

Lower respiratory infections among hospitalized children in New Caledonia and relevance of induced sputum analysis

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Key points

Of 108 cases enrolled, 87.9% had a pathogen detected. Viruses were detected in 81.6% of cases, including pathogens never described before in New Caledonia. Bacteriological detection remains challenging and induced sputum analysis did not appear to significantly improve detection.

Abstract

We conducted a prospective study over a one year period in New Caledonia to determine the pathogens associated with hospitalized lower respiratory infections (LRI) in children.

Bacteriological infection was based on IS and blood culture, urinary antigen detection, PCR on induced sputum (IS) and nasopharyngeal specimens for atypical bacteria, and serology on paired sera. Respiratory viruses were detected on respiratory specimens by immunofluorescence and PCR and by serology on paired sera. Pathogens were detected in 87.9% of the 108 hospitalized cases. Viruses represented 81.6% of the 152 pathogens detected. Respiratory syncytial virus and rhinovirus were the most frequent, accounting for 32.2% and 24.3% of the pathogens identified, respectively. Only 26.3% of 99 IS specimens collected were determined to be good quality. IS appeared of limited value for bacteriological diagnosis yielding only 13 bacterial cases and PCR results of IS and nasopharyngeal specimens had high concordance for viruses ($\kappa = 0.90$).

INTRODUCTION

According to WHO estimates, infectious diseases and especially pneumonia were the leading causes of death globally in children younger than 5 years of age in 2008 [1]. Accurate and rapid diagnosis of the etiology of lower respiratory infections (LRI) is challenging, especially in children; up-to-date epidemiological data about LRI are therefore essential to determine first line antibiotic therapy that can be used in each country and to identify potential new pathogens to be targeted by future vaccines [2].

Little is known about respiratory pathogens responsible for LRI in New Caledonia [3]. Chest physiotherapy is recommended for infants with bronchiolitis as standard of care [4] and is also often used in patients with difficulty breathing due to pneumonia. Besides being a therapeutic procedure, chest physiotherapy can also be used to obtain induced sputum specimens. Recently, it has been established that induced sputum analysis could be relevant for the diagnosis of community-acquired pneumonia in children [5]. The objectives of this study were to (i) evaluate the relevance of IS for the etiological diagnosis of LRI in children

and (ii) evaluate the relevance of nasopharyngeal specimens collected from cases by comparing them to specimens collected from controls.

METHODS

Site description

New Caledonia is a French archipelago located in the South Pacific with a population of 245,580 inhabitants (2009 census) [6]. One quarter of the population is under 15 years old and the ethnic distribution within this age group is as follows: Melanesian (44.1%), European (27.8%), Polynesian (10.9%), people of mixed ethnicity (13.8%), Indonesian (0.6%) and other ethnic groups (2.8%). This multicultural population is mainly urban; 33.3% have a rural lifestyle and 2.1% live in tribal villages. New Caledonia has two marked seasons: a warm and wet season (December to March) and a cooler one (June to September). The population has good access to the health care system which is of European standards. However, medical consultation is sometimes delayed for people living in tribal villages and/or for those who use traditional medicine as first point of care.

Study design

This was a case-control study conducted at the New Caledonia Hospital (Noumea).

Case population

Hospitalized children (1 month - 15 years) admitted with LRI (*i.e.* pneumonia or bronchiolitis) were enrolled prospectively. Criteria described in Table 1 were used for patient enrollment. LRI was classified as not severe or severe using the WHO criteria [7].

Control population

Children (1 month - 15 years) consulting at the hospital (Emergency Department admission, hospitalization, or appointment at the outpatient ward) without respiratory symptoms were checked for matching eligibility with cases (Table 1).

Ethics

The protocol was approved by the Institutional Review Board of the Johns Hopkins Bloomberg School of Public Health (Baltimore, MD) and the Health Authorities in France (Agence Française de Sécurité Sanitaire des Aliments et des Produits de Santé, Comité de Protection des Personnes Sud-Ouest et Outre-Mer III) and in New Caledonia.

Signed written informed parental consent was obtained before enrolment.

