Respiratory Syncytial Virus Infection is Associated with Increased Bacterial Load in the Upper Respiratory Tract in Young Children

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Abstract

Background: Respiratory syncytial virus (RSV) is the leading cause of severe respiratory tract infection requiring hospitalization among infants and young children. Furthermore, RSV infection has been shown to stimulate increased bacterial load in the nasopharynx and to promote bacterial otitis media and/or pneumonia.

Objective: To assess the diversity and prevalence of bacterial pathogens associated with viral infections of the respiratory tract among young children.

Study design: During the 2012 seasonal RSV epidemic in South East Queensland, Australia, 201 nasopharyngeal aspirates (NPAs) collected from symptomatic children under five years of age and sent for pathology laboratory screening were examined. Samples were analysed for common viral and bacterial pathogens by quantitative PCR and culturing techniques. For a subset of 29 samples the complete microbial community was profiled using culture-independent PCR and pyrosequencing of the 16S ribosomal RNA gene.

Results: RSV infection was confirmed in 67 patients, of which 49 were positive for high bacterial load in the upper respiratory tract. Bacterial detection was significantly higher amongst RSV positive samples (73.1%) than amongst RSV negative samples (56.7%) (p=0.03) and was independent of other viral pathogens. The predominant bacterial species detected during RSV infections were Moraxella catarrhalis (22), Streptococcus pneumoniae (17), Haemophilus influenzae (5) and Staphylococcus aureus (6). Notably, microbial profiling analysis showed that during either RSV or rhinovirus infections a single bacterial species can constitute between 80 and 95% of the bacterial community present.

Conclusions: In nasopharyngeal samples collected from symptomatic children, high levels of bacteria were found more commonly in the presence of RSV infections. Most significantly, RSV infection was associated with a 3-fold increase in S. pneumoniae detection.

Keywords: Respiratory syncytial virus; Streptococcus Pneumoniae; Paediatric respiratory infection; Co-infection

Abbreviations: ARIs: Acute Respiratory Infections; HAdV: Human Adenovirus; hMPV: Human Metapneumovirus; HRV: Human Rhinovirus; IFAV/IFBV: Influenza Virus A/B; NPAs: Nasopharyngeal Aspirates; PICO: Paediatric Intensive Care Unit; PICU: Paediatric Intensive Care Unit; PIV1-3: Parainfluenza Virus 1-3; PQC: Pathology Queensland Central; QIIME: Quantitative Insights Into Microbial Ecology; RSV: Respiratory Syncytial Virus; UPGMA: Unweighted Pair Group Method with Arithmetic Mean

Introduction

Respiratory viral infections that affect the lower respiratory tract during early childhood are often associated with secondary bacterial infections including Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis and Staphylococcus aureus [1,2]. While colonization with these bacterial species is common during childhood and often independent of symptoms [3,4], respiratory viral infections have been shown to stimulate a substantial increase in bacterial load that can lead to bacterial otitis media or pneumonia [5-9].

Globally, Respiratory Syncytial Virus (RSV) is the leading cause of severe Acute Respiratory Infections (ARIs) requiring hospitalization among infants and young children. RSV infects over 90% of children by the age of two and causes cold-like symptoms that frequently progress to lower respiratory disease such as bronchiolitis and pneumonia [10]. Approximately 0.5% of RSV infections require hospitalization [11], with RSV estimated to be responsible for 3 million hospitalizations and 200,000 deaths annually [10]. RSV is known to be associated with bacterial co-infections of the upper and lower respiratory tract [1,2,12-18], however it remains unclear whether a correlation exists between bacterial co-infection and RSV disease severity. While a number of...
studies have failed to find any correlation with severity [16,19], others have shown that patients with RSV/bacterial co-infections are more likely to be admitted to a Paediatric Intensive Care Unit (PICU) [20] and of patients admitted to the PICU, those with RSV/bacterial co-infection require ventilator support for longer than those with RSV alone [1,17].

Previous reports attempting to define the prevalence of viral/bacterial co-infections during ARI and pneumonia have provided varying estimates (23-66%) [1,2,8,12-18,21,22]. While differences are to be expected, given the numerous environmental factors including differences in populations, geographic locations and seasonality that are likely to play a role, a large percentage of this variation may also be attributed to differences in sample collection, screening methods and the classification used to define positive samples.

