Official study title:

Impact of the Gut Microbiome on Immune response and disease severity in acute Respiratory Syncytial Virus (RSV) infection in Vietnamese children: A prospective observational study

ClinicalTrials.gov Identifier (NCT Number):

NCTXXXXXXXX (to be added once assigned)

Organization's Unique Protocol ID:

41/BVNTW-HĐĐĐ

Sponsor:

Vietnam National Children's Hospital (VNCH), Hanoi, Vietnam

Responsible Party:

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Document Type:

Study Protocol and Statistical Analysis Plan

Document Version:

Version 1.0

Document Date:

28 Jan 2026

STUDY PROTOCOL

Title: Impact of the Gut Microbiome on Immune response and disease severity in acute Respiratory Syncytial Virus (RSV) infection in Vietnamese children: A prospective observational study

1. Background and Rationale

Respiratory Syncytial Virus (RSV) is the leading cause of severe lower respiratory tract infection in infants and young children, responsible for substantial global morbidity and mortality, particularly in low- and middle-income countries. In Vietnam, RSV accounts for nearly half of hospitalizations for respiratory infections in children under two years of age. Notably, approximately 65% of severe RSV cases occur in previously healthy children without identifiable risk factors, highlighting a critical gap in early risk prediction and disease stratification.

Emerging evidence supports the role of the **gut–lung axis**, whereby gut microbiota modulate systemic and pulmonary immune responses through microbial metabolites and immune signaling pathways. Alterations in gut microbiota composition and associated cytokine responses have been implicated in the severity of viral respiratory infections, including RSV. However, no integrated microbiome–immune studies of RSV have been conducted in Vietnam or the broader Asian region, despite this region bearing the highest disease burden.

2. Study Objectives

2.1 Primary Objectives

- To characterize differences in gut microbiota composition between children with severe and mild RSV infection

2.2 Secondary Objectives

- To describe systemic immune responses by quantifying key serum cytokines in RSV-infected children.

- To analyze the relationships between gut microbiota profiles, immune responses, and clinical severity of RSV infection.
- To identify candidate microbiota-immune biomarkers associated with severe RSV disease.

3. Study Design

- Study Type: Observational
- Observational Model: Prospective Cohort
- Time Perspective: Prospective
- Sample Size

  Total: 250 children aged 1–24 months

  Severe RSV: 125 cases

  Mild RSV: 125 cases

4. Study Population

Infants with acute lower respiratory tract infection and RT-PCR-confirmed RSV presenting to Vietnam National Children's Hospital

5. Eligibility Criteria

5.1 Inclusion Criteria

- Age 1–24 months
- Laboratory-confirmed RSV infection by RT-PCR
- Acute lower respiratory tract infection
- Informed consent from parent or legal guardian

5.2 Exclusion Criteria

- Prematurity <32 weeks gestation or birthweight <1500 g
- Chronic conditions (e.g., congenital heart disease, chronic lung disease, chronic liver/kidney disease)
- Primary or acquired immunodeficiency
- Severe malnutrition (weight-for-age Z-score < −3 SD)
- Antibiotic use within 2 weeks before admission
- Probiotic use within 4 weeks before admission

- Co-infection with other pathogens (viral/bacterial)
- Stool sample not obtained within 24 hours of admission

6. Study drocedures and data collection

RSV diagnosis: Real-time RT-PCR from respiratory specimens

Clinical data collection: by CRF

Gut microbiota analysis:

- Stool samples collected within 24 hours of admission
- 16S rRNA gene sequencing (V3–V4 region) using Illumina MiSeq

Immune response assessment:

- Quantification of key serum cytokines (e.g., IL-6, IL-8, TNF-α, IFN-γ, IL-10, IL-17, IL-22) using ELISA

Clinical severity assessment:

- Based on oxygen requirement, respiratory support, and PICU admission

Follow-up assessments will be performed at predefined time points up to Day 28 after PICU admission.

7. Outcome Measures

7.1 Primary Outcomes

- Differences in gut microbiota diversity and composition between severe and mild RSV cases

7.2 Secondary Outcomes

- Cytokine profiles associated with RSV severity
- Correlations between microbiota features, immune responses, and clinical severity
- Identification of integrated microbiota–immune biomarkers predictive of severe disease
- ICU-free days, ventilator-free days, vasopressor-free days

8. Ethical Considerations

The study is conducted in accordance with the Declaration of Helsinki. Approval has been obtained from the Institutional Review Board of Vietnam National Children's Hospital. Written informed consent is obtained prior to study participation.

STATISTICAL ANALYSIS PLAN

This study is a prospective observational, cross-sectional analysis with integrated biological sampling. All analyses are exploratory and hypothesis-generating.

- All statistical tests will be two-sided
- Significance level:
    - Primary analyses: $\alpha = 0.05$
    - High-dimensional microbiome analyses: False Discovery Rate (FDR) correction using Benjamini–Hochberg method
- Effect sizes and 95% confidence intervals (CI) will be reported alongside p-values
- Analyses will be performed using STATA or R (version ≥4.3) and QIIME2 for microbiome data

No interim analyses or stopping rules are planned, as there is no intervention**.**

1. Baseline Characteristics

- Continuous variables:
    - Mean ± SD (normal distribution)
    - Median (IQR) (non-normal distribution)
- Categorical variables:
    - Frequency and percentage

Group comparisons (severe vs mild RSV):

- Student's t-test or Mann–Whitney U test
- Chi-square test or Fisher's exact test

2. Primary Analysis

The primary analysis will compare children with severe RSV to those with mild RSV.

- Outcome:

  RSV severity (severe vs mild)
- Main exposures:
    - Gut microbiota diversity and composition

    - Serum cytokine profiles
- Methods:
    - Alpha diversity indices (Shannon, Simpson) compared using non-parametric tests
    - Beta diversity assessed using UniFrac distances and PERMANOVA
    - Differential bacterial taxa identified using multivariable count-based models with false discovery rate (FDR) correction

3. Immune response analysis

- Serum cytokine concentrations will be compared between severity groups using non-parametric methods
- Cytokines will be log-transformed if required
- Immune patterns will be explored using dimension-reduction and clustering methods

4. Integrated microbiome–immune analysis

- Associations between gut microbiota features and cytokine levels will be assessed using Spearman correlation, with FDR adjustment
- Microbiome–immune interaction networks will be constructed to identify key taxa and immune mediators linked to disease severity

Multivariable modeling and biomarker identification

- Logistic regression models will be used to identify independent predictors of severe RSV
- Sequential models will incorporate:
    1. Clinical variables
    2. Immune markers
    3. Microbiota features
- Model performance will be evaluated using ROC curves and area under the curve (AUC)
- Penalized regression methods will be applied to explore parsimonious biomarker panels

5. Sensitivity and subgroup analyses

Analyses will be repeated in key subgroups to assess robustness:

- Age (<6 months vs ≥6 months)
- Prior antibiotic exposure (yes/no)
- Feeding type (exclusive breastfeeding vs others**)**

6. Missing data

- Missing data will be described
- Primary analyses will use available data
- Sensitivity analyses will be performed if missingness is substantial