Jpn. J. Infect. Dis., 65, 122-125, 2012

Original Article

Prevalence of β-Lactamase-Negative Ampicillin-Resistant *Haemophilus influenzae* Isolated from Patients of a Teaching Hospital in Thailand

Aroonlug Lulitanond[1]*, Aroonwadee Chanawong[1], Keskaew Pienthaweechai[1], Pipat Sribenjalux[1], Ratree Tavichakorntrakool[1], Chotechana Wilailuckana[1], Pirom Puang-Ngern[2], and Pairshompoo Saetung[3]

[1]*Centre for Research and Development of Medical Diagnostic Laboratories,*
[3]*Faculty of Associated Medical Sciences, and*
[2]*Clinical Microbiology Laboratory Unit, Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand*

(Received July 9, 2011. Accepted December 5, 2011)

SUMMARY: The aim of this study was to investigate the prevalence of β-lactamase-negative ampicillin-resistant (BLNAR) *Haemophilus influenzae* isolated from patients of a teaching hospital in Thailand. Eighty-eight isolates of *H. influenzae* were collected between September 2005 and March 2008. All isolates were identified and characterized for biotypes and capsular types. The β-lactamase production of these isolates was examined, and their susceptibility to the following 12 antimicrobial agents was determined: ampicillin (AMP), amoxicillin-clavulanate (AMC), cefotaxime (CTX), cefuroxime (CXM), meropenem (MEM), clarithromycin (CLR), telithromycin (TEL), tetracycline (TET), ciprofloxacin (CIP), levofloxacin (LEV), trimethoprim-sulfamethoxazole (SXT), and chloramphenicol (CHL). Of the 88 *H. influenzae* isolates, 69 (78.4%), 13 (14.8%), 4 (4.5%), and 2 (2.3%) were from the respiratory tract, pus, the genital tract, and blood, respectively. Half of the isolates were biotype II (44 isolates, 50%). The other half comprised biotypes I (23 isolates, 26.1%), III (15 isolates, 17.1%), and IV (6 isolates, 6.8%). All isolates were capsular non-typeable, except for 2 isolates that were type f. Antimicrobial susceptibility showed that all isolates were susceptible to AMC, CTX, MEM, TEL, CIP, and LEV (100%), whereas 96.6%, 94.3%, 80.7%, 68.2%, 50.0%, and 44.3% were susceptible to CXM, CLR, CHL, TET, AMP, and SXT, respectively. The β-lactamase-production rate of *H. influenzae* isolates was 40.9%, and the prevalence of BLNAR was 18.2%.

INTRODUCTION

Haemophilus influenzae is a Gram-negative bacterium isolated from the upper respiratory tract of certain normal humans. It is a major cause of bacterial meningitis in children aged 5 months to 5 years and is also a significant agent of respiratory tract infections, including acute otitis media, sinusitis, pneumonia, and other serious infections in children and adults (1). In elderly individuals, particularly those with underlying lung disease, this organism can cause severe pneumonia. Of all 6 serotypes (a–f) of *H. influenzae*, serotype b caused the most invasive diseases prior to the introduction of the *H. influenzae* type b (Hib) vaccine (2,3). Since 1988, when the conjugate vaccine was introduced, the incidence of invasive Hib disease has been reduced dramatically (4). However, Hib immunization has yet to be included in routine childhood vaccinations in many countries, including Thailand, and *H. influenzae* remains one of the most important causes of community-acquired pneumonia (CAP) (5). In the past, ampicillin had been recommended as the drug of choice for *H. influenzae* infection. However, the first ampicillin-resistant *H. influenzae* was reported in 1974 in several countries (6). The major mechanism of this resistance was the production of plasmid-mediated β-lactamases (7). Non-β-lactamase-mediated resistance to ampicillin in *H. influenzae* was first reported in the early 1980s (8). This β-lactamase-negative ampicillin-resistant (BLNAR) determinant was associated with the alteration of bacterial penicillin-binding proteins (PBPs) (9) as a result of *ftsI* gene mutation (10). Prevalence of BLNAR among *H. influenzae* has been increasing in various countries in Europe (11) and Asia (12). The increasing development of bacterial resistance would limit treatment options. This study aimed to investigate the current situation of antimicrobial susceptibility patterns and the prevalence of BLNAR among *H. influenzae* isolated from patients of a teaching hospital in Thailand.

