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Nasopharyngeal density of respiratory viruses in childhood pneumonia in a highly vaccinated setting: findings from a case–control study

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ABSTRACT

Background Detection of pneumonia-causing respiratory viruses in the nasopharynx of asymptomatic children has made their actual contribution to pneumonia unclear. We compared nasopharyngeal viral density between children with and without pneumonia to understand if viral density could be used to diagnose pneumonia.

Methods Nasopharyngeal swabs (NPS) were collected from hospitalised pneumonia cases at Princess Margaret Hospital (PMH) and contemporaneous age-matched controls at PMH outpatient clinics and a local immunisation clinic in Perth, Australia. The density (copies/mL) of respiratory syncytial virus (RSV), influenza A virus (InfA), human metapneumovirus (HMPV) and rhinovirus in NPS was determined using quantitative PCR. Linear regression analysis was done to assess the trend between viral density and age in months. The association between viral density and disease status was examined using logistic regression. Area under receiver operating characteristic (AUROC) curves were assessed to determine optimal discriminatory viral density cut-offs.

Results Through May 2015 to October 2017, 230 pneumonia cases and 230 controls were enrolled. Median nasopharyngeal density for any respiratory virus was not substantially higher in cases than controls ($p>0.05$ for each). A decreasing density trend with increasing age was observed—the trend was statistically significant for RSV (regression coefficient −0.04, $p=0.004$) but not for other viruses. After adjusting for demographics and other viral densities, for every $\log_{10}$ copies/mL density increase, the odds of being a case increased by six times for RSV, three times for HMPV and two times for InfA. The AUROC curves were <0.70 for each virus, suggesting poor case–control discrimination based on viral density.

Conclusion The nasopharyngeal density of respiratory viruses was not significantly higher in children with pneumonia than those without; however, the odds of being a case increased with increased density for some viruses. The utility of viral density, alone, in defining pneumonia was limited.

Key messages

- Does nasopharyngeal respiratory virus density vary between children with and without pneumonia and is there any optimal density threshold for common respiratory viruses that could help clinicians to diagnose viral pneumonia cases?
- The nasopharyngeal density of common respiratory viruses, alone, is not reliable to diagnose viral pneumonia cases effectively in clinical settings.
- This is the largest age–frequency matched case–control study in Australia and similar high-income countries during the post-pneumococcal vaccine era showing that although the absolute nasopharyngeal density of common respiratory viruses was not substantially higher in pneumonia cases than controls, the increased density of respiratory syncytial virus, human metapneumovirus and influenza A virus was associated with an increased risk of being a pneumonia case when adjusted for demographics and other viral densities.

INTRODUCTION

Globally, pneumonia is responsible for 120 million cases and 0.9 million paediatric deaths each year among young children under 5 years. The burden is highest in low-income countries.[1–3] Although deaths are rare in high-income countries, pneumonia-associated hospitalisation is common. In Australia, the incidence of pneumonia is 5–8 cases/1000 years, with Aboriginal children at 10 times higher risk than non-Aboriginal children.[4 5]

Identification of pathogens in blood or lower airway specimens provides the most specific evidence of pneumonia aetiology, but specimen collection is invasive and rarely practised for paediatric patients. Given the ease of collection, swabs from the upper respiratory tract are frequently tested for aetiology. However, the relationship between pathogen detection in the nasopharynx and lower respiratory tract infection including pneumonia has low specificity because many

of these pathogens are present in the nasopharynx as commensal and are detected in children without respiratory symptoms.[6 7] Pathogen density in the nasopharynx could provide additional information and may further aid distinguishing asymptomatic from symptomatic infection. A number of studies, mostly from low-income settings with limited pneumococcal conjugate vaccine (PCV) coverage, report higher nasopharyngeal viral density in pneumonia cases compared with asymptomatic children or children with mild respiratory infections[8–14], while some studies found no difference.[15 16] Increased nasopharyngeal viral density for some viruses has been associated with clinical severity in children with acute respiratory infection.[13 17] Limited evidence exists to inform a reliable density cut-off for specific viruses to diagnose pneumonia cases at clinical settings.[8 10 12 18–20]

We conducted a prospective case–control study among children with radiologically confirmed pneumonia and contemporaneous children without pneumonia in Western Australia (WA),[21] a high-income setting with >90% coverage for paediatric vaccines.[22] We investigated the nasopharyngeal density of common respiratory viruses and compared between children with and without pneumonia. We sought to assess an optimal nasopharyngeal density threshold for different respiratory viruses that could be used to diagnose pneumonia cases effectively.

