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The Effect of RSV Infection on the Respiratory Microbiome of Adults

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Abstract

Respiratory syncytial virus (RSV) is the most common cause of acute lower respiratory tract infection in infants. It is being increasingly recognised as a cause of morbidity and mortality in the elderly. Microbial dysbiosis in the respiratory tract has been hypothesized to predispose individuals to severe RSV infection. This study explores changes in the bacterial community over the course of a controlled human challenge study.

From 37 healthy adult patients exposed to a challenge inoculum of RSV, throat swabs were collected daily for 10 days during quarantine and on days 14 and 28 post quarantine. Swabs were processed for bacterial and viral quantification and 16S rRNA gene sequencing.

Over the course of the study three clinical outcomes were observed; clinical cold (n = 17), asymptomatic infection (n = 6) or no infection (n = 14). These three outcome groups had no significant differences in the bacterial load, diversity or community composition at baseline. Over the twenty-eight days following RSV inoculation no significant changes in the bacterial community were observed between the outcome groups.

This study of healthy adults revealed no major changes in the bacterial community of the respiratory tracts following RSV inoculation, suggesting that this microbial community is resilient to viral perturbations.

Introduction

Respiratory syncytial virus (RSV) is the most common cause of acute lower respiratory tract infection in infants, resulting in regular seasonal surges in admission to hospitals throughout the world [1]. In addition to its impact in childhood, it is increasingly recognised as a cause of morbidity and mortality in elderly frail persons [2]. RSV is highly contagious and options for control or prevention of infection are limited [3].

In contrast to other viral infections, such as influenza and rhinovirus that induce a long lasting protective immune response, reinfection with an identical RSV strain has been demonstrated in adult volunteers [4]. As a result despite extensive work, a safe and effective vaccine has thus far proven elusive [2].

Severe bronchiolitis in infants is associated with recurrent wheeze and asthma diagnosis in later childhood [5]. One possible explanation is that RSV infection causes durable changes in the microbial community within the lungs and that this has secondary effects on physiology and immunity. However, it is also possible that disordered microbial communities (dysbiosis) predispose to severe RSV disease [6]. Indeed, longitudinal birth cohort studies have demonstrated that propensity for frequent respiratory tract infections in early life are associated with a perturbed respiratory microbiota, dominated by *Moraxella,* that may precede respiratory viral infections. These studies indicate associations between the respiratory commensal microbiota and respiratory viral infection [7]. However, the issues of cause and effect have been difficult to resolve in these observational studies that recruit symptomatic cases of natural RSV infection or children with recurrent wheezing syndromes. Human volunteer challenge studies enable the sequence of events to be followed prospectively although they are confined to adult volunteers.

To determine the effect of RSV infection on the respiratory microbiome, we infected healthy adults with an established RSV challenge inoculum, Memphis 37 (RSV-A M37). We anticipated that infection during this controlled human challenge study with RSV-A M37 would result in significant changes in the bacterial community within the respiratory tract being observed.

Results

Our 37 volunteers (Table 1) showed one of three outcomes: clinical cold with RSV detection and/or virus-specific IgA production (n = 17), asymptomatic infection (n = 6) or no infection (n = 14).

Table 1. Patient demographics for the 37 patients included in the study.

	Clinical Cold (Infected)	**Uninfected**	**Asymptomatic Infected**
N	17	14	6
Age (Median (Min- Max))	20 (18 - 50)	22 (19 - 39)	20 (18-27)
Sex (Female)	6	6	3

Microbial changes over time

As previously reported, prior to viral inoculation (baseline)[8], there were no significant difference in bacterial alpha diversity (Richness, $P = 0.948$; Shannon-Weiner, $P = 0.263$; Simpsons, $P = 0.166$) or beta diversity (Bray-Curtis dissimilarity, $R^2 = 0.055$, $P = 0.087$).

Changes in viral load, bacterial load and bacterial diversity over the course of the study are shown in Figure 1. Significant differences between viral loads were seen between the uninfected and clinical cold groups at Days 5, 6, 7 and 8 (P.adjusted < 0.001).

Significant differences were also seen between the asymptomatic infected group and the clinical cold group on Days 7 and 8 (P.adjusted = 0.03 and 0.04 respectively); the mean viral load at day 2 was higher in the asymptomatic infected group even though they did not go on to develop clinical colds.

