Nasopharyngeal Resistome Study Statistical Analysis Plan V1.0

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Protocol Summary: Study Design and Research Questions

Study type: A multi-site, extended observational case-control study with a preliminary cohort component.

Research Question: Does hospital admission and antibiotic treatment for pneumonia generate a persistent hospital-acquired nasopharyngeal resistome (NPR) in children in Blantyre, and is this associated with adverse health outcomes in a resource limited setting?

Primary Study Objectives:

1. To measure how the diversity and relative abundance of the NPR of children hospitalised with pneumonia differs from the NPR of healthy children in the community.
2. To measure how the diversity and relative abundance of the NPR of children hospitalised with pneumonia differs from the NPR of children with pneumonia treated with antibiotics in the community.
3. To investigate whether participants re-hospitalised with pneumonia within a 3-month period have a persistent hospital-acquired NPR pattern.

Secondary Study Objective:

4. To measure the impact of exposure to a hospital environment on the development of a 'hospital-acquired' NPR.

Exploratory Study Objective:

5. To measure how the diversity and relative abundance of the NPR of children presenting with pneumonia differs between those with and without prior antibiotic use, based on urine bioassay.

Methodology: We will recruit 350 children aged 12-36 months in four groups: (i) healthy children in the community, (ii) children with pneumonia in the community, (iii) children hospitalised with pneumonia and (iv) children re-hospitalised with pneumonia. This is illustrated in Figure 1 (below). This observational study will be conducted in Ndirande community, at Ndirande community healthcare centre and at Queen Elizabeth Central Hospital (QECH). Nasopharyngeal swabs (NPS) and urine samples will be taken at recruitment and follow-up study visits; NPS will undergo metagenomic sequencing to identify the nasopharyngeal microbiome (NPM) and NPR, urine samples will undergo a bioassay to detect antibiotic activity, indicating recent antibiotic exposure. Demographics, co-morbidities, pneumonia severity, antibiotic exposure and health outcomes will be recorded at study visits.

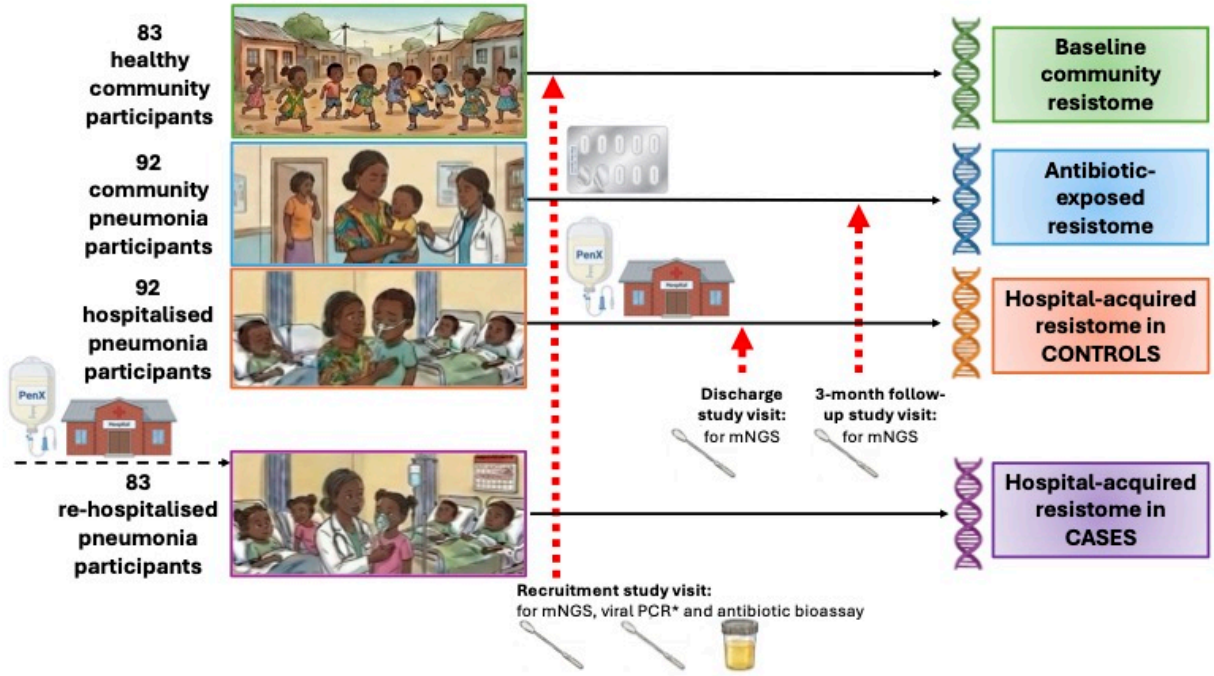

Figure 1. Study design and sampling frame. Red arrows show study visits. Small icons show antibiotic and hospital exposure. Hospitalised pneumonia participants (orange boxes) that are not re-hospitalised within 3 months of discharge form the control group for the case-control comparison. *Only participants with pneumonia will have a second NPS taken for viral PCR at recruitment. mNGS, shotgun metagenomic sequencing.

Participants will be frequency-matched for sex across the healthy community, community pneumonia, hospitalised pneumonia and re-hospitalised pneumonia groups for the statistical analysis. Figure 1 illustrates the key groups for comparison in the statistical analysis.

Children hospitalised at QECH with pneumonia (without any hospital admission in the preceding 6 months), who are not re-hospitalised within 3 months of recruitment will form the controls (see Figure 1); any hospitalised participant that is re-hospitalised will be excluded from the control group for the case-control analysis. Children admitted to QECH with pneumonia within 3 months of a previous hospital admission for pneumonia, meningitis or an infection managed with intravenous beta-lactam and/or aminoglycoside antibiotic will form the cases.

Expected results: The study is expected to describe NPR evolution following antibiotic selective pressure and residential healthcare exposure for pneumonia in children in Blantyre. It will provide contemporary data on AMR carriage in the community, hospital a re-admission rates with pneumonia and antibiotic use patterns.

Study Endpoints

Diversity is a measure used in microbial ecology to quantify the variety of microorganisms in a community. For the analysis of resistome composition, diversity is a measure of antibiotic resistance gene (ARG) richness, the number of different ARGs within a microbial community, and evenness or relative abundance of ARG distribution within the community. Alpha diversity quantifies the ARG richness and evenness within one sample or individual; beta diversity compares ARG richness and evenness between communities. In

this study, alpha diversity of the NPR within each sample will be measured using the Shannon Diversity Index, a continuous metric, where a higher number indicates a greater level of ARG diversity and evenness within the sample.

The Bray-Curtis Dissimilarity Index will be calculated to quantify the beta diversity, or compositional (dis)similarity, between the NPR of the four different cohorts. This compares the abundance of ARGs between two samples, by considering both size (overall abundance per sample), shape (abundance of each type of ARG) and the presence or absence of ARGs within a microbial community. Values range between 0 and 1, where 0 indicates that two samples have the same composition (they share all their ARGs) and 1 indicates the two samples do not share any ARGs.