*Corresponding author: Mailing address: Department of Clinical Microbiology, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen Province 40002, Thailand. Tel & Fax: +66-43-202086. E-mail: arolul@kku.ac.th

MATERIALS AND METHODS

Bacterial strains: Eighty-eight non-duplicate *H. influenzae* isolates were collected from patients at Srinagarind Hospital, a 900-bed hospital in northeastern Thailand, between September 2005 and March 2008. All isolates were identified by the X (hemin) and V (nicotinamide adenine dinucleotide) factors requirement

test using X, V, and XV paper strips (Becton and Dickinson, Sparks, Md., USA), the porphyrin test, and conventional biochemical tests (13).

Biotypes and capsular types: All isolates were characterized for biotype by indole, urease, and ornithine decarboxylase tests (13). In addition, their capsular types were determined using multiplex PCR assays for the serotype-specific capsular polysaccharide biosynthesis gene (*cap*) and transport gene (*bexA*), as described previously (14). Reference strains for specific serotypes of *H. influenzae* included the following: *H. influenzae* ATCC 9006 for serotype a, *H. influenzae* ATCC10211 for serotype b, *H. influenzae* ATCC 9007 for serotype c, *H. influenzae* ATCC 9332 for serotype d, *H. influenzae* ATCC 8142 for serotype e, and *H. influenzae* ATCC 9833 for serotype f.

Antimicrobial susceptibility testing: A disk diffusion test for 12 antimicrobial agents (Oxoid, Hampshire, UK)—ampicillin (10 μg), amoxicillin-clavulanate (20/10 μg), clarithromycin (15 μg), cefotaxime (30 μg), cefuroxime (30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), levofloxacin (5 μg), telithromycin (15 μg), tetracycline (30 μg), trimethoprim-sulfamethoxazole (1.25/23.75 μg), and meropenem (10 μg)—was performed on Haemophilus test medium (Oxoid), as per the Clinical and Laboratory Standards Institute (CLSI) (15), using *H. influenzae* ATCC 49247 as a drug-sensitive control.

β-Lactamase test: All isolates were tested for β-lactamase production using the penicillin disk cloverleaf method (16) and confirmed by using the nitrocefin stick test (Oxoid).

H. influenzae isolates that had an ampicillin inhibition zone diameter $\leq$18 mm (resistant) (15) but were negative for β-lactamase were judged to be BLNAR.

RESULTS

Source of clinical isolates: Among the 88 *H. influen-*

Table 1. Biotypes and capsular types of the *H. influenzae* isolates

Source of isolates (no.)	No. of isolates										
	Biotype				Capsular type						
	I	II	III	IV	Non-type	a	b	c	d	e	f
Respiratory tract (69)	20	35	11	3	68						1
Pus (13)	2	6	3	2	13						
Genital tract (4)		2	1	1	4						
Blood (2)	1	1			1						1
Total (88)	23	44	15	6	86						2

Table 2. Antimicrobial susceptibility of *H. influenzae* isolates

Source of isolates	No. of isolates	No. of isolates susceptible to each antimicrobial agent (%)											
		AMP	AMC	CHL	CIP	CLR	CTX	CXM	LEV	MEM	SXT	TEL	TET
β-lactamase-producing group													
Respiratory tract	29	0	29	17	29	28	29	29	29	29	5	29	16
Pus	4	0	4	3	4	4	4	4	4	4	3	4	2
Genital tract	3	0	3	2	3	3	3	3	3	3	2	3	2
Total	36 (40.9)	0 (0)	36 (100)	22 (61.1)	36 (100)	35 (97.2)	36 (100)	36 (100)	36 (100)	36 (100)	10 (27.8)	36 (100)	20 (55.6)
BLNAS group													
Respiratory tract	34	34	34	34	34	33	34	31	34	34	21	34	27
Pus	7	7	7	7	7	7	7	7	7	7	4	7	7
Genital tract	1	1	1	0	1	1	1	1	1	1	1	1	1
Blood	2	2	2	2	2	1	2	2	2	2	1	2	2
Total	44 (50)	44 (100)	44 (100)	43 (97.7)	44 (100)	42 (95.5)	44 (100)	41 (93.2)	44 (100)	44 (100)	27 (61.4)	44 (100)	37 (84.1)
BLNAR group													
Respiratory tract	6	0	6	4	6	5	6	6	6	6	2	6	2
Pus	2	0	2	2	2	1	2	2	2	2	0	2	1
Total	8 (9.1)	0 (0)	8* (100)	6 (75)	8 (100)	6 (75)	8* (100)	8* (100)	8 (100)	8 (100)	2 (25)	8 (100)	3 (37.5)
Total	88 (100)	44 (50)	88 (100)	71 (80.7)	88 (100)	83 (94.3)	88 (100)	85 (96.6)	88 (100)	88 (100)	39 (44.3)	88 (100)	60 (68.2)

