

# Title: Epidemiology and transmission of *Streptococcus pneumoniae* and Respiratory syncytial virus in children and elderly: a household-based prospective cohort study

## Background & Rationale

*Streptococcus pneumoniae* (*S. pneumoniae*) is a common pathogen that causes community-acquired infections, such as bacteremia, meningitis, pneumonia, and otitis media. After the introduction of pneumococcal conjugate vaccines (PCVs), the population and ecological environment of *S. pneumoniae* have been disrupted with impact on the dominant serotypes and antibiotic resistance [1]. PCV13 coverage has been >90% since 2015, and the incidence of invasive pneumococcal disease (IPD) in children <5 years of age is reduced by 70%, and serogroup 15 isolates caused most IPD in Taiwan [2]. The use of PCVs has dramatically decreased incidence rates of diseases caused by vaccine-targeted serotypes in children and the rates of pneumococcal disease caused by non-vaccine serotypes (NVTs) have risen [3]. Besides, serotype distribution of adult IPD in Taiwan has evolved after the introduction of PCV in children, indicating an indirect impact in adults [4]. This increase in NVT IPD has also exist and in those aged 65 years and over has offset the reduction in vaccine-type IPD since PCV13 introduction in England [5].

Respiratory syncytial virus (RSV) infection causes lower respiratory tract infection such as bronchiolitis and pneumonia and is the leading cause of hospitalization of infants and young children [6-8]. After testing for viruses, bacteria, and fungi in children with severe hospitalized pneumonia, the study found that 61% of severe pneumonia cases are caused by viruses led by RSV, which alone accounted for 31% of cases [9]. In recent years, improvement in the sensitivity and specificity of molecular methods has provided new opportunities to delineate the causative CAP pathogens [10, 11]. In addition to *S. pneumoniae*, non-typeable *Haemophilus influenzae*, and *Moraxella catarrhalis* are the common etiologies [12, 13], RSV is associated with the common bacterial pathogens causing AOM [14]. The nasopharyngeal microbiota composition characterized by *H. influenzae* and *S. pneumoniae*, is associated with a distinct host inflammatory immune response and enhanced disease severity as defined by more frequent need for RSV hospitalization. Altogether, the data suggest that viral-bacterial interactions within the ecological respiratory niche modulate the systemic host immune response to RSV, thereby potentially influencing disease phenotypes [15]. This has also helped to uncover the interplay among the different pathogens during acute respiratory infections [11, 16].

In the post-pneumococcal conjugate vaccine era, seasonal increases in RSV, influenza virus, and human metapneumovirus infections in children were associated with increased pediatric admissions with invasive pneumococcal disease, especially pneumonia caused by nonvaccine serotypes [17]. Co-detection of RSV and *S. pneumoniae* in the nasopharynx was associated with more severe ARI, suggesting that *S. pneumoniae* colonization plays a pathogenic role in young children [18]. Also, pneumococcal colonization can enhance subsequent RSV infection [19]. Besides, invasive serotype colonization was significantly lower in RSV positive CAP, whereas colonization with noninvasive serotypes such as serotype 33 F tended to be higher in RSV positive CAP [20]. The serotype of *S. pneumoniae* is changing in the post-pneumococcal conjugate vaccine era in Taiwan, the effect of RSV infection for the efficacy of pneumococcal conjugate vaccine needs further study.

Asymptomatic carriage of *S. pneumoniae* in the nasopharynx is a prerequisite for disease. In the previous study, adults acquire *S. pneumoniae* relatively more in the household than other age groups, and transmissions within household were mostly seen from older to younger siblings [21]. Oropharyngeal colonization is 65.8% of *S. pneumoniae* autolysin (lytA) gene sequence [22]. RSV has become an increasingly documented cause of illness in adults and optimal diagnosis requires reverse transcription polymerase chain reaction (RT-PCR) assays or serology [23].

Close contacts within households may present potential opportunities for RSV transmission [24]. Studies have shown that family members of infants, such as older siblings and parents, are an important RSV infection source in the infants [25, 26]. In about 50% of households, more than one person is infected, and repeat infections in the same individual from homologous or heterologous RSV subtypes within the same season are documented [27]. The identification of RSV infections and increased awareness of the prominent role this respiratory virus has in relatively severe illness in adults by using molecular testing [28, 29]. Besides, the direct interaction between RSV protein and the pneumococcus might result in increased bacterial virulence and worsening disease outcome in respiratory infection [30]. On the other hand, *S. pneumoniae* colonization can enhance subsequent RSV infection [19]. The interactions occurring between *S. pneumoniae* and RSV during respiratory infections and pneumococcal acquisition indicate that pneumococcal serotypes have different ability to cause infection as well as coinfections [31].

