Atypical bacterial pathogen infection in children with acute bronchiolitis in northeast Thailand

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KEYWORDS
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Background: Atypical bacterial pathogens—including Mycoplasma pneumoniae, Chlamydophila pneumoniae, and Chlamydia trachomatis—are important infectious agents of the respiratory system. Most current information pertains to adults and little is known about the role of these organisms in lower respiratory tract infections among young children with acute bronchiolitis.

Methods: This study detected these pathogens in the nasopharyngeal secretions of children between 1 month and 2 years of age admitted with acute bronchiolitis to hospitals in Khon Kaen, northeast Thailand. The M pneumoniae and C pneumoniae in the nasopharyngeal secretions were detected using multiplex and nested-polymerase chain reaction (PCR), whereas PCR and restriction fragment length polymorphism were used to investigate C trachomatis. These samples were also tested by multiplex reverse transcriptase PCR for respiratory viruses, including respiratory syncytial virus (RSV), influenza A, influenza B, and human metapneumovirus.

Results: Of the 170 samples taken from hospitalized children with acute bronchiolitis, 12.9% were infected with atypical bacteria and 85.3% with respiratory viruses. RSV was the most common causative viral agent found in 64.7% of the samples. M pneumoniae was the most common atypical bacterial pathogen (14/170, 8.2%) and most of the patients infected with it were between 6 and less than 12 months of age (71 cases). Of the infected cases in this age group, 7 of 14 were infected with M pneumoniae and 4 of 4 with C pneumoniae. Both
Introduction

Acute bronchiolitis is the most common lower respiratory tract infection and the most frequent cause of hospitalization in young children. The most common pathogen is respiratory syncytial virus (RSV), which occurs as a yearly winter epidemic with various symptoms, ranging from mild upper respiratory tract infection to severe bronchiolitis with hyperinflated lungs and hypoxemia.1

The atypical bacterial pathogens—*Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Chlamydia trachomatis*—are recognized as respiratory pathogens. They are all small bacteria and cannot be detected using routine culturing methods.

*M pneumoniae* is a well-known childhood pathogen and is highly transmissible. Most infections caused by this organism are relatively minor (including pharyngitis, tracheobronchitis, bronchiolitis, and croup) with one-fifth being asymptomatic. Acute infections with this organism may promote the exacerbation of asthmatic symptoms and may be accompanied by wheezing in children not having asthma.2

*C pneumoniae* was also reported in acute lower respiratory infection with mild dyspnea and wheezing in the pediatric population.3 Acute *C pneumoniae* infections have been associated with the presence and/or exacerbation of asthma in children.4

*C trachomatis* is transmitted by infected women to their infants at birth via contact with infected cervicovaginal secretion. If the infection is not detected and treated, such infected infants may develop conjunctivitis, bronchiolitis, and pneumonia.5 It should therefore not be ruled out in infants less than 6 months of age with clinical symptoms of lower respiratory tract disease for which no other pathogen can be found.

One of the main difficulties in dealing with lower respiratory tract infections in pediatric patients is correctly identifying the infecting agent. Culture, antigen screening, and serological methods are only helpful in about one of three cases.6 *Chlamydia* and *mycoplasma* are common bacteria that may cause rhinitis, pharyngitis, bronchitis, and pneumonia,6 and bronchiolitis may predispose some infants to developing childhood asthma or asthma exacerbation. Despite their ubiquity, *chlamydia* and *mycoplasma* are among the least frequently diagnosed respiratory pathogens in the clinical setting mainly because of the lack of standardized, rapid, and specific diagnostic tests.8

*Chlamydia* and *mycoplasma* are fastidious and difficult to grow in culture, so they require either specialized cell culture techniques or a long period of incubation before their presence can be confirmed or excluded. The interpretation of serological tests used for *C pneumoniae*, *C trachomatis*, and *M pneumoniae* diagnoses is also problematic because a large proportion of the population has preexisting IgG antibodies from prior exposure(s); therefore, diagnosis of infections caused by these organisms is usually confirmed with the polymerase chain reaction (PCR) technique.