AMP, ampicillin; AMC, amoxicillin-clavulanate; CHL, chloramphenicol; CIP, ciprofloxacin; CLR, clarithromycin; CTX, cefotaxime; CXM, cefuroxime; LEV, levofloxacin; MEM, meropenem; SXT, trimethoprim-sulfamethoxazole; TEL, telithromycin; TET, tetracycline; BLNAS, β-lactamase-negative ampicillin-susceptible; BLNAR, β-lactamase-negative ampicillin-resistant.
*CLSI recommends reporting as resistant, although the BLNAR strains give in vitro susceptibility to these agents.

zae isolates, 69 (78.4%) were from the respiratory tract, 13 (14.8%) from pus, 4 (4.5%) from the genital tract, and 2 (2.3%) from blood. Of these isolates, 80 (90.9%) were from adults, the remaining isolates were from children between 6 months and 6 years of age.

Biotypes and capsular types: The predominant biotype of the *H. influenzae* isolates was type II (44 isolates, 50%) followed by type I (23 isolates, 26.1%), type III (15 isolates, 17.1%), and type IV (6 isolates, 6.8%) as shown in Table 1. Using primers targeted to all 6 capsule-specific (*cap*) genes and the capsular export gene (*bexA*), most of the *H. influenzae* isolates were nontypeable strains (86 of 88 isolates, 97.7%). The prevalence of encapsulated *H. influenzae* was only 2.3% (2 of 88 isolates). The 2 isolates of *H. influenzae* were type f. One isolate was from a child's blood culture, whereas the other isolate was from an adult's sputum (Table 1).

Antimicrobial susceptibility and β-lactamase production: The in vitro activities of the 12 antimicrobial agents tested against the 88 *H. influenzae* isolates are summarized in Table 2. Amoxicillin-clavulanate, cefotaxime, ciprofloxacin, levofloxacin, telithromycin, and meropenem were the most active agents, and all isolates were fully susceptible (100%) to these agents. Susceptibility to cefuroxime was 96.6%, followed by clarithromycin (94.3%), chloramphenicol (80.7%), tetracycline (68.2%), ampicillin (50.0%), and trimethoprim-sulfamethoxazole (44.3%). Of the 88 isolates, 36 (40.9%) were β-lactamase positive ampicillin-resistant (BLPAR), 8 (18.2%) were BLNAR. All BLNAR *H. influenzae* isolates had no inhibition zone with the ampicillin disk, whereas the 2 representative isolates had an ampicillin MIC of 32 μg/ml (data not shown).

DISCUSSION

H. influenzae is one of the most common causes of meningitis and pneumonia in infants and children under 5 years old (2). Among the 6 serotypes of *H. influenzae* isolates, Hib is the most virulent (17). However, since the highly effective and safe protein-polysaccharide conjugate Hib vaccine was introduced in 1988, the incidence of Hib disease among children in this age group has decreased (18,19). Although the Hib vaccine is not included in routine childhood vaccination programs in our area, we found that most of the *H. influenzae* causing respiratory tract infections were noncapsulated strains, biotype II. This finding is consistent with several previous reports (1,20,21). In the present study, only 2 isolates were capsular type f biotype II, one of which was from a child. Serotype f and, to a lesser extent, serotype e have been reported predominantly among nontype b *H. influenzae* infections in both adults and children (22). Invasive infections caused by noncapsulated *H. influenzae* occurred mainly in neonates and elderly persons. The infection developed rapidly and followed a fulminant course with a high fatality rate (23). The clinical presentation of both serotype f and serotype e *H. influenzae* diseases was similar to that of noncapsulated *H. influenzae* infections in that almost 50% of cases occurred among persons ≥65 years of age (24). Most patients (78%) included in this study were diagnosed with pneumonia, and 3.5% of these pneumonia patients were children. Unfortunately, patient information and clinical outcome for each case in the present study were not available.