Data and specimen collection

Upon enrolment, standardized questionnaires were used to record demographic and epidemiological data, clinical and radiological features and medical history for cases and controls. Cases were assessed after 48 hours and at hospital discharge for follow-up. A senior radiologist reviewed all the chest-X-rays for confirmation.

Specimens were collected shortly after admission. For cases, two different respiratory tract specimens were collected: nasopharyngeal specimen - NPS - (nasopharyngeal aspiration - NPA - or 2 nasopharyngeal flocked swabs - NPFS - if parents or children refused NPA) and induced sputum (IS). IS was collected by a chest physiotherapist who cleared the upper airway, then used the increased exhalation technique (IET) to clear the distal airways with assisted cough (AC) to facilitate large-airway clearance [4]. The manual compression of the infant's thorax used in IET is aimed at achieving distal airway flow limitation at low lung volume to facilitate mucus clearance [8].

NPA and IS specimens were divided into 2 parts: one in a sterile vial for bacterial culture and one in a viral transport medium (VTM) for virus detection. NPFS (Copan Diagnostics) were placed into STGG (skimmed milk / tryptone / glucose / glycerol) medium [9] and VTM. Blood was collected for culture, serology (acute serum and convalescent serum 4-6 weeks after admission), antibacterial activity detection and procalcitonin measurement. Urine was collected for urinary antigen detection. For controls, one respiratory sample (NPA or 2 NPFS if parents or children refused NPA) was collected and processed as described above. Acute and convalescent sera were also collected.

Diagnostic methods

Tests were performed in Institut Pasteur of New Caledonia (Noumea, New Caledonia), except serology testing for viruses and *Chlamydia pneumoniae* detection, performed in Canterbury Health Laboratories (Christchurch, New Zealand).

Microbiological laboratory tests

Microbiological tests were carried out on respiratory specimens according to the French Society of Microbiology (SFM) criteria [10]. IS samples were Gram stained and leukocytes and epithelial cell counts recorded. Only good quality IS (>25 granulocytes and <25 epithelial cells per low power field) were retained for culture [11]. IS and NPA specimens were digested by adding 0.1% dithiothreitol (Digest-EUR, Eurobio) in equal amount to the sample volume and then cultured at 37°C on blood agar with nalidixic acid, on chocolate agar. For IS, cultures were counted, with results expressed as colony-forming units per millilitre (CFU/mL) and interpreted using the SFM standards. NPFS were Gram stained and cultured at 37°C on the same agar media as IS or NPA. Blood cultures were done using the BacT/ALERT®3D system (bioMérieux) with pediatric bottles. *Legionella pneumophila* serotype 1 antigens were

detected in non-concentrated urine using an immunochromatographic assay (Binax NOW[®], Binax Inc.).

Immunofluorescence detection

Digested samples (for IS and NPA) or VTM (for NPFS) were centrifuged. Epithelial cells were then plotted onto multiwell microscope slides, fixed and stained using Monofluo[®] Kit (Biorad) allowing detection of influenza A and B viruses, PIV3 and AdV and Monofluo[®] Screen (Biorad) for RSV detection.

Nucleic acid extraction and molecular analysis of IS and NPS

RNA and DNA contained in IS and NPS were extracted from VTM on the EasyMag system (bioMérieux).

Real-time PCR was performed on both IS and NPS for parallel detection of *Chlamydomphila pneumoniae* and *Mycoplasma pneumoniae* [12] and for detection of *Bordetella pertussis* as previously described [13], using the LightCycler[®] (Roche Diagnostics).

Detection of 18 respiratory viruses (influenza A, B and C viruses, PIV 1, 2, 3 and 4, human metapneumovirus [hMPV], RSV, human coronaviruses [hCoV] 229E, OC43, NL63, HKU1 and the severe acute respiratory syndrome-associated coronavirus [SARS-CoV], human rhinovirus [hRhV], human bocavirus [hBoV], AdV and enterovirus [EnV]) was performed on both IS and NPS using previously published multiplex RT-PCR protocol with slight modifications [14, 15].

