Original Article

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

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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), cefturoxime (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.

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 ≤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-
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 non-typeable 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, 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-lactamas 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-lactamas 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-lactam 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-
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Conflict of interest None to declare.

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