Bacterial load in the asymptomatic infected group fluctuated more than in other groups in the first four days of infection (Figure 1), although these changes were not significant and may reflect the low number of cases in the asymptomatic group (n = 6). No significant differences in bacterial load were observed between groups over the course of viral infection, and there were also no significant differences in any of the alpha diversity measures (richness, Shannon-Weiner and Simpsons). We also examined species turnover over time [9] but found no significant differences between the three groups.

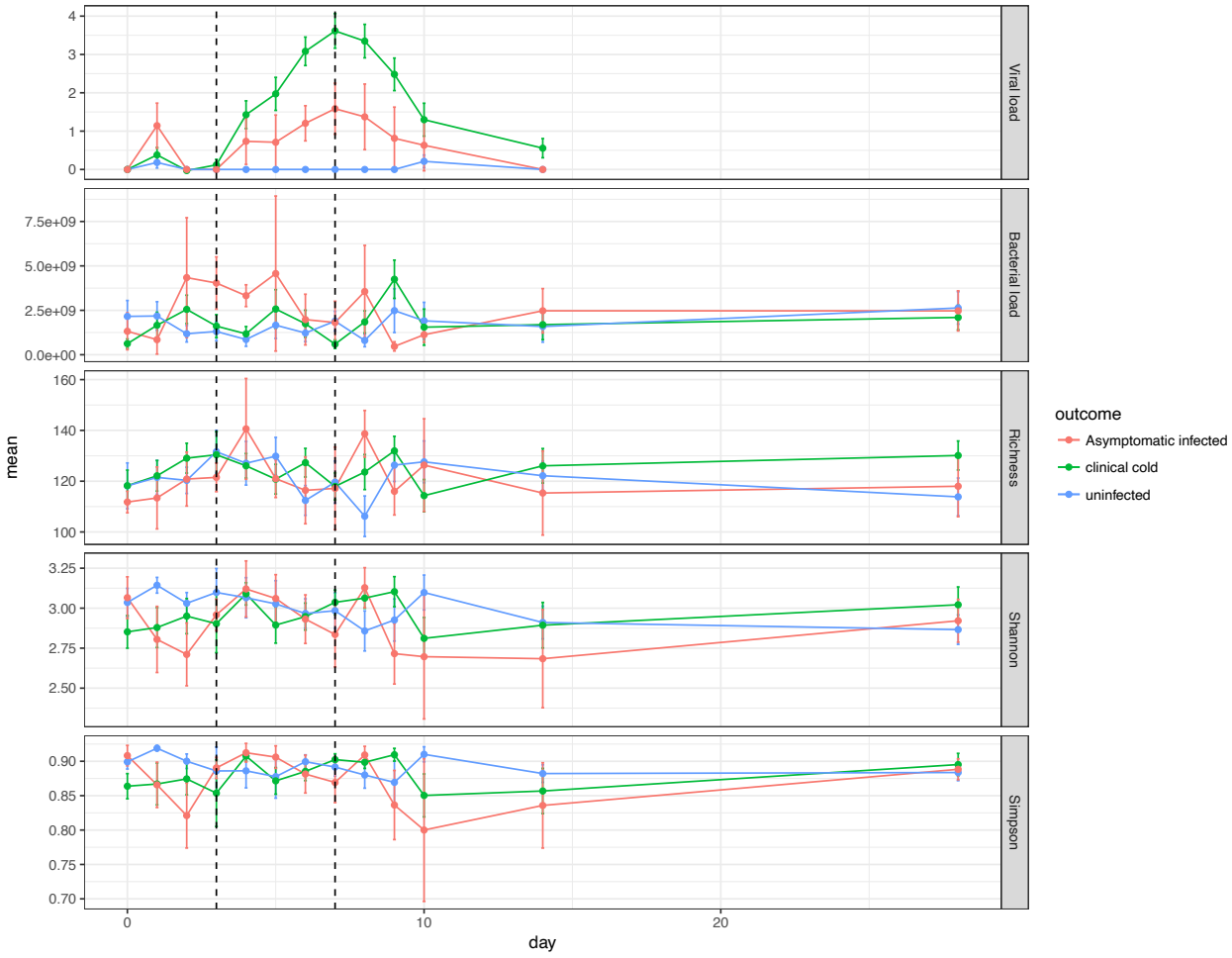

Figure1. Mean change in viral load (by qPCR), bacterial load (by qPCR) and alpha diversity (three measures Richness, Shannon, Simpsons) over the course of the study for the 3 groups; Clinical Cold (Infected), Asymptomatic Infected, Uninfected.