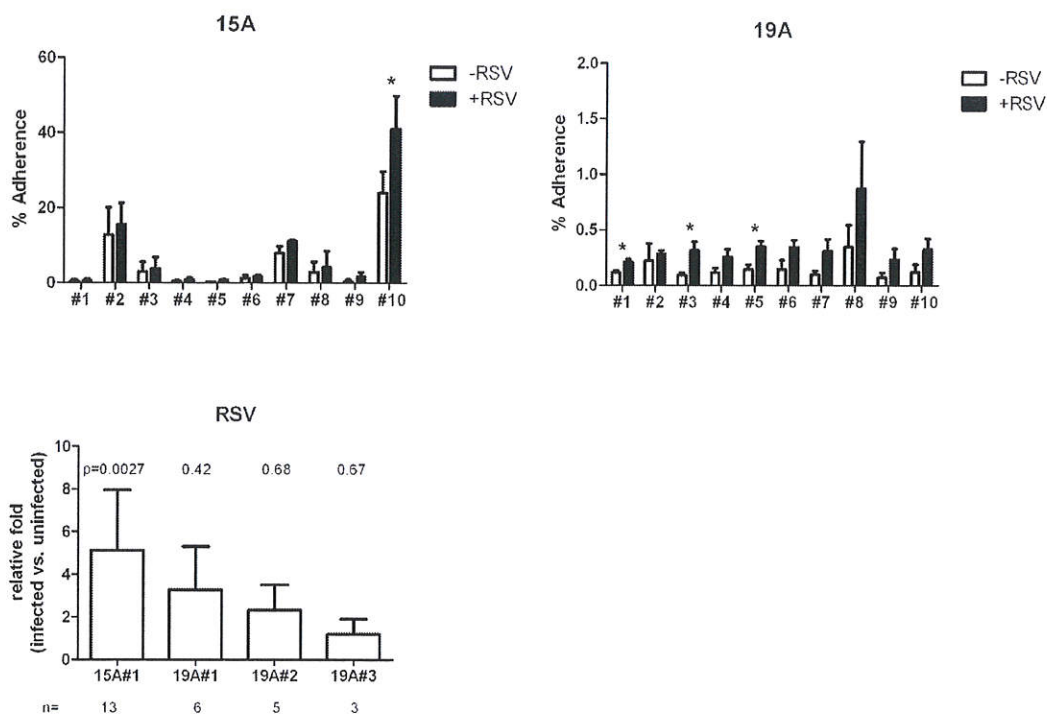


## Significance

Strategies to prevent these interactions to reduce the incidence of *S. pneumoniae* and RSV infection are very important in children as well as the elderly. The elderly might colonize with *S. pneumoniae* first and then get the RSV acquisition from the younger households. Vaccine delivery should be justified by detailed understanding of *S. pneumoniae* and RSV transmission dynamics. The determinants include carriage rate, age distribution, infection severity, and presence of concurrent *S. pneumoniae* or RSV in the same household. This household-based prospective cohort study aims to establish the household transmission of RSV and *S. pneumoniae* especially in the elderly and infants/children as well as inter-relationship between *S. pneumoniae* and RSV. We hope our data can be utilized in the AI fields to develop a model to predict the transmissions in the exceedingly early stage of disease of *S. pneumoniae* and RSV and strategy of prevention.

## Preliminary Data

Our previous data suggest that interactions between RSV and *S. pneumoniae*. RSV infections can increase the ability of adherence of *S. pneumoniae* 15 A in Hep2 cell.



We selected 13 patients for the RSV genotype and 16S ribosomal RNA test. The 16SrRNA revealed *S. pneumoniae* was the predominant bacterial in the nasopharyngeal site.

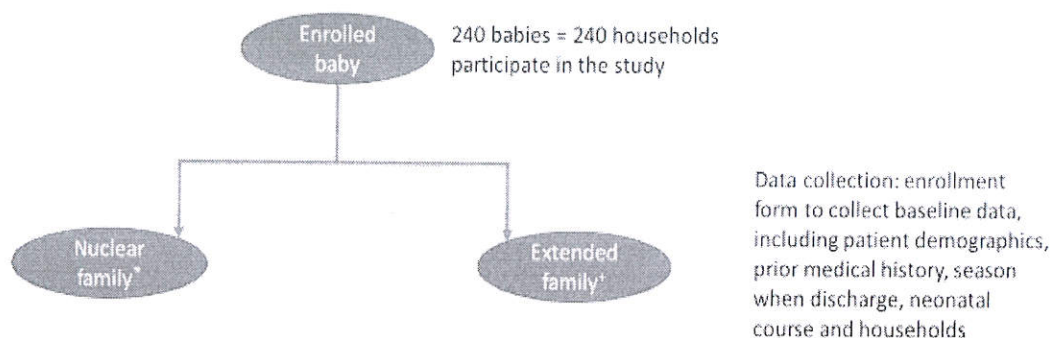
RSV	RSV genotype	16SrRNA
A	ON1	<i>Streptococcus pneumoniae</i>
B	BA9	<i>Streptococcus pneumoniae</i>
A	ON1	<i>Streptococcus pneumoniae</i>
B	BA9	<i>Haemophilus influenzae</i>
A	ON1	<i>Streptococcus salivarius</i>
B	BA9	<i>Streptococcus thermophilus</i>
A	ON1	<i>Streptococcus pneumoniae</i>
B	BA9	<i>Streptococcus lactarius</i>
B	BA9	<i>Streptococcus pneumoniae</i>
A	ON1	<i>Streptococcus pneumoniae</i>
B	BA9	<i>Streptococcus pneumoniae</i>
B	BA9	<i>Propionibacterium acnes</i>
B	BA9	<i>Streptococcus pneumoniae</i>



## Study site and population

A household-based prospective cohort study will be conducted from 2021 to 2025 in northern Taiwan. We will invite 240 households, having a baby who is discharged from MacKay children's Hospital.