The study will focus on two primary endpoints in analysis of NPM and NPR composition post-antibiotic and hospital exposure:

1) Difference in beta diversity
2) Difference in relative abundance of ARG classes for first line antibiotics used to treat pneumonia

Secondary endpoints will be used to analyse secondary outcomes when comparing NPM and NPR composition post-antibiotic and hospital exposure:

3) Difference in alpha diversity
4) Difference in relative abundance of individual ARGs.

Study Time End-points

The study end-point is recruitment for community healthy and hospital readmission participants for all analyses. The study end-point for the primary analysis is at 3-months post-recruitment for community pneumonia and hospital pneumonia participants, which is likely to represent a new post-antibiotic baseline. This time-point was selected as studies of paediatric infective-asthma exacerbations have demonstrated microbiome recovery to a new baseline within 3 months (Hou et al., 2022); similarly, pneumococcal carriage surveillance revealed a multi-drug-resistant lineage in global pneumococcal sequencing cluster 1 (GPSC1) at 100 days post-antibiotic exposure, suggesting an enduring impact of antibiotic selection pressure on the resistome (Tonkin-Hill et al., 2022). The hospital readmission cohort represent a sampling time-point within 3-months of hospital discharge. For primary analyses, this group will be compared with the 3-month end-point in the hospital pneumonia cohort.

Secondary analyses will include comparison of resistome composition between the cohorts at the following timepoints:

- Recruitment of community pneumonia participants. The recruitment NPS in this cohort is likely to represent an acute ‘antibiotic-exposed’ resistome; 70% of patients report self-administration of antibiotics prior to seeking healthcare assessment in Malawi (Limwado et al., 2024).
- Recruitment and discharge for hospital pneumonia participants.

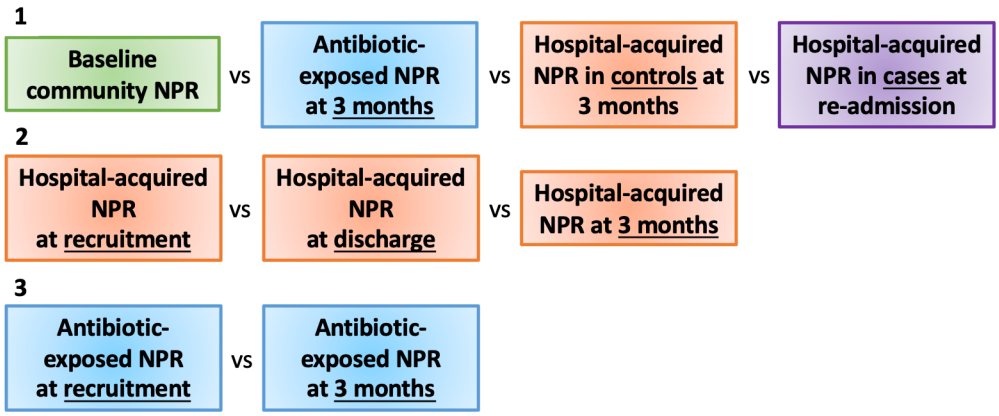

Figure 2. An illustration of the planned statistical analysis in the primary study outcomes to address the study objectives, through comparison of NPR composition between and within cohorts.

Quality Control for Metagenomic Sequencing Data

There is no published gold standard for quality control of shotgun metagenomic sequence data. The following pre-defined analysis thresholds will be applied to bacterial reads in the metagenomic sequence data to ensure high-confidence alignments to taxa present in the NCBI database:

1) >90% identity matches of reads and assembled contigs to reference sequences.
2) Alignment lengths ≥ 50bp to reduce false positive alignments.

Reads will be normalised using Reads Per Kilobase per Million (RPKM) mapped reads, permitting quantitative comparison of the relative abundance of bacterial and antibiotic resistance gene (ARG) reads between samples, by dividing both transcript length and total number of reads. RPKM is calculated by dividing the number of reads mapping to a particular gene by its length in kilobases, then multiplying by 1,000,000 and dividing by the total number of mapped reads in the sample; this adjusts for biases that may arise during sequencing and analysis, including differences in gene length and sequencing depth (Zhang et al., 2022). Normalised reads will undergo a centred log-ratio transformation to account for compositionality prior to differential abundance comparisons across groups (Aitchison, 1982).

A minimum of three types of negative control will be included in the study design to permit monitoring of contaminants throughout sample collection and processing: (i) sampling blank controls, to detect contaminant DNA introduced during the sampling procedure, (ii) DNA extraction blank controls, to monitor contaminant DNA content in extraction kits and (iii) no-template amplification controls, to monitor contaminant DNA present in reagents and laboratory environment during library preparation and sequencing (Eisenhofer et al., 2019).

Study Outcomes

Primary Outcomes:

1. To estimate the difference in beta diversity and relative abundance of ARG classes in the NPR between healthy community and hospital readmission cohorts at enrolment, and community pneumonia and hospital pneumonia cohorts at the 3-month study end-point.
2. To estimate the change in beta diversity and relative abundance of ARG classes in the NPR between recruitment, discharge and 3-month study time-points in the hospital pneumonia cohort.
3. To estimate the change in beta diversity and relative abundance of ARG classes in the NPR between recruitment and the 3-month study end-point in the community pneumonia cohort.

Secondary Outcomes: NPR Analysis

1. To estimate the difference in alpha diversity and relative abundance of individual ARGs in the NPR between healthy community and hospital readmission cohorts at enrolment, and community pneumonia and hospital pneumonia cohorts at the 3-month study end-point.
2. To estimate the change in alpha diversity and relative abundance of individual ARGs in the NPR between recruitment, discharge and 3-month study time-points in the hospital pneumonia cohort.
3. To estimate the change in alpha diversity and relative abundance of individual ARGs in the NPR between recruitment and the 3-month study end-point in the community pneumonia cohort.