Most studies conducted to date have focused solely on the detection of a handful of common pathogens through targeted PCR screening or detection of those that are amenable to culture. Such approaches to bacterial identification are not ideal as disease-associated species may not readily grow in culture and thus be excluded from individual analyses, while culturable but less relevant agents may be included. Targeted approaches often result in a significant proportion (~25%) of samples testing negative for both bacterial and viral pathogens despite clear ARI symptoms [9,18,21]. The failure to quantify bacterial load in addition to presence/absence can also complicate differentiation between commensal carriage and increased bacterial load that may contribute to pathogenesis and ultimately to appropriate decisions about individual patient management. Consequently, current detection rates for viral/bacterial co-infections may either substantially under- or over-estimate the true situation.

The development of culture-independent, molecular approaches for the study of microbial communities provides the opportunity to investigate microbial diversity without limiting analysis to those species which are able to be cultivated in the laboratory. Techniques such as deep sequencing of 16S ribosomal RNA genes are able to reveal far greater microbial diversity than culture-based studies [23], although such techniques are still limited by their inability to differentiate beyond the family or genus level to the level of individual species. Several studies have used culture-independent techniques to characterize the microbiota of the respiratory tract, in various states of health and disease including chronic lung diseases, such as Cystic Fibrosis [24-26] and Chronic Obstructive Pulmonary Disease [27-29] as well as acute respiratory infections [30-32]. To date, there appear to be no studies in the literature specifically examining both viral carriage and microbiota composition in paediatric ARIs.

**Objectives**

The aim of this study was to examine the prevalence of viral/bacterial co-infections of the respiratory tract during early childhood and to identify associations between individual viruses and bacterial pathogens. To achieve this aim we conducted a detailed analysis of 201 Nasopharyngeal Aspirates (NPAs) sent for pathology laboratory screening and collected from children under the age of five with ARI symptoms. Samples were screened for common respiratory viral infections by real-time PCR (rtPCR) and for bacteria amenable to detection via culturing methods. In order to directly compare the information obtained through culture-independent vs dependent screening, a subset of 29 samples was screened by culture-independent pyrosequencing of the 16S rRNA gene to provide a less biased profile of the bacterial communities present in the nasopharynx during ARI.

**Materials and Methods**

**Study population**

We accessed 201 NPAs from children under the age of five that were sent to Pathology Queensland Central (PQC) at the Royal Brisbane and Women’s Hospital for routine screening of respiratory virus presence. These samples were collected in clinical settings from hospitals in South East of Queensland, Australia over an eight week period spanning the seasonal RSV epidemic, from late March to mid May 2012. NPAs were mixed with glycerol to a final concentration of 25% and stored at -80°C. Samples were de-identified with information of patient sex and date of birth being accessible. This study was approved by the Children's Health Services Queensland Human Research Ethics Committee, HREC reference number: HREC/12/QRCH/196.

**Viral testing**

All 201 samples were tested by Queensland Health Pathology Services (QHPS) for RSV, parainfluenzae viruses-1,-2,-3 (PIV1-3), influenza virus A and B, Human Adenovirus (HAdV) and Human Metapneumovirus (hMPV) via rtPCR as per QHPS routine procedures. NPAs were either extracted neat or diluted in VTM if the volume was low. 200 µl was extracted on either the MagNApure (Roche) or the X-TractorGene (Qiagen) using a total nucleic acid extraction kit and eluted in 100 µl. Real-time PCR assays were performed by combining extracts with appropriate mastermix into 96 well plates and run on an ABI7500 instrument using the cycling conditions: 50°C for 20 minutes (1 cycle), 95°C for 15 minutes (1 cycle), 95°C for 15 sec and 60°C for 60 sec (45 cycles) [33-36]. Additionally, the presence of human rhinovirus (HRV) was detected via real-time RT-PCR as previously reported [37].

**Culture-dependent bacterial testing**

Each sample (10 µl) was spread onto a 5% blood agar plate with an optochin disk to allow the presumptive identification of *S. pneumoniae* and a 5% chocolate agar plate with an X-V Factor disk to allow the presumptive identification of *H. influenzae* and incubated overnight at 37°C. Only plates with greater than 100 colonies (i.e. over 10,000 colony forming units (cfu)/ml) with conserved morphological features were considered as positive for bacterial growth. Representative colonies were then characterized by 16S rRNA gene sequencing. Briefly, bacterial cells were lysed by suspending 1-2 colonies in 100 µl of water and boiling for 5 mins prior to PCR amplification and sequencing of the 16S rRNA gene with primers 27F (5’AGAGTTTGATCCTGGCTCAG) and 514R (5’CCGCGGCTGCTTGGCAC).

