## Letter to the Editor

# Molecular Epidemiology and Clinical Features of *Haemophilus influenzae* among Hospitalized Children with Community-acquired Pneumonia in Chengde, China*

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Community-acquired pneumonia (CAP) is an acute lung infection that is caused by several different pathogens and is associated with significant morbidity and mortality. The high global incidence of CAP poses a heavy disease and economic burden to patients, especially children. Respiratory illnesses such as pneumonia and influenza are the fourth leading cause of death in China[1]. The top 3 etiologic pathogens of CAP in the Asia-Pacific region are *Streptococcus pneumoniae*, *Haemophilus influenzae* (*H. influenzae*), and *Mycoplasma pneumoniae* (*M. pneumoniae*). A study of CAP-associated pathogens from 2001 to 2013 showed *M. pneumoniae* as the most commonly isolated pathogen, especially in children. *H. influenzae* infection was most common (20.6%) between 2001 and 2003, but data on *H. influenzae* were missing from 2010 because the focus of CAP etiology had turned to *M. pneumoniae* and respiratory viruses[2].

*H. influenzae* is a Gram-negative bacterium that can be divided into typeable and non-typeable *H. influenzae* (NTHi) based on the presence or absence of a polysaccharide capsule. Typeable *H. influenzae* are further divided into 6 serotypes (a to f) based on the capsular polysaccharide antigen, with *H. influenzae* type b (Hib) representing the most common cause of bacterial pneumonia. While the incidence of Hib has declined dramatically since the widespread introduction of Hib-conjugate vaccines, the incidence of NTHi has increased significantly. Globally, NTHi are recognized as the most common causative pathogens in all ages, and they are confirmed pneumonia pathogens in children[3]. Therefore, the aim of this study was to investigate the molecular epidemiology and clinical features of CAP caused by *H. influenzae* among hospitalized children in Chengde, China.

A total of 333 children aged 5 months to 14 years who were hospitalized with CAP in the Department of Pediatrics of a Clinical Teaching Hospital affiliated to Chengde Medical University were enrolled in this study from November 2017 to May 2018. We collected throat swabs from all patients and reviewed medical records for information on age, sex, residence, peak and duration of fever, as well as hospitalization length. Laboratory test data including white blood cell (WBC), neutrophil, and lymphocyte count as well as C-reactive protein (CRP) and lactate dehydrogenase (LDH) concentrations were also recorded. This study was approved by the Ethics Committee of Chengde Medical University with the reference number 2017020 and was performed in accordance with the principles set forth in the Declaration of Helsinki. The collection of samples used in this study was authorized by the guardians of the hospitalized children with prior informed consent.

Bacterial DNA and viral nucleic acids (DNA or RNA) were extracted from throat swabs using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) or

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Viral Nucleic Acid Extraction Kit II (Geneaid, New Taipei City, Taiwan, China) according to manufacturer instructions. The *bexA* gene of *H. influenzae* was selected as the target gene and the prevalence of *H. influenzae* was detected using real-time PCR. The forward and reverse primers (TGCGGTAGTGTTAGAAAATGGTATTATG and GGACAAACATCACAAGCGGTTA, respectively) used in this study were described previously[4]. Real-time PCR was prepared using 2 × *TansStart*® Top Green qPCR SuperMix (TRANS, Beijing, China) in a final volume of 20 μL containing 5 μL of template DNA and conducted *via* the MyiQ2 real-time PCR detection system (Bio-Rad, California, USA). Real-time PCR detection was conducted according to the following protocol: initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation for 30 seconds at 95 °C and annealing for 30 seconds at 55 °C. In order to assess co-infection, atypical pathogens such as *M. pneumoniae*, *Chlamydophila pneumoniae* (*C. pneumoniae*), and *Legionella pneumophila* (*L. pneumophila*); bacterial pathogens including group A Streptococcus (GAS), *Klebsiella pneumoniae* (*K. pneumoniae*), *Staphylococcus aureus* (*S. aureus*), and *Pseudomonas aeruginosa* (*P. aeruginosa*); and viral pathogens including Influenza A/B/C viruses, parainfluenza virus (PIV) 1, 2, and 3, Adenovirus (AdV), human bocavirus (HBoV), human rhinovirus (HRV), human metapneumovirus (hMPV), respiratory syncytial virus (RSV), and human coronavirus (HCoV) were also detected. Co-infection was defined as testing positive for *H. influenzae* and presenting with at least one bacterial or viral pathogen.