To our knowledge, there are few reports about atypical bacterial pathogen infection in children with acute bronchiolitis, especially in northeast Thailand. We, therefore, conducted our study using a PCR technique to estimate the prevalence of *C pneumoniae*, *C trachomatis*, and *M pneumoniae* infections in pediatric patients with acute bronchiolitis admitted to either of two hospitals in Khon Kaen province, Thailand.

Methods

Subjects and sample collection

This study was approved by the Khon Kaen University Ethics Committee for Human Research, as per the Helsinki Declaration. This study was part of a randomized, clinical trial evaluating the efficacy of dexamethasone for the treatment of acute bronchiolitis (registered in ClinicalTrials.gov identification number: NCT00122785).

Between April 2002 and August 2004, children with acute bronchiolitis hospitalized at either of two hospitals in Khon Kaen, northeast Thailand, were recruited with informed, written, parental consent. Acute bronchiolitis was defined as the first episode of wheezing associated with tachypnea, increased respiratory effort, and an upper respiratory tract infection. Patients were eligible for the study if they met the following criteria: being between 4 weeks and 24 months of age and being admitted with their first episode of wheezing within 7 days. Patients were excluded from the study if they had (1) a known history of asthma; (2) a previous history of intubation; (3) a history of bronchopulmonary dysplasia or chronic lung disease; (4) an underlying congenital heart disease or any immunodeficiency; or (5) been on any steroid treatment within the previous 2 weeks.

On admission, a sample of nasopharyngeal secretion was taken from each child’s nostril by a previously described technique.10 The secretion obtained was immediately put into a tube containing viral transport media and then was sent to a laboratory for processing. Aliquots of samples were stored at −70°C until used.
Atypical bacteria study

DNA extraction
DNA was extracted from a 200-μL aliquot of sample using a PUREGENE DNA purification kit (Genta Systems, Minneapolis, MN, USA) as per the manufacturer's instructions. The integrity of the DNA was confirmed by amplification of a housekeeping gene (β-globin) from the DNA samples, using the PC04/GH20 primers (Invitrogen Life Technologies, Carlsbad, CA, USA).

Multiplex and nested PCRs for detection of M pneumoniae and C pneumoniae
Multiplex PCR was used for simultaneous detection of M pneumoniae and C pneumoniae. The chosen primers of M pneumoniae enclo"d a specific 144 bp fragment: MP5-1 (5'-GAA GCT TAT GGT ACA CCT TGG-3') and MP5-2 (5'-ATT ACC ATC TTT GGT AGG-3'). The primers for C pneumoniae—specific for the Omp1 gene—were: Cpn1 (5'-GTT TAC GGT CCA AGA CCT TGT T-3', nt 453–473) and Cpn2 (5'-TCC AAT GTA TGG CAC TAA AGA-3', nt 858–838, 405 bp).

Every sample with a negative result using multiplex PCR was reanalyzed using specific nested PCRs under the same conditions for the two pathogens. The specific nested PCRs were performed in parallel with the primers Cpn1 and Cpn2 (5'-ATT GAT GGT CGC AGA CTT TGT T-3', 339 bp) for C pneumoniae and primers MUH-1 and MUH-2 (104 bp) for M pneumoniae. All of the amplification products were analyzed using 2% (wt/vol) agarose gel electrophoresis followed by ethidium bromide staining. A no-template negative control and a low-concentration of each of M pneumoniae or C pneumoniae DNA positive control were done with each run.

PCR and restriction fragment length polymorphism analysis for detection of C trachomatis
Primers corresponding to the Omp2 gene (i.e. Ch1: 5'-ATG TCC AAA CTC ATG AGA CGA G-3' and Ch2: 5'-CTT TTA AGA GGT TTT ACC CA-3' were used. Standard amplification conditions for the primers Ch1 and Ch2 were used. Each PCR product (603 bp) was separated on 1.5% agarose gel and then was subjected to restriction fragment length polymorphism using restriction enzyme Alu I (Promega, Madison, WI, USA). Digestion was performed by incubating 10 μL of PCR product with 1 U of enzyme, 2 μL of 10× buffer, and 7 μL of water for 1 hour at 37°C. The PCR products were analyzed using electrophoresis on a 4% agarose gel, stained with ethidium bromide, and compared with the predicted fragments. For C trachomatis, the fragment lengths of 158, 119 and 114, and 84 and 77 bp were accurately predicted.