Correlation of bacterial diversity and cytokine measures

Repeated measure correlations were carried out between bacterial diversity measures (richness, Shannon-Weiner or Simpsons) and cytokine measurements in nasal mucosal lining fluid (IFN-α, IFN-β, IFN-γ, IL-15, IL-1β, IL-6, IP10 (CXCL10), MIG (CXCL9), and TNF-α). No significant correlations were observed when the whole data set was compared or when it was subdivided based on clinical symptoms. In addition, no significant correlations were found between bacterial diversity measures and patient symptom score or viral load (data not shown).

Bacterial community association with viral infection

RSV M37 was administered after initial baseline testing on Day 0. As shown in Figure 1, between administration of virus and Day 3 little virus was detected in the participants' respiratory tracts. At Day 3 viral load started to increase in patients who went on to develop symptoms. Day 7 was the peak of viral infection and viral load fell after this point. At Day 28 no patients had any sign of clinical infection and viral load was not measured, as previously described [8].

Indicator species analysis and DESeq2 were used to investigate if any individual OTUs were associated with the outcome of viral infection at these time points.

At baseline, indicator species and DESeq2 analyses identified several OTUs significantly associated with outcome. Four OTUs were found to overlap between the two analyses. To investigate this further, the abundance of significant OTUs were compared between clinical outcomes using a Wilcoxon sign rank test with Bonferroni correction for multiple testing (n = 21). Eleven OTUs were found to have a significant difference in means ($P > 0.05$) although no OTU was significant post correction for multiple testing.

At Day 3 no OTUs were found to be significantly different between clinical outcome groups. At Day 7 however a single *Haemophilus* OTU, Haemophilus_1650, was found to be significantly increased in the uninfected group using both indicator species and DESeq2 analysis. Differences in Haemophilus_1650 over the study time course were not significant by the Wilcoxon sign rank test. At Day 7 the difference in Haemophilus_1650 did not hold up to Bonferroni correction for multiple testing (P.adjusted = 0.37).

Throat swabs were collected from all patients at Day 28 and whilst indicator species analysis found no significant OTUs associated with viral outcome, DESeq2 analysis indicated 15 OTUs of interest. Fourteen of these OTUs were significantly associated with the uninfected group whilst only a single OTU was found to be associated with those recently recovered from clinical cold. All identified OTUs however, were found to be in low abundance and were not significant under further testing (e.g. Wilcoxon sign rank test).

Discussion

Analysis of the respiratory microbiome in healthy adults revealed that, contrary to results reported in children, no significant changes in the bacterial community were seen over the course of RSV infection.

After inoculation with RSV-A M37, seventeen of the 37 patients in the study went on to develop clinical symptoms. In the asymptomatic population the majority of these participants cleared the viral infection within 3 days (n = 14), however there were a small number of asymptomatic infected individuals (n = 6). Throat swabs, collected daily during the quarantine period of 10 days and then further swabs taken post quarantine at Days 14 and 28, revealed no significant differences in the bacterial load or diversity associated with patient groups over the viral infection cycle.

Initial investigation of differences in the bacterial community at baseline revealed no significant differences in bacterial load, diversity or community composition suggesting that the bacterial community at baseline did not have a significant effect on the course of viral infection. A post- hoc power analysis based on the effect size seen in the baseline samples (f = 0.23) suggested that using the conventional power of

0.80, n = 62 per group would be required to identify significant differences in Shannon- Weiner diversity between the 3 groups. A larger study may be able to detect more subtle changes in the respiratory bacterial community.

Indicator species analysis and DESeq2 were used to investigate OTUs that may influence the outcome of viral infection. This analysis was carried out at the key time points in the viral infection, these being baseline, days 3 (the start of concerted viral replication), 7 (peak viral replication) and 28. Although several OTUs were statistically significant by these analyses, none stood up to further analysis with control for multiple testing.

No significant correlations between changes in the microbial community and changes in cytokine measurements were seen, suggesting that the microbial community within the respiratory tract is resilient to RSV mediated changes in inflammatory environment of the upper respiratory tract. This result may however also be due to the small number of participants within the study.