Categorical and continuous variables were described as counts (percentages) or median (interquartile range, IQR). Categorical variables were compared using the Chi-squared or Fisher's exact test, and continuous variables were compared using the Mann-Whitney U-test. All statistical analyses were performed using SPSS v.19.0 (IBM Corp., Armonk, NY). Results were considered statistically significant if $P < 0.05$.

Among the 333 children hospitalized with CAP, 209 were positive for *H. influenzae.* Of these, 86 (41.1%) were singly infected with *H. influenzae* and 123 (58.9%) were co-infected (98, 22, and 3 children had double, triple, and quadruple bacterial infections, respectively). A total of 125 children were infected with GAS, 32 of whom were singly infected, and 64 of whom were co-infected with GAS and *H. influenzae*. Of 66 children infected with *K. pneumoniae,* only 10 were singly infected, 30 were co-infected with *K. pneumoniae* and *H. influenzae*, and 4 were co-infected with *K. pneumoniae* and GAS. Finally, of 20 children infected with *S. aureus*, only 1 child was singly infected, and 4 were co-infected with both *S. aureus* and *H. influenzae*. Our results show that co-infections—especially double and triple bacterial infections—were common events in hospitalized children with CAP (Table 1).

Although *H. influenzae* was detected throughout the study period of November 2017 to May 2018, the months of December 2017 and January 2018 had the highest detection rates, with 57 and 40 children diagnosed, respectively (Figure 1A). The prevalence of *K. pneumoniae*, GAS, and *S. aureus* was concentrated from November 2017 to January 2018 (Figure 1A), and 107 males and 102 females were infected with *H. influenzae* between November 2017

**Table 1.** Detection and co-infections of *H. influenzae*, GAS, *K. pneumoniae* and *S. aureus* among hospitalized children with CAP

| Bacteria | *H. inluenzae* | GAS | *K. pneumonia* | *S. aureus* |
|---|---|---|---|---|
| *H. inluenzae* (*n*) | 86 | 64 | 30 | 4 |
| GAS (*n*) | | 32 | 4 | 1 |
| *K. pneumonia* (*n*) | | | 10 | 2 |
| *S. aureus* (*n*) | | | | 1 |
| Total | 209 | 125 | 66 | 20 |
| Single bacteria, *n* (%) | 86 (41.1) | 32 (25.6) | 10 (15.2) | 1 (5.0) |
| Co-infections, *n* (%) | 123 (58.9) | 93 (74.4) | 56 (84.8) | 19 (95.0) |
| Double bacteria, *n* (%) | 98 (46.9) | 69 (55.2) | 36 (54.5) | 7 (35.0) |
| Triple bacteria, *n* (%) | 22 (10.5) | 21 (16.8) | 17 (25.8) | 9 (45.0) |
| Quadruple bacteria, *n* (%) | 3 (1.4) | 3 (2.4) | 3 (4.5) | 3 (15.0) |

and May 2018 (Figure 1B). These children mainly resided in urban regions ($n$ = 175) (Figure 1C).

We divided the 333 hospitalized children into two groups according to age: < 5 years old ($n$ = 168) and ≥ 5 years old ($n$ = 165). Results showed that 109 children infected with *H. influenzae* were aged < 5 years and 100 were ≥ 5 years ($P$ = 0.821). Out of 125 children infected with GAS, 71 were < 5 years old and 54 were ≥ 5 years old, and children < 5 years old were the main target for infection with GAS ($P$ < 0.0001). Out of 66 children infected with *K. pneumoniae*, 32 were < 5 years old and 34 were ≥ 5 years old, with children ≥ 5 years old acting as the main targets for *K. pneumoniae* infection ($P$ = 0.039). There was no difference in the distribution of *S. aureus* between two the groups ($P$ = 0.400) (Supplementary Table S1 available in www.besjournal.com).

We further divided the 333 children into 2 groups according to disease severity. Overall, there were 293 mild cases and 40 severe cases. Further stratification showed that 229 (78.2%) mild cases and 31 (77.5%) severe cases were positive for at least one of the following pathogens: *H. influenzae*, GAS, *K. pneumoniae* and *S. aureus*. We found 78 children in the 'mild case' group and 8 in the 'severe case' group who were singly infected with *H. influenzae.* Co-infection was also common in mild and severe case groups, with 115 and 16 children affected, respectively (Supplementary Table S2 available in www.besjournal.com).