**Community profiling**

Total DNA was extracted from a subset of 29 samples using either the PureLink Genomic DNA Purification Kit (Life technologies - Invitrogen division) or the High Pure Viral Nucleic Acid Isolation Kit (Roche) as per the manufacturer’s instructions. The overall concentration of DNA extracted from each sample was quantified by measuring absorbance at 260nm, while quantitative PCR of the 16S rRNA gene was used to estimate the microbial load for each sample. Quantitative PCR used primers 1406F (5’GYACWCACCGCCCGT) and 1525R (5’AGGAGGTGWTCCARCC). To identify organisms present in each sample, 454 pyrosequencing of the 16S rRNA gene was performed.
performed. For sequencing, copies of the 16S rRNA gene present in each sample were amplified with the primers 803F and 1392R containing the 454 adaptors [38]. Each reverse primer contained a unique barcode sequence (5-8 bp in length) to allow the identification of each sample during the multiplex sequencing process. A two-step PCR process was used to maximize PCR product [39]. In the first step, 50 µl PCR reactions were performed using 10 µl of extracted DNA with 25 µl of MyTaq HS Mix (Bioline, Sydney) and 1 µl each of 10 µM native 803F and 1392R primers. In the second round of PCR, 2 µl of the product from the first step was used as template in 10-cycle 50 µl PCR reactions, using the barcoded, pyrosequencing versions of the 803F and 1392R primers. Amplified DNA was then sequenced at the Australian Centre for Ecogenomics using 454 GS-FLX Titanium pyrosequencing.

Reads were trimmed to 350 bp and those under this length or of low quality were removed. Chimeric sequences were removed via UCLUST [40] and homopolymer sequencing errors were corrected via Acacia [41]. Quantitative Insights into Microbial Ecology (QIIME) was used to select operational taxonomic units (OTUs) [42], with a UCLUST cluster similarity threshold of 97%. OTU taxonomy was then assigned using BLAST with the Greengenes database [40,43,44] and the read counts normalized across the 29 samples. OTUs that were not identified in the Greengenes database were searched against the GenBank NR (non-redundant) database and non-specific amplifications of host DNA were removed.

Measures of alpha diversity were calculated, to assess richness (number of species) and evenness (relative abundance of species) of the microbial community within each sample. These metrics included rarefaction curves of the observed number of species, Shannon indices, Simpson indices and Chao1 estimates. Average Shannon indices, Simpson indices and Chao1 estimates for each cohort were calculated from the first step was used as template in 10-cycle 50 µl PCR reactions, using the barcoded, pyrosequencing versions of the 803F and 1392R primers. Amplified DNA was then sequenced at the Australian Centre for Ecogenomics using 454 GS-FLX Titanium pyrosequencing.

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Statistical Analysis

Fisher's exact test was used to analyse the statistical significance of the results, using a two-sided alternative hypothesis. Contingency tables were built to compare viral and bacterial infection categories. Odds ratios were calculated to assess the strength of various associations and are reported along with 95% confidence intervals.

Results

Viral detection

A total of 201 samples were collected from children under the age of five years with a median age of 9.3 months. 153 samples (76.1%) were positive for at least one common respiratory virus with up to three viruses detected in individual samples. RSV was the most common virus identified among children less than six months old (RSV 31.3%; HRV 19.3%), while HRV was the most common among children between 6 months and 5 years of age (HRV 35.6%; RSV 19.5%) (Table 1).

Bacterial detection

Of 201 samples 126 (62.7%) were defined as positive for bacterial colonization, under criteria used to differentiate between high level bacterial colonization (>10,000 cfu/ml) and normal, low level growth of nasal microbiota in the respiratory tract. The most common bacteria identified by culture-based methods were *M. catarrhalis* in 54 samples (26.9%), *S. pneumoniae* in 28 samples (13.9%), *S. aureus* in 23 samples