Serology

Particle agglutination (Serodia MycoII, Fujirebio) was performed to detect Immunoglobulin M (IgM) antibodies to *Mycoplasma pneumoniae*. We used ELISA immuno-assays (Euroimmun) to detect antibodies to influenza A and B viruses, AdV, RSV and PIV1-3 and immunofluorescence (MRL Diagnostics, Cypress) for antibodies to *Chlamydia pneumoniae*.

Detection of pre-hospital antibiotic use

Acute serum was screened for antibacterial activity to detect pre-hospital antibiotic use as described elsewhere [16, 17].

Procalcitonin measurement

Procalcitonin levels were measured using the Vidas[®] B.R.A.H.M.S PCT assay (bioMérieux).

Criteria for determination of microbial infection

In the case population, viral infection was defined as (i) a respiratory virus detected from IS and/or NPS by immunofluorescence, real-time PCR or multiplex RT-PCR, (ii) a seroconversion (four-fold or greater rise) in reciprocal antibody titers to a respiratory virus on paired serum samples. Bacterial infection was defined as (i) a usual respiratory pathogen (*Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus*, *Moraxella catarrhalis*) cultured from blood, (ii) a usual respiratory pathogen cultured from good quality IS, (iii) a urinary antigen tested positive for *L. pneumophila*, (iv) a PCR for *M. pneumoniae*, *C. pneumoniae* or *B. pertussis* positive from IS or NPS, (v) IgM reciprocal antibody titers ≥ 160 for *M. pneumoniae* in acute and/or convalescent serum (vi) a seroconversion (four-fold or greater rise) in reciprocal antibody titers to *C. pneumoniae*.

In the control population, infection was defined as (i) a usual respiratory pathogen detected in the NPS by culture, immunofluorescence, PCR or multiplex RT-PCR, (ii) a seroconversion or a four-fold or greater rise in reciprocal antibody titers for respiratory viruses observed on paired serum samples, (iii) IgM reciprocal antibody titers ≥ 160 for *M. pneumoniae* in acute and/or convalescent serum (iv) a seroconversion (four-fold or greater rise) in reciprocal antibody titers to *C. pneumoniae*.

Statistics

We calculated medians and interquartile ranges (IQRs) for continuous variables and absolute numbers and proportions for categorical variables. Comparisons between groups were conducted using Student's t-tests or Wilcoxon rank-sum test for continuous variables and Chi-squared or Fisher's exact tests for categorical variables, as appropriate. All reported p-values were 2-sided, and $p < 0.05$ was considered significant. A simple kappa coefficient was calculated to assess the agreement between the induced sputum and nasopharyngeal samples. Statistical analyses were performed using Stata 11.0 (StataCorp LP, College Station).

RESULTS

Patients

Of 251 children hospitalized with LRI during the study period, 108 (43%) were enrolled in the study (69 bronchiolitis and 39 pneumonia). The others were not screened, not eligible for the study or refused to consent.

Patient characteristics are summarized in Table 2. Patients with severe LRI (pneumonia and bronchiolitis) were younger ($p=0.036$) and more likely not to live in tribal villages ($n = 49$ vs $n = 20$; $p=0.057$). Melanesian patients were overrepresented in the study compared to the

general population of New Caledonia (64.2% vs 44.1%, $p<0.001$). There was no significant association between ethnic group and LRI severity. None of the cases died.

Induced sputum was obtained from 99 (91.6%) cases, of which 97 (97.9%) also had an NPS collected. Twenty six (26.3%) IS were of good quality and 25 had a matching NPS. Pre-hospital antibiotic use was tested in 94 (87%) cases and antibacterial activity found in 26 sera (27.7%).

Pathogen detection

Of 108 cases enrolled, 95 (87.9%) had a pathogen detected (Figure 1). Yield was higher in cases with bronchiolitis than in those with pneumonia (92.7% vs 79.5%, $p=0.04$). A viral pathogen was found in 90 (83.3%) cases and a bacterial pathogen in 25 (23.1%). We identified 51 monomicrobial infections and 44 polymicrobial infections (Table 3). Most of the bacteria were detected by culture (1 blood culture positive for *M. catarrhalis* and 13 from good-quality IS culture), 11 were detected by serology and 3 by PCR. No urinary samples tested positive for *L. pneumophila* serotype 1.