Around 20 babies per months for 12 months adding up to 240 babies in total. The estimation is based on historical discharge number from NBR MacKay Memorial Hospital Taipei. The number is also proved feasible in previous studies.



\* nuclear family: a couple and their dependent children, regarded as a basic social unit.

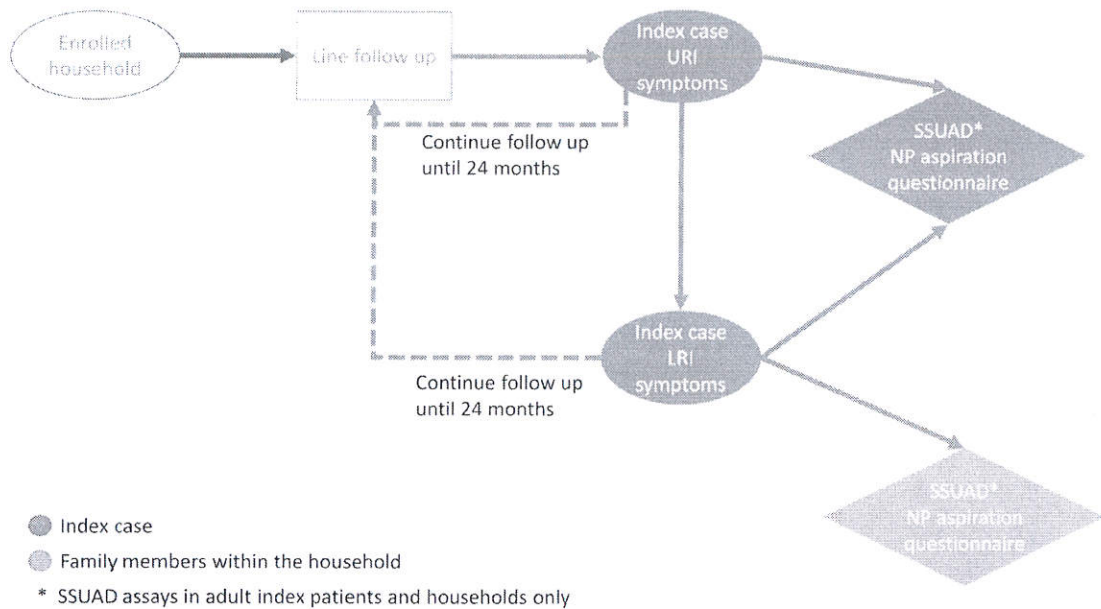
+ extended family: a family that extends beyond the nuclear family, including grandparents, aunts, uncles, and other relatives, who all live nearby or in one household.

## Patient recruitment and management

The households will be classified into nuclear family type and extended family type. The “Nuclear family type” is defined as husband and wife with baby (aged less than 5 months) and with or without children (2–5) and the “Extended family type” (three-generation family) is defined as husband and wife with baby (aged less than 5 months), children (2–5), and grandparents aged > 65 years old. The exclusion criteria are co-morbid medical conditions of the baby such as chronic lung disease, cyanotic congenital heart disease, neuromuscular disease and a primary immunodeficiency. We will initially complete an enrollment form to collect baseline data, including patient demographics, prior medical history, season when discharge, neonatal course and households. A LINE-based management will be done weekly by study team to see if there are any respiratory symptoms in household. In addition to the monthly physician weekly LINE response, the research nurse will contact the parents or legal guardians by telephone monthly for the 24 months to obtain data on changes in baseline information, and specific facts regarding possible respiratory infections after the last contact.







## Study endpoint

The primary endpoint is the *S. pneumoniae* and RSV isolation rates in children and their household contacts of different family type. The secondary endpoint is the serotype of *S. pneumoniae* and RSV in post-PCV13 era.

## Study design

If the households present with **respiratory symptoms**, the visit will be arranged as early as possible. When they presenting to the emergency department, outpatient clinic, or inpatient ward, the urine will be collected for the serotype-specific urinary antigen detection (SSUAD) assays in adult households and the nasopharyngeal or throat swab for PCR will be performed to study serotype of *S. pneumoniae* and RSV subgroup. If the subjects who have clinical syndromes suggestive of pneumonia or bronchopneumonia, they will be asked to participate in the further study along with their household family members. Nasopharyngeal aspiration, urine, blood, and induced sputum will be collected for cultures, PCR and serological test. Clinical manifestations, disease course, and outcomes will be recorded. Family members in the same household will asked to undergo screening with a nasopharyngeal or throat swab and urine sample. We will trace back 2-week contact history of children in nuclear family who get RSV or pneumococcal infections, especially focus on other family members. The data will be further analyzed to evaluate the influence of short period and long period contact.