METHODS

Study population

From May 2015 to October 2017, children aged ≤17 years hospitalised at the Princess Margaret Hospital for Children (PMH) with X-ray confirmed community-acquired pneumonia (CAP) were prospectively enrolled as cases. During the study period, PMH was the only tertiary paediatric hospital in the state of WA (total population of 2.6 million[23]). Children attending PMH outpatient orthopaedics or allergy and immunology clinics and a large urban immunisation clinic were concurrently enrolled as controls. Controls were enrolled throughout the study period and frequency-matched to the number of cases enrolled in each of the four age groups (≤1 year, 1–5 years, 6–9 years and ≥10 years) in the preceding 2 weeks. Controls with mild respiratory symptoms (eg, rhinorrhoea or cough) were not specifically excluded but children attending hospital or clinic for treatment or follow-up of a lower or upper respiratory illness (including all respiratory or ear, nose and throat clinic attendees) were excluded.Written informed consent was

Table 1 Background characteristics of cases and controls, Perth, Australia, May 2015 to October 2017

Parameter	Case (%) (N=230)	Control (%) (N=230)	Total (%) (N=460)
Demographic and clinical data			
Age			
≤12 months	21 (9.1)	23 (10)	44 (9.5)
1–5 years	126 (54.7)	157 (68.2)	283 (61.5)
6–9 years	60 (26.1)	27 (11.7)	87 (18.9)
10–17 years	23 (10)	23 (10)	46 (10)
Male sex	120 (52.1)	122 (53.0)	242 (52.6)
Aboriginal	21 (9.1)	2 (0.8)	23 (5.0)
Premature	32 (13.9)	17 (7.3)	49 (10.6)
Smoker at household	38 (16.5)	22 (9.5)	60 (13.0)
Exposure to antibiotics*	109 (47.3)	5 (2.1)	114 (24.7)
Any comorbidity	34 (14.7)	10 (4.3)	44 (9.5)
Immunodeficiency†	7 (3.0)	1 (0.4)	8 (1.7)
Immunocompromised condition	5 (2.1)	0 (0.0)	5 (1.1)
Congenital abnormality‡	17 (7.3)	4 (1.7)	21 (4.6)
Chronic respiratory illness	9 (3.9)	2 (0.8)	11 (2.3)
Chronic neuromuscular disorder illness	9 (3.9)	2 (0.8)	11 (2.3)
Other§	1 (0.4)	0 (0.0)	1 (0.2)

*In the 7 days prior to enrolment.
†IgG subclass deficiency (n=1); low IgA (n=1); T-cell deficiency (n=1), mannose-binding lectin deficiency (n=2), DiGeorge's syndrome (n=2).
‡Capillary malfunction syndrome (n=1); Beckwith-Wiedemann syndrome and congenital hypothyroidism (n=2); atrioventricular septal defect (n=1); Down syndrome (n=3); Sotos syndrome (n=1); spinal muscular atrophy type 2 (n=1); developmental delay (n=1); congenital heart disease (n=2); Prader–Willi syndrome (n=1); congenital sensorineural deafness (n=1); Ehlers–Danlos syndrome (n=1); gastroschisis (n=1); cleft lip (n=1).
§Intracranial shunt (n=1).

required from the parent or legal guardian of each participant and assent was obtained from those participants aged ≥7 years old. After enrolment, demographic and existing comorbidities were recorded using a structured questionnaire for both cases and controls. The study protocol describing the eligibility criteria and sample size calculation for the study has been published.[21]

Patient and public involvement

The parents of the study participants were not involved in the development of study design, recruitment or implementation of the study. However, during enrolment the research team assured that the identity of any participant would not be disclosed in dissemination of results or in any publication from this study.

