

# HAEMOPHILUS INFLUENZAE FROM PATIENTS AT THE LARGEST UNIVERSITY TERTIARY CARE CENTER, THAILAND 2012 - 2015

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**Abstract.** *Haemophilus influenzae* was isolated from 556 different patients, mostly 10 years or under, at a tertiary referral hospital in Bangkok, Thailand during 2012 - 2015. Peak period of detection was from January to March. Thirty-nine percent of the isolates were  $\beta$ -lactamase positive.  $\beta$ -Lactamase-negative ampicillin-resistant *H. influenzae* (BLNAR) constituted 2% of  $\beta$ -lactamase-negative cases. *H. influenzae* was susceptible to ampicillin (58%), amoxicillin/clavulanate (99%), cefotaxime (100%), ceftriaxone (100%), cefuroxime (99%), ciprofloxacin (99%), chloramphenicol (86%), tetracycline (75%), and trimethoprim-sulphamethoxazole (52%).  $\beta$ -Lactamase-producing isolates (72%) showed high minimal inhibitory concentration (MIC) to ampicillin (128-516  $\mu$ g/ml) and all BLNAR isolates low ampicillin MIC (2-16  $\mu$ g/ml). These findings indicate that the level of ampicillin resistance in *H. influenzae* depended on differences in resistance mechanism.

**Keywords:** *Haemophilus influenzae*, antibiogram,  $\beta$ -lactamase, Thailand

## INTRODUCTION

*Haemophilus influenzae* is an important pathogen causing various types of infections, including community-acquired pneumonia, acute otitis media and acute epiglottitis (Tristram *et al*, 2007; WPRO, 2012). *H. influenzae* is also one of the most important pathogens that causes bacteraemia and meningitis in children (Srifuengfung *et al*, 2007; Shuel and Tsang, 2009). *H. influenzae* serotype b (Hib) is responsible for 8 million cases of serious illness and

an estimated 371,000 deaths annually (WPRO, 2012). *H. influenzae* is especially of concern in developing countries, compared to its lesser overall impact in developed countries (WPRO, 2012).

Currently use of Hib conjugate vaccine in Thailand is extremely limited (mostly in private hospitals), as it is not on the Expanded Program of Immunization for Thailand due to its high cost (Srifuengfung *et al*, 2016). Treatment of *H. influenzae* is primarily by  $\beta$ -lactams, but resistance is a problem, with resistance to ampicillin varying from 10% to 60%, depending on geographic region, and is predominantly mediated by TEM-1 or ROB-1  $\beta$ -lactamase (Bae *et al*, 2010). Another cause of  $\beta$ -lactam resistance is mutation in *ftsI* encoding penicillin-binding protein 3 (PBP3), which leads to altered PBP with low affinity

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for  $\beta$ -lactams, known as  $\beta$ -lactamase-negative ampicillin-resistant *H. influenzae* (BLNAR) (Shuel and Tsang, 2009). BLNAR is relatively rare in North America, but high prevalence has been documented in Japan and Korea (Hasegawa *et al*, 2006; Kim *et al*, 2007; Shuel and Tsang, 2009). Rare *H. influenzae* isolates containing both  $\beta$ -lactamase and PBP3 mutation have been reported (Tristram *et al*, 2007). The prevalence of BLNAR warrants added attention during treatment because BLNAR should be considered resistant to amoxicillin-clavulanate, ampicillin-sulbactam, cefaclor, cefamandole, cefatamet, cefonicid, cefprozil, cefuroxime, loracarbef, and piperacillin-tazobactam, despite apparent *in vitro* susceptibility (CLSI, 2015).

This study determined prevalence and antibiogram profiles of *H. influenzae* isolates obtained from patients at a national tertiary referral hospital in Thailand during 2012 to 2015. Such data are important to avoid empiric therapy so that further development of drug resistance is minimized.