The antimicrobial susceptibility test showed a high proportion of resistance to trimethoprim-sulfamethoxazole (55.7%), followed by ampicillin (50.0%) and tetracycline (31.8%). Multidrug-resistance (resistant to 3 or more antimicrobial agents) among these isolates was 20.5%. β-Lactamase production reported in the present study was quite high (40.9%) compared to that in Germany (3%) (25,26), but was lower than that in Korea (65%) (26). Resistance to β-lactams among *H. influenzae* is mainly mediated by constitutive β-lactamase production. To date, only 2 types of β-lactamases have been described for this pathogen: TEM-1 and ROB-1 (7,27). Although there are geographical differences, TEM-1 is considerably more prevalent than ROB-1 among β-lactamase-positive *H. influenzae* strains worldwide (28). Both enzymes belong to class A serine β-lactamases and confer high-level resistance to penicillin and ampicillin, but ROB-1 confers additional resistance to cefaclor (29). In the present study, only 18.2% of the ampicillin-resistant strains were β-lactamase non-producers (BLNAR). This, however, may be an underestimation since the disk diffusion assay cannot detect *H. influenzae* strains with low BLNAR. High-BLNAR isolates could effectively be identified by the standard disk diffusion method, whereas low-BLNAR isolates could be characterized as ampicillin susceptible (30). Therefore, CLSI has recommended the microbroth dilution method for BLNAR detection among *H. influenzae* isolates (15). However, some low-BLNAR isolates have shown a low ampicillin MIC (0.5 μg/ml) (30). Thus, the suspected BLNAR isolates should be further confirmed by PCR amplification and nucleotide sequence analysis of the *ftsI* gene mutation (10,31,32). Prevalence of BLNAR has been reported differently in various countries worldwide, ranging from 0.3% in Western European countries (33) to 40% in Japan (12). This resistance is associated with lowered affinity to β-lactams by alteration of PBPs 3, 4, and 5 (34). Such alteration leads to the loss of susceptibility to aminopenicillin alone or combined with β-lactamase inhibitors, such as amoxicillin-clavulanate and some cephalosporins (cefaclor, cefetamet, cefonicid, cefprozil, cefuroxime, and loracarbef). CLSI recommends reporting such altered *H. influenzae* as resistant, although the BLNAR strains have in vitro susceptibility to these agents (15). It has been reported that 60%–73% of BLNAR isolates were resistant to amoxicillin-clavulanate (30,32). All the BLNAR isolates in the present study were susceptible to amoxicillin-clavulanate. This may be because small number of *H. influenzae* isolates were included in this study.

In summary, this study showed the complete susceptibility of *H. influenzae* isolates to amoxicillin-clavulanate, cefotaxime, ciprofloxacin, levofloxacin, meropenem, and telithromycin. These agents are therefore highly active against *H. influenzae* and are most suitable for empirical use. However, a number of BLNAR isolates were detected among *H. influenzae* in this area. Treatment with β-lactams in such cases should be considered. Therefore, reports of BLNAR would provide useful information for appropriate antimicrobi-

al therapy.

Acknowledgments This work was partially supported by Khon Kaen University.

We thank: (1) the staff of the Clinical Microbiology Laboratory Unit of Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, for collecting the clinical isolates; (2) the Faculty of Associated Medical Sciences and Centre for Research and Development of Medical Diagnostic Laboratories (CMDL), Khon Kaen University, for their support; and (3) Prapaphan Srimunta, Marinee Watcharasutanon, Sukanya Suttiboriban, and Jaikaew Numchumpa, for their technical help.

