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Respiratory Viruses in Nepalese Children With and Without Pneumonia

A Case-Control Study

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Background: The causative role of respiratory viruses detected in upper airway secretions in childhood pneumonia needs further investigation.

Objective: To measure the association between infection with respiratory RNA viruses and pneumonia in children.

Methods: From March 2006 to July 2007, we conducted a case-control study of 680 pneumonia cases (WHO criteria) and 680 randomly selected, concurrently sampled age-matched controls among children aged 2–35 months in Bhaktapur, Nepal. A nasopharyngeal aspirate from each child was examined for 7 respiratory viruses using reverse transcription polymerase chain reaction. We calculated the matched odds ratios (MORs) for the detection of the individual viruses from a case compared with a control as measures of pathogenicity using conditional logistic regression.

Results: At least 1 virus was recovered in 248 (36.5%) cases and 48 (7.1%) controls. The MOR varied from 2.0 to 13.0; the highest associations were observed for parainfluenza virus type 3 (MOR 13.0; 95% confidence interval [CI] 6.0–28.0), respiratory syncytial virus (MOR 10.7; CI 4.6–24.6), and influenza A (MOR 6.3; CI 1.9–21.4). We observed that the association was lower for children age 2–5 months compared with older children for parainfluenza virus type 3 (*P* value for interaction 0.002).

Conclusions: All of the 7 respiratory viruses were associated with pneumonia, but their pathogenicity varied. Parainfluenza type 3, RSV, and influenza A were most strongly associated with pneumonia.

Key Words: lower respiratory tract infection, respiratory syncytial virus, parainfluenza virus, influenza virus, human metapneumovirus, multiplex reverse transcription PCR

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Identification of respiratory viruses in specimens from the upper respiratory tract of children is associated with symptoms of respiratory illness. However, the role of each virus in the etiology of lower respiratory tract infections needs further investigation.

Polymerase chain reaction (PCR) has to a great extent replaced conventional methods in routine diagnosis of common respiratory infections[1] and has improved sensitivity.[2,3] This, and the wider application of expanded diagnostic panels, has led to an increase in the proportion of pathogen-positive respiratory specimens from patients[4,5] as well as from asymptomatic individuals,[6] especially in young children.[6,7] Furthermore, the interpretation of a positive PCR test has been complicated by the simultaneous increase in the detection of multiple viral agents in airway specimens from symptomatic individuals.[8,9] Thus, there is a need to measure the degree to which the presence of the different respiratory viruses in upper airway secretions is associated with pneumonia.

To address this issue, we conducted a case-control study embedded in a larger clinical trial on pneumonia where we also undertook a comprehensive study of viral etiology among children 2–35 months of age in Bhaktapur, Nepal.[10,11] We here report pathogenicity estimates of 7 common respiratory viruses identified using reverse transcription PCR.

SUBJECTS AND METHODS

Study Subjects

Both cases and controls were recruited from an open cohort of children less than 3 years of age that were under monthly active surveillance for respiratory illness. The design and methods of the surveillance and the inclusion of the cases have been described.[10,11] For this study we recruited children with pneumonia who were 2–35 months old from the municipality of Bhaktapur presenting at our study clinic from March 25, 2006, to July 9, 2007. Study doctors screened children for fast breathing and lower chest wall indrawing and classified illness according to the standard World Health Organization (WHO) algorithm for acute respiratory infection. Pneumonia was defined as cough or difficult breathing combined with fast breathing, ie, ≥50 breaths/min for children 2–11 months old or ≥40 breaths/min for children ≥12 months old, while severe pneumonia was defined as cough or difficult breathing with lower chest wall indrawing.[12] Cases with other severe illness, documented tuberculosis, congenital heart disease, dysentery, severe anemia (defined as hemoglobin <7 mg/L), or severe malnutrition (defined as <70% NCHS median weight for height) were not included in the study. Those with a history of cough for more than 14 days or who had got antibiotics within the last 48 hours were also no included. These exclusion criteria also applied to control children, except that hemoglobin was not routinely measured in controls. Children

could be enrolled as a case or as a control in the current study again only after 2 months.

One control was randomly selected for each case from a list of children under surveillance that was updated monthly. The control was individually matched on age (in months) of the case. After inclusion of the case, a fieldworker made a home visit to the selected potential control child the same or the following day. If parents consented to the child's participation, the child was referred to the study clinic to be examined for eligibility. Children who did not meet the criteria for pneumonia were eligible as controls. If the child did not present at the clinic after 2 home visits, or was not found or not eligible for other reasons, another randomly selected age-matched child was approached.