Virus study

RNA extraction
An aliquot of the same samples was also detected for respiratory viruses, including RSV, influenza A, influenza B, and human metapneumovirus (hMPV). The RNA was extracted from 140 μL of nasopharyngeal secretion using a QIAamp viral RNA mini kit (Qiagen, Germany).

Multiplex reverse transcriptase PCR
The extracted RNA was tested for RSV, influenza A, influenza B, and hMPV using multiplex reverse transcriptase (RT)-PCR as described by Bellau-Pujol et al. Briefly, 5 μL of RNA was added to 20 μL reaction mixtures of the one-step RT-PCR kit (Qiagen, Germany) and 0.4 μM of each of viral-specific forward and reverse primers. cDNAs of the viruses—provided by Professor Dr. François Freymuth (Caen University Hospital, France)—were used as the positive controls. Then, the one-step RT-PCR products (2 μL) were subjected to heminested multiplex PCR, performed in a 25-μL volume containing 10× buffer (New England Biolabs, Ipswich, MA, USA), 0.2 mM dNTPs, another set of primers, and 1 U of Taq DNA polymerase (New England Biolabs).

Results

During the study period, 170 children were diagnosed with acute bronchiolitis needing hospitalization; their parents gave consent and so the children were eligible for collection of nasopharyngeal secretions, which were used for detection of atypical bacteria, including M pneumoniae, C pneumoniae, and C trachomatis, as well as detection of respiratory viruses, such as RSV, influenza A, influenza B, and hMPV.

The baseline demographic data of all children and subgroups are presented in Table 1. The mean age was

### Table 1 Baseline demographic data of the study population

<table>
<thead>
<tr>
<th>Demographic data</th>
<th>Total (n = 170)</th>
<th>RSV (n = 110)</th>
<th>Bacteria (n = 22)</th>
<th>Student t test (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mo)</td>
<td>10.7 ± 5.7</td>
<td>10.2 ± 5.7</td>
<td>9.8 ± 5.8</td>
<td>0.000</td>
</tr>
<tr>
<td>Sex, male</td>
<td>107 (62.9)</td>
<td>65 (59.1)</td>
<td>15 (68.2)</td>
<td>0.215</td>
</tr>
<tr>
<td>Breastfeeding</td>
<td>160 (94.1)</td>
<td>102 (92.7)</td>
<td>21 (95.4)</td>
<td>0.323</td>
</tr>
<tr>
<td>Atopic history—parents</td>
<td>49 (28.8)</td>
<td>34 (30.9)</td>
<td>6 (27.3)</td>
<td>0.369</td>
</tr>
<tr>
<td>Passive smoker</td>
<td>102 (60)</td>
<td>63 (57.3)</td>
<td>19 (86.4)</td>
<td>0.005</td>
</tr>
<tr>
<td>Fever before admission</td>
<td>155 (91.2)</td>
<td>99 (90.0)</td>
<td>20 (90.9)</td>
<td>0.450</td>
</tr>
<tr>
<td>Clinical scorea</td>
<td>7.1 ± 1.4 (5–11)</td>
<td>7.0 ± 1.4 (5–11)</td>
<td>7.2 ± 1.1 (5–9)</td>
<td>0.000</td>
</tr>
<tr>
<td>O₂ saturation &lt; 95% at enrollment</td>
<td>83 (48.8)</td>
<td>57 (51.8)</td>
<td>14 (63.6)</td>
<td>0.157</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation, n(%) or mean ± standard deviation (range).

The clinical score is based on four respiratory variables and is scored using the following scales: respiratory rate (0–3 points), wheezing (0–3 points), cyanosis (0–3 points), and accessory muscle use (0–3 points).