Secondary Outcomes: NPR Analysis (Dependent on primary outcome analysis)

4. *(Dependent outcome) If there is a change in NPR composition between discharge and the 3-month study end-point in the hospital pneumonia cohort:* To estimate the difference in the following measures of NPR composition between the hospital pneumonia cohort at discharge, the healthy community and hospital readmission cohorts at enrolment, and the community pneumonia cohort at the 3-month study end-point:
    a) ARG richness and evenness (alpha diversity) of the NPR
    b) Overall community composition (beta diversity) of the NPR
    c) Relative abundance of ARG classes in the NPR
    d) Relative abundance of individual ARGs in the NPR
5. *(Dependent outcome) If there is a change in NPR composition between enrolment and the 3-month study end-point in the community pneumonia cohort:* To estimate the difference in the following measures of NPR composition between the community pneumonia cohort at enrolment, the healthy community and hospital readmission cohorts at enrolment, and the hospital pneumonia cohort at the 3-month study end-point:
    a) ARG richness and evenness (alpha diversity) of the NPR
    b) Overall community composition (beta diversity) of the NPR
    c) Relative abundance of ARG classes in the NPR
    d) Relative abundance of individual ARGs in the NPR

6. *(Dependent outcome) If there is a change in NPR composition between discharge and the 3-month study end-point in the hospital pneumonia cohort, and a change in the NPR composition between enrolment and the 3-month study end-point in the community pneumonia cohort:* To estimate the difference in the following measures of NPR composition between the healthy community, community pneumonia and hospital readmission cohorts at enrolment, and the hospital pneumonia cohort at discharge:
    e) ARG richness and evenness (alpha diversity) of the NPR
    f) Overall community composition (beta diversity) of the NPR
    g) Relative abundance of ARG classes in the NPR
    h) Relative abundance of individual ARGs in the NPR

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Secondary Outcomes: NPM Analysis

7. To estimate the difference in the following measures of NPM composition between the healthy community and hospital readmission cohorts at enrolment, and community pneumonia and hospital pneumonia cohorts at the 3-month study end-point:
    a) Species richness and evenness (alpha diversity) of the NPM
    b) Overall taxonomic composition (beta diversity) of the NPM
    c) Relative abundance of bacterial taxa in the NPM
8. To estimate the change in the following measures of NPM composition between recruitment, discharge and 3-month study time-points in the hospital pneumonia cohort:
    a) Species richness and evenness (alpha diversity) of the NPM
    b) Overall taxonomic composition (beta diversity) of the NPM
    c) Relative abundance of bacterial taxa in the NPM
9. To estimate the change in the following measures of NPM composition between recruitment and the 3-month study end-point in the community pneumonia cohort:
    a) Species richness and evenness (alpha diversity) of the NPM
    b) Overall taxonomic composition (beta diversity) of the NPM
    c) Relative abundance of bacterial taxa in the NPM

Secondary Outcomes: Health Outcomes

10. To estimate the difference in the incidence of adverse health outcomes between the community pneumonia cohort and hospital pneumonia cohort at the 3-month study end-point:
    a) Repeat healthcare clinic attendances
    b) Repeat antibiotic prescriptions
    c) Hospitalisation
    d) Mortality

Exploratory Outcomes

11. To estimate the association between a hospital-acquired NPR and recurrent hospitalisation with pneumonia.
12. To estimate difference in NPR composition between the healthy community and hospital readmission cohorts at enrolment, and community pneumonia and hospital

pneumonia cohorts at the 3-month study end-point based on spectral clustering of Bray-Curtis dissimilarity indices.

13. To estimate how the duration of hospitalisation will impact the following measures of NPR composition at discharge:
    a) ARG richness and evenness (alpha diversity) of the NPR
    b) Overall community composition (beta diversity) of the NPR
    c) Relative abundance of ARG classes in the NPR
14. To estimate how the use of shared bed-spaces during hospitalisation will impact the following measures of NPR composition at discharge:
    a) ARG richness and evenness (alpha diversity) of the NPR
    b) Overall community composition (beta diversity) of the NPR
    c) Relative abundance of ARG classes in the NPR
15. To estimate how prior antibiotic use, detected by urine bioassay, will impact the following measures of NPR composition at recruitment within each study group:
    a) ARG richness and evenness (alpha diversity) of the NPR
    b) Overall community composition (beta diversity) of the NPR
    c) Relative abundance of ARG classes in the NPR
16. To estimate the change in the relative abundance of mobile genetic elements (MGE) in the NPR of the hospital pneumonia cohort at enrolment and discharge time points.

Sample Size

In total we aim to recruit 350 study participants: 83 healthy community participants, 92 participants with pneumonia in the community, 92 participants admitted to hospital with pneumonia and 83 participants re-admitted to hospital with pneumonia.
There is no established method for sample size calculation in resistome studies; we use an approach for microbiome studies (Ferdous et al., 2022). Accepting 90% power, 5% type I error (95% confidence interval) and the assumptions below, we've calculated that we will require a sample size of 83 participants in each group to detect a medium effect size (Δ= 0.505), using the formula $n=2(Z_{1-\alpha/2}+ Z_{1-\beta})^2/\Delta^2$ (Ferdous et al., 2022). Study groups with a sample size of 92 participants permit a 10% loss to follow up.

Assumptions:

- Differences in gut plasmidome composition have been detected with 41 participants per group (Willmann et al., 2019), but NPR has a lower biomass than gut resistome, therefore the resistome is likely to be less diverse, with a lower relative abundance of ARGs.
- We have used the measured difference in relative abundance of a specific macrolide ARG (ermX) 3.5 years after mass-azithromycin administration (see table 1, unpublished data) as a medium effect size that maybe clinically relevant (Δ= 0.505).
- No adjustment for level of significance for multiple comparisons has been made. However, a false discovery rate will be set using the Benjamini-Hochberg method.

Table 1. Data on which the sample size calculation is based, showing increase in the relative abundance of ermX, a macrolide antibiotic resistance gene, in children 3.5 years post azithromycin mass-drug administration. Reads per million, RPM (measure of relative abundance of an ARG, normalised for the number of DNA reads in the sample)

ermX	Mean RPM*	Standard deviation	Effect size
Azithomycin exposed group	0.34	0.10	0.505
Placebo exposed group	0.29	0.07	

Statistical approach

General Considerations

The reporting of this study will be prepared in accordance with the STROBE guidelines (von Elm et al., 2007). All continuous variables will be summarised using the following descriptive statistics:

- N (size of analysis population without missing values)
- Arithmetic mean
- Standard deviation (SD)
- Median
- 25th percentile value (P25), 75th percentile value (P75) and interquartile range (IQR)
- Minimum and maximum (where relevant)

The proportion/percentage of observed levels will be reported for all categorical measures. Where appropriate, corresponding exact 95% confidence intervals (CIs) for proportions will be included.

Reporting Conventions

P-values ≥ 0.001 and ≤ 0.999 will be reported to 3 decimal places; *p*-values less than 0.001 will be reported as "< 0.001". The mean, standard deviation, median, IQR and other statistics will be reported to 3 decimal places. Minimum and maximum values will use the same number of decimal places as the original data. Proportions and percentages will be presented as 2 decimal places; values greater than zero but < 0.01 will be presented as "< 0.01", and values greater than 99.99% but less than 100% will be reported as "> 99.99%". Estimated parameters, not on the same scale as raw observations (e.g. regression coefficients) will be reported to 3 significant figures.

Missing Data

While every effort will be made to follow-up enrolled participants, there may be loss-to-follow-up. The primary analyses, including examination of longitudinal resistome evolution, will only include participants with complete follow-up data. Analyses for all secondary outcomes will include all participants. Missing data will be fully described.

Technical Details

The R environment for statistical computing (v4.2.2 or later) will be used for all analyses. All analysis code will be made publicly available on GitHub.