For viruses, 42 were detected by immunofluorescence, 108 by PCR and 11 by serology. PCR on IS allowed detection of 99 (64.3%) pathogens (97 viruses and 2 bacteria).

The seasonal distribution of the two most frequently detected viruses, RSV and hRhV, is shown in Figure 2. The incidence of LRI, especially bronchiolitis, was higher from March to June, associated with the seasonal distribution of RSV. Four influenza viruses were detected during southern winter (August and September).

Findings in controls

For each case, we aimed to enroll one control, matched on age and date of admission. Control enrollment proved challenging due to the difficulty in obtaining informed consent from children without LRI and the novelty of conducting research in this pediatric department. Twenty two controls were enrolled of which 13 (59.1%) had at least one pathogen detected. Bacteria were detected in 5 (22.7%) controls, viruses in 6 (27.3%) and both bacteria and viruses in 2 (9.1%). Culture of NPS yielded 8 bacteria in 7 controls. We detected 10 viruses in 8 controls (8 by PCR, 1 by serology, 1 by PCR and immunofluorescence).

Comparison of results in paired induced sputum and nasopharyngeal specimen samples

For commonly found viruses (RSV and hRhV), the agreement between IS and NPS samples was high ($\kappa > 0.9$; Table 4); numbers were too small for reliable determination of agreement for other viruses detected, but agreement for any virus detected was high ($\kappa = 0.89$). For bacteria, the agreement between the samples was lower, both when all IS are compared regardless of quality ($\kappa = 0.38$ for any bacterial pathogen, and when subset for only pairs containing good-quality IS samples ($\kappa = 0.43$)).

DISCUSSION

This study is the first description of pathogens associated with LRI in hospitalized children in New Caledonia. The yield of 87.9% with a pathogen detected is consistent with published results in other settings [5, 18]. This high yield is mostly due to the elevated number of viral pathogens detected, as 83.3% of cases had evidence of viral infection. Bacteria represented 18.4% of pathogens detected but we didn't use PCR to detect classical bacteria as we did for viruses so the low rate of bacterial detection could be the consequence of the decreased sensitivity of cultures compared to PCR. It may also result from the fact that 64% of cases

enrolled had a clinical diagnosis of bronchiolitis, which is more likely to be of viral origin. The present study produced four main findings regarding LRI of viral origin in children in New Caledonia. First, novel molecular tools enabled us to detect newer viruses including hMPV, hCoV and hBoV, which had been previously described in respiratory infections but never before identified in our setting. Second, RSV infections were clearly seasonal and peaked at the beginning of the cool season, likely promoted by community life (beginning of the school year). Third, the seasonal distribution of influenza in children appeared to be concordant with previous findings in adults, in which the majority of cases occurred between June and September [3]; however, our ability to assess flu seasonality was limited because the total number of influenza cases observed during our study period was surprisingly small and all were associated with viral types B and C. None of these cases was accompanied by a pneumococcal super-infection, which differs from other descriptions [3, 19] but may reflect our small numbers. This finding is important as it emphasizes the value of long-term continuous surveillance to monitor trends in influenza and other respiratory viruses over time. Finally, hRhV was detected throughout the year and was as common in cases as in controls, calling into question its role as a LRI pathogen.

We most likely missed a great number of bacterial cases due to several important factors. First many bacterial pneumonia cases are non-bacteremic, yielding negative blood cultures despite excellent laboratory performance, even in the absence of prior antibiotics. Second, we obtained very few good-quality IS specimens appropriate for microbiological investigation despite our study's emphasis on collecting these specimens. Third, culture-based methods can be affected by prior antibiotic administration and 28% of our patients had evidence of antibiotic use.

In our study, the yield from induced sputum was not as high as previously described [5]. We obtained specimens from >90% of cases with LRI but the quality of the specimens was disappointing most of the time. In the subgroup of cases with pneumonia, 26.5% of IS (9/34) were of good-quality compared with 75.2% in a study from Lahti *et al.* (Turku, Finland) published in 2008 [5]. The different methods used for sputum induction and the frequent turnover of the physiotherapists in our study could be responsible for discrepancies observed with previous studies.

