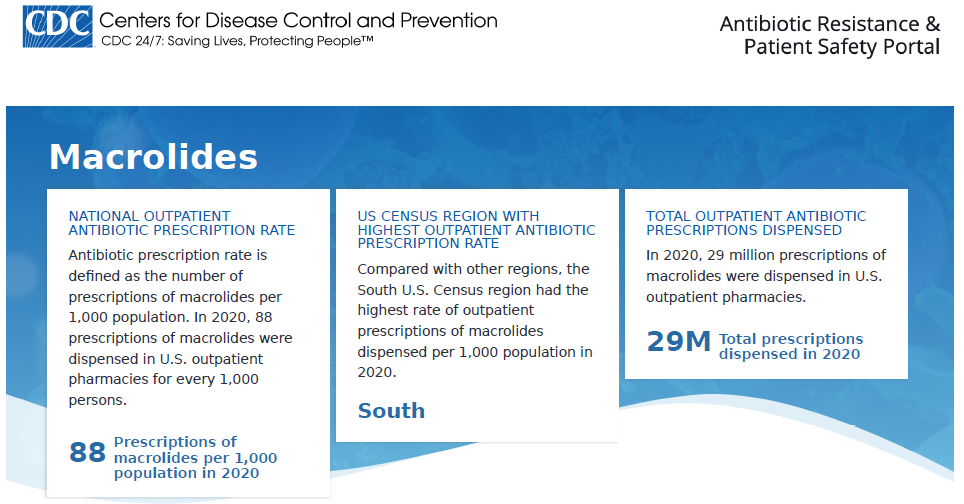
Australia



Europe



USA



(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?

(2)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.

We agree

We originally had discussion of both the clinical stability of the patient population as well as the fact that the close contacts did not have a chronic lung disease.

Patients and close contacts were stable at the time of sample collection and close contacts did not have a chronic lung disease. The risk of transmission of resistant bacterial populations would therefore be less than between two people with chronic lung diseases during periods of exacerbation. For example, periods of exacerbation can include increased coughing,37, 38 which has been shown to promote dissemination of potentially resistant bacteria through the production of cough aerosols.39, 40 Further, during periods of stability, intact commensal microbial systems, including those in the oropharynx, resist colonisation by external bacterial populations.41 This protection is greatly reduced when microbiota are disrupted, such as during respiratory viral infection.42 The risks of onward transmission of resistant bacterial populations might therefore vary according to the health of both azithromycin recipients and close contacts.

**[Preparation]**

**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.

**[Preparation]**

1. Thank you for the suggestion on GEE 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

Bin

1. **Mughini-Gras 2019:** Humans to human transmission are the main source of community-acquired β-lactam-resistant E.coli
   1. Most community-acquired β-lactam-resistant E.coli carriage was attributed to human-to-human transmission within or between households in the open community (60·1%, 95% credible interval 40·0–73·5)
2. **Knox 2015\_Trends in microbiology:** Several studies have highlighted the role of the household as the primary reservoir for S. aureus in the community 24, 25, 41, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58. The events that follow a CA-MRSA infection in a household include an increase in: (i) the risk of infections among other household members 26, 44, 45, 48, 49, 50, 51; (ii) MRSA colonization among other household members 46, 47, 52, 53, 54, 55, 56, 57, 59; and (iii) contamination of environmental surfaces 24, 25, 58. These reports have described epidemic clones that ‘ping pong’ among family members 26, 51, resulting in high rates of recurrent infection. Eradicating S. aureus carriage from household members and the environment in an effort to reduce the frequency of these infections has achieved mixed results 60, 61.
3. Staphylococcus aureus nasopharyngeal carriage rates and antimicrobial susceptibility patterns among health care workers and their household contacts
4. Transmission of methicillin-resistant Staphylococcus aureus within a household
5. Prevalence of nasal colonization among patients with community-associated methicillin-resistant Staphylococcus aureus infection and their household contacts
6. Staphylococcus aureus nasal colonization among pediatric cystic fibrosis patients and their household contacts
7. Molecular epidemiology and household transmission of community-associated methicillin-resistant Staphylococcus aureus in Hong Kong