Specimen collection and laboratory procedure

A nasopharyngeal swab (NPS) (FLOQSwabs; Copan Diagnostics, Murrieta, California, USA) was collected from cases and controls into 1 mL skim–milk–tryptone–glucose–glycerol broth following standardised procedure.[24] For cases, the swab was collected <36 hours of hospital presentation. After NPS collection from cases and controls, samples were stored at −80°C until tested. Specimens that had detectable nucleic acid for influenza A (InfA), respiratory syncytial virus (RSV), human metapneumovirus (HMPV) or rhinovirus (RV) using a multiplex real-time polymerase-chain-reaction (rt-PCR) assay[25 26] underwent density assessment using quantitative PCR (qPCR) following methods published previously.[21 27] In brief, standard curves were generated for each virus from serial 10-fold dilutions of nucleic acid extracted from reference strains. The pathogen density in each specimen was quantified by interpolating from the appropriate standard curve. All viral quantification assays were validated in-house according to the minimum information for publication of quantitative real-time PCR experiments (MIQE) and National Pathology Accreditation Advisory Council (NPAAC) guidelines.[27]

Data analysis

Viral density was measured and expressed in copies/mL and transformed to $\log_{10}$ unit for analysis. The mean (range) density of each virus, if normally distributed, otherwise median (IQR) was determined and compared between pneumonia cases and controls. For continuous normally distributed data, Student's t-test was used to compare means; Wilcoxon rank-sum test was used to compare medians. Linear regression analysis was done to assess the trend between viral density and age in months. Logistic regression was used to determine the increase in odds for being a case (vs a control) with 1 $\log_{10}$ (copies/mL) increase in viral density. Multivariate logistic regression was used to adjust for underlying differences between cases and controls in demography, pre-exposure to antibiotics (in the 7 days preceding enrolment) and nasopharyngeal density of other viruses. To enable logistic regression analyses, negative samples or positive samples but undetermined quantity were assigned with half of lowest limit of quantification (LLQ) for the qPCR assay.

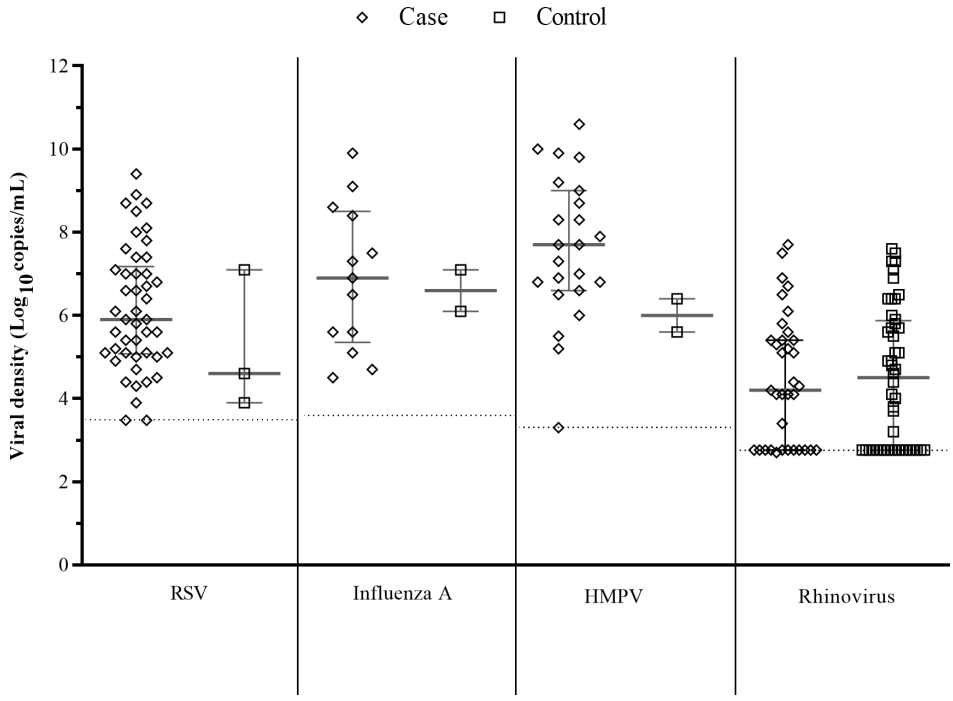

Figure 1 Distribution of log-transformed nasopharyngeal densities (copies/mL) of respiratory viruses in nasopharyngeal swab from cases and controls, Perth, Australia, May 2015 to October 2017. Grey solid dash, median with IQR; dotted dash line, lowest limit of quantification for the virus. HMPV, human metapneumovirus; RSV, respiratory syncytial virus.