## MATERIALS AND METHODS

### Source of samples

Five hundred and fifty-six *H. influenzae* isolates from patients were obtained from the Bacteriology Laboratory, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand during January 1, 2012 - December 31, 2015. Siriraj Hospital is a tertiary care university hospital and the largest hospital in Thailand, with 2,600 beds and 100,000 admissions yearly. Multiple isolates from different sites in the same patient were counted as a single sample. Sputum was acceptable for culture if it contained > 25 polymor-

phonuclear cells and < 25 epithelial cells per light microscope field (10x10 = 100x magnification). For quantitative culture of bronchoalveolar lavage fluid (BAL), a 1- $\mu$ l standard calibrated loop was used and a cut-off value of 10<sup>4</sup> CFU/ml *H. influenzae* is interpreted as a positive culture. *H. influenzae* from various clinical specimens was isolated and identified by typical colony morphology and X and V growth factor requirements according to standard microbiological techniques (Tille, 2014). Isolates were stored at -70°C in 5% brain-heart infusion broth containing 20% (v/v) glycerol until used.

### Determination of antibiogram profile

Antimicrobial susceptibility tests of 9 antimicrobial agents (Table 2) were performed using a disk diffusion method (CLSI, 2015) and *Haemophilus* Test Medium (HTM) (Oxoid, Hampshire, UK). Minimal inhibitory concentration (MIC) of ampicillin was performed by E-test (AB Biodisk, Solna, Sweden). *H. influenzae* ATCC 49247 was used as control.  $\beta$ -Lactamase-negative isolates showing an inhibition zone diameter  $\leq$  18 mm (resistant to ampicillin) are considered BLNAR (CLSI, 2015).

### Assay for $\beta$ -lactamase and *bla*<sub>TEM</sub>

Test for  $\beta$ -lactamase was performed using nitrocefin disk (Becton, Dickinson, Franklin Lakes, NJ). *H. influenzae* ATCC 49247 and *Staphylococcus aureus* ATCC 25923 was used as a negative and a positive control, respectively.

Presence of *bla*<sub>TEM</sub> (in 30 randomly selected  $\beta$ -lactamase-producing *H. influenzae* isolates) was detected by PCR. Total genomic DNA was extracted from a loopful of colonies of each isolate taken from 16-18 hour culture on chocolate agar at 35°C under an atmosphere of 5% CO<sub>2</sub> using Puregene DNA Purifi-

Table 1  
Prevalence of  $\beta$ -lactamase and  $\beta$ -lactamase-negative ampicillin-resistant *Haemophilus influenzae* (BLNAR) from patients, Siriraj Hospital, Bangkok, 2012-2015.

| Year  | Age of patients   | Male: female | Number of isolates | $\beta$ -Lactamase status |                   | BLNAR<br>n (%) <sup>a</sup> |
|-------|-------------------|--------------|--------------------|---------------------------|-------------------|-----------------------------|
|       |                   |              |                    | Positive<br>n (%)         | Negative<br>n (%) |                             |
| 2012  | 1 month-92 years  | 1.1:1.0      | 141                | 58 (41)                   | 83 (59)           | 1 (1)                       |
| 2013  | 1 month-94 years  | 1.1:1.0      | 144                | 49 (34)                   | 95 (66)           | 3 (3)                       |
| 2014  | 10 days-96 years  | 1.2:1.0      | 155                | 61 (39)                   | 94 (61)           | 2 (2)                       |
| 2015  | 2 months-88 years | 0.9:1.0      | 116                | 47 (40)                   | 69 (60)           | 2 (3)                       |
| Total |                   |              | 556                | 215 (39)                  | 341 (61)          | 8 (2)                       |

<sup>a</sup>Relative to  $\beta$ -lactamase negative.