We compared co-infection with *H. influenzae* and atypical pathogens (*M. pneumoniae and C. pneumoniae*), bacterial pathogens (*H. influenzae*, GAS, *K. pneumoniae,* and *S. aureus*), and viruses (RSV, hMPV, HBoV, HCoV, and AdV) across age groups. Our results show that 15 (13.8%) children < 5 years old and 11 (11.0%) children ≥ 5 years old were singly infected with *H. influenzae*, 39 (18.7%) were co-infected with atypical pathogens (22 children with a percentage of 20.2 were < 5 years old), 83 (39.7%) were co-infected with at least one bacterial pathogen (44 children with percentage of 44.0 were ≥ 5 years old), 16 (7.7%) were co-infected with at least one viral pathogen, and a total of 45 (21.5%) children were co-infected with at least one bacterial and one viral pathogen (26 children with percentage of 23.9 were < 5 years old) (Supplementary Table S3 available in www.besjournal.com).

There were no differences across sex between severe and mild case groups (11 *vs.* 96; $P$ = 0.576). Children with severe cases infected with *H. influenzae* tended to be older than mild cases [median, 7 (IQR, 6–8) years *vs.* 4 (IQR, 3–6) years; $P$ < 0.0001]. There was no difference in the peak of fever between the two groups [39.5 (IQR, 39.0–40.0) °C *vs.* 39.3 (IQR, 38.8–39.7) °C; $P$ = 0.159]; however, the duration of fever in the severe case group was longer than that of the mild case group [7 (IQR, 4.5–10.0) d; $P$ = 0.029]. There was no difference in the number of days of cough ($P$ = 0.116) and rales ($P$ = 0.912) between the two groups. The patients in the severe case group had a longer duration of hospitalization [9 (IQR, 7.5–12.0) d; $P$ < 0.0001], a

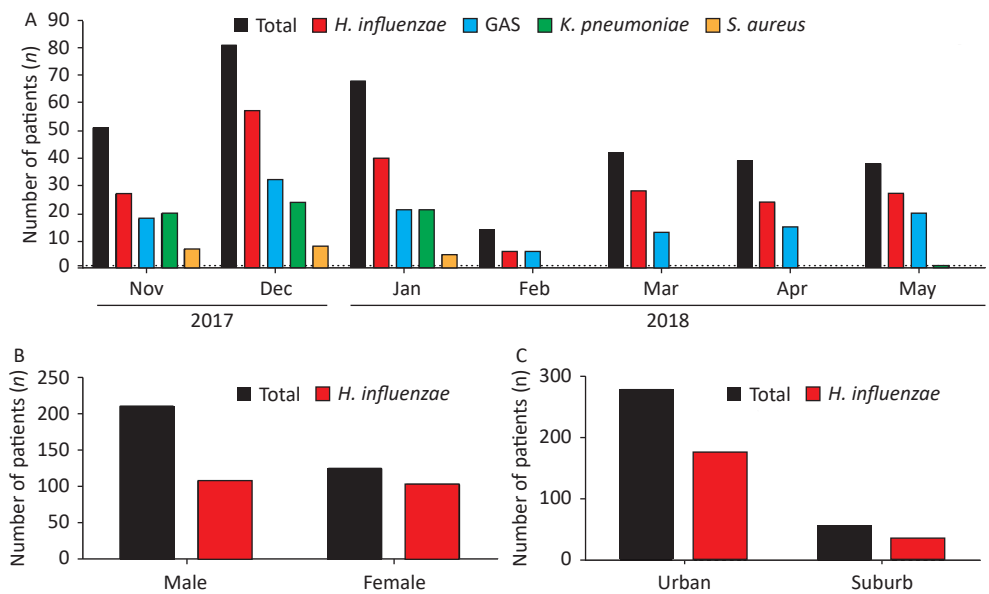


**Figure 1.** Prevalence of *H. influenzae* among hospitalized children with CAP.

higher ratio of neutrophils [65.1 (IQR, 60.4%–70.4%); *P* = 0.005] and concentration of CRP [2.1 (IQR, 1.3–14.8) mg/L; *P* = 0.041], and lower ratio of lymphocytes [24.0 (IQR, 20.6%–30.3%); *P* = 0.010] than the mild case group. There was no difference in WBC count [7.6 (IQR, 6.4–8.8) $\times 10^9$ cells/L *vs.* 7.8 (IQR, 5.7–11.5) $\times$ $10^9$ cells/L; *P* = 0.360] or LDH concentration [249 (IQR, 218–304) U/L *vs.* 264 (IQR, 228–320) U/L; *P* = 0.505] between the two groups (Table 2).