Virology

Nasopharyngeal aspirates (NPAs) were obtained using a sterile, disposable suction catheter (Pennine Healthcare Ltd., Derbyshire, UK) with a suction trap (trachea suction set, Unomedical a/s, Birkerød, Denmark) connected to a foot pump (Ambu Uni-Suction Pump, Ambu A/S, Ballerup, Denmark), transported to the laboratory in Kathmandu, processed and divided into aliquots as previously described.[10] One aliquot of each specimen was kept at 2–8°C before analysis in our research laboratory at the Institute of Medicine, Tribhuvan University, Kathmandu, while remaining aliquots were frozen at −70°C and transported on dry ice to Norway for quality control analyses. The aliquot analyzed in Nepal was tested for respiratory syncytial virus (RSV), influenza A and B, parainfluenza virus (PIV) types 1, 2, and 3 and human metapneumovirus (hMPV) using a commercially available multiplex reverse transcription PCR assay (Hexaplex Plus, Prodesse Inc., Waukeshaw, WI), as described elsewhere.[10]

Statistical Analyses

The data were double entered and compared daily using Visual Microsoft FoxPro version 6.0 (Microsoft Corporation, Redmond, WA). Statistical analyses were performed using Stata/MP 10.0 for Macintosh (Stata corporation, College Station, TX). Proportions in the baseline table were compared using logistic regression. In an unmatched design, the pathogenicity odds ratio (OR) for each virus is the odds of detecting a pathogen-positive specimen from a child with pneumonia divided by the odds of detecting a pathogen-positive specimen from a control child. We sought to identify possible confounders, such as the presence of other viruses, gender, breast-feeding status, birth weight, stunting, wasting, and whether the child had been delivered in a hospital, using unconditional logistic regression including age categories as factors. The analyses included 1360 specimens from 1059 children of whom 808 were enrolled once, 210 twice, 33 thrice, 7 4 times, and 1 child 5 times as a case or a control. We explored the degree to which any dependence of repeated observations in the 251 reenrolled children affected the variance estimates using the cluster option in Stata. This adjustment only marginally affected the precision of the unmatched ORs; hence, repeated measurements were not taken into account when we in subsequent conditional logistic regression analyses of the 1360 enrollments calculated the matched OR (MOR). Because we sampled each control concurrently, ie, shortly after the corresponding case had been identified and because children previously included as a case or a control could be included again in the study, and this MOR is a direct rather than a biased estimate of the incidence rate ratio for the given pathogen[13] (KJ Rothman, personal communication). We also explored whether the MORs were different in children below and above 6 months of age for the 2 most common viruses. The *P* values for such possible interactions were obtained from the unconditional models. Statistical significance was defined as a *P* value <0.05. Anthropometric measures were expressed as Z-scores, which were generated using the WHO Child Growth Standards.[14]

Ethics

The study had ethical clearance from the Research Ethics Committee of the Institute of Medicine at Tribhuvan University in Kathmandu, Nepal. The implementation of the project was in agreement with the international ethical principles for medical research involving human subjects as stated in the latest version of the Helsinki Declaration. Informed consent for participation was obtained from the guardian of the child, usually of the parents. A witnessed verbal informed consent was obtained from those who were illiterate. A register of the witnesses was kept.

RESULTS

Control Selection

We approached 1955 potential controls for the 726 cases enrolled in the study. For 60% of the cases, we approached 1–2 children as potential controls, for 30% of the cases we approached 3–5 children, for 9% of the cases 6–10 children, while for 1% of the cases 11–15 children were approached before an eligible control could be included. The most common reasons for a child not being selected as a control were that the family was either traveling (n = 301) or had moved (n = 240). Ninety-four children had taken antibiotics during the last 48 hours, while 10 had died, 7 suffered from dysentery, and for 258, no reason was given for why the parents did not bring their child to the study clinic despite having accepted to do so. There were 165 who were not included for other reasons mainly because of recent inclusion (ie, <2 months ago) in the study. There were 154 children who did not participate because their caregivers did not consent. We enrolled 44% of the controls within 3 days of having enrolled the corresponding case, 75% within 1 week, 90% within 2 weeks, and 98% within 3 weeks. For 20 cases, no control was obtained within reasonable time. Furthermore, 8 pairs were excluded from the analyses due to protocol deviations. Another 3 pairs were excluded because of missing NPA and 15 to inhibition of the PCR assay in either the case or in the control specimen. The final analyses were thus based on data from 680 matched pairs.