Participant Characteristics

We will list the participant's characteristics by the observation group within which they were enrolled, see Tables 2 to 5.

Table 2. (MOCK TABLE) Study participant characteristics

	Total (N = 350)	Healthy community (N = 83)	Community pneumonia (N = 92)	1st admission with pneumonia (N = 92)	Re-admission with pneumonia (N = 83)
Age (months)					
Mean (SD)	N (SD)	N (SD)	N (SD)	N (SD)	N (SD)
Sex					
Female	N (%)	N (%)	N (%)	N (%)	N (%)
Male	N (%)	N (%)	N (%)	N (%)	N (%)
Co-morbidities					
Present	N (%)	N (%)	N (%)	N (%)	N (%)
Mode of delivery					
NVD	N (%)	N (%)	N (%)	N (%)	N (%)
C-section	N (%)	N (%)	N (%)	N (%)	N (%)
Exclusively breastfed 1st 3 months of life					
Yes	N (%)	N (%)	N (%)	N (%)	N (%)
Indoor household pollutant exposure					
Yes	N (%)	N (%)	N (%)	N (%)	N (%)
Received PCV?					
1 dose	N (%)	N (%)	N (%)	N (%)	N (%)
2 doses	N (%)	N (%)	N (%)	N (%)	N (%)
3 doses	N (%)	N (%)	N (%)	N (%)	N (%)
No	N (%)	N (%)	N (%)	N (%)	N (%)
Received HiB vaccine?					
1 dose	N (%)	N (%)	N (%)	N (%)	N (%)
2 doses	N (%)	N (%)	N (%)	N (%)	N (%)
3 doses	N (%)	N (%)	N (%)	N (%)	N (%)
No	N (%)	N (%)	N (%)	N (%)	N (%)
MUAC (cm)					
Mean (SD)	N (SD)	N (SD)	N (SD)	N (SD)	N (SD)
NPS viral PCR result					
Influenza A	N (%)	N (%)	N (%)	N (%)	N (%)
Influenza B	N (%)	N (%)	N (%)	N (%)	N (%)
RSV subtype A	N (%)	N (%)	N (%)	N (%)	N (%)
RSV subtype B	N (%)	N (%)	N (%)	N (%)	N (%)
Negative	N (%)	N (%)	N (%)	N (%)	N (%)
Healthcare seeking behaviour					
Time taken to seek healthcare (hours)					
< 1	N (%)	N (%)	N (%)	N (%)	N (%)
1-6	N (%)	N (%)	N (%)	N (%)	N (%)
7-12	N (%)	N (%)	N (%)	N (%)	N (%)
> 12 hours	N (%)	N (%)	N (%)	N (%)	N (%)
Parent educational level					
Primary school	N (%)	N (%)	N (%)	N (%)	N (%)
Secondary school	N (%)	N (%)	N (%)	N (%)	N (%)
College/University	N (%)	N (%)	N (%)	N (%)	N (%)
Unknown	N (%)	N (%)	N (%)	N (%)	N (%)
Prior antibiotic exposure					
Antibiotic exposure in preceding 7 days					
Yes	N (%)	N (%)	N (%)	N (%)	N (%)
Duration of preceding antibiotic exposure (days)					
Mean (SD)	N (SD)	N (SD)	N (SD)	N (SD)	N (SD)
Class of preceding antibiotic exposure					
Beta-lactam	N (%)	N (%)	N (%)	N (%)	N (%)
Macrolide	N (%)	N (%)	N (%)	N (%)	N (%)
Tetracycline	N (%)	N (%)	N (%)	N (%)	N (%)
Phenicol	N (%)	N (%)	N (%)	N (%)	N (%)
Aminoglycoside	N (%)	N (%)	N (%)	N (%)	N (%)
Other	N (%)	N (%)	N (%)	N (%)	N (%)

Table 3. (MOCK TABLE) Hospitalised participant characteristics

	Total (N = 175)	1st admission with pneumonia (N = 92)	Re-admission with pneumonia (N = 83)
Haemoglobin (g/dL)			
Mean (SD)	N (SD)	N (SD)	N (SD)
Malaria point-of-care test positive			
Yes	N (%)	N (%)	N (%)
Time since last hospital admission (days)			
Mean (SD)	N (SD)	N (SD)	N (SD)
Markers of pneumonia severity			
Central cyanosis	N (%)	N (%)	N (%)
Oxygen saturations < 90%	N (%)	N (%)	N (%)
Inability to feed	N (%)	N (%)	N (%)
Vomiting	N (%)	N (%)	N (%)
Convulsions	N (%)	N (%)	N (%)
Lethargy/impaired conscious level	N (%)	N (%)	N (%)
Duration of hospital admission (days)			
Mean (SD)	N (SD)	N (SD)	N (SD)
Duration of inpatient bed-sharing (days)			
Mean (SD)	N (SD)	N (SD)	N (SD)
Number of inpatients bed-sharing			
Mean (SD)	N (SD)	N (SD)	N (SD)

Table 4. (MOCK TABLE) Antibiotic prescriptions for pneumonia treatment

	Total (N = 267)	Community pneumonia (N = 92)	1st admission with pneumonia (N = 92)	Re-admission with pneumonia (N = 83)
Total duration of antibiotic prescribed for pneumonia (days)				
Mean (SD)	N (SD)	N (SD)	N (SD)	N (SD)
Class of outpatient antibiotic prescription				
Beta-lactam	N (%)	N (%)	N (%)	N (%)
Macrolide	N (%)	N (%)	N (%)	N (%)
Tetracycline	N (%)	N (%)	N (%)	N (%)
Chloramphenicol	N (%)	N (%)	N (%)	N (%)
Aminoglycoside	N (%)	N (%)	N (%)	N (%)
Other	N (%)	N (%)	N (%)	N (%)
Duration of outpatient antibiotic prescription (days)				
Mean (SD)	N (SD)	N (SD)	N (SD)	N (SD)
Class of inpatient antibiotic prescription				
Beta-lactam	N (%)	N (%)	N (%)	N (%)
Macrolide	N (%)	N (%)	N (%)	N (%)
Tetracycline	N (%)	N (%)	N (%)	N (%)
Phenicol	N (%)	N (%)	N (%)	N (%)
Aminoglycoside	N (%)	N (%)	N (%)	N (%)
Other	N (%)	N (%)	N (%)	N (%)
Duration of inpatient antibiotic prescription (days)				
Mean (SD)	N (SD)	N (SD)	N (SD)	N (SD)