In a previous study of 156 children, 107 were positive for at least one pathogen, and 64 (41%) were infected with *H. influenzae* in Romania from June 2016 to May 2017[5]. Another study of 2,970 patients found 176 cases of *H. influenzae* and 49 *H. influenzae* co-infections in the German prospective cohort study CAPNETZ from October 2002 to July 2013[6]. *H. influenzae* is the dominant pathogen in UK adults with non-severe CAP and chronic lung disease[7]. In our study, 209 hospitalized children were infected with *H. influenzae*, but only 86 (41.1%) of those were singly infected with *H. influenzae*. We also examined 3 atypical pathogens, 5 bacterial pathogens, and 8 viral pathogens in these specimens, and our results show that *M. pneumoniae* (*n* = 221) and *H. influenzae* were the dominant pathogens. Regarding viral pathogens, 80 children were positive for hMPV and 22 children were positive for HBoV[8], with co-infection accounting for a higher proportion among these detected pathogens. This result indicates that co-infection is a common cause of pneumonia.

A total of 8,571 invasive *H. influenzae* cases were reported in England and Wales during 2000–2013, but only 1,585 cases were aged between 1 month and 10 years old. Among 362 *H. influenzae* invasive isolates during 2009–2013, there were 214 NTHi, 25 Hib, and 52 non-type b *H. influenzae* (36 Hif, 14 Hie, 1 Hia, and 1 Hic) cases[9]. This result indicated that while NTHi was the dominant pathogen, other non-type b *H. influenzae* were also present, warranting surveillance in clinical cases. The *bexA* gene was used to determine the presence of a polysaccharide capsule, and the type of *H. influenzae* (a–f) was confirmed via PCR and slide agglutination test using capsule-specific primers and monovalent a–f antisera, respectively. NTHi should be PCR-negative for *bexA* and Hib-specific targets without a detectable capsule[9]. The previous study also confirmed that NTHi isolates lacked both *bexA* and capsule-specific genes[10]. In our study, we used the *bexA* gene as a target to screen for the prevalence of *H. influenzae* and confirmed positivity in 209 children. However, one limitation of our study is that we could not determine the serotypes of *H. influenzae*. Therefore, further studies are needed to determine the exact bacteriologic characteristics of these *H. influenzae* strains.

**Table 2.** Clinical features of hospitalized children infected with *H. influenzae* according to disease severity

| Characteristics | Severe cases (*n* = 24) | Mild cases (*n* = 185) | *P* |
|---|---|---|---|
| Demographic and clinical presentation | | | |
| Males, *n* (%) | 11 (45.8) | 96 (51.9) | 0.576 |
| Age (years), median (IQR) | 7 (6–8) | 4 (3–6) | < 0.001 |
| Peak of fever (°C), median (IQR) | 39.5 (39.0–40.0) | 39.3 (38.8–39.7) | 0.159 |
| Duration of fever (d), median (IQR) | 7 (4.5–10.0) | 5 (3.5–7.0) | 0.029 |
| Cough (d), median (IQR) | 13 (6–20) | 10 (8–13) | 0.116 |
| Rales, *n* (%) | 13 (54.2) | 98 (53.0) | 0.912 |
| Clinical outcome | | | |
| Duration of hospitalization (d), median (IQR) | 9 (7.5–12.0) | 6 (5–7) | < 0.001 |
| Laboratory data | | | |
| WBC ($\times 10^9$cells/L), median (*IQR*) | 7.6 (6.4–8.8) | 7.8 (5.7–11.5) | 0.360 |
| Neutrophils (%), median (IQR) | 65.1 (60.4–70.4) | 59.2 (46.8–67.8) | 0.005 |
| Lymphocytes (%), median (IQR) | 24.0 (20.6–30.3) | 29.8 (21.6–41.9) | 0.010 |
| CRP (mg/L), median (IQR) | 2.1 (1.3–14.8) | 1.7 (0.5–7.0) | 0.041 |
| LDH (U/L), median (IQR) | 249 (218–304) | 264 (228–320) | 0.505 |