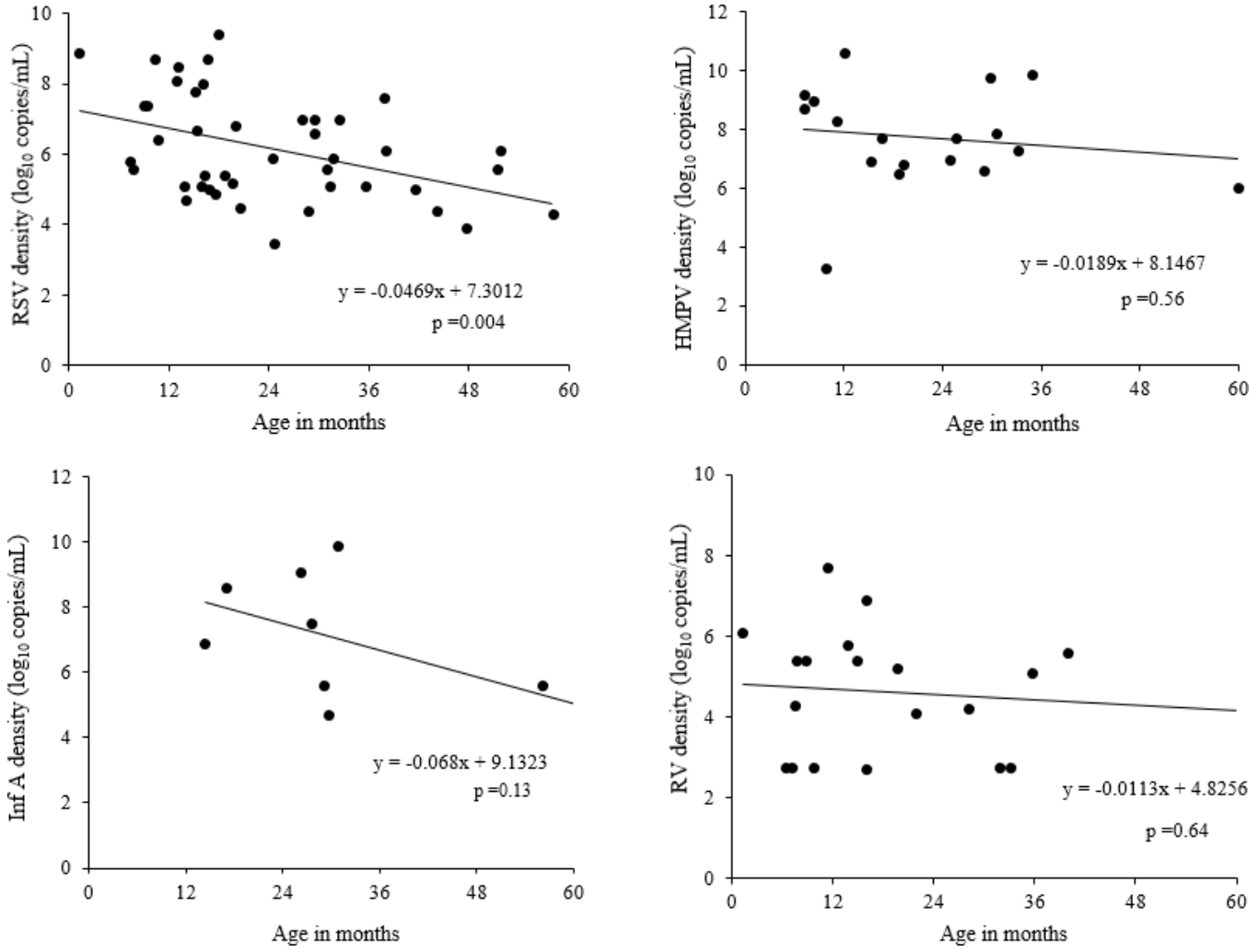

Figure 2 Distribution of nasopharyngeal density of respiratory viruses by age in months in pneumonia cases* under 5 years, Perth, Australia, May 2015 to October 2017. *The density analysis was limited to cases under 5 years since the majority of virus positive cases (ranged 60%–93%) were <5 years. HMPV, human metapneumovirus; Inf A, influenza A; RSV, respiratory syncytial virus; RV, rhinovirus.

The LLQ was 6000 copies/mL for RSV, 7000 copies/mL for InfA and 1147 copies/mL for RV and HMPV. The utility of specific viral density for diagnosing pneumonia case was assessed using receiver operating characteristic (ROC) curves. An area under the ROC (AUROC) curve value of 0.9–1.0 was considered as excellent discriminator, 0.8–0.9 as good, 0.7–0.8 as fair, 0.6–0.7 as poor and 0.5–0.6 as failed discriminator.[28] The Youden index was calculated to determine the optimal diagnostic cut-off to distinguish cases from controls for viruses with AUROC value >0.7 (at least fair discriminator).[29] All analyses were conducted using STATA V.13.0 (StataCorp) and figures were produced using GraphPad Prism V.5.0 (GraphPad Software, California, USA).