Previous studies have focused on exploring the microbiome in infants, showing a positive association between *Haemophilus, Moraxella* and *Streptococcus* dominated nasal microbiota and infection severity [10-12]. Our study of healthy adults did not confirm these reported microbial changes. This may be due to the maturity and relative stability of the immune system and microbial community in these individuals, unlike those of the infant population. However, these studies had larger sample sizes than the current study, a larger study would be required to confirm the resilience of the bacterial community in the adult population during RSV infection.

Controlled human infection challenges are essential to understand disease pathogenesis and underpin eventual vaccine development. However, there are

substantial ethical issues associated with exposing healthy individuals to infectious risk. As a result, these studies must be conscientiously designed to minimise risks, while participants volunteers must be fully indemnified against any potential harm [13]. When considering these parameters, sample sizes are necessarily limited. This provides a challenge for the analysis of highly variable microbiome data, making interpretation difficult.

Despite these limitations, the present study provides important information about the resilience of the microbiome in healthy adults to viral perturbations and suggests that co-morbidities may have an important effect on the bacterial community within the lungs of vulnerable individuals, as observed in studies of natural infections.

Methods

Study design and sampling

Thirty-seven healthy non-smoking individuals aged between 18 and 50 years old were recruited into the study (Table 1) [14]. Participants were screened for immunodeficiencies, respiratory diseases and medications [8].

Baseline samples (Nasosorption [15], nasal lavage fluid, blood and throat swabs) were collected prior to participant inoculation with 10^4 plaque-forming units of RSV A Memphis 37 (RSV-A M37) by intranasal drops. Subjects were quarantined for 10 days post infection and sampled daily. Subjects returned for further sampling at days 14 and 28 post infection. The study was approved by the UK National Research Ethics Services (study numbers 10/H0711/94 and 11/LO/1826) and performed in accordance with the Declaration of Helsinki, written informed consent was provided by all subjects.

Antibody assay

Endpoint titre of IgA against RSV lysate was determined from nasal wash samples as previously described [14].

Cytokine and chemokine inflammatory mediators (IFN-α, IFN-β, IFN-γ, IL-15, IL-1β, IL-6, IP10 (CXCL10), MIG (CXCL9), TNF-α) within nasosorption eluates were quantified by multiplex high sensitivity immunoassay (MesoScale Discovery, Rockville, USA). Nasosorption samples were eluted as previously described [15] and stored at -80°C prior to analysis.

DNA extraction

DNA extraction was performed on throat swabs using the MPBio FastDNA^{TM} spin kit for soil as per the manufacturer's instructions. Blank swabs were extracted and sequenced to control for any contamination as previously described [16].

Quantitative PCR

Viral infection was quantified by qPCR of nasal lavage fluid as previously described [17]. Bacterial load was quantified by SYBR green qPCR as previously described [16]. All qPCR reactions were performed in triplicate.

Bacterial sequencing

16S rRNA gene sequencing was performed on the Illumina MiSeq platform using dual barcode fusion primers targeting the V4 region of the 16S rRNA gene as described previously [16]. Extraction controls, PCR negative and mock communities were included on all sequencing runs. Sequences were submitted to the European Nucleotide Database, project number PRJEB28323.

Sequence processing was carried out using QIIME and UCLUST to assign taxonomic identification to operational taxanomic units (OTU) using the Silva 115 NR database, as previously described [16]. OTU and tree files were output into R for further analysis.

Statistics

All further analysis was carried out in R, version 3.3.2 using Phyloseq, version 1.22.3. Permutational multivariate analysis of variance using Bray-Curtis dissimilarity matrix was carried out in Vegan, version 2.4-6. Changes over time were investigated using ANOVA with Tukey HSD post hoc testing. Repeated measures correlations were carried out using rmcorr, version 0.3.0.

OTU differences were explored using indicator species analysis (Indicspecies, version 1.7.6) and DESeq2 (DESeq2, version 1.18.1). Indicator species analysis, with 10,000 iterations, was performed on the rarefied data, while DESeq2 used un-rarefied data due to internal normalization within the package.

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Author contributions

P.J.M.O., C.C., and M.F.M conceived the study, with input from W.O.C.C.. M.S.H., A.P., and C.C. performed volunteer infections and collected samples. M.S.H., R.S.T., L.C., P.J. and C.C. collected human antibody and microbial data. Microbiome data was analyzed by L.C. and P.J. The manuscript was written by L.C. with input from all authors.