Table 5. (MOCK TABLE) Participant characteristics at 3-month follow up

	Total (N = 267)	Community pneumonia (N = 92)	1st admission with pneumonia (N = 92)
3-month mortality (count)			
Yes	N (%)	N (%)	N (%)
Re-presentation to hospital or healthcare centre with pneumonia in past 3-months?			
Yes	N (%)	N (%)	N (%)
Re-admission to hospital with pneumonia in past 3-months?			
Yes	N (%)	N (%)	N (%)
Duration of re-hospitalisation (days)			
Mean (SD)	N (SD)	N (SD)	N (SD)
Additional outpatient antibiotic prescription in past 3-months for pneumonia			
Yes	N (%)	N (%)	N (%)
Number of additional outpatient antibiotic prescription in past 3-months for pneumonia			
Mean (SD)	N (SD)	N (SD)	N (SD)
Class of additional outpatient antibiotic prescription			
Beta-lactam	N (%)	N (%)	N (%)
Macrolide	N (%)	N (%)	N (%)
Tetracycline	N (%)	N (%)	N (%)
Phenicol	N (%)	N (%)	N (%)
Aminoglycoside	N (%)	N (%)	N (%)
Other	N (%)	N (%)	N (%)
Duration of additional outpatient antibiotic prescription (days)			
Mean (SD)	N (SD)	N (SD)	N (SD)

Analysis of Primary Outcomes

The Bray-Curtis Dissimilarity Index will be calculated to quantify the beta diversity, or compositional (dis)similarity between the NPR of the four study cohorts. This will be used to generate Non-metric Multidimensional Scaling (NMDS) plots to visualise the (dis)similarity between the NPR composition of the different cohorts (see Figure 3); these plots map items based on distance, to represent the pairwise dissimilarity between objects in a two-dimensional space. The Bray-Curtis Dissimilarity Index will be compared between groups using a permutational multivariate analysis of variance (PERMANOVA).

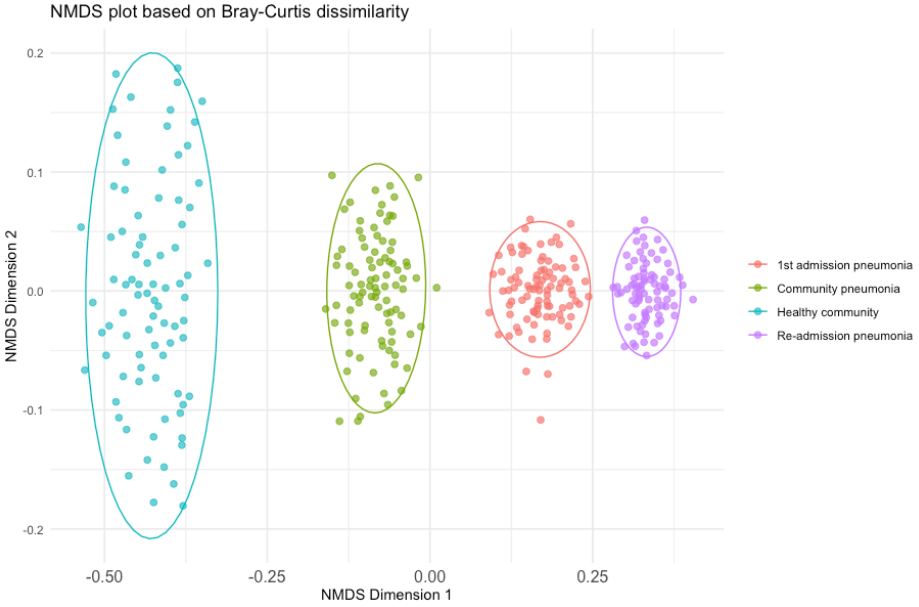

Figure 3. (MOCK FIGURE) NMDS plot comparing Bray Curtis dissimilarity index between the four study groups.

The relative abundance of ARG classes will be compared between cohorts visually using stacked bar plots (see Figure 4) and/or heatmaps with antibiotic usage data (see Figure 5), and statistically using the Kruskal-Wallis test, or Wilcoxon Signed-Rank test for paired samples, to compare the median values between cohorts with false discovery rate correction.

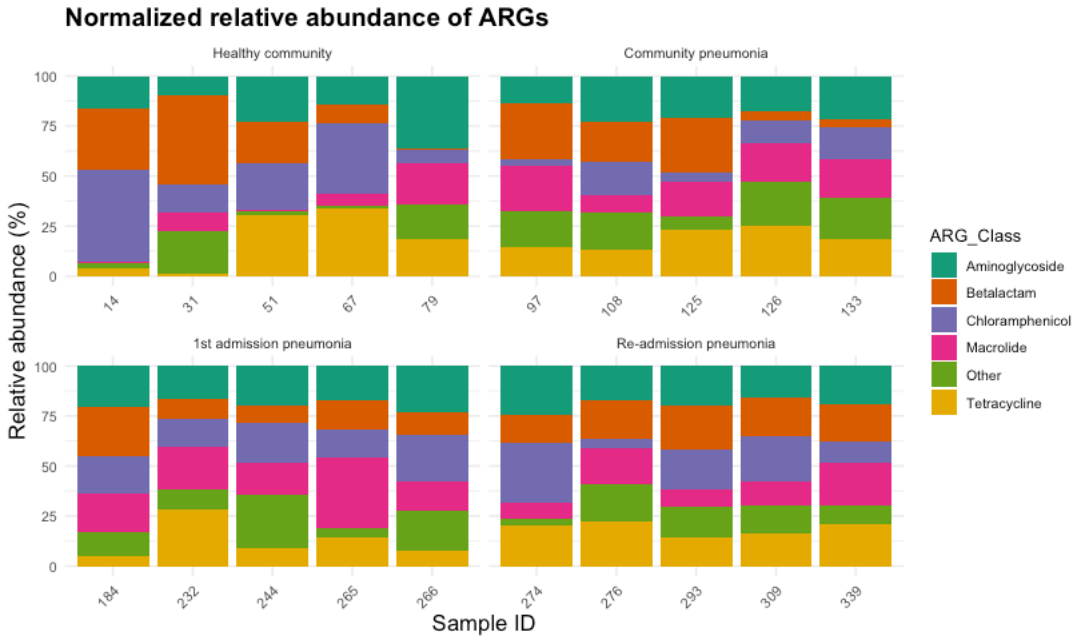

Figure 4. (MOCK FIGURE) Bar chart comparing the relative abundance of ARG classes between samples in healthy community, community pneumonia, 1st admission pneumonia and re-admission pneumonia groups.