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| Table 1: Viral detection: Number of cases (percentage). |
|-----------------------------------------------|
| **Viral detection**                           |
| **0-6 months**                               |
| **6-12 months**                              |
| **12-24 months**                             |
| **24-60 months**                             |
| **Total**                                     |
| **Number of samples**                        |
| **83**                                       |
| **41**                                       |
| **36**                                       |
| **41**                                       |
| **201**                                      |
| **Bacterial positive**                       |
| **57 (68.7)**                                |
| **27 (65.8)**                                |
| **27 (75.0)**                                |
| **15 (36.6)**                                |
| **126 (62.7)**                               |
| **M. catarrhalis**                           |
| **21 (25.3)**                                |
| **16 (39.0)**                                |
| **11 (30.6)**                                |
| **6 (14.6)**                                 |
| **54 (26.9)**                                |
| **S. pneumoniae**                            |
| **8 (9.6)**                                  |
| **7 (17.1)**                                 |
| **8 (22.2)**                                 |
| **5 (12.2)**                                 |
| **28 (13.9)**                                |
| **S. aureus**                                |
| **15 (18.7)**                                |
| **1 (2.4)**                                  |
| **3 (8.3)**                                  |
| **2 (4.9)**                                  |
| **23 (11.4)**                                |
| **H. influenzae**                            |
| **3 (3.6)**                                  |
| **1 (2.4)**                                  |
| **4 (11.1)**                                 |
| **1 (2.2)**                                  |
| **9 (4.5)**                                  |
| **Other**                                    |
| **23 (27.7)**                                |
| **9 (22.0)**                                 |
| **8 (22.2)**                                 |
| **4 (9.8)**                                  |
| **44 (21.9)**                                |
| **Bacterial negative**                       |
| **26 (31.3)**                                |
| **14 (34.2)**                                |
| **9 (25.0)**                                 |
| **26 (63.4)**                                |
| **75 (37.3)**                                |
| **1 bacteria**                               |
| **36 (43.4)**                                |
| **19 (46.3)**                                |
| **15 (38.2)**                                |
| **26 (61.8)**                                |
| **85 (42.3)**                                |
| **2 or more bacteria**                       |
| **21 (25.3)**                                |
| **8 (19.5)**                                 |
| **8 (20.5)**                                 |
| **4 (9.8)**                                  |
| **41 (20.4)**                                |

*Includes: Klebsiella spp., Bacillus spp., Neisseria spp., Haemophilus spp., Rothia spp., S. millis, S. dysgalactiae, S. haemolyticus, S. epidermidis, S. lugdunensis, S. warneri, M. nonliquefaciens, Stenotrophomonas spp., Enterococcus faecalis, Gammaproteobacteria spp., Hafnia alvei, Obesumbacterium spp., Serratia marcescens, Enterobacter spp., Acinetobacter calcoaceticus and E. coli.

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infections were associated with bacterial detection compared with that RSV is associated with bacterial detection; 77.5% of single RSV positive for the co-detection of bacteria. 65.3% of single viral infections and 65.6% of multiple viral infections of multiple viral pathogens did not affect bacterial detection, with samples were positive for bacteria (p=0.17). Furthermore, the presence of multiple viral pathogens did not affect bacterial detection, with 54.2% of virus-negative samples. One or more of the four most commonly detected bacteria (M. catarrhalis, H. influenzae and S. aureus) were found in 40 of 67 RSV positive samples and 56 of 134 (41.8%) samples that was negative for RSV. The odds ratio for bacterial detection increased to 4.95 (95% CI=1.31-18.66) between the ages of six months and two years. Within this age range, 23 of 26 RSV positive samples (88.5%) were positive for bacteria, most notably M. catarrhalis (12 of 26 samples, 46.2%) and S. pneumoniae (10 of 26, 38.5%). The odds ratio for detection associated with RSV infection was calculated to be 3.80 (95%CI=1.67-8.69) for all children under 5 and 5.75 (95%CI=1.71-19.38) for children aged between 6 and 24 months (Tables 4 and 5).

**Microbial community profiling**

To assess changes in the microbiota of the nasopharynx induced by viral infection, 29 samples were selected at random from each of three sample groups based on viral detection (10 RSV-positive, 10 HRV-positive and 9 virus-negative samples). Sequencing was successful in all cases with between 665 and 15783 reads for each sample (mean: 5397). Relative abundances of the most frequent bacterial families identified in each sample group are shown in Figure 1, while the individual taxa contributing greater than 5% of the microbial community in each sample are shown in Table 6.

**Table 3**: Viral/bacterial co-detection: Number of cases (percentage).

|                          | RSV pos (67 samples) | RSV neg (134 samples) | Odds ratio | 95% CI |
|--------------------------|----------------------|------------------------|------------|--------|
| Any bacteria             | 49 (73.1)            | 76 (56.7)              | 2.08       | 1.10-3.94 |
| M. catarrhalis           | 22 (32.8)            | 32 (23.9)              | 1.56       | 0.82-2.97 |
| S. pneumoniae            | 17 (25.4)            | 11 (8.2)               | 3.80       | 1.67-6.89 |
| S. aureus                | 6 (9.0)              | 17 (12.7)              | 0.68       | 0.25-1.81 |
| H. influenzae            | 5 (7.5)              | 4 (3.0)                | 2.62       | 0.68-10.10 |
| Other                    | 13 (19.4)            | 31 (23.7)              | 0.80       | 0.39-1.65 |