Population Characteristics

The mean age of cases and controls was 13.5 months and 13.7 months, respectively. More boys than girls were included in both groups (Table 1). More controls than cases had been delivered at a hospital but their mean birth weights were almost identical. Compared with controls, cases were more frequently wasted and stunted and also had lower mean weight for length/height Z-score. Other background characteristics were evenly distributed between cases and controls. T1

Characteristics of Pneumonia Episodes

The clinical characteristics of the 680 pneumonia cases are given in Table 2. There were only 28 (4.1%) cases of severe pneumonia. Pneumonia episodes occurred throughout the year with epidemic peaks in June and September 2006, each peak coinciding either with epidemics of RSV or PIV type 3. Unlike the preceding 2 winter seasons,[10] there was no winter epidemic of pneumonia during the current study period. T2

Symptoms in Controls

Among the 680 controls, 272 (40.0%) had no respiratory symptoms, ie, no complaint of cough, difficult breathing, nasal discharge, fever, ear ache, or ear discharge. Of the 164 (24.1%)

TABLE 1. Background Characteristics of 680 Pneumonia Cases Aged 2–35 Months and 680 Age-Matched Controls

Characteristic	Cases			Controls		
	N	Value		N	Value	
Mean age in months (SD)	680	13.5	(8.1)	680	13.7	(8.1)
Boys (%)	680	393	(57.8)	680	376	(55.3)
Mean birth weight, g (SD)*	533	2851	(465.3)	605	2881	(445.3)
Low birth weight (≤2500 g) (%)	533	78	(14.6)	605	80	(13.2)
Hospital delivery (%)	678	549	(81.0)	680	605	(89.0)
Currently breastfed (%)	680	594	(87.4)	680	592	(87.1)
Literate mother (%)	678	527	(77.7)	679	545	(80.3)
Literate father (%)	678	649	(95.7)	680	660	(97.1)
Ownership of land	677	257	(38.0)	678	284	(41.9)
Newar caste	677	370	(54.7)	678	388	(57.2)
Staying inside factory compound	677	112	(16.6)	678	115	(17.0)
Living in extended family	676	295	(43.6)	678	323	(47.6)
Mean WHZ (SD)†	678	−0.18	(1.03)	679	0.13	(0.96)
Mean HAZ (SD)	678	−1.23	(1.22)	679	−1.10	(1.24)
Wasted (<-2 WHZ) (%)	678	26	(3.8)	679	9	(1.3)
Stunted (<-2 HAZ) (%)	678	181	(26.7)	679	143	(21.1)

*Written documentation from birth certificate of 473 cases and 547 controls, remaining records from mother's recall.
†Calculated using the WHO Child Growth Standards (14).
WHZ indicates weight for length/height Z-score; HAZ, length/height for age Z-score.

TABLE 2. Clinical Characteristics of 680 Pneumonia Cases in Children 2–35 Months of Age Diagnosed From March 2006 to July 2007 in Bhaktapur, Nepal

Characteristic	Cases		
	N	Value	
Median number of days with cough at presentation (IQR)	680	3	(2 to 4)
Reported nasal discharge (%)	679	588	(86.6)
History of ear discharge	680	2	(0.30)
Diarrhea and/or vomiting	680	105	(15.4)
Mean respiratory rate in breaths/min (SD)*			
2–11 months	311	57	(5.4)
12–35 months	369	49	(6.1)
Axillary temperature (%)			
≥37.5°C	679	253	(37.3)
≥38.5°C	679	80	(11.8)
Wheezing (%)	680	349	(51.3)
Crepitation (%)	680	176	(25.9)
Lower chest indrawing (%)	680	28	(4.1)
Oxygen saturation (%)†			
<93%	680	188	(27.7)
<90%	680	4	(0.59)
CRP in mg/L			
Median (IQR)	566	11	(<8 to 24)
>40 mg/L (%)	566	71	(12.5)
>80 mg/L (%)	566	15	(2.7)

*The lower of 2 respiratory rate counts was used.
†The higher of 2 SpO_2 measurements was used.
IQR indicates interquartile range; CRP, C-reactive protein.