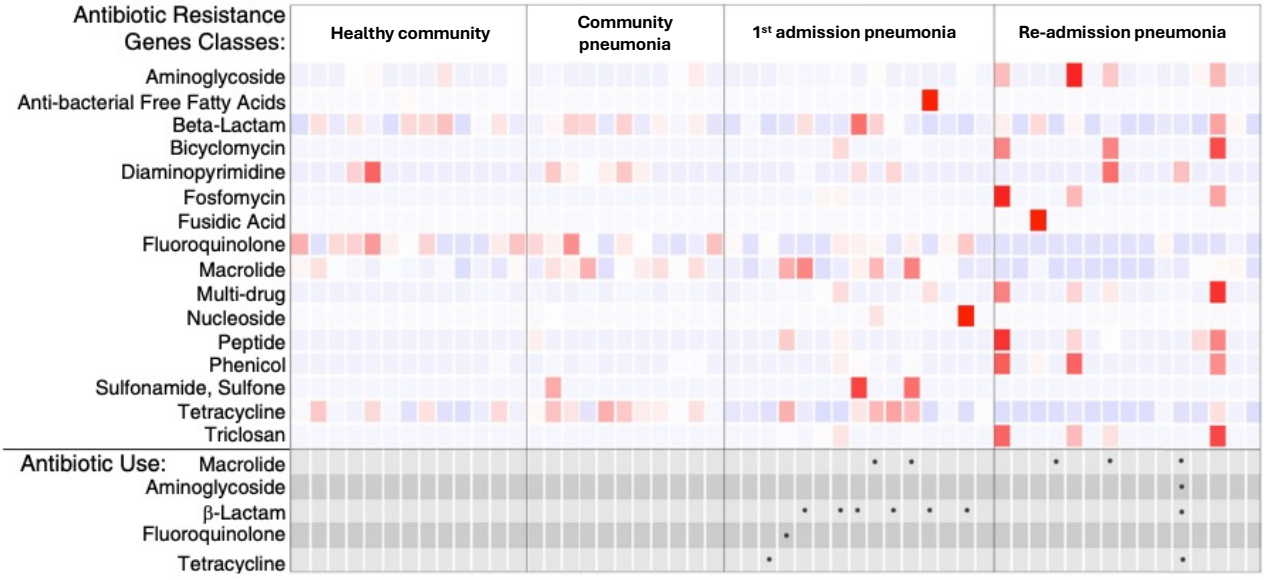

Figure 5. (MOCK FIGURE) Heatmap comparing the relative abundance of ARG classes between samples in healthy community, community pneumonia, 1st admission pneumonia and re-admission pneumonia groups, with antibiotic consumption data recorded below.

Primary Outcome Expectations:

1. There will be a difference in the overall composition of the NPR (beta diversity) between all the cohorts. There will be an increase in the relative abundance of ARG classes that produce AMR to first line antibiotics used to treat pneumonia within the NPR between healthy community and all pneumonia participants; hospitalised cohorts will have the highest relative abundance of these ARG classes.
2. There will be a difference in the overall composition of the NPR (beta diversity) between recruitment and discharge time-points in hospital pneumonia participants, but no difference between discharge and 3-month follow up time-points. There will be an increase in the relative abundance of ARG classes that produce AMR to first line antibiotics used to treat pneumonia within the NPR between recruitment and discharge time-points in hospital pneumonia participants. There will be no difference in the relative abundance of these ARG classes between discharge and 3-month follow up time-points.
3. There will be no difference in the overall composition of the NPR (beta diversity) between recruitment and 3-month follow up in community pneumonia participants. There will be no difference in the relative abundance of ARG classes that produce AMR to first line antibiotics used to treat pneumonia within the NPR between recruitment and 3-month follow up in community pneumonia participants.

The statistical methods and visual comparisons used to test these hypotheses (primary study outcomes 1 to 3) are summarised in Table 7, below.

Analyses of Secondary Outcomes

The relative abundance of individual ARGs will be compared between cohorts as detailed for the ARG classes, above. The median Shannon Diversity index will be calculated to estimate the alpha diversity of the NPR in each study cohort; this will be compared between study cohorts visually using box-plots (see Figure 6), and statistically using the Kruskal-Wallis test, or Wilcoxon Signed-Rank test for paired samples (different time-points from the same study cohort) with false discovery rate correction.

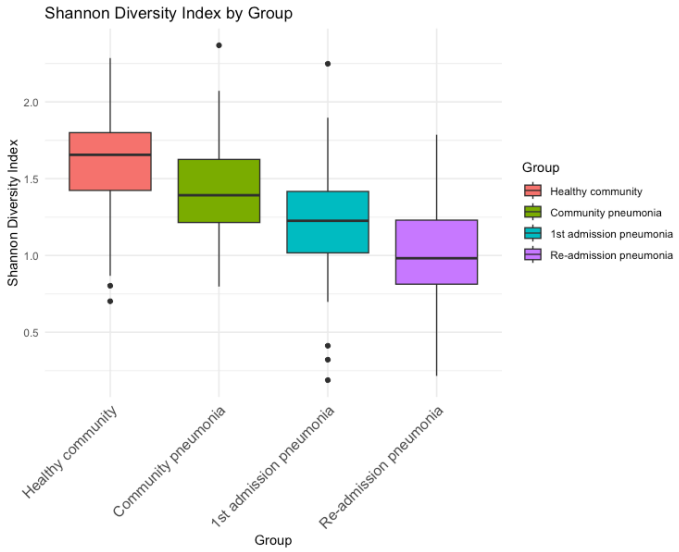

Figure 6. (MOCK FIGURE) Boxplot comparing median Shannon diversity index for bacterial species diversity across the four study groups.

Secondary Outcome Expectations:

1. There will be a difference in the alpha diversity of the NPR between the cohorts; healthy community participants will have the least diverse NPR and hospitalised cohorts will have the most diverse NPR. There will be an increase in the relative abundance of individual ARGs that produce AMR to first line antibiotics used to treat pneumonia within the NPR between healthy community and all pneumonia participants; hospitalised cohorts will have the highest relative abundance of these ARGs.
2. There will be no difference in the alpha diversity of the NPR between discharge and 3-month follow up in hospitalised pneumonia participants; the NPR will be more diverse at discharge than recruitment. There will be an increase in the relative abundance of individual ARGs that produce AMR to first line antibiotics used to treat pneumonia within the NPR between recruitment and discharge time-points in hospital pneumonia participants. There will be no difference in the relative abundance of these ARGs between discharge and 3-month follow up time-points.
3. There will be no difference in the alpha diversity of the NPR between recruitment and 3-month follow up in community pneumonia participants. There will be no difference in the relative abundance of individual ARGs that produce AMR to first line antibiotics used to treat pneumonia within the NPR between recruitment and 3-month follow up in community pneumonia participants.
4. a) There will be a difference in the alpha diversity of the NPR between the cohorts; healthy community and community pneumonia participants will have a less diverse NPR than hospital participants at discharge. Hospital readmission participants will have the most diverse NPR.
   b) There will be a difference in the overall composition of the NPR (beta diversity) between all the cohorts.
   c)/d) There will be an increase in the relative abundance of ARG classes and individual ARGs that produce AMR to first line antibiotics used to treat pneumonia within the NPR between healthy community and all pneumonia participants; hospital readmission participants will have the highest relative abundance of these ARG classes.
5. a) There will be a difference in the alpha diversity of the NPR between the cohorts; community pneumonia participants at enrolment will have a more diverse NPR than healthy community participants. Hospital readmission participants will have the most diverse NPR.
   b) There will be a difference in the overall composition of the NPR (beta diversity) between all the cohorts.
   c)/d) There will be an increase in the relative abundance of ARG classes and individual ARGs that produce AMR to first line antibiotics used to treat pneumonia within the NPR between healthy community and all pneumonia participants; hospitalised participants will have a higher relative abundance of these ARG classes than community pneumonia participants at enrolment.
6. a) There will be a difference in the alpha diversity of the NPR between the cohorts; healthy community participants will have the least diverse NPR. Hospital readmission participants will have the most diverse NPR. Community pneumonia participants at enrolment will have a less diverse NPR than hospital participants at discharge.

b) There will be a difference in the overall composition of the NPR (beta diversity) between all the cohorts.
c)/d) There will be an increase in the relative abundance of ARG classes and individual ARGs that produce AMR to first line antibiotics used to treat pneumonia within the NPR between healthy community and all pneumonia participants; hospitalised participants will have a higher relative abundance of these ARG classes than community pneumonia participants at enrolment.