**Table 4**: Bacteria associated with RSV infection (all age groups): Number of cases (percentage).

|                          | RSV pos (26 samples) | RSV neg (51 samples) | Odds ratio | 95% CI |
|--------------------------|----------------------|----------------------|------------|--------|
| Any bacteria             | 23 (88.5)            | 31 (60.8)            | 4.95       | 1.31-18.66 |
| M. catarrhalis           | 12 (46.2)            | 15 (29.4)            | 2.06       | 0.77-5.47 |
| S. pneumoniae            | 10 (38.5)            | 5 (9.8)              | 5.75       | 1.71-19.38 |
| S. aureus                | 1 (3.8)              | 3 (5.9)              | 0.64       | 0.06-6.48 |
| H. influenzae            | 2 (7.7)              | 3 (5.9)              | 1.33       | 0.21-8.52 |
| Other                    | 5 (19.2)             | 12 (23.5)            | 0.77       | 0.24-2.49 |

**Table 5**: Bacteria associated with RSV infection (6-24 months): Number of cases (percentage).

H. influenzae peaked in the 12-24 month age group. Children under the age of two were approximately twice as likely to be positive for high bacterial colonization as those over two (69.4% vs 36.6%; p = 0.0001). In 85 samples (42.3%) a single bacterial species was identified while in 41 samples (20.4%) two or more species of bacteria were present. No significant positive associations between the detection of bacterial species were identified, however we found a negative association between S. pneumoniae and S. aureus, as these were never detected in the same sample (p=0.050), a finding that has been previously reported [45-49] (Table 2).

**Viral/bacterial co-detection**

Potential viral and bacterial pathogens were detected together in 100 samples (49.8%), while virus alone was detected in 53 samples (26.4%) and bacteria alone in 26 samples (12.9%). No potential pathogens were identified in 22 samples (10.9%), which was lower than for previous studies [9,14,18,21,50]. Detection of any virus was not significantly associated with bacterial detection. 65.4% of virus-positive samples were also positive for bacteria, while 54.2% of virus-negative samples were positive for bacteria (p=0.17). Furthermore, the presence of multiple viral pathogens did not affect bacterial detection, with 65.3% of single viral infections and 65.6% of multiple viral infections positive for the co-detection of bacteria.

When comparing individual viral infections (Table 3) it is apparent that RSV is associated with bacterial detection; 77.5% of single RSV infections were associated with bacterial detection compared with 60.3% of single HRV infections and 54.2% of viral negative samples (p=0.057 and 0.019, respectively). Most significantly, single RSV infection was associated with S. pneumoniae detection, with 24.5% of RSV infections positive for S. pneumoniae compared to 6.9% of HRV and 8.3% of virus negative samples (p=0.014 and 0.053). The association between RSV infection and bacterial detection was most striking in children between the ages of six months and two years (Table 3). RSV was also associated with an increase in M. catarrhalis and H. influenzae detection compared to HRV and virus-negative samples, however the trend was not significant (p=0.53 and 0.06 for M. catarrhalis, and p=0.24 and 0.44 for H. influenzae) (Table 3).

Taking into account all samples positive for RSV, the odds ratio of bacterial co-detection is 2.08 (95% CI=1.10-3.94), when compared to all samples negative for RSV, irrespective of other viral pathogens. Bacterial co-infection was detected in 49 of 67 RSV positive samples (73.1%), compared with 51 of 86 (59.3%) of other viral infections and 26 of 48 (54.2%) of viral negative samples. One or more of the four most commonly detected bacteria (M. catarrhalis, S. pneumoniae, H. influenzae and S. aureus) were found in 40 of 67 (59.7%) RSV infections and 56 of 134 (41.8%) samples that was negative for RSV.
### Table 6: Comparison of culture dependent-screen and microbiota analysis.

