The Journal of Microbiology, August 2009, p. 494-497 Vol. 47, No. 4
DOI 10.1007/s12275-009-0002-4

Genotypic Characteristics of *Haemophilus influenzae* Isolates from Pediatric Pneumonia Patients in Chengdu City, Sichuan, China

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(Received December 24, 2008 / Accepted April 6, 2009)

Two hundred and seventy-three *Haemophilus influenzae* strains isolated from pediatric pneumonia patients in China were studied. We used Multilocus Sequence Typing (MLST) to analyze genotypic characteristics. All strains were biotyped and serotyped. Relatedness and patterns of genes among isolates were determined by the analysis of MLST and eBURST. *H. influenzae* primarily causes acute pneumonia in children under 1 year old. Nontypeable *H. influenzae* was responsible for most cases of pediatric pneumonia. All 273 strains were classified into eight biotypes. They mostly belonged to the I, II, and III biotypes (17.6%, 43.6%, and 22.7%, respectively). 62 strains (22.7%) produced β-lactamase. We found 28 novel alleles. Fifty different STs were found by MLST, of which 39 were novel. These were ST477 through ST508 and ST521 through ST527. Group 17 and predicted founders 503 were new groups in this study. No STs correlated with strains from Korea, which is adjacent to China. The *H. influenzae* strains from China appeared to have heterogeneous ST types patterns which may be the reason no outbreaks or epidemics of *H. influenzae* infections have occurred in Chengdu city, Sichuan, China.

***Keywords*:** *Haemophilus influenzae*, multilocus sequence typing, biotyping, serotyping

Haemophilus influenzae is a pathogen found exclusively in humans. It causes a wide range of illnesses, from the upper respiratory tract infections to serious invasive diseases, such as pneumonia, septicemia, and meningitis. The first introduction of polyribosylribitol phosphate Hib conjugate vaccine to China occurred in 1998. However, no data of active laboratory-based surveillance for invasive *H. influenzae* diseases was available. This study was to determine the characteristics of molecular epidemiology of *H. influenzae* in China using serotyping, biotyping, and MLST (multilocus sequence typing). MLST has a great advantage over other typing methods because isolates characterized in different laboratories can be readily compared, and the allelic profiles of isolates and associated epidemiological information can be held in a single database that can be investigated over the Internet (Meats *et al*., 2003; Urwin and Maiden, 2003).

Materials and Methods

Bacterial strains

The tracheal exudates were collected by tracheal intubation which were placed into the lower respiratory tract from nasopharynx. The samples were drew into a sterile tube by machine suction. All specimens were immediately streaked onto a chocolate agar plate. Plates were then incubated at 37°C with 5% CO_2 for 24 h. *H. influenzae* strains were originally defined on the basis of typical colony morphology on chocolate agar, Gram stain, requirement for V and X factors, hemolysis on horse blood agar, oxidase and catalase, production of β-lactamase, and reaction in the porphyrin test. Seven *H. influenzae* strains (serotype a, M4741; serotype b, M5216; serotype c, M6542; serotype d, M6548; serotype e, M9418; serotype f, M6297 and nontypeable M5209) from the American Center for Disease Contol were used as reference strains. The isolates were identified again using the API®NH system (BIOMÉRIEUX®SA, France).

Biotyping and serotyping

All strains were biotyped according to Kilian's classification (Kilian, 1976) using the API®NH system (testing for urease activity, indole production, and ornithine decarboxylase activity). Serotyping was performed using a slide agglutination assay with serotype-specific antisera for types a through f (BD Co. Ltd., USA). PCR to identify *H. influenzae* strains was performed, as previously described (van Ketel *et al*., 1990; Falla *et al*., 1994).

Multilocus sequence typing

MLST was performed as previously described by Meats *et al*. (2003) and included the 7 genes, *adk*, *atpG*, *frdB*, *fucK*, *mdh*, *pgi*, and *recA* (http://haemophilus.mlst.net). The results of DNA sequencing for each of the seven genes for each ST were concatenated. The eBURST algorithm and the

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Table 1. Distribution of the 8 biotypes, 50 STs, and β-lactamase production

Biotype	No. of strains (%)	Produced β-lactamase (%)		STs (No.)
		+	−	
I	48 (17.6)	5 (10.4)	43 (89.6)	124, 436, 477, 478, 480, 483, 487, 490, 491, 499, 504, 507
II	119 (43.6)	34 (28.6)	85 (71.4)	57, 136(2), 140, 408(2), 422, 436(2), 479, 480(3), 481, 487, 488, 492, 494, 495, 496(2), 497, 498, 500, 501, 505, 521, 522, 524, 525, 526, 527
III	62 (22.7)	13 (21)	49 (79)	107, 196, 262, 482, 484, 485, 503, 508, 523
IV	20 (7.3)	8 (40)	12 (60)	245, 486(4), 502
V	16 (5.9)	0 (0)	16 (100)	478(2), 493
VI	1 (0.4)	0 (0)	1 (100)	506
VII	5 (1.8)	2 (40)	3 (60)	
VIII	2 (0.8)	0 (0)	2 (100)	
Total	273	62 (22.7)	211 (77.3)	