7. a) There will be a difference in the alpha diversity of the NPM between the cohorts; healthy community participants will have the most diverse NPM. Hospitalised participants will have the least diverse NPM.
   b) There will be a difference in the overall composition of the NPM (beta diversity) between all the cohorts.
   c) There will be a difference in the relative abundance of bacterial taxa within the NPM between healthy community and all pneumonia participants. Healthy community participants will have the highest relative abundance of commensal bacterial taxa. Hospital readmission participants will have the highest relative abundance of pathogenic bacterial taxa.
8. a) There will be no difference in the alpha diversity of the NPM between discharge and 3-month follow up in hospitalised pneumonia participants; the NPM will be less diverse at discharge than recruitment.
   b) There will be a difference in the overall composition of the NPM (beta diversity) between recruitment and discharge time-points in hospital pneumonia participants, but no difference between discharge and 3-month follow up time-points.
   c) There will be a difference in the relative abundance of bacterial taxa within the NPM between recruitment and discharge time-points in hospital pneumonia participants, but no difference between discharge and 3-month follow up time-points.
9. a) There will be no difference in the alpha diversity of the NPM between recruitment and 3-month follow up in community pneumonia participants.
   b) There will be no difference in the overall composition of the NPM (beta diversity) between recruitment and 3-month follow up in community pneumonia participants.
   c) There will be no difference in the relative abundance of bacterial taxa within the NPM between recruitment and 3-month follow up in community pneumonia participants.

The statistical methods and visual comparisons used to test these hypotheses (secondary study outcomes 1 to 9) are summarised in Table 8, below.

10. The hospital pneumonia cohort will have a higher rate of repeat health clinic attendances, antibiotic prescriptions and hospitalisation at the 3-month study end-point than the community pneumonia cohort.

The rate of repeat health clinic attendance, antibiotic prescription, hospitalisation and mortality within 3 months will be compared between the community pneumonia and hospitalised pneumonia cohorts visually using a bar chart, and statistically using a Wilcoxon Rank Sum test with false discovery rate correction.

Exploratory Outcome Expectations:

11. There will be an association between a persistent 'hospital-acquired NPR' and recurrent hospitalisation with pneumonia.

This hypothesis will be tested using a logistic regression model to test for association between NPR composition and re-hospitalisation with pneumonia using 'cases' in the hospital re-admission group frequency matched by age and sex to 'controls' in the 1st hospital admission group, and stratified to assess for effect modification by the following variables:

- Respiratory co-morbidities
- Severity of pneumonia (WHO classification)
- Co-infection with viral pathogen (positive viral PCR for Influenza A/B and Respiratory Syncytial Virus)

The NPR composition of study participants in the 'case' and 'control' groups will be classified into a binary variable: 'hospital-acquired NPR' and 'non-hospital acquired NPR', based on the diversity and relative abundance of ARGs. The cut offs for this classification will be determined by the median diversity and relative abundance of ARG classes in the healthy community, community pneumonia and hospitalised pneumonia cohorts. Potential confounders and sources of interaction will be identified by testing the association between recorded variables and the binary outcome variable 'hospital acquired NPR', and included in the multivariable models when $p<0.1$. The results from the logistic regression model will be summarised in tabular form, see Table 6 below.

Table 6 (MOCK TABLE) Summary of the logistic regression model fit. The reference group are the 'controls': no re-admission within 3 months of hospitalisation with pneumonia.

	Odds ratio	***P* score**	**95% CI**
Hospital-acquired NPR			
Respiratory co-morbidity			
Severe pneumonia			
RSV co-infection			

12. The NPR of each cohort at the specified time points will have a distinct ARG profile, as determined by spectral clustering using Bray-Curtis dissimilarity indices.

Spectral clustering will be performed on Bray-Curtis indices between participant ARG profiles, with stability and robustness of the clusters evaluated by maximising the average silhouette score, to determine whether cohorts can be distinguished by clusters of distinct resistance gene profiles.

13. a) Participants with a longer duration of hospitalisation will have a more diverse NPR.
    b) Participants with a longer duration of hospitalisation will have a higher relative abundance of ARG classes and individual ARGs that produce AMR to first line antibiotics used to treat pneumonia within the NPR.

These hypotheses will be tested using simple linear regression models to test for association between NPR composition (beta diversity measured by Bray Curtis dissimilarity index and relative abundance of specific ARG classes measured by RPKM) and duration of hospitalisation, stratified to assess for effect modification by the following variables:

- Respiratory co-morbidities
- Severity of pneumonia (WHO classification)

14. a) Participants from shared bed spaces will have a more diverse NPR (alpha diversity) when compared to participants that did not share a bed-space.
    b) Participants from shared bed-spaces will have a different overall NPR composition (beta diversity) when compared to participants that did not share a bed-space.
    c) Participants from shared bed-spaces will have a higher relative abundance of ARG classes and individual ARGs associated with environmental gram-negative organisms when compared to participants that did not share a bed-space.

15. There will be a difference in the overall composition of the NPR (beta diversity) between participants with and without antibiotic use prior to recruitment, as detected by the urine antibiotic bioassay, within each study group. Participants with prior antibiotic use detected will have lower alpha diversity and a higher relative abundance of ARG classes used as first-line for antibiotic therapy for pneumonia.

The statistical methods and visual comparisons used to test these hypotheses are summarised in Table 8, below.

16. There will be an increase in the relative abundance of MGE between enrolment and discharge in the NPR of hospitalised participants.

This hypothesis will be tested by visually comparing the relative abundance of MGEs between enrolment and discharge time points, using stacked bar plots of RPKM, and statistically by comparing the median relative abundance using the Wilcoxon Signed-Rank test for paired samples with false discovery rate correction.

Table 7. Illustrates for each primary outcome, the sample groups being compared, hypotheses being tested, methods of data visualisation and statistical tests being used.