Case report form will be used to collect information about the family members, including demographic data, the number of bedrooms in the house, amount of contact time with the patient, presence and pattern of current or recent signs and symptoms and contact history with persons outside of the household who had clinical syndromes suggestive of *S. pneumoniae* or RSV infection.

Follow-up telephone interviews repeated questions about signs and symptoms at 2, 4, and 8 weeks. If any household family member reported experiencing signs or symptoms suggesting *S. pneumoniae* or RSV infection during the follow-up period, clinical assessment and laboratory investigation will be repeated.



	Data collection	Specimen collection
Enrollment	1. information consent form 2. baseline data, including patient demographics, prior medical history, season when discharge, neonatal course and households	
Line follow up period	Checking if there are any respiratory symptoms	
Index case presents symptoms suggesting upper respiratory tract infection		Index case 1. Serotype-specific urinary antigen detection (SSUAD) assays 2. Nasopharyngeal or throat swab for PCR will be performed to study serotype of <i>S. pneumoniae</i> and RSV subgroup. RSV will be sequenced to ascertain F protein heterogeneity.
Index case presents symptoms suggesting lowerer respiratory tract infection	Index case 1. Clinical manifestations, disease course, and outcomes will be record.  Family members 1. Questionnaire-based interviews will be used to collect information bedrooms in the house, amount of contact time with the patient, presence and pattern of current or recent signs and symptoms and contact history with persons outside the household who had clinical syndromes suggestive of <i>S. pneumoniae</i> or RSV infection.	Index case 1. Routine clinical examination of nasopharyngeal aspiration, urine, blood and induced sputum will be collected for culturws, PCR and serological tests.  Family members 1. Family members in the same household will ased to undergo screeing with a nasopharyngeal or throat swab and urine sample.
Post first episode	Follow-up interviews repeated questions about signs and symptoms at 2, 4, and 8 weeks. If any household family member reported experiencing signs or symptoms suggesting <i>S. pneumoniae</i> or RSV infection during the follow-up period, clinical assessment and laboratory investigation will be repeated.	

## LINE management

Establish a LINE group with the parent or guardian. The family will response back weekly or when they have acute respiratory infection (ARI) symptoms/signs. We will contact them monthly to fill out a Google Questionnaire.

## Serotype-specific urinary antigen detection (SSUAD) assays

Urine specimens of adult households will be collected and stored at  $-80^{\circ}\text{C}$  for SSUAD) assays.

## RSV PCR for RSVA and RSVB

Direct molecular detection of RSV Types A & B from nasopharyngeal swabs will be done by using HiberGene's RSV A/B Combo. The F protein gene will be amplified by RT-PCR using primers described in our previous study [32]. RSV genotypes will be assigned after alignment with reference sequences and subsequent phylogenetic analysis using MEGA software (Version MEGA 7.0.26). For this purpose, the best-fit nucleotide substitution model will be calculated. Phylogenetic trees will be constructed using the maximum likelihood algorithm and the General time-reversible model with a gamma distribution of 5. To test the tree stability bootstrap values of 1,000 will be used.

## Antimicrobial Nonsusceptibility Testing

Antimicrobial susceptibility testing will be performed according to guidelines issued by the Clinical and Laboratory Standards Institute (CLSI) regarding broth microdilution and breakpoint criteria. Both intermediate and resistant isolates will be categorized as nonsusceptible for the purposes of the analysis, unless otherwise specified. Seven antimicrobial agents, each representing a different class, will be tested: penicillin G (parenteral, nonmeningitis), ceftriaxone (nonmeningitis), clindamycin, erythromycin, tetracycline, levofloxacin, and vancomycin. A multidrug resistance (MDR) phenotype was defined as either intermediate or resistance to  $\geq 3$  of these 7 antimicrobial agents.



## Multiplex PCR for viruses and *S. pneumoniae* serotyping

Patients will be arranged for nasopharyngeal/oropharyngeal (NP/OP) swabs collected within 48 hours after symptoms developed and tested using multiplex polymerase chain reaction (PCR) for a range of viruses [8] and for 92 pneumococcal serotypes [33]. Serotyping of isolated *S. pneumoniae* will be performed by Quellung reaction (Statens Serum Institute, Copenhagen, Denmark) or PCR. Genomic DNA of bacterial cells will be extracted from a fresh overnight culture by Viogene Geno Plus Genomic DNA Extraction Miniprep System (Taiwan) according to the manufacturer's instruction. *CpsB* gene is amplified and sequenced for PCR seq Typing[34]. The amplifying and sequencing primers are as follow: cps F 5'-GCA ATG CCA GAC AGT AAC CTC TAT-3', and cps R, 5'-CCT GCC TGC AAG TCT TGA TT-3'. The reaction mixture contains 1 µl genomic DNA extract, 0.4 µM each primer and 2X Premix (SapphireAmp® Fast PCR Master Mix, Takara), makes up to a final volume of 25 µl with DNase/RNase-free distilled water. The reaction cycles are an initial denaturation step at 95°C for 5 min, followed by 30 amplification cycles of 95°C for 30 S, 65°C for 30 S, and 72°C for 1 min. Purified amplicons are sent for sequencing to Genomics Biotechnology Co., Ltd (New Taipei City, Taiwan). Sequencing will be performed using the Sanger sequencing technique. Serotype will be decided based on the serotype with the highest BLAST bit score identity of the *CpsB* gene sequence from GenBank.