Competing interests

The author declare no competing interests.

Data availability

Sequences were submitted to the European Nucleotide Database and fully available under project number PRJEB28323.

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Figures

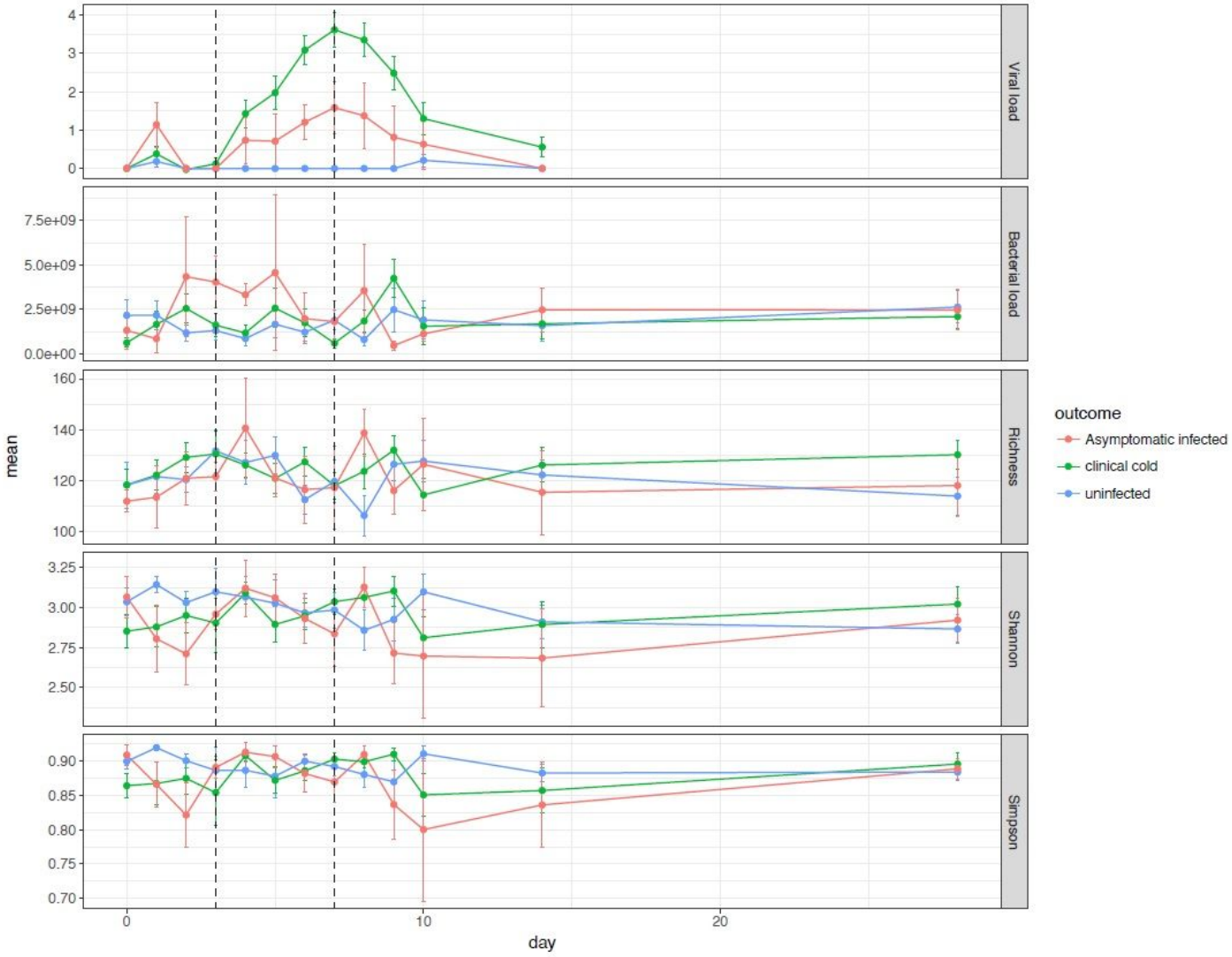

Figure 1

Mean change in viral load (by qPCR), bacterial load (by qPCR) and alpha diversity (three measures Richness, Shannon, Simpsons) over the course of the study for the 3 groups; Clinical Cold (Infected), Asymptomatic Infected, Uninfected.