Primary Study Outcome	Group 1	Group 2	Group 3	Group 4	Hypothesis	Statistical analysis plan
1	Healthy community	Community pneumonia, NPS at 3-month follow up	Hospital pneumonia, NPS at 3-month follow up	Re-hospitalised pneumonia, NPS at enrolment	There will be a difference in the following measures of NPR composition between groups: a) Beta diversity b) Relative abundance of ARG classes	a) Beta diversity • Visually compare the Bray-Curtis Dissimilarity Index between groups using NMDS plots. • Statistically compare the Bray-Curtis Dissimilarity Index between groups using PERMANOVA (Outcome 1), or paired PERMANOVA (Outcomes 2 and 3). b) Relative abundance of ARG classes • Visually compare the relative abundance between groups using stacked bar plots of RPKM. • Statistically compare the median relative abundance between all four groups using the Kruskal-Wallis test with false discovery rate correction (Outcome 1). • Statistically compare the median relative abundance between groups of two using the Wilcoxon Signed-Rank test for paired samples with false discovery rate correction (Outcomes 2 and 3)
2	Hospital pneumonia, NPS at enrolment	Hospital pneumonia, NPS at discharge	Hospital pneumonia, NPS at 3-month follow up	N/A	There will be a difference in measures a) band b) of NPR composition between groups 1 and 2, and groups 1 and 3, but ***no*** statistically significant difference between groups 2 and 3.	
3	Community pneumonia, NPS at enrolment	Community pneumonia, NPS at 3-month follow up	N/A	N/A	There will be ***no difference*** in the measures a) and b) of NPR composition between groups 1 and 2.	

Table 8. Illustrates the sample groups being compared, hypotheses being tested, methods of data visualisation and statistical tests being used for secondary and exploratory outcomes (where this is not detailed in the main body of text).

Secondary Study Outcome	Group 1	Group 2	Group 3	Group 4	Hypothesis	Statistical analysis plan
1	Healthy community	Community pneumonia, NPS at 3-month follow up	Hospital pneumonia, NPS at 3-month follow up	Re-hospitalised readmission pneumonia, NPS at enrolment	There will be a difference in the following measures of NPR composition between groups: a) Alpha diversity b) Relative abundance of individual ARGs	a) Alpha diversity • Visually compare the median Shannon Diversity Index of the NPR between groups using a boxplot. • Statistically compare the median Shannon Diversity Index of the NPR between all four groups using a Kruskal Wallis test with false discovery rate correction (Outcome 1). • Statistically compare the median Shannon Diversity Index of the NPR between groups of two using the Wilcoxon Signed-Rank test for paired samples with false discovery rate correction (Outcomes 2 and 3). b) Relative abundance of individual ARGs • Visually compare the relative abundance between groups using stacked bar plots of RPKM. • Statistically compare the median relative abundance between all four groups using the Kruskal-Wallis test with false discovery rate correction (Outcome 1). • Statistically compare the median relative abundance between groups of two using the Wilcoxon Signed-Rank test for paired samples with false discovery rate correction (Outcomes 2 and 3).
2	Hospital pneumonia, NPS at enrolment	Hospital pneumonia, NPS at discharge	Hospital pneumonia, NPS at 3-month follow up	N/A	There will be a difference in measures a) to b) of NPR composition between groups 1 and 2, and groups 1 and 3, but ***no*** statistically significant difference between groups 2 and 3.	
3	Community pneumonia, NPS at enrolment	Community pneumonia, NPS at 3-month follow up	N/A	N/A	There will be ***no difference*** in the following measures a) to b) of NPR composition between groups 1 and 2.	
4	Healthy community	Community pneumonia, NPS at 3-month follow up	Hospital pneumonia, NPS at discharge	Re-hospitalised pneumonia, NPS at enrolment	There will be a difference in the following measures of NPR composition between groups: a) Alpha diversity b) Beta diversity c) Relative abundance of ARG classes d) Relative abundance of individual ARGs	a) Alpha diversity • Visually compare the median Shannon Diversity Index of the NPR or NPM between groups using a boxplot. • Statistically compare the median Shannon Diversity Index of the NPR or NPM between all four groups using a Kruskal Wallis test with false discovery rate correction (Outcomes 4 to 7, and 15). • Statistically compare the median Shannon Diversity Index of the NPM between groups of two using the Wilcoxon Signed-Rank test for paired samples with false discovery rate correction (Outcomes 8 and 9). b) Beta diversity • Visually compare the Bray-Curtis Dissimilarity Index between groups using NMDS plots.
5	Healthy community	Community pneumonia, NPS at enrolment	Hospital pneumonia, NPS at 3-month follow up	Re-hospitalised pneumonia, NPS at enrolment		
6	Healthy community	Community pneumonia, NPS at Enrolment	Hospital pneumonia, NPS at discharge	Re-hospitalised pneumonia, NPS at enrolment		

15	Healthy community					• Statistically compare the Bray-Curtis Dissimilarity Index between groups using PERMANOVA (Outcomes 4 to 7, and 16), or paired PERMANOVA (Outcomes 8 and 9). c/d) Relative abundance of ARG classes/individual ARGs for the NPR; relative abundance of bacterial taxa for the NPM • Visually compare the relative abundance between groups using stacked bar plots of RPKM. • Statistically compare the median relative abundance between all four groups using the Kruskal-Wallis test with false discovery rate correction (Outcomes 4 to 7, and 16). • Statistically compare the median relative abundance between groups of two using the Wilcoxon Signed-Rank test for paired samples with false discovery rate correction (Outcomes 8 and 9).
	Antibiotic use detected	No antibiotic use detected	N/A	N/A		
	Community pneumonia, NPS at enrolment					
	Antibiotic use detected	No antibiotic use detected	N/A	N/A		
	Hospital pneumonia, NPS at enrolment					
	Antibiotic use detected	No antibiotic use detected	N/A	N/A		
	Re-hospitalised pneumonia, NPS at enrolment					
	Antibiotic use detected	No antibiotic use detected	N/A	N/A		
16	Hospital pneumonia, NPS at discharge					
	Shared bed-space	No shared bed-space	N/A	N/A		
7	Healthy community	Community pneumonia, NPS at 3-month follow up	Hospital pneumonia, NPS at 3-month follow up	Re-hospitalised pneumonia, NPS at enrolment	There will be a difference in the following measures of NPM composition between groups: a) Alpha diversity b) Beta diversity c) Relative abundance of bacterial taxa	
8	Hospital pneumonia, NPS at enrolment	Hospital pneumonia, NPS at discharge	Hospital pneumonia, NPS at 3-month follow up	N/A	There will be a difference in measures a) to c) of NPM composition between groups 1 and 2, and groups 1 and 3, but ***no*** statistically significant difference between groups 2 and 3.	
9	Community pneumonia, NPS at enrolment	Community pneumonia, NPS at 3-month follow up	N/A	N/A	There will be ***no difference*** in the measures a) to c) of NPM composition between groups 1 and 2.	

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