cation Kit (Genra Systems, Big Lake, MN). Primers were TEM-F (5'-ATGAG-TATTCAACATTTCCG-3') and TEM-R (5'-CTGACAGTTACCAATGCTTA-3') (Rasheed *et al*, 1997). PCR was conducted in 25- $\mu$ l reaction mixture containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 0.5  $\mu$ M each primer, 200  $\mu$ M each dNTP, and 1 U *Taq* DNA polymerase (DyNAzyme™ II, Roche, Indianapolis, IN). Thermocycling was performed in a GeneAmp PCR System 2400 (Perkin-Elmer, San Jose, CA) as follows: 94°C for 5 minutes; followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute; with a final heating at 72°C for 7 minutes. DNA of pUC19 containing *bla*<sub>TEM</sub> was used as a positive control. Amplicons (863 bp) were analyzed by 1% agarose gel-electrophoresis, and one randomly selected amplicon was cloned using StrataClone PCR Cloning Kit (Agilent Technologies, Stratagene Products Division, Santa Clara, CA). DNA insert was sequenced using TEM-F and TEM-R primers (BioDesign, Pathum Thani, Thailand). DNA sequence was analyzed using BLAST software (<http://blast.ncbi.nlm.nih.gov/BLAST>) and multiple sequence

alignment using Bioedit program (<http://bioedit.software.informer.com/7.2/>). The sequence of our full length *bla*<sub>TEM-1</sub> was 100% identical to *E. coli* plasmid pC15-1a and was deposited with GenBank, accession no. NC005327 (Phoomniyom, 2011).

## RESULTS

Patients' age ranged from 10 days to 96 years (Table 1). Mean age ( $\pm$  standard deviation) was 41.5 ( $\pm$  30.9) years. Sources of clinical specimens were sputum (61%), adenoid biopsy tissue (11%), bronchus/bronchial wash/BAL (10%), eye (7%), cervix/vagina (4%), ear (4%), sinus aspirate (2%), nasal pus/discharge (1%), and cerebrospinal fluid, pleural fluid, pus, and nasal mass (< 1% each). For BAL quantitative culture, a range of *H. influenzae* from 2x10<sup>4</sup> to  $\geq$  10<sup>5</sup> CFU/ml were obtained, with  $\geq$  10<sup>5</sup> CFU/ml being the most common bacterial count.

Two hundred and fifteen of 556 (39%) *H. influenzae* samples were  $\beta$ -lactamase positive, with BLNAR prevalence of 1%. *H. influenzae* was isolated mostly from patients aged  $\leq$  10 years (Fig 1) and the highest median number of *H. influenzae*

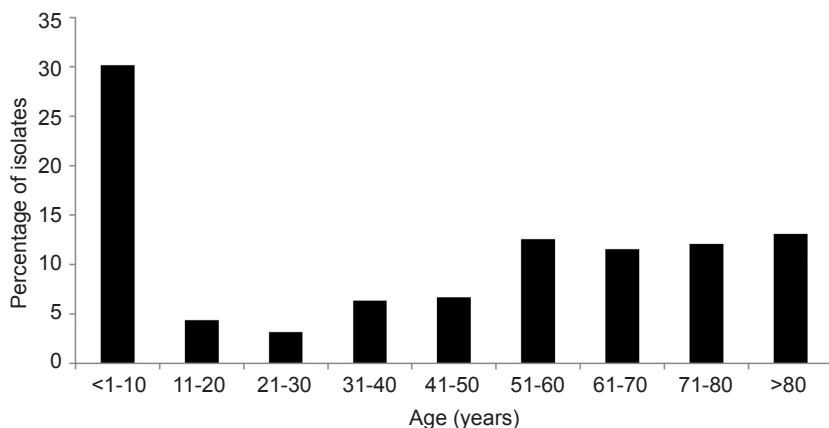


Fig 1—Age distribution of *Haemophilus influenzae*-infected patients, Siriraj Hospital, Bangkok, 2012-2015.