| Patient | Age         | Culture-dependant screen | Taxa contributing to >5% of microbiota | Microbial biomass (ng/µl) |
|---------|-------------|---------------------------|----------------------------------------|--------------------------|
| 0540    | 1 month     | Gammaproteobacteria       | Staphylococcus (77.67)                 | 338.77                   |
| 8213    | 2 months    | Negative                  | Enterobacteriaceae (50.67)             | 0.51                     |
| 2921    | 2 months    | Klebsiella sp             | Prevotella (28.65)                     | 0.29                     |
| 9636    | 4 months    | Negative                  | Haemophilus (48.67)                    | 0.41                     |
| 0908    | 9 months    | M. catarrhalis            | Moraxella (92.33)                      | 13.98                    |
| 1002    | 11 months   | S. aureus                 | Streptococcus (64.00)                  | 0.87                     |
| 5582    | 1 year      | S. pneumoniae+M. catarrhalis | Streptococcus (93.50)           | 18.86                    |
| 9851    | 2 years, 9 months | S. pneumoniae+E. faecalis | Neisseria (18.67)                   | 0.84                     |
| 5149    | 2 years, 9 months | M. catarrhalis+Klebsiella sp | Moraxella (87.33)              | 26.06                    |
| 4323    | 3 years     | M. catarrhalis            | Moraxella (39.83)                    | 2.83                     |
| 4543    | 3 months    | Negative                  | Stenotrophomonas (30.83)              | 0.27                     |
| 4362    | 4 months    | S. aureus                 | Staphylococcus (94.67)               | 136.37                   |
| 6594    | 6 months    | S. aureus+S. mitis        | Streptococcus (54.33)                 | 1.86                     |
| 3456    | 7 months    | Negative                  | Flavobacteriaceae (24.33)             | 0.46                     |
| 3374    | 7 months    | S. pneumoniae+M. catarrhalis | Streptococcus (65.17)             | 1.68                     |
| 9428    | 10 months   | M. catarrhalis            | Moraxella (81.67)                    | 52.63                    |
| 3066    | 1 year, 8 months | E. coli                | Carnobacteriaceae (80.33)            | 0.85                     |
| 2144    | 1 year, 11 months | M. catarrhalis          | Moraxella (93.33)                    | 236.36                   |
| 4308    | 2 years, 10 months | S. dysgalactiae+Rothia sp | Neisseria (54.50)                   | 32.72                    |
| 2110    | 3 years     | Negative                  | Flavobacteriaceae (19.67)             | 0.44                     |
| 5110    | 1 month     | Negative                  | Haemophilus (33.00)                   | 3.56                     |
| 9905    | 1 month     | Negative                  | Streptococcus (65.67)                 | 0.71                     |
| 7569    | 1 month     | S. pneumoniae+M. catarrhalis | Streptococcus (49.00)             | 6.67                     |
| 8664    | 2 months    | S. aureus                 | Corynebacterium (96.50)               | 3.74                     |
| 9360    | 2 months    | Enterobacteriaceae         | Leclercia (89.33)                    | 159.64                   |
| 0734    | 2 months    | S. maltophilia            | Acinetobacter (84.17)                | 6.89                     |
| 1742    | 4 months    | Negative                  | Moraxella (89.33)                    | 34.16                    |
| 1276    | 1 year, 4 months | Negative                | Streptococcus (71.00)                | 0.46                     |
| 5784    | 4 years, 5 months | S. aureus               | Staphylococcus (40.17)               | 3.19                     |

**HRV positive**

| Patient | Age         | Culture-dependant screen | Taxa contributing to >5% of microbiota | Microbial biomass (ng/µl) |
|---------|-------------|---------------------------|----------------------------------------|--------------------------|
| 5784    | 2 years, 10 months | S. dysgalactiae+Rothia sp | Neisseria (54.50)                   | 32.72                    |
| 2784    | 4 years, 5 months | S. aureus               | Staphylococcus (40.17)               | 3.19                     |

**Virus negative**

| Patient | Age         | Culture-dependant screen | Taxa contributing to >5% of microbiota | Microbial biomass (ng/µl) |
|---------|-------------|---------------------------|----------------------------------------|--------------------------|
| 5110    | 1 month     | Negative                  | Haemophilus (33.00)                   | 3.56                     |
| 9905    | 1 month     | Negative                  | Streptococcus (65.67)                 | 0.71                     |
| 7569    | 1 month     | S. pneumoniae+M. catarrhalis | Streptococcus (49.00)             | 6.67                     |
| 8664    | 2 months    | S. aureus                 | Corynebacterium (96.50)               | 3.74                     |
| 9360    | 2 months    | Enterobacteriaceae         | Leclercia (89.33)                    | 159.64                   |
| 0734    | 2 months    | S. maltophilia            | Acinetobacter (84.17)                | 6.89                     |
| 1742    | 4 months    | Negative                  | Moraxella (89.33)                    | 34.16                    |
| 1276    | 1 year, 4 months | Negative                | Streptococcus (71.00)                | 0.46                     |
| 5784    | 4 years, 5 months | S. aureus               | Staphylococcus (40.17)               | 3.19                     |