Our results showed the prevalence of *H. influenzae* and *M. pneumoniae* is higher in hospitalized children with CAP in Chengde between 2017 and 2018, and these results indicate current pneumonia surveillance should focus not only on viral pathogens but should also establish a bacterial pathogen profile. Additionally, strengthening the surveillance of non-type b *H. influenzae* and NTHi in CAP would provide important information in controlling and preventing *H. influenzae*-associated CAP.

The authors declare that they have no competing interests.

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**Supplementary Table S1.** Age distribution of *H. influenzae*, GAS, *K. pneumoniae* and *S. aureus* among hospitalized children with CAP

| Bacteria | < 5 years (*n* = 168) | | ≥ 5 years (*n* = 165) | | *P* |
|---|---|---|---|---|---|
| | Total | Co-infections | Total | Co-infections | |
| *H.inluenzae*, *n* (%) | 109 (64.9) | 65 (59.6) | 100 (60.6) | 58 (58.0) | 0.821 |
| GAS, *n* (%) | 71 (42.3) | 52 (73.2) | 54 (32.7) | 41 (75.9) | < 0.001 |
| *K. pneumoniae*, *n* (%) | 32 (19.0) | 27 (84.4) | 34 (20.6) | 29 (85.3) | 0.039 |
| *S. aureus*, *n* (%) | 12 (7.1) | 12 (100.0) | 8 (4.8) | 7 (87.5) | 0.400 |

**Supplementary Table S2.** Distribution of *H. influenzae*, GAS, *K. pneumoniae* and *S. aureus* according to disease severity

| Bacteria | Mild cases (*N* = 293) *n* (%) | Severe cases (*N* = 40) *n* (%) | *P* | *OR* (95% *CI*) |
|---|---|---|---|---|
| Negative | 64 (21.8) | 9 (22.5) | 0.925 | 0.963 (0.436–2.126) |
| Positive | 229 (78.2) | 31 (77.5) | 0.925 | 1.039 (0.470–2.294) |
| *H.inluenzae* | 78 (26.6) | 8 (20.0) | 0.370 | 1.451 (0.641–3.285) |
| GAS | 27 (9.2) | 5 (12.5) | 0.565 | 0.711 (0.257–1.965) |
| *K. pneumoniae* | 8 (2.7) | 2 (5.0) | 0.343 | 0.533 (0.109–2.605) |
| *S. aureu* | 1 (0.3) | 0 (0.0) | 1.000 | – |
| Co-infection | 115 (39.2) | 16 (40.0) | 0.927 | 0.969 (0.494–1.903) |

**Supplementary Table S3.** Co-infections of bacterial and viral pathogens with *H. influenzae* according to age group

| Bacteria | < 5 years (*N* = 109) *n* (%) | ≥ 5 years (*N* = 100) *n* (%) | Total (*N* = 209) *n* (%) | *P* | *OR* (95% *CI*) |
|---|---|---|---|---|---|
| *H. influenzae* | 15 (13.8) | 11 (11.0) | 26 (12.4) | 0.546 | 1.291 (0.563–2.962) |
| *H. influenzae* + *M. pneumoniae*/*C. pneumoniae* | 22 (20.2) | 17 (17.0) | 39 (18.7) | 0.555 | 1.235 (0.613–2.488) |
| *H. influenzae* + bacteria* | 39 (35.8) | 44 (44.0) | 83 (39.7) | 0.225 | 0.709 (0.407–1.237) |
| *H. influenzae* + viruses# | 7 (6.4) | 9 (9.0) | 16 (7.7) | 0.484 | 0.694 (0.248–1.939) |
| *H. influenzae* + bacteria + viruses | 26 (23.9) | 19 (19.0) | 45 (21.5) | 0.394 | 1.335 (0.686–2.600) |

***Note.*** *Bacteria including *H.inluenzae*, GAS, *K.pneumonia* and *S.aureus*. #Viruses including RSV, hMPV, HBoV, HCoV and AdV.