RESULTS

Study population and nasopharyngeal viral detection

We enrolled 230 cases and 230 controls during the study period. More cases than controls were Aboriginal, were born premature, had at least one smoker in the household and had prior exposure to antibiotics ($p<0.05$ for each) (table 1). A higher proportion of cases (47%) than controls (2%) had exposure to antibiotics in the 7 days prior to enrolment and nearly all cases (227/230, 98.7%) received antibiotic during hospitalisation. Based on diagnostic laboratory results performed on blood and pleural fluid samples collected for clinical care, 30 cases had confirmed bacterial pneumonia (detection of bacteria in blood or pleural fluid) including 15 with empyema, 9 with bacteraemia and 6 with both.[30]

Nasopharyngeal viral density distribution and discriminatory analysis

The log-transformed median nasopharyngeal densities for RSV, HMPV and InfA were higher in cases than controls, whereas RV densities were lower in cases than controls—none of these differences were statistically significant ($p>0.05$ for each) (figure 1). The viral densities did

Table 2 Unadjusted and adjusted OR of log-transformed density of respiratory pathogens in the nasopharynx in comparison between cases and controls, Perth, Australia, May 2015 to October 2017

	Respiratory virus, n (%)		OR per 1 log_{10} increase in copies/mL (95% CI)	
Pathogen	Case (N=230)	Control (N=230)	Unadjusted	Adjusted*
RSV	46 (20.0)	3 (1.3)	3.85 (1.90 to 7.77)	**6.23 (2.51 to 15.41)**
HMPV	23 (10.0)	2 (0.8)	2.03 (1.27 to 3.25)	**2.83 (1.57 to 5.11)**
Influenza A	13 (5.6)	2 (0.8)	1.74 (1.06 to 2.85)	**2.00 (1.16 to 3.44)**
Rhinovirus	35 (15.2)	48 (20.8)	0.88 (0.72 to 1.07)	1.13 (0.88 to 1.47)

Bold values under "Adjusted" column indicate p value <0.05

*Adjusted for age groups, gender, aboriginal status, smoker at household, prematurity, antibiotic in preceding 7 days and density of other viruses in nasopharynx.
HMPV, human metapneumovirus; RSV, respiratory syncytial virus.

not vary among cases and controls when analysed independently in younger (≤5 years) and older (>5 years) age group (online supplementary table 1). Among the cases, for all viruses, a decreasing density trend with increasing age was observed—the trend was statistically significant for RSV (regression coefficient −0.04, p=0.004) but not for other viruses (figure 2). The AUROC was 0.68 for RSV density, 0.53 for InfA density, 0.61 for HMPV density and 0.51 for RV density, suggesting that nasopharyngeal viral density provides a poor discrimination between cases and controls (online supplementary figure 1), hence, optimal viral cut-off density to distinguish cases were not determined.

Relationship between nasopharyngeal density for respiratory viruses and disease status

Logistic regression analysis showed that the odds of being a pneumonia case increased with every unit increase in log_{10} copies/mL of RSV, HMPV and InfA independently and when adjusted for demographic factors, antibiotic exposure prior to enrolment and the density of other viruses in the nasopharynx (table 2).

DISCUSSION

This evaluation was nested in the first prospective pneumonia case–control study among Australian children with radiologically confirmed pneumonia (cases) and contemporaneous children without pneumonia (controls) to determine whether the density of common respiratory viruses in the nasopharynx is higher among children with pneumonia than controls. Our results suggest, after adjusting for demographic factors, prior antibiotic treatment and the density of other viruses, that an increase in pathogen density for RSV, InfA and HMPV increases the odds for being a case. The viral density is higher in young children and it decreases with age. The poor discriminating value for each virus limited our aim to assess optimal density threshold for a virus to define pneumonia case—suggesting that nasopharyngeal viral density, alone, could not be used to define a viral pneumonia case effectively.