## *S. pneumoniae* and RSV interaction metagenomic study

### Sample preprocessing

The collection tube will be kept on ice during the whole process. The whole process would be not over than 30 minutes to keep the stability of microbiome profile. For sample quality control, the standard samples are defined as the presence of a white sputum plug on visual inspection by clinicians. First, to break disulphide bonds in mucin, 4 ml dithiothreitol 0.1% (DDT) per gram sputum will be added after collection. Then we will homogenize the samples with Eppendorf homogenizer or vortex for 15 minutes at 4°C. Then we will add phosphate-buffered saline (PBS) and filter the sample to increase homogenization. Lastly, the samples will undergo 15 minutes centrifugation at 4°C, 450 g (RCF). The supernatants will be removed and the samples will be aliquoted in 0.5 ml tubes, and stored at -80°C for DNA extraction. The present study was approved by the institutional review board of all participated hospitals. Written informed consent was obtained from every participant and had been fully explained.

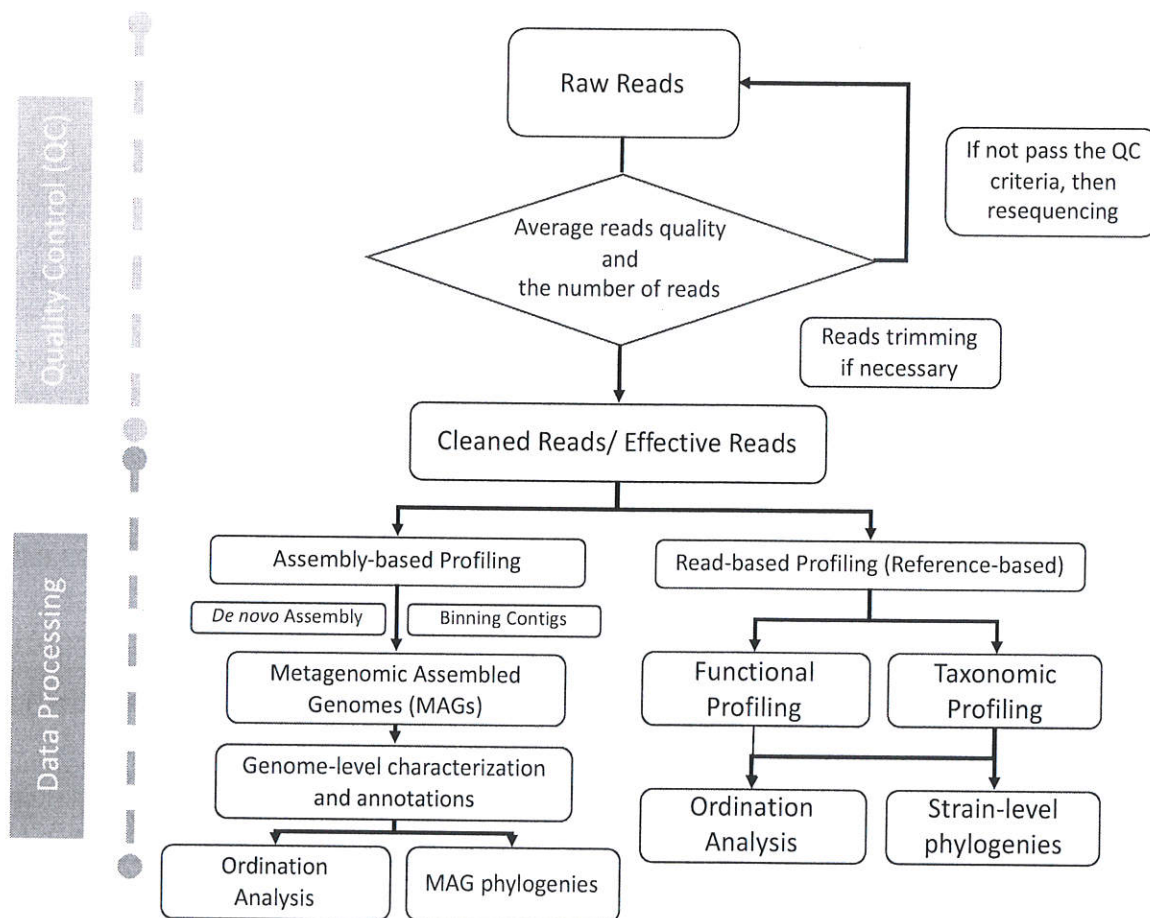
### DNA extraction and shotgun metagenomic sequencing

Microbiota DNA was extracted from preprocessed sputum samples using DNeasy PowerMax Soil Kit (QIAGEN Inc., USA). All lysis, separation of impurities and purification procedures were followed the manufacturer protocol of DNeasy PowerMax Soil Kit. The DNA samples will be evaluated for the quality via NanoDrop Microvolume Spectrophotometers. The qualified DNA will be used for library preparation by using Nextera XT library preparation kit (Illumina) according to the manufacturer's instructions. The shotgun sequencing will be performed on the Illumina NovaSeq 6000 platform, targeting >8 Gb sequence per sample with 150 bp pair-end mode.