Conflict of interest None to declare.

REFERENCES

1. Ladhani, S., Slack, M.P., Heath, P.T., et al. (2010): Invasive *Haemophilus influenzae* disease, Europe, 1996–2006. Emerg. Infect. Dis., 16, 455–463.
2. Watt, J.P., Wolfson, L.J., O'Brien, K.L., et al. (2009): Burden of disease caused by *Haemophilus influenzae* type b in children younger than 5 years: global estimates. Lancet, 374, 903–911.
3. Peltola, H. (1999): Spectrum and burden of severe *Haemophilus influenzae* type b diseases in Asia. Bull. World Health Organ., 77, 878–887.
4. Peltola, H. (1998): *Haemophilus influenzae* type b disease and vaccination in Europe: lessons learned. Pediatr. Infect. Dis. J., 17 (9 Suppl.), S126–132.
5. Apisarnthanarak, A. and Mundy, L.M. (2005): Etiology of community-acquired pneumonia. Clin. Chest Med., 26, 47–55.
6. Thornsberry, C. and Kieven, L.A. (1974): Antimicrobial susceptibility of *Haemohpilus influenzae*. Antimicrob. Agents Chemother., 6, 620–624.
7. Medeiros, A.A. and O'Brien, T.F. (1975): Ampicillin-resistant *Haemophilus influenzae* type b possessing a TEM-type β-lactamase but little permeability barrier to ampicillin. Lancet, I, 716–719.
8. Markowitz, S.M. (1980): Isolation of an ampicillin-resistant, non-β-lactamase-producing strain of *Haemophilus influenzae*. Antimicrobial. Agents Chemother., 17, 80–83.
9. Parr, T.R., Jr. and Bryan, L.E. (1984): Mechanism of resistance of an ampicillin-resistant, β-lactamase-negative clinical isolate of *Haemophilus influenzae* type b to β-lactam antibiotics. Antimicrob. Agents Chemother., 25, 747–753.
10. Ubukata, K., Shibasaki, Y., Yamamoto, K., et al. (2001): Association of amino acid substitutions in penicillin-binding protein 3 with β-lactam resistance in β-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. Antimicrob. Agents Chemother., 45, 1693–1699.
11. Fluit, A.C., Florijn, A., Verhoef, J., et al. (2005): Susceptibility of European β-lactamase-positive and -negative *Haemophilus influenzae* isolates from the periods 1997/1998 and 2002/2003. J. Antimicrob. Chemother., 56, 133–138.
12. Hasegawa, K., Yamamoto, K., Chiba, N., et al. (2003): Diversity of ampicillin-resistance genes in *Haemophilus influenzae* in Japan and the United States. Microb. Drug Resist., 9, 39–46.
13. Garcia, L.S., Procop, G.W., Roberts, G.D., et al. (1998): *Haemophilus*. p. 556–563. *In* Forbes, B.A., D.F. Sahm, and A.S. Weissfeld (eds.), Diagnostic Microbiology. 10th ed. Mosby, St. Louis, USA.
14. Falla, T.J., Crook, D.W., Brophy, L.N., et al. (1994): PCR for capsular typing of *Haemophilus influenzae*. J. Clin. Microbiol., 32, 2382–2386.
15. Clinical and Laboratory Standards Institute (2010): Performance standards for antimicrobial susceptibility testing, 30th informational supplement M100-S20. Clinical and Laboratory Standards Institute, Wayne, Pa., USA.
16. Lee, W.-S. and Komarmy, L. (1976): New method for detecting in vitro inactivation of penicillins by *Haemophilus influenzae* and *Staphylococcus aureus*. Antimicrob. Agents Chemother., 10, 564–566.
17. Centers for Disease Control and Prevention (2008): Continued shortage of *Haemophilus influenzae* type b (Hib) conjugate vaccines and potential implications for Hib surveillance—United States, 2008. Morbid. Mortal. Wkly. Rep., 57, 1252–1255.
18. Murphy, T.V., White, K.E., Pastor, P., et al. (1993): Declining incidence of *Haemophilus influenzae* type b disease since introduction of vaccination. JAMA, 269, 246–248.