Overall agreement between IS and NPS samples was high for viral findings, as nearly one third of controls without respiratory infection had at least one virus present in their upper-respiratory airways, suggesting that the presence of a virus in NPS may not be sufficient to ascribe etiology. Even for RSV, the difference in viral prevalence between cases and controls was not statistically significant ($p=0.30$), although the number of RSV detected was small. Seven viruses were found in IS only suggesting that IS analysis could be of relevance for viral detection in some cases. Culture of good-quality induced sputum yielded only 13 bacteria of 3 different species (*S. pneumoniae*, *H. influenzae*, *M. catarrhalis*); of note, all of these bacteria are also known to be involved in nasopharyngeal colonization in healthy children and in children with upper respiratory tract infection [20, 21], as confirmed in our study by a prevalence of 31.8% of bacterial colonization among controls. The fact that 4 of the bacteria isolated from IS were also cultured from the matched NPS indicates that we cannot exclude the possibility of contamination by upper respiratory secretions. Therefore, it would be interesting to determine if a pathogen isolated from a good-quality IS is more likely to be responsible for the respiratory infection if absent from the NPS collected simultaneously. In order to progress in the interpretation of findings in paired IS and NPS, a comparison with a gold standard method (*e.g.* transthoracic needle aspiration) is needed.

Though we have emphasized the limitations of pathogen detection to ascribe an etiological diagnosis, our findings may nevertheless reflect a true predominance of viruses among cases of hospitalized LRI. In other settings with good primary care, high Hib and pneumococcal vaccine coverage and high access to antibiotics, most pediatric respiratory illness of bacterial origin will be prevented or treated in the outpatient setting and never require hospitalization. In a trial of *Haemophilus influenzae* type b (Hib) vaccine in Lombok, Indonesia, which used active surveillance to identify sick children at home and treated them in village clinics early in the course of illness, the vaccine showed no protective effect against hospitalized LRI, suggesting that Hib cases were treated early and thus did not progress to severe disease [22]. Similar results were observed for protection against hospitalized LRI by pneumococcal conjugate vaccine in a trial among American Indians (personal communication, K. O'Brien). Together these results indicate that viruses cause the majority of severe, hospitalized cases of LRI in locations with good access to outpatient antibiotic treatment and support the etiological findings from our study.

In conclusion, newly developed techniques (*e.g.* PCR) allowed high rates of detection of viral pathogens as described elsewhere [23]. Bacterial detection remained challenging and IS analysis did not appear to significantly improve detection over nasopharyngeal samples in our study, mainly because of difficulties in obtaining good-quality specimens. Further studies are needed in order to help distinguish between infection and colonization, by comparing findings in NPS and IS with gold-standard methods.

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References

- [1] Black RE, Cousens S, Johnson HL, et al. Child Health Epidemiology Reference Group of WHO and UNICEF. Global, regional, and national causes of child mortality in 2008: a systematic analysis. *Lancet* **2010**; 375:1969-87.
- [2] Scott JA, Brooks WA, Peiris JS, Holtzman D, Mulholland EK. Pneumonia research to reduce childhood mortality in the developing world. *J Clin Invest* **2008**; 118:1291-300.
- [3] Mermond S, Berlioz-Arthaud A, Estivals M, Baumann F, Levenes H, Martin PM. Aetiology of community-acquired pneumonia in hospitalized adult patients in New Caledonia. *Trop Med Int Health* **2010**;15:1517-24.
- [4] Agence Nationale d'Accréditation et d'Evaluation en Santé. Consensus conference on the management of bronchiolitis in infants. *Arch Pediatr* **2001**; 8(suppl 1):1s–196s. Available at: <http://www.has-sante.fr/portail/upload/docs/application/pdf/Bronchiolitis.pdf>. Accessed 7 April 2010.
- [5] Lahti E, Peltola V, Waris M, et al. Induced sputum in the diagnosis of childhood community-acquired pneumonia. *Thorax* **2009**; 64:252-7.
- [6] Institut de la Statistique et des Etudes Economiques. Recensement de la population 2009. Available at: <http://www.isee.nc/population/population.html>. Accessed 12 April 2010.
- [7] World Health Organization. Pocket book of hospital care for children: guidelines for the management of common illnesses with limited resources. **2005**. Available at: <http://whqlibdoc.who.int/publications/2005/9241546700.pdf> . Accessed 7 April 2010.
- [8] Gajdos V, Katsahian S, Beydon N, et al. Effectiveness of chest physiotherapy in infants hospitalized with acute bronchiolitis: a multicenter, randomized, controlled trial. *PLoS Med* **2010**;28:7.