RSV = respiratory syncytial virus.
were separated into three groups (Table 3). Children distress at admission.

Table 2. All of these children had moderate respiratory tis were detected, four were infected with Of the 22 children in whom atypical bacterial etiologies was the most common etiologic organism (64.7%) followed 12.9% of the cases (22/170). Of the respiratory viruses, RSV incidence (41.8%). In our study, between 6 and less than 12 months of age had the highest was the most common etiologic organism (64.7%) followed by influenza A (12.9%), influenza B (4.1%), and hMPV (3.5%). Of the 22 children in whom atypical bacterial etiologies were detected, four were infected with C trachomatis—the clinical characteristics of which are presented in Table 2. All of these children had moderate respiratory distress at admission.

According to age, the children with acute bronchiolitis were separated into three groups (Table 3). Children between 6 and less than 12 months of age had the highest incidence (41.8%). In our study, M pneumoniae was the most common causal organism found (8.2%, 14/170 cases) among the bacterial pathogens and most frequently in children older than 6 months of age. C pneumoniae was detected in four cases of acute bronchiolitis (2.4%) all of whom were between 6 and less than 12 months of age. Four cases of acute bronchiolitis (2.4%) were positive for C trachomatis, which was the most frequent cause in children between 1 and less than 6 months of age.

Twenty cases of the atypical bacterial bronchiolitis had coinfection with viral respiratory pathogen (Table 4). Indeed, almost all of the M pneumoniae (13/14) and C pneumoniae (3/4) cases were coinfected with viral respiratory pathogens. Coinfection with RSV was the most frequent. All cases of positive C trachomatis infection were detected in patients infected with RSV.

Discussion

Recent evidence indicates that infections by intracellular pathogens, such as chlamydia and mycoplasma, may cause acute and chronic wheezing in some individuals. The highest percentage of the serum samples positive for M pneumoniae-specific antibodies occurred in patients with asthma (60%), a full two-fold greater than in the control subjects. Specific anti-C pneumoniae antibodies were also observed but in a smaller percentage (i.e. 13.3% of children with asthma).18 Using PCR, Freymuth et al.4 detected M pneumoniae and C pneumoniae in 8% of 132 children with acute exacerbation of asthma. Recently, Biscardi et al.19 found M pneumoniae infection in 20% and C pneumoniae infection in 3.4% of 119 children hospitalized for severe asthma.

We were therefore interested in the infection of these atypical bacteria in acute bronchiolitis because bronchiolitis may especially predispose some infants to develop childhood asthma or asthma exacerbation. Hence, we investigated the prevalence of M pneumoniae and chlamydia (including C pneumoniae and C trachomatis) using PCR-based techniques to highlight the role of these organisms in respiratory tract infections, especially in children with acute bronchiolitis.

All 170 samples of children, hospitalized for acute bronchiolitis, were evaluated in this study conducted in Khon Kaen province, northeast Thailand. The nasopharyngeal secretion was sampled at the time of admission. Two-thirds (145/170) of the children had viral etiologies, including RSV, influenza virus, and hMPV. The association of hMPV and RSV in children younger than 2 years of age with acute bronchiolitis was previously reported.10 After using atypical bacterial pathogen detection techniques, M pneumoniae infection was found in 8.2% of the patients, whereas acute C pneumoniae infection was found in 2.4%; both occurred mostly in children between 6 and less than 12 months of age (Table 3).

Ouchi et al.20 evaluated 1,104 Japanese children with acute lower respiratory tract infections; and of these, 149 (13.5%) had acute C pneumoniae infections, 118 (10.7%) had acute M pneumoniae infections, and 27 (2.4%) had both. M pneumoniae was more common than C pneumoniae

<table>
<thead>
<tr>
<th>Age group (mo)</th>
<th>$n$</th>
<th>M pneumoniae</th>
<th>C pneumoniae</th>
<th>C trachomatis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 to &lt;6</td>
<td>36</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>6 to &lt;12</td>
<td>71</td>
<td>7</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>12–24</td>
<td>63</td>
<td>5</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>170</td>
<td>14 (8.2%)</td>
<td>4 (2.4%)</td>
<td>4 (2.4%)</td>
</tr>
</tbody>
</table>

$C$ trachomatis = Chlamydia trachomatis; $C$ pneumoniae = Chlamydophila pneumoniae; $M$ pneumoniae = Mycoplasma pneumoniae.
among patients with pneumonia, whereas C pneumoniae was more common in patients with bronchiolitis. C pneumoniae was more common among younger children and in those who presented with wheezing.