TABLE 3. Distribution of Respiratory Viruses Identified in 680 Pneumonia Cases in Nepalese Children Aged 2–35 Months and 680 Age-Matched, Concurrently Sampled Controls With and Without Any Respiratory Symptoms

Virus†	Cases With Pneumonia (n = 680)		Controls With Respiratory Symptoms* (n = 408)		Controls Without Respiratory Symptoms* (n = 272)	
	n	(%)	n	(%)	n	(%)
RSV	66	(9.7)	5	(1.2)	3	(1.1)
Influenza A	20	(2.9)	4	(1.0)	0	(0.0)
Influenza B	20	(2.9)	7	(1.7)	2	(0.7)
PIV type 1	28	(4.1)	5	(1.2)	2	(0.7)
PIV type 2	6	(0.9)	2	(0.5)	1	(0.4)
PIV type 3	96	(14.1)	10	(2.4)	2	(0.7)
hMPV	23	(3.4)	6	(1.5)	1	(0.4)
Any virus	248	(36.5)	37	(9.0)	11	(4.0)

*Respiratory symptoms: complaint of cough, difficult breathing, nasal discharge, fever, ear ache or ear discharge.
†Viruses were identified by reverse transcriptase polymerase chain reaction in nasopharyngeal aspirates.
RSV indicates respiratory syncytial virus; PIV, parainfluenza virus; hMPV, human metapneumovirus.

control children who had a history of cough, the median duration of cough was 3 days (interquartile range, 2–4). Fifty-two (7.6%) controls were reported to have difficult breathing. Nasal discharge was reported in 350 (51.9%) of the 674 controls where this information was available, and 202 (57.7%) of them had no other respiratory symptoms. History of fever was reported in 34 (5.0%) control children, while only 6 had an axillary temperature ≥37.5°C. Sixty-eight (10%) had a history of diarrhea and/or vomiting.

Frequency and Seasonality of Viral Infections

At least 1 virus was recovered in 248 (36.5%) cases and 48 (7.1%) controls (Table 3). Overall, the most common viruses in cases were PIV type 3 (14.1%) and RSV (9.7%). Among the 28 cases with severe pneumonia, 11 had any virus detected of which 7 were RSV positive. Both RSV and PIV type 3 were observed in well-defined epidemics during the study period. A PIV type 3 epidemic occurred in spring-summer 2006, peaking in June, while an RSV epidemic peaked in September the same year. More than 1 virus was identified in 11 (1.6%) of the cases and in 2 (0.3%) of the controls. The most common codetection was PIV type 3 combined with hMPV, which was seen among 7 of the 9 cases with a codetection. Overall, one or more of the viruses were more frequently detected in controls with respiratory symptoms (9.1%) than in controls without such complaints (4.0%) ($P = 0.007$), but

TABLE 4. Respiratory Viruses Identified by Reverse Transcriptase Polymerase Chain Reaction in Nasopharyngeal Aspirates and Their Association With Pneumonia in 680 Age-Matched Case-Control Pairs

Virus	Concordant Pairs		Discordant Pairs		MOR	95% CI
	Case−/Control−	Case+/Control+	Case+/Control−	Case−/Control+		
RSV*	608	2	64	6	10.7	4.6–24.6
2–5 months	112	0	14	2	7.0	1.6–30.8
6–35 months	496	2	50	4	12.5	4.5–34.6
Influenza A	657	1	19	3	6.3	1.9–21.4
Influenza B	651	0	20	9	2.2	1.0–4.9
PIV type 1	645	0	28	7	4.0	1.7–9.2
PIV type 2	671	0	6	3	2.0	0.5–8.0
PIV type 3†	577	5	91	7	13.0	6.0–28.0
2–5 months	111	2	11	4	2.8	0.9–8.6
6–35 months	466	3	80	3	26.7	8.4–84.4
hMPV	651	1	22	6	3.7	1.5–9.0
Any virus	407	23	225	25	9.0	6.0–13.6

*The *P* value for interaction between RSV and age category was 0.77.

†The *P* value for interaction between PIV type 3 and age category was 0.002.

MOR indicates matched odds ratio; RSV, respiratory syncytial virus; PIV, parainfluenza virus; hMPV, human metapneumovirus.

this difference was not statistically significant for any of the individual viruses.

Viral Association With Pneumonia

The matched odds ratio (MOR) for presence of the individual viruses in NPA specimens from a case compared with a control varied from 2.0 (PIV type 2) to 13.0 (PIV type 3) (Table 4). The estimate for RSV was comparable to that of PIV type 3. The MOR varied by age category. We observed a substantially lower pathogenicity for PIV type 3 in the 2–5 months age group (*P* for interaction 0.002). The RSV pathogenicity point estimate was also lower in the younger than the older age category but this interaction was far from statistically significant ($P = 0.77$).