+, β-lactamase-producing strains
−, β-lactamase-non-producing strains

H. influenzae database at http://eburst.mlst.net were used to examine the relatedness and patterns of evolutionary descent among isolates (Meats *et al*., 2003; Feil *et al*., 2004). The sampling strains (included collecting years, age groups, and biotyping series) for the study of MLST were analyzed by Chi-square test or the Fisher exact test. *P* value <0.05 was considered statistically significant.

Results

Analysis by biotyping, serotyping, and PCR methods

A total of 273 *H. influenzae* strains were isolated and identified from January 2004 to March 2007. Of these, 17.6% (48/273) were isolated in 2004, 23.8% (65/273) were isolated in 2005, 30.0% (82/273) were isolated in 2006, and 28.6% (78/273) were isolated in 2007. The male to female patient ratio for *H. influenzae* infection was 1.5:1. Among all cases, 77.7% (212/273) were found in 1 year old, 8.4% (23/273) were found in 1 to 2 years old, 5.9% (16/273) were found in 2 to 3 years old, and 8.1% (22/273) were found in 4 to 9 years old.

Eight biotypes were identified among the 273 *H. influenzae* isolates. 17.6% (48/273) belonged to biotype I, 43.6% (119/273) belonged to biotype II, 22.7% (62/273) belonged to biotype III, 7.3% (20/273) belonged to biotype IV, 5.9% (16/273) belonged to biotype V, 0.4% (1/273) belonged to biotype VI, 1.8% (5/273) belonged to biotype VII, and 0.7% (2/273) belonged to biotype VIII. A total of 62 strains (22.7%) produced β-lactamase. The β-lactamase positive strains were distributed among different biotypes strains: 10.4% (5/48) in biotype I, 28.6% (34/119) in biotype II, 21.0% (13/62) in biotype III, 40.0% (8/20) in biotype IV, and 40.0% (2/5) in biotype VII (Table 1). Only one strain was serotype f by slide agglutination assay and PCR method. There were 80 strains that were positive ambiguous by sera agglutination: 2.93% (8/273) in type a, 6.59 (18/273) in type b, 1.83% (5/273) in type c, 9.52% (26/273) in type d, 2.20% (6/273) in type e, and 6.23% (17/273) in type f. These were all negative according to PCR method. The other strains were not typeable by slide agglutination assay or by PCR method.

Analysis of MLST

We selected 63 strains, which were mainly collected from November through April each year for three years, to study the characteristics of genetics by MLST. The collected strains

Table 2. Statistical analyses for the MLST analysis of 273 strains

Year	No. of strains (%)			Biotype	No. of strains (%)			Age	No. of strains (%)		
	Collecting	MLST	$\chi^2_{0.05}$, P		Collecting	MLST	$\chi^2_{0.05}$, P		Collecting	MLST	$\chi^2_{0.05}$, P
2004	48 (17.6)	16 (25.4)	1.24, $P>0.05$	I	48 (17.6)	12 (19.1)	0.27, $P>0.05$	~1	212 (77.7)	51 (81.0)	0.60, $P>0.05$
2005	65 (23.8)	21 (33.3)	1.34, $P>0.05$	II	119 (43.6)	31 (49.2)	0.79, $P>0.05$	~2	23 (8.4)	5 (7.9)	0.16, $P>0.05$
2006	82 (30.0)	18 (28.6)	0.22, $P>0.05$	III	62 (22.7)	9 (14.3)	1.53, $P>0.05$	~3	16 (5.9)	3 (4.8)	0.34, $P>0.05$
2007	78 (28.6)	8 (12.7)	2.89, $P>0.05$	IV	20 (7.3)	6 (9.5)	0.69, $P>0.05$	4~	22 (8.1)	4 (6.3)	0.56, $P>0.05$
				V	16 (5.9)	3 (4.8)	0.34, $P>0.05$				
				VI	1 (0.4)	1 (1.6)					
				VII	5 (1.8)	1 (1.6)					
				VIII	2 (0.7)	0 (0)					
Total	273	63	$\chi^2_{0.05}=3.84$		273	63	$\chi^2_{0.05}=3.84$		273	63	$\chi^2_{0.05}=3.84$

were not statistically different from the 273 strains in collecting years, age groups, or biotyping series. The strains chosen for MLST analysis represent the population characters of all strains (Table 2).