## Shotgun metagenomic sequencing analyses pipeline

In the beginning, all the raw shotgun sequencing files will be under quality control with multiQC and trimmomatic to filter out the adapters and the reads with low quality score. Then we will use FastQ Screen to filter the reads that map on the host genomes and focus on the effective reads that belonged to bacterial genomes. For the comprehensively shotgun metagenomic analysis, we will generally adopt the two main strategies of microbiome identification of shotgun sequencing nowadays: read-based profiling and assembly-based profiling (See Figure). For the assembly approach, we will use MegaHIT as assembler to obtain contigs for each sample. When assembly, MegaHIT will use a memory-efficient succinct de Bruijn graph representation combined with a range of k-mers for iteratively improving assembly. The contigs will undergo quality control and gene prediction using QUAST. The contigs that passed the quality control will be used for further phylogenies, ordination and functional analysis that mentioned below. For the read-based approach, we will use MetaPhlAn2 with the default setting for taxonomic profiling and relative abundance calculation. For further strain-level analysis, we will use PanPhlAn to identify strain-specific gene sets from species' pangenome for strain identification. Functional profiling will be performed with HUMAnN2. In brief, HUMAnN2 include the following steps for gene family/pathways abundance and pathway coverage calculation: mapping metagenomic reads to the pangenomes of species by, annotating the aligned sequence to UniRef90 families and using DIAMOND for the unaligned sequence. For deciding the optimal combination of numerous metagenomic analytic software, we have considered the computing resource, our previous analysis experience and the simulation comparisons results from previous research to design an efficient and comprehensive analytic pipeline.



### Power/Sample Size:

We design the study to have a power of 80% at a 5% significance level to determine an incidence of household-acquired *S. pneumoniae* infection are 29-46% in nuclear family and 38-50% in extended family according to the previous study [35]. The sample size in each group will be 93 patients in each group [36]. We will enroll 120 households in each group for the possible 15% loss rate in 2 years.



## Statistical Methods

## Infected model

Households with at least 1 ARI case during the study period will be included for data analysis. A household with ARI in different years will be treated as 2 different households. Because household visits will be conducted weekly, we will collect specimens for ARI episodes as possible. We will identify these ARI through symptom records. The ARI cases in RSV or *S. pneumoniae* whose dates of onset are within 10 days before or after RSV or *S. pneumoniae* onset will be identified and defined as preceding and subsequent ARI, respectively. We will apply a Bayesian approach to estimate acquisition and clearance rates for RSV or *S. pneumoniae* in a susceptible-infected-susceptible model [37]. The inference relies on repeated measurements of the current status of being a non-carrier (susceptible) or a carrier (infected) of one of the pathogens. We will collect the measurements with sampling intervals. The index cases are the first members of the household to have clinically apparent illness confirmed by laboratory studies. The secondary cases are defined as other family members whose respiratory symptoms occurred later than the index cases' illness. Identified source of infection within the household is defined as the first case in the household who displayed clinically apparent disease and who have clear contact history with individuals outside the household who had illnesses suggestive of *S. pneumoniae* or RSV infection. The infection transmission interval will be defined as the time between the onset of disease for the first case in the household and the onset of disease in a secondary case [38].

## Hierarchical generalized linear mixed effect models (HGLMEM)

In order to assess variables that are significantly impacted in *S. pneumoniae* or RSV infection status across time points in 2 different households, we will fit several random and fixed effects three-level longitudinal generalized hierarchical linear mixed effect models (HGLMEM) with restricted maximum likelihood (REML) estimation method [39, 40]. HGLMEM will be used to evaluate longitudinal *S. pneumoniae* or RSV infection status measurement across time points (level 1) that are nested-within individual subject (level 2) and nested-within household (level 3). Because the model can assume random time effects of measurement, HGLMEM will be a practical strategy for analyzing our data sets. Random effects account for within-subject correlation and will be fitted an unstructured (UN) covariance structure. The structure implies that all different members of a cluster are differently correlated with each other across time. Fixed effects such as the clinical covariates (including clinical data, candidate markers, and target genes), baseline assessment in *S. pneumoniae* or RSV infection status, and household group  $\times$  time interaction term will be all included to reflect differential patterns of change over time for the two different household groups. Different fixed effects covariates' combination hierarchical models will be built in the three-level HGLMEM. The HGLMEM will be used to compare the change of *S. pneumoniae* or RSV infection status across time points and to survey the potential associations between these outcomes and independent variables.

Data will be summarized using descriptive statistics. Continuous variables will be expressed as median with interquartile range (IQR) and compared using Mann-Whitney U test. Categorical variables will be presented as frequency and percentage and compared using chi-square or Fisher's exact test, as appropriate. A *P* value less than 0.05 will be considered statistically significant.