DISCUSSION

Very few studies report on the actual associations between the PCR detection of common respiratory viruses and pneumonia in children[15] and to our knowledge; no study has reported on such associations from developing countries. Our study had the advantage that cases and controls were matched on age and time of inclusion. The risk of acquiring these infections varies greatly with age and, because of the epidemic nature of these infections,[10] with season. We found that all but one of the 7 viruses recovered in NPA from our study children were significantly associated with pneumonia. PIV type 2 was considerably less commonly isolated from our patients and the precision of its pathogenicity estimate accordingly poor. Our results are in agreement with those of the prospective birth cohort study in 263 Australian children that reported similar associations for viral lower respiratory tract infections during the first year of life.[15]

Among the 7 viruses included in our study, PIV type 3, RSV, and influenza A were most strongly associated with pneumonia. These were also the most prevalent viruses in the larger study in which this case–control study was embedded.[10] The proportions of these viruses in the control specimens were 1.8%, 1.2%, and 0.6%, respectively. In other studies the proportion of RSV-positive respiratory samples from asymptomatic children using PCR ranges from 0% to 5%, while for other common viruses, such as PIV, influenza, and hMPV, proportions range from 0% to 2%.[6,16] Results are not immediately comparable between studies, as they will depend on the age group studied, the case definition used, as well as the selection procedures for controls. The fact that these viruses are not commonly found in control children, as in our study in Nepal, indicates a strong causal relationship between their presence in the upper respiratory tract and current illness.

We observed that the pathogenicity of PIV type 3 varied with age, being substantially lower in the youngest age group (2–5 months) compared with the older children (6–35 months). A similar but weaker age-dependent difference, albeit far from statistically significant, was seen for RSV. Passively acquired maternal antibodies could partially protect these younger infants from pneumonia during the first months of life.[17–19]

The proportion of virus-positive NPAs in controls was higher for all the 7 viruses if the child had respiratory symptoms than if they had no such symptoms. However, comparing the proportions that were positive for any of the 7 viruses, the difference between the controls with (9.1%) and without (4.0%) any respiratory symptoms was substantially smaller than the difference between the cases (36.5%) and the controls (7.1%). This is in line with a previous study that showed that the association between detection of RSV, PIV, and hMPV, and clinical illness was more pronounced for lower than for upper respiratory tract infection when compared with healthy controls.[15]

The duration of pneumonia and the period where a virus may be detected is not completely overlapping. Viruses may be detectable by PCR in specimens from the upper respiratory tract both before onset of symptoms and occasionally for days and weeks after clinical illness.[3,6,20] These positive results could, however, occur in both cases and controls and thereby lead to lower estimates of association. Based on the small proportions of positive samples in the controls, we believe that this does not substantially affect our estimates.

The cases were recruited from patients presenting to our study clinic, while the controls were identified from a surveillance list that was updated monthly. There is a substantial population of migrant families in Bhaktapur, who were less likely than the indigenous population to be registered on our list of households in the study area. As a result, children from migrant families could have been less likely to be selected as controls compared with children from the more stable population, and any difference in socioeconomic status between these 2 groups could have introduced a bias in our estimates. From the population characteristics listed in Table 1, we observed a difference in anthropometric measures between the cases and controls, as well as the proportion

of children that were delivered in a hospital. However, adjusting for these factors in the unconditional logistic regression analyses had negligible effects on the pathogenicity odds ratios (data not shown), making such selection an unlikely source of bias.

We would have liked to include a wider array of respiratory pathogens in our detection panel. Thus, other relevant microorganisms, such as adenovirus, rhinovirus, enterovirus, coronavirus, bocavirus, as well as *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* are all of interest. Several of these pathogens are frequently found in asymptomatic children[6] and often involved in coinfections,[5] but few reports derive from proper cohort[15] or case–control studies.[7]

This is the first case–control study measuring the associations between pneumonia and common respiratory viruses detected by PCR in young children of a developing country. Our analyses show that the pathogenicity of the 6 most common viruses is high but varies in these otherwise healthy children 2–35 months of age. PIV type 3 was less pathogenic in children below 6 months of age than in those who were older. By quantifying the degree to which these 7 common respiratory viruses are associated with pneumonia, this study could contribute to define the burden of childhood pneumonia attributable to the different viral agents.

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