19. Bisgard, K.M., Kao, A., Leake, J., et al. (1998): *Haemophilus influenzae* invasive disease in the United States, 1994–1995: near disappearance of a child vaccine preventable disease. Emerg. Infect. Dis., 4, 229–237.
20. Huong, P.L.T., Thi, N.T., Anh, D.D., et al. (2006): Genetic and phenotypic characterization of *Haemophilus influenzae* type b isolated from children with meningitis and their family members in Vietnam. Jpn. J. Infect. Dis., 59, 111–116.
21. Mojgani, N., Rahbar, M., Taqizadeh, M., et al. (2011): Biotyping, capsular typing, and antibiotic resistance pattern of *Haemophilus influenzae* strains in Iran. Jpn. J. Infect. Dis., 64, 66–68.
22. Dworkin, M.S., Park, L. and Borchardt, S.M. (2007): The changing epidemiology of invasive *Haemophilus influenzae* disease, especially in persons $\geq$65 years old. Clin. Infect. Dis., 44, 810–816.
23. Heath, P.T., Booy, R., Azzopardi, H.J., et al. (2001): Non-type b *Haemophilus influenzae* disease: clinical and epidemiologic characteristics in the *Haemophilus influenzae* type b vaccine era. Pediatr. Infect. Dis. J., 20, 300–305.
24. Campos, J., Hernando, M., Roman, F., et al. (2004): Analysis of invasive *Haemophilus influenzae* infections after extensive vaccination against *H. influenzae* type b. J. Clin. Microbiol., 42, 524–529.
25. Tristram, S., Jacobs, M.R. and Appelbaum, P.C. (2007): Antimicrobial resistance in *Haemophilus influenzae*. Clin. Microbiol. Rev., 20, 368–389.
26. Hoban, D. and Felmingham, D. (2002): The PROTEKT surveillance study: antimicrobial susceptibility of *Haemophilus influenzae* and *Moraxella catarrhalis* from community-acquired respiratory tract infections. J. Antimicrob. Chemother., 50 (Suppl. S1), 49–59.
27. Rubin, L.G., Medeiros, A.A., Yolken, R.H., et al. (1981): Ampicillin treatment failure of apparently β-lactamase-negative *Haemophilus influenzae* type b meningitis due to novel β-lactamase. Lancet, Ii, 1008–1010.
28. Farrell, D.J., Morrissey, I., Bakker, S., et al. (2005): Global distribution of TEM-1 and ROB-1 β-lactamases in *Haemophilus influenzae*. J. Antimicrob. Chemother., 56, 773–776.
29. Karlowsky, J.A., Verma, G., Zhanel, G.G., et al. (2000): Presence of ROB-1 β-lactamase correlates with cefaclor resistance among recent isolates of *Haemophilus influenzae*. J. Antimicrob. Chemother., 45, 871–875.
30. Garcia-Cobos, S., Campos, J., Roman, F., et al. (2008): Low β-lactamase-negative ampicillin-resistant *Haemophilus influenzae* strains are best detected by testing amoxicillin susceptibility by the broth microdilution method. Antimicrob. Agents Chemother., 52, 2407–2414.
31. Harimaya, A., Yokota, S., Sato, K., et al. (2008): Remarkably high prevalence of *ftsI* gene mutations in *Haemophilus influenzae* isolates from upper respiratory tract infections in children of the Sapporo district, Japan. J. Infect. Chemother., 14, 223–227.
32. Bae, S., Lee, J., Lee, J., et al. (2010): Antimicrobial resistance in *Haemophilus influenzae* respiratory tract isolates in Korea: results of a nationwide acute respiratory infections surveillance. Antimicrob. Agents Chemother., 54, 65–71.
33. Kayser, F.H., Morenzoni, G. and Santanam, P. (1990): The second European collaborative study on the frequency of antimicrobial resistance in *Haemophilus influenzae*. Euro. J. Clin. Microbiol. Infect. Dis., 9, 810–817.
34. Mendelman, P.M., Chaffin, D.O. and Kalaitzoglou. G. (1990): Penicillin binding proteins and ampicillin resistance in *Haemophilus influenzae*. J. Antimicrob. Chemother., 25, 525–534.