Statistical analyses for interaction are also carried out via R software and QIIME2. In the present proposal, only the species-level taxonomic data will be used to calculate the alpha (richness, Shannon-Weaver index) and beta diversity that based on Jaccard distance or Bray-Curtis distance matrix as suggested by previous research. The beta diversity will be presented via principal coordinate analysis (PCoA, avoiding negative eigenvalues), or via non-metric multidimensional scaling (NMDS). Univariate analyses will be carried out with Wilcoxon/Kruskal-Wallis tests. For global/ individual microbiota composition differences comparison, permutational analysis of variance (PERMANOVA) and analysis of composition of microbiomes (ANCOM) methods in QIIME2 will be used. We will also perform linear discriminant analysis (LDA) effect size (LEfSe) for differential abundance analysis. We will use *ggplot2* package in R software, QIIME2 and GraPhIAn for results visualization.



## Reference

1. Huang YC, Lin CF, Ting PJ, et al. Respiratory pathogens - Some altered antibiotic susceptibility after implementation of pneumococcus vaccine and antibiotic control strategies. *J Microbiol Immunol Infect* **2019**.
2. Lu CY, Chiang CS, Chiu CH, et al. Successful Control of *Streptococcus pneumoniae* 19A Replacement With a Catch-up Primary Vaccination Program in Taiwan. *Clin Infect Dis* **2019**; 69(9): 1581-7.
3. Feikin DR, Kagucia EW, Loo JD, et al. Serotype-specific changes in invasive pneumococcal disease after pneumococcal conjugate vaccine introduction: a pooled analysis of multiple surveillance sites. *PLoS Med* **2013**; 10(9): e1001517.
4. Chi HC, Hsieh YC, Tsai MH, et al. Impact of pneumococcal conjugate vaccine in children on the serotypic epidemiology of adult invasive pneumococcal diseases in Taiwan. *J Microbiol Immunol Infect* **2018**; 51(3): 332-6.
5. Ladhani SN, Collins S, Djennad A, et al. Rapid increase in non-vaccine serotypes causing invasive pneumococcal disease in England and Wales, 2000-17: a prospective national observational cohort study. *Lancet Infect Dis* **2018**; 18(4): 441-51.
6. Hall CB, Weinberg GA, Iwane MK, et al. The burden of respiratory syncytial virus infection in young children. *N Engl J Med* **2009**; 360(6): 588-98.
7. Obando-Pacheco P, Justicia-Grande AJ, Rivero-Calle I, et al. Respiratory Syncytial Virus Seasonality: A Global Overview. *J Infect Dis* **2018**; 217(9): 1356-64.
8. Lin CY, Hwang D, Chiu NC, et al. Increased Detection of Viruses in Children with Respiratory Tract Infection Using PCR. *International journal of environmental research and public health* **2020**; 17(2).
9. Causes of severe pneumonia requiring hospital admission in children without HIV infection from Africa and Asia: the PERCH multi-country case-control study. *Lancet* **2019**; 394(10200): 757-79.
10. Wolf DG, Greenberg D, Shemer-Avni Y, Givon-Lavi N, Bar-Ziv J, Dagan R. Association of human metapneumovirus with radiologically diagnosed community-acquired alveolar pneumonia in young children. *J Pediatr* **2010**; 156(1): 115-20.
11. Jain S, Williams DJ, Arnold SR, et al. Community-acquired pneumonia requiring hospitalization among U.S. children. *N Engl J Med* **2015**; 372(9): 835-45.
12. Chiu NC, Lin HY, Hsu CH, Huang FY, Lee KS, Chi H. Epidemiological and microbiological characteristics of culture-proven acute otitis media in Taiwanese children. *J Formos Med Assoc* **2012**; 111(10): 536-41.
13. Kung YH, Chiu NC, Lee KS, et al. Bacterial etiology of acute otitis media in the era prior to universal pneumococcal vaccination in Taiwanese children. *J Microbiol Immunol Infect* **2014**; 47(3): 239-44.
14. Patel JA, Nguyen DT, Revai K, Chonmaitree T. Role of respiratory syncytial virus in acute otitis media: implications for vaccine development. *Vaccine* **2007**; 25(9): 1683-9.
15. de Steenhuijsen P, Pitsers WA, Heinonen S, Hasrat R, et al. Nasopharyngeal Microbiota, Host Transcriptome, and Disease Severity in Children with Respiratory Syncytial Virus Infection. *Am J Respir Crit Care Med* **2016**; 194(9): 1104-15.
16. Tsolia MN, Psarras S, Bossios A, et al. Etiology of community-acquired pneumonia in hospitalized school-age children: evidence for high prevalence of viral infections. *Clin Infect Dis* **2004**; 39(5): 681-6.
17. Ampofo K, Bender J, Sheng X, et al. Seasonal invasive pneumococcal disease in children: role of preceding respiratory viral infection. *Pediatrics* **2008**; 122(2): 229-37.
18. Brealey JC, Chappell KJ, Galbraith S, et al. *Streptococcus pneumoniae* colonization of the nasopharynx is associated with increased severity during respiratory syncytial virus infection in young children. *Respirology* **2018**; 23(2): 220-7.
19. Nguyen DT, Louwen R, Elberse K, et al. *Streptococcus pneumoniae* Enhances Human Respiratory Syncytial Virus Infection In Vitro and In Vivo. *PLoS One* **2015**; 10(5): e0127098.
20. Greenberg D, Givon-Lavi N, Faingelernt Y, et al. Nasopharyngeal Pneumococcal Carriage During Childhood Community-Acquired Alveolar Pneumonia: Relationship Between Specific Serotypes and Coinfecting Viruses. *J Infect Dis* **2017**; 215(7): 1111-6.
21. Althouse BM, Hammitt LL, Grant L, et al. Identifying transmission routes of *Streptococcus pneumoniae* and sources of acquisitions in high transmission communities. *Epidemiol Infect* **2017**; 145(13): 2750-8.
22. Tramuto F, Amodio E, Calamusa G, Restivo V, Costantino C, Vitale F. Pneumococcal Colonization in the Familial Context and Implications for Anti-Pneumococcal Immunization in Adults: Results from the BINOCOLO Project in Sicily. *International journal of molecular sciences* **2017**; 18(1).
23. Walsh EE, Falsey AR. Respiratory syncytial virus infection in adult populations. *Infect Disord Drug Targets* **2012**; 12(2): 98-102.
24. Agoti CN, Munywoki PK, Phan MVT, et al. Transmission patterns and evolution of respiratory syncytial virus in a community outbreak identified by genomic analysis. *Virus evolution* **2017**; 3(1): vex006.
25. Crowcroft NS, Zambon M, Harrison TG, Mok Q, Heath P, Miller E. Respiratory syncytial virus infection in infants admitted to paediatric intensive care units in London, and in their families. *Eur J Pediatr* **2008**; 167(4): 395-9.



