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ORIGINAL ARTICLE

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

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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 agents 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

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M pneumoniae (13/14) and *C pneumoniae* (4/4) had etiologies indicating viral coinfections. Four (2.4%) of all of the cases had *C trachomatis* infections and all of these were infected with RSV, including three patients less than 6 months of age.
Conclusion: These results suggest that in children with virus-induced acute bronchiolitis coinfection with *M pneumoniae*, *C pneumoniae*, or *C trachomatis* can be expressed differently in each age group. These atypical bacteria may be the important infectious agents that induce severe illness of acute bronchiolitis.

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*, *Chlamydophila 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,[7] 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)[9]; 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 $-70^{\circ}C$ until used.

Atypical bacteria study

DNA extraction

DNA was extracted from a 200-μL aliquot of sample using a PUREGENE DNA purification kit (Gentra 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* enclosed a specific 144 bp fragment: MP5-1 (5′-GAA GCT TAT GGT ACA GGT TGG-3′) and MP5-2 (5′-ATT ACC ATC CTT GTT GTA AGG-3′).[11] The primers for *C pneumonia*—specific for the *Omp1* gene—were: Cpn1 (5′-GTT CAA TCT CGT TGG TTT ATT-3′, nt 453—473) and Cpn3 (5′-TCC AAT GTA TGG CAC TAA AGA-3′, nt 858—838, 405 bp).[12]

Every sample with a negative result using multiplex PCR was reanalyzed using specific nested PCRs under the same conditions to differentiate between 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*[12] and primers MUH-1 and MUH-2 (104 bp)[13] 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 ATC AGA CGA G-3′ and Ch2: 5′-CCT TCT TTA AGA GGT TTT ACC CA-3′ were used. Standard amplification conditions for the primers Ch1 and Ch2 were used.[14] Each PCR product (603 bp) was separated on 1.5% agarose gel and was then investigated with 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.[15] 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

Demographic data	Total ($n = 170$)	RSV ($n = 110$)	Bacteria ($n = 22$)	Student t test (p)
Age (mo)	10.7 ± 5.7	10.2 ± 5.7	9.8 ± 5.8	0.000
Sex, male	107 (62.9)	65 (59.1)	15 (68.2)	0.215
Breastfeeding	160 (94.1)	102 (92.7)	21 (95.4)	0.323
Atopic history—parents	49 (28.8)	34 (30.9)	6 (27.3)	0.369
Passive smoker	102 (60)	63 (57.3)	19 (86.4)	0.005
Fever before admission	155 (91.2)	99 (90.0)	20 (90.9)	0.450
Clinical score[a]	7.1 ± 1.4 (5—11)	7.0 ± 1.4 (5—11)	7.2 ± 1.1 (5—9)	0.000
O_2 saturation < 95% at enrollment	83 (48.8)	57 (51.8)	14 (63.6)	0.157

[a] Modified from De Boeck et al.[16] and Tal et al.[17]

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.

Table 2 Clinical manifestations of *C trachomatis* infants

Clinical manifestations	Infant I	Infant II	Infant III	Infant IV
Age (mo)	1.7	1.7	2.5	23.5
Sex	Male	Male	Male	Female
Atopic history	No	No	Yes	Yes
Clinical score at enrollment[a]	6	6	7	8
O_2 saturation at enrollment (%)	92	95	99	93
Symptoms before enrollment (d)	5	7	3	4
Respiratory distress before enrollment (hr)	48	168	12	48

[a] Modified from De Boeck et al.[16] and Tal et al.[17]
C trachomatis = Chlamydia trachomatis.

about 10 months. All of the children had moderate to severe respiratory distress at admission. In cases of acute bronchiolitis, respiratory viruses were found in 85.3% (145/170) of the cases, whereas atypical bacteria (i.e. *M pneumoniae*, *C pneumoniae*, *and C trachomatis*) were found in 12.9% of the cases (22/170). Of the respiratory viruses, RSV 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 3 Atypical bacterial infection in 170 nasopharyngeal samples of children with acute bronchiolitis

Age group (mo)	*n*	*M pneumoniae*	*C pneumoniae*	*C trachomatis*
1 to <6	36	2	0	3
6 to <12	71	7	4	0
12–24	63	5	0	1
Total	170	14 (8.2%)	4 (2.4%)	4 (2.4%)

C trachomatis = *Chlamydia trachomatis*; *C pneumoniae* = *Chlamydophila pneumoniae*; *M pneumoniae* = *Mycoplasma pneumoniae*.

Table 4 Coinfection by atypical bacterial pathogen with viral respiratory pathogens in children with acute bronchiolitis

Pathogens	RSV	Influenza A	Influenza B	hMPV	RSV and influenza A	Total, *n* (%)
M pneumoniae (14 cases)	6	2	2	2	1	13 (92.9)
C pneumoniae (4 cases)	3	0	0	0	0	3 (75)
C trachomatis (4 cases)	4	0	0	0	0	4 (100)

C trachomatis = *Chlamydia trachomatis*; *C pneumoniae* = *Chlamydophila pneumoniae*; hMPV = human metapneumovirus; *M pneumoniae* = *Mycoplasma pneumoniae*; RSV = respiratory syncytial virus.

among patients with pneumonia, whereas *C pneumoniae* was more common in patients with bronchitis. *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.[21,22]

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.[23] who reported that 3% of *C trachomatis* infections in 100 children between 3 months and 12 years of age were admitted to the pediatric 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.[24,25] Notwithstanding, individual infectious agents have been associated with the development of chronic lung disease and exacerbation of asthma.[26–29] Coinfection of two atypical bacterial agents—possessing chronic sequelae potential—may result in a protracted illness,[30] 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.[31] 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.[32] 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.[19], 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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