The detailed results from MLST analysis and the sources of the isolates used in this study were submitted to the *H. influenzae* MLST database (http://haemophilus.mlst.net). A total of 28 novel alleles were found: 3 for *adK* (102, 103, 104), 3 for *frdB* (100, 101, 102), 1 for *fucK* (56), 10 for *mdh* (146, 147, 148, 149, 150, 151, 152, 153, 154, 156), 7 for *pgi* (124, 125, 126, 127, 128, 129, 130), and 4 for *recA* (89, 90, 91, 92). A total 50 different STs were obtained, of which 39 were novel. STs were named ST477 through ST508 and ST521 through ST527. There were four strains in both ST480 and ST486 and three strains in ST478. The STs of ST136, ST408, ST436, ST487, and ST496 were represented by two strains each.

There were 825 strains of *H. influenzae*, comprised of 543 STs, that were submitted to http://haemophilus.mlst.net from now. These strains were classified into 73 groups and 39 predicted founders according to the numbers of identical loci for group def=6 (eBURST V3). In this study, 6 of 73 strains belonged to group 2 and predicted founders 3, 5 to group 11 and predicted founders 422; 4 to group 17 and predicted founders 503, and 1 to groups 5, 6, 9, 15, 29 and predicted founders 124, 1, 259, 57, 321. Two strains of group 36 and predicted founders were multiple candidates. 54% (34/63) were singletons. Group 17 and predicted founders 503 was new group and predicted founders in this study. Some strains correlated to that were from Finland (ST57), United Kingdom (ST107, ST196, ST436), The Netherlands (ST245), United State of America (ST124, ST136, ST140, ST262) and Canada (ST408, ST422). None of STs correlated to the strains from Korea, which is adjacent to China by the analysis of MLST (http://haemophilus.mlst.net).

In China, the predominant epidemiological STs were ST486 and 480. The mainly epidemiological groups were 2, 11, and 17. The mainly epidemiological predicted founders were 3, 422, and 503. 73% (46/63) strains were single group.

Four strains of biotype II belonged to ST480 with β-lactamase producing activities. There were four strains of biotype VI belonging to ST486, none of which could produce β-lactamase.

Discussion

H. influenzae is one of the most common pathogenic bacteria in children with lower respiratory tract infections, followed by *Streptococcus pneumoniae* and *Klebsiella pneumoniae* in Shanghai and Chengdu city of China (Huang *et al*., 2006, 2008). In this study we found that the majority of strains in children with pneumonia were biotype I, biotype II and biotype III. 22.7% of the strains produced β-lactamase. The incidence of β-lactamase production was only 12.0% in patients with acute upper respiratory tract infection under 5 years old in Shanghai, Guangzhou, and Beijing city from 2000 to 2002 (Shen *et al*., 2007). The incidence of β-lactamase producing strains manifested an ascending tendency in China from 2004 to 2007.

In this study, we confirmed a high heterogeneity of nontypeable *H. influenzae* strains (NTHi) by the MLST analysis. They appeared to be separate and diverse populations. In some bacteria, diseases were caused by a specific clone complex, which spread and caused an outbreak. Those genotypes are favoured by selection and expand, thus creating an 'epidemic' population structure (Shao *et al*., 2006). The heterogeneous characteristics of the NTHi strains may be the reason that no outbreak or epidemics occurred among children of Chengdu city. These observations help to understand phenotypic and genotypic characteristics of invasive *H. influenzae* isolates which cause pneumonia in children (Tsang *et al*., 2006).

Conventionally, serotyping of *H. influenzae* was performed by using slide agglutination testing. This technique was simple and gave quick result. However, it may be unreliable. It cannot be used to distinguish between noncapsulate strains and capsule-deficient mutants of type b strains (b^- strains) because some of these bacteria exhibited nonspecific agglutination. This discrepancy may be due to the low specificity of antiserum which results in difficulty in interpreting the agglutination test (Falla *et al*., 1994; CDC, 2002; Anyanwu *et al*., 2003). The findings of this study is consistent with previous data. We believe that serotyping should be confirmed by PCR genotyping to minimize the errors of conventional serotyping using the slide agglutination test (CDC, 2002) because PCR sero-genotyping is more sensitive and specific than traditional slide agglutination assays.

Nontypeable *H.influenzae* is also responsible for pediatric pneumonia (Heath *et al*., 2001; Anyanwu *et al*., 2003; LaClaire *et al*., 2003). We found that 77.7% cases occurred under 1 year old. 83.9% (229/273) strains belonged to biotypes I, II, or III. But these strains showed diversity of ST types by the MLST. It was different from the invasive *H. influenzae* serotype b (Hib) strains. The Hib strains were mostly biotype I and ST44, ST54, and ST6. The Hib and NTHi strains had different characteristics in the biotypes, serotypes and STs.

Acknowledgements

We thank wholeheartedly the members of Daniel Godoy at Imperial College who participated in this study and who were involved with the STs analysis on http://haemophilus.mlst.net.

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