**Table 6: Comparison of culture dependent-screen and microbiota analysis.**
Bacteria from the bacterial families Streptococcaceae, Moraxellaceae, Staphylococcaceae, Enterobacteriaceae, Pasteurellaceae, Prevotellaceae and Xanthomonadaceae were frequent across RSV positive, HRV-positive and virus-negative samples (Figure 1). Neisseriaceae, Gemellaceae and Fusobacteriaceae families were commonly associated with virus-positive samples, while bacteria from the Micrococcaceae family were present in virus-negative samples. Corynebacteriaceae were dominant in virus-negative samples, although the family was present in virus-positive samples as well. In contrast, a greater percentage of bacteria in virus positive samples were accounted for by Flavobacteriaceae, Comamonadaceae, Idiomarinaeae and Pseudomonadaceae species. Enterococcaceae were specific to RSV-positive samples, Veillonellaceae were common to RSV-positive and virus-negative samples and Carnobacteriaceae were shared between HRV-positive and virus-negative samples.

Reflecting the community profiling results at the family level, several bacterial genera were common to all three cohorts, including Moraxella, Staphylococcus, Streptococcus, Haemophilus, Corynebacterium, Prevotella, Leclercia, Stenotrophomonas and Pseudomonas (Table 6). Neisseria was almost exclusively in virus-positive samples. Unspecified Idiomarinaeae species were observed in all three cohorts, always in association with Diaphorobacter, Pseudomonas and Stenotrophomonas, although these three genera also occurred independently.

Table 6 shows the major taxa contributing to the microbial community. Of particular note are three RSV positive samples, three HRV positive samples and one virus-negative sample in which Moraxella, Streptococcus or Staphylococcus were found to represent between 80 to 90% of the bacteria present in the nasopharynx. In each of these samples the total microbial biomass was approximately 10-fold higher than the median value for all 29 samples (Table 6).

Comparison between culturing and community profiling results

Comparison between the culture-dependent and -independent screens is limited by the inability to differentiate taxa to the level of species in the community profiles. Table 6 provides a comparison between the culture-dependent and -independent screens for the individual samples. Overall the correlation between the two screens with regard to detection of the most widely recognized pathogenic genera, Streptococcus, Moraxella, Haemophilus and Staphylococcus, was fair. However, as expected, the culture-independent microbiota analysis was both more sensitive for the detection of bacteria amenable to culturing and for detection of genera that were unable to be cultured. The culture-independent screen identified Streptococcus (in six samples), Moraxella (in two samples), Haemophilus (in three samples) and Staphylococcus (in three samples) that were either not detected or produced less than the defined cut off at 10,000 cfu/ml in the culture-dependent screen. It also should be stressed that in these instances identified genera do not necessarily correlate with the species amenable to culture identified in other samples. Additionally in some instances (samples 2921, 6594, 3066) bacteria producing greater than 10,000 cfu/ml were shown to constitute only a small fraction of the total microbial biomass while in other instances (samples 9636, 1276) bacteria producing less than 10,000 cfu/ml were constituted a high proportion of the microbial biomass.

Discussion

Globally, RSV is the leading cause of severe respiratory tract infections requiring hospitalization during early childhood. Various studies have now shown that RSV infection is often associated with bacterial co-infection [1,2,12-18]. Our results show that approximately 75% of RSV infections are associated with a high load of potentially pathogenic bacteria in the nasopharynx (greater than 10,000 cfu/ml of NPA). This proportion of RSV/bacterial co-detection was significantly higher than for other viral infections and virus-negative samples. The prevalence of bacterial detection associated with RSV was found to peak among children aged between six and 24 months with close to 90% testing positive for high bacterial prevalence.

The most common bacteria associated with RSV were M. catarrhalis (32.8%), S. pneumoniae (25.4%) and H. influenzae (7.5%). One or more of these three pathogens were found in 55% of all RSV infections and 73% of RSV infections in children between the ages of six and 24 months. While RSV was shown to be associated with increased bacterial prevalence when compared to other acute respiratory infections amongst the same age group, unfortunately samples from healthy children were not available for direct comparison or from samples outside of the "RSV season". It has previously been shown that the colonization rates for such bacteria among healthy children are high (M. catarrhalis: 22-36%, S. pneumoniae: 17-38%, H. influenzae: 12-32%), and comparable to those determined by this study in association with RSV infection [4,47,49,51-53]. However, differences in the methods of sample collection, culturing and the definition of bacterial load considered positive mean that direct comparison between our results and those of healthy children is difficult.

Previous studies attempting to determine prevalence of bacterial co-infection associated with RSV infection have produced estimates varying between 16% and 55% [1,2,9,12,14-18,21,50]. The majority of these studies examined bacteria within samples collected from the lower respiratory tract or measured circulating antibodies to respiratory bacteria. Brunstein et al. [18] was the only other study to focus on the detection of bacteria in the upper airways. A comparable
level of bacterial prevalence associated with RSV infection was found for *S. pneumoniae* (17.3%) and *H. influenzae* (11.4%), however other bacteria of interest were not examined.