26. Otomaru H, Kamigaki T, Tamaki R, et al. Transmission of Respiratory Syncytial Virus Among Children Under 5 Years in Households of Rural Communities, the Philippines. *Open Forum Infect Dis* **2019**; 6(3): ofz045.
27. Munywoki PK, Koech DC, Agoti CN, et al. The source of respiratory syncytial virus infection in infants: a household cohort study in rural Kenya. *J Infect Dis* **2014**; 209(11): 1685-92.
28. Kim L, Rha B, Abramson JS, et al. Identifying Gaps in Respiratory Syncytial Virus Disease Epidemiology in the United States Prior to the Introduction of Vaccines. *Clin Infect Dis* **2017**; 65(6): 1020-5.
29. Midgley CM, Haynes AK, Baumgardner JL, et al. Determining the Seasonality of Respiratory Syncytial Virus in the United States: The Impact of Increased Molecular Testing. *J Infect Dis* **2017**; 216(3): 345-55.
30. Smith CM, Sandrini S, Datta S, et al. Respiratory syncytial virus increases the virulence of *Streptococcus pneumoniae* by binding to penicillin binding protein 1a. A new paradigm in respiratory infection. *Am J Respir Crit Care Med* **2014**; 190(2): 196-207.
31. Yan T, Tang X, Sun L, Tian R, Li Z, Liu G. Co infection of respiratory syncytial viruses (RSV) and *Streptococcus pneumoniae* modulates pathogenesis and dependent of serotype and phase variant. *Microb Pathog* **2020**; 104126.
32. Chi H, Liu HF, Weng LC, et al. Molecular epidemiology and phylodynamics of the human respiratory syncytial virus fusion protein in northern Taiwan. *PLoS One* **2013**; 8(5): e64012.
33. Pai R, Gertz RE, Beall B. Sequential multiplex PCR approach for determining capsular serotypes of *Streptococcus pneumoniae* isolates. *J Clin Microbiol* **2006**; 44(1): 124-31.
34. Leung MH, Bryson K, Freystatter K, et al. Sequotyping: serotyping *Streptococcus pneumoniae* by a single PCR sequencing strategy. *J Clin Microbiol* **2012**; 50(7): 2419-27.
35. Melegaro A, Gay NJ, Medley GF. Estimating the transmission parameters of pneumococcal carriage in households. *Epidemiol Infect* **2004**; 132(3): 433-41.
36. Menashe I, Rosenberg PS, Chen BE. PGA: power calculator for case-control genetic association analyses. *BMC genetics* **2008**; 9: 36.
37. Erasto P, Hoti F, Auranen K. Modeling transmission of multitype infectious agents: application to carriage of *Streptococcus pneumoniae*. *Stat Med* **2012**; 31(14): 1450-63.
38. Chang LY, Tsao KC, Hsia SH, et al. Transmission and clinical features of enterovirus 71 infections in household contacts in Taiwan. *JAMA* **2004**; 291(2): 222-7.
39. Wang KS, Liu X, Ategbale M, et al. Generalized Linear Mixed Model Analysis of Urban-Rural Differences in Social and Behavioral Factors for Colorectal Cancer Screening. *Asian Pac J Cancer Prev* **2017**; 18(9): 2581-9.
40. Glorioso TJ, Grunwald GK, Ho PM, Maddox TM. Reference effect measures for quantifying, comparing and visualizing variation from random and fixed effects in non-normal multilevel models, with applications to site variation in medical procedure use and outcomes. *BMC Med Res Methodol* **2018**; 18(1): 74.

5