Previous epidemiological studies that assessed nasopharyngeal density found higher viral density in pneumonia cases than in children with no or mild respiratory infections.[8 9 17 18 31] Our findings were similar to published data although the density differences between children with and without pneumonia were not statistically significant. The potential explanation could be that the common respiratory viruses such as RSV, HMPV and InfA were rarely detected in our control population,[32] so the power of our analysis to assess the differences in viral densities between cases and control was limited. RV was detected in greater proportion in controls than cases in our study population and also the density were higher in controls than cases—contrast findings to previous case–control studies.[8 9 32] While the overall absolute density was not significantly higher in cases than controls, we found that cases had increased RSV, InfA and HMPV density when compared with controls after adjusting for demographic factors and the nasopharyngeal density of other pathogens, consistent with previous case–control studies including the large case–control study (Pneumonia Etiology Research for Child Health (PERCH)) in multiple low-middle income settings.[8] These findings further strengthen the current understanding that RSV, InfA and HMPV are important viral contributors to childhood pneumonia in this and similar settings.[9 33 34] None of the previous pneumonia aetiology studies in developed settings, to our knowledge, had explored the relationship between age and nasopharyngeal viral density in pneumonia children. Our data, consistent to PERCH study,[8] showed a decreasing viral density trend with increasing age, confirming young children not only have disproportionately greater rate of viral pneumonia but also have higher viral density,[35] suggesting that preventive programmes towards the young children would provide greatest benefit.

While a positive relationship between viral density and pneumonia had been demonstrated, we note that

nasopharyngeal viral density has limited clinical usefulness for distinguishing symptomatic from asymptomatic infection. The nasopharyngeal viral densities in pneumonia cases and controls frequently overlapped in this study and we could not determine a reliable density threshold for specific viruses enabling to define pneumonia cases. These findings are consistent with findings from the PERCH study. This data demonstrate that despite the heterogeneity in study settings there are limited variability in pathogen density distribution to be used for clinical diagnosis of pneumonia.[8–10 19]

The major strengths of this case–control study involve methodological improvement to previous studies including the comparison of viral density between pneumonia cases and contemporaneous age–frequency matched children without pneumonia and assessment of absolute viral density using a standard curve of known quantity. Despite these strengths, our findings have some limitations. The density and composition of pathogens in the nasopharynx are likely to vary over the course of illness.[36] We collected specimens only once from each participant and thus could not determine whether our specimen represented the peak pathogen density. Collection of specimens at multiple time points could inform the temporal changes of density and also the synergistic relationship between viruses. Our approach of using half of LLQ as viral quantityfor undetermined positive cases and negative cases for enabling multivariateregression warrants cautious interpretation. Since there were only a fewpositive virus samples in control group, having a model restricted to onlychildren with acceptable viral quantity would have, otherwise, very low power. Density assessment in specimens from the lower respiratory airway would be more sensitive, but routine collection from children is not feasible.[6] From a clinical perspective, having an additional control group comprising children with upper respiratory tract infection could help facilitate analysis for discriminating pneumonia cases from those non-pneumonia respiratory tract infections. However, similar densities for most viruses between controls with and without upper respiratory infection have been previously reported.[8]

To date, this study reports the most comprehensive assessment of nasopharyngeal density of respiratory viruses in children with pneumonia and without pneumonia in Australia and similar high-income countries during PCV era. While the absolute nasopharyngeal densities for common respiratory viruses were not substantially higher among children with pneumonia than those without pneumonia in Western Australia, a positive relationship between nasopharyngeal pathogen density for RSV, HMPV and InfA and pneumonia diagnosis has been determined after adjustment by demographics and density of other viruses. The increasing nasopharyngeal viral density at young age warrants development of targeted prevention strategies. The utility of viral density as a tool to guide clinicians in diagnosing pneumonia cases effectively is limited and needs further investigation. Given the complexity of potential relationships between disease development and pathogen density, we suggest more sophisticated analytical approaches including Bayesian network modelling to explore if viral density data in combination with other clinical and immunological parameters could develop a diagnostic algorithm that reliably distinguish viral pneumonia and guide appropriate management plan through judicious antiviral and antibiotic therapies.

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Contributors MUB: participant enrolment, data collection, specimen collection, data analysis, first draft manuscript and subsequent versions. TS: conceive the study, supervise data collection, supervise data analysis and critical review of manuscript. CS: laboratory testing and critical review of manuscript. JL: laboratory testing and critical review of manuscript. MB: conceive the study, support data collection and critical review of manuscript. AM: conceive the study, support data collection and critical review of manuscript. PR: conceive the study and critical review of manuscript. AJ: conceive the study and critical review of manuscript. DS: conceive the study, supervise laboratory testing, interpret laboratory finding and critical review of manuscript. CB: conceive the study, supervise data collection, interpret laboratory analysis, supervise data analysis and critical review of manuscript.

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