The viral and culture-dependent bacterial screens we have employed in this study provided increased power in determining a presumptive causative agent compared to previous studies. Only 22 of 201 (10.9%) samples were negative for both viral and bacterial pathogens, compared to typically around 25% of samples tested by previous studies which have employed methods that limit their scope by focusing solely on specific pathogens [9,18,21,50].

The use of the culture-independent microbiota analysis can further increase the ability to detect the presence of potentially pathogenic bacteria that are unable to be cultured using routine methods, e.g. *Flavobacteriaceae*, *Idiomarinaceae*, *Comamonadaceae*, *Neisseria* and *Pseudomonas*. We found that virus-positive samples more frequently profiled high numbers of *Flavobacteraeae* and *Flavobacteriaceae* within *Comamonadaceae*, which have been detected previously in virus-positive NPAs [32], and *Neisseria* and *Pseudomonas*, which have been detected in lower respiratory tract infections [30,31,54]. While *Flavobacteriaceae* and *Neisseria* species have also been detected in the healthy nasopharynx [55], *Comamonadaceae* have, rarely, been found in human chronic lung conditions [56,57]. *Idiomarinaceae* were also present more frequently in virus-positive samples. This family contains Gram-negative, aerobic organisms that are usually isolated from saline habitats and to our knowledge have not been previously reported in human infections. Their presence in virus-positive samples from paediatric ARIs is surprising and warrants further investigation. Since the majority of the *Idiomarinaceae* reads were from an unclassified genus, their presence could be suggestive of a novel human-associated phylotype.

The use of culture-independent microbiota analysis was found to be more sensitive than the culture-dependent screen as a number of samples harbored species of bacteria that in theory should have been detected by bacterial culture. In some of these cases bacteria were present but below the limit of 10,000 cfu/ml set for positive detection. Discrepancies between the results of the culture-dependent and -independent screens highlight the inherent difficulty in detection of bacteria through culture-dependent methods. In some instances species were shown to constitute a considerable percentage of total bacterial biomass but failed to be identified in the culture dependent screen. The reliance on live bacterial isolates for identification is presumably a major obstacle affecting the culture-dependent screen. Bacterial lysis initiated by the host's immune response or antibiotic use may affect bacterial viability. Additionally storage of samples at -80°C in 25% glycerol may have reduced bacterial viability in some instances. By coupling the larger culture-dependent screen with a more focused culture-dependent screen we were able to put together a more comprehensive understanding of the specific effect of viral infection on bacterial communities within the nasopharynx. Strikingly, community profiling data revealed that in 4 of 10 RSV infections, as well as 3 of 10 HRV infections, an individual bacterial genus constituted between 75 and 95% of the total bacterial population of the nasopharynx. The species identified were *M. catarrhalis*, *S. pneumoniae* and *S. aureus* in the case of RSV and *M. catarrhalis* and *S. aureus* in the case of HRV. Furthermore, the total bacterial load in these samples estimated by rtPCR was substantially higher than for other samples suggesting that a large growth of these individual species accompanied viral infection as has been previously suggested [58-63]. The substantial changes to the flora of the nasopharynx accompanying viral infection should be further investigated as such changes would be likely to affect the induction of a targeted immune response, contribute to the presentation of symptoms and severity and increase the likelihood of progression to secondary bacterial pneumonia or otitis media.

The clinical significance of these findings is an important question that cannot be addressed by the present study as we did not have access to clinical information. Previously it has been suggested patients with RSV/bacterial co-infections were more likely to be admitted to the PICU [20] and to require ventilatory support for longer than those with only RSV [1,17,20].

The strikingly high prevalence of RSV/bacterial co-infections, along with the potential for such bacteria to proliferate to the point of constituting up to 95% of the total bacterial community during viral infection, highlights the possibility that RSV infection could conceivably facilitate bacterial transmission and colonization of new hosts. As both RSV and common bacterial pathogens of the respiratory tract are transmitted through direct contact with nasal secretions, it appears logical that both may therefore be expelled and subsequently acquired by new hosts [64]. In support of this hypothesis it has been shown that infection with influenza virus is able to enhance both susceptibility and efficient transmission of *S. pneumoniae* in animal models [63-68]. While our study has highlighted an association between RSV and bacterial pathogens in the nasopharynx of young children in a clinical setting, a prospective study will be required to understand the clinical significance of this observation.

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