In an Argentinean study, 49 of 255 (19.2%) children between 1 and 18 months of age—without evidence of viral or bacterial infections but with clinical and radiological evidence of acute lower respiratory distress—were tested serologically for a recent C trachomatis infection. The results were positive in 28 of 166 (16.9%) children with bronchiolitis and in 18 of 89 (20.2%) with pneumonia. C trachomatis infection was detected in all age groups up to 18 months. Thirty of 49 infections were in children older than 3 months of age and 16 in children older than 6 months. These results suggest that C trachomatis infection may be associated with bronchiolitis and pneumonia in children between the 1 and 18 months of age, a proportion of which may be horizontally transmitted. 

In our study, C trachomatis infection was detected in only 2.35% because most of the C trachomatis cases (3/4 cases) were found in children less than 6 months of age (Table 3). It was found in only one case of a child between 12 and 24 months of age. This difference may depend on the method of determination; however, our study corresponds to a study in Turkey by Bütün et al. who reported that 3% of C trachomatis infections in 100 children between 3 months and 12 years of age were admitted to the pediatrric outpatient department with respiratory symptoms, such as fever, cough, and respiratory distress.

Coinfection by viruses and bacteria in the respiratory airways is common although their role in the outcome of illness is controverted. Notwithstanding, individual infectious agents have been associated with the development of chronic lung disease and exacerbation of asthma. Coinfection of two atypical bacterial agents—possessing chronic sequelae potential—may result in a protracted illness, more severe illness, and/or a poor long-term outcome. In our study, all of the cases of C trachomatis infection were coinfected with RSV. M pneumoniae and C pneumoniae were also detected in coinfection with viral etiologies, especially RSV (Table 4). The range mean (standard deviation) of clinical scores between atypical bacterial infected cases (severe) and RSV-induced bronchiolitis (moderate) were statistically different. Their clinical manifestations, however, were not different (Tables 1 and 2) as all of the children had moderate to severe respiratory distress on admission.

Bacterial coinfections occurring in respiratory viral infections were reported by Lehtinen et al. who studied the phenomenon in a total of 220 children with viral wheezing between 3 months and 16 years of age. Rhinovirus (32%), RSV (31%), and enteroviruses (31%) were the most common causative viruses. Serologic evidence of bacterial coinfection was found in 18% of the children, in whom Streptococcus pneumoniae (8%) and M pneumoniae (5%) infection were the most common. In contrast, Esposito et al. used serologic investigative techniques and PCR to demonstrate M pneumoniae in 22.5% and C pneumoniae in 15.5% of children with acute wheezing compared with 7.5% and 2.5%, respectively, in healthy control subjects. When the children who were infected with either organism were treated with clarithromycin, improvement in the course of disease was observed, which supports the hypothesis that these atypical organisms exacerbate asthma. Biscardi et al., however, found M pneumoniae infection in 20% and C pneumoniae infection in 3.4% of 119 children hospitalized for severe asthma, and the benefits of antibiotic treatment were questionable. In our study, the number of atypical bacterial infections was small because it resulted from a limited number of patients with acute childhood bronchiolitis. This limitation may therefore affect the difference in their clinical manifestations.

In conclusion, our study indicates that atypical bacteria are being detected as coinfections in children with viral-induced bronchiolitis or wheezing, which varies according to age group. These results suggest that the atypical bacteria may be important coinfection agents of respiratory viruses, which may induce severe illness of acute childhood bronchiolitis. Further investigation may be warranted, especially of more severe cases.

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References