- [9] O'Brien KL, Nohynek H; World Health Organization Pneumococcal Vaccine Trials Carriage Working Group. Report from a WHO Working Group: standard method for detecting upper respiratory carriage of *Streptococcus pneumoniae*. *Pediatr Infect Dis J* **2003**; 22:e1–e11.
- [10] Société Française de Microbiologie. Diagnostic microbiologique des infections broncho-pulmonaires. In REMIC: Société Française de Microbiologie Ed; **2010**,93-98.
- [11] Geckler RW, Gremillion DH, McAllister CK, Ellenbogen C. Microscopic and bacteriological comparison of paired sputa and transtracheal aspirates. *J Clin Microbiol* **1977**; 6:396-9.
- [12] Raggam RB, Leitner E, Berg J, Mühlbauer G, Marth E, Kessler HH. Single-run, parallel detection of DNA from three pneumonia-producing bacteria by real-time polymerase chain reaction. *J Mol Diagn* **2005**;7:133-8.
- [13] Ménard A, Lehours P, Sarlangue J, Bébéar C, Mégraud F, de Barbeyrac B. Development of a real-time PCR for the identification of *Bordetella pertussis* and *Bordetella parapertussis*. *Clin Microbiol Infect* **2007**;13:419-23
- [14] Wang W, Ren P, Sheng J, et al. Simultaneous detection of respiratory viruses in children with acute respiratory infection using two different multiplex reverse transcription-PCR assays. *J Virol Methods* **2009**; 162:40-5.
- [15] Buecher C, Mardy S, Wang W, et al. Use of a multiplex PCR/RT-PCR approach to assess the viral causes of influenza-like illnesses in Cambodia during three consecutive dry seasons. *J Med Virol*. **2010**; 82:1762-72.
- [16] Berkley JA, Loew BS, Mwangi I, et al. Bacteremia among children admitted to a rural hospital in Kenya. *N Engl J Med* **2005**; 352:39-47.

- [17] Rhodes J, Hyder JA, Peruski LF, et al. Antibiotic use in Thailand: quantifying impact on blood culture yield and estimates of pneumococcal bacteremia incidence. *Am J Trop Med Hyg.* **2010**; 83:301-6.
- [18] Michelow IC, Olsen K, Lozano J, et al. Epidemiology and clinical characteristics of community-acquired pneumonia in hospitalized children. *Pediatrics* **2004**; 113:701-7.
- [19] O'Brien KL, Walters MI, Sellman J, et al. Severe pneumococcal pneumonia in previously healthy children: the role of preceding influenza infection. *Clin Infect Dis* **2000**; 30:784-9.
- [20] Harrison LM, Morris JA, Telford DR, Brown SM, Jones K. The nasopharyngeal bacterial flora in infancy: effects of age, gender, season, viral upper respiratory tract infection and sleeping position. *FEMS Immunol Med Microbiol* **1999**; 25:19-28.
- [21] Charvériat MA, Chomarat M, Watson M, Garin B. Nasopharyngeal carriage of *Streptococcus pneumoniae* in healthy children, 2 to 24 months of age, in New-Caledonia. *Med Mal Infect* **2005**; 35:500-6.
- [22] Gessner BD, Sutanto A, Linehan M, et al. Incidences of vaccine-preventable *Haemophilus influenzae* type b pneumonia and meningitis in Indonesian children: hamlet-randomised vaccine-probe trial. *Lancet* **2005**; 365:43-52.
- [23] Ruuskanen O, Lahti E, Jennings LC, Murdoch DR. Viral pneumonia. *Lancet* **2011**; 377(9773):1264-75.