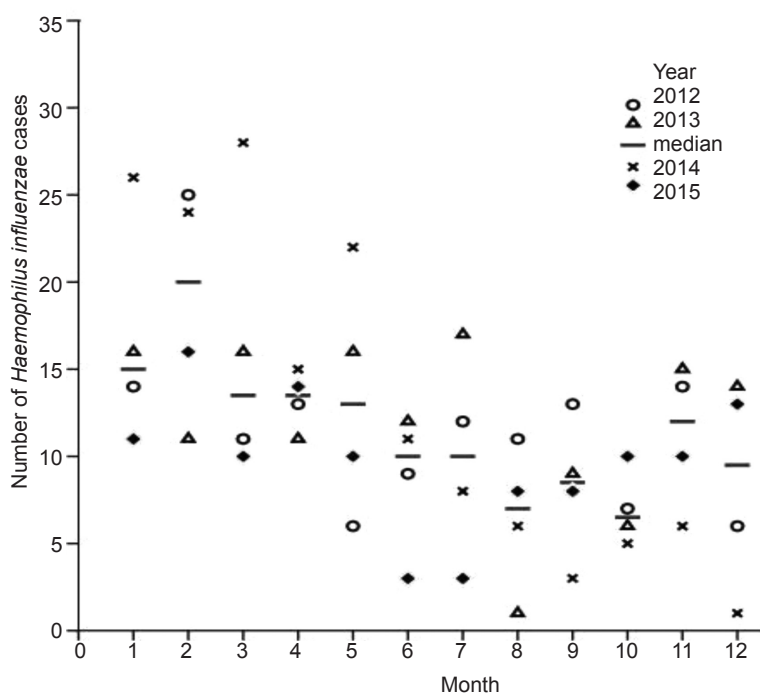


Fig 2—Monthly prevalence of *Haemophilus influenzae* cases, Siriraj Hospital, Bangkok, 2012-2015.

cases was obtained in February (month 2) over the period of the survey (Fig 2).

The majority (> 50%) of *H. influenzae* was susceptible to ampicillin, amoxicillin/clavulanate, cefotaxime, ceftriax-

one, cefuroxime, ciprofloxacin, chloramphenicol, tetracycline, and trimethoprim-sulphamethoxazole, and highest resistance (47%) to trimethoprim-sulphamethoxazole (Table 2).  $\beta$ -Lactamase-producing isolates (73%) showed high MIC (128 -516  $\mu\text{g/ml}$ ) and all BLNAR isolates showed low MIC (2-16  $\mu\text{g/ml}$ ) to ampicillin. Antibigram profiles of BLNAR isolates for the other drugs indicated susceptibility to amoxicillin/clavulanate (25%), cefotaxime (100%), ceftriaxone (100%), cefuroxime (75%), ciprofloxacin (100%), chloramphenicol (100%), tetracycline (100%), and trimethoprim-sulphamethoxazole (37.5%).

All 30 randomly picked *H. influenzae* isolates tested positive for  $\beta$ -lactamase carried blaTEM (data not shown). DNA sequence of one 863-bp amplicon revealed 100% nucleotide and amino acid sequence

(coding region of 286 amino acids) identity with published sequence of bla<sub>TEM-1</sub> of a number of bacteria, eg, *Enterobacter aerogenes* plasmid pYS10  $\beta$ -lactamase (data not shown).

Table 2  
Antibiogram profile of 556 *Haemophilus influenzae* isolates from patients, Siriraj Hospital, Bangkok, 2012-2015.

| Antimicrobial agent                         | Susceptibility, n (%) |              |            |
|---|-----------------------|--------------|------------|
|   | Susceptible           | Intermediate | Resistant  |
| Ampicillin (10 µg)                          | 323 (58)              | 1 (< 1)      | 232 (41)   |
| Amoxicillin/clavulanate <sup>a</sup>        | 550 (99)              | -            | 6 (1)      |
| Cefotaxime (30 µg)                          | 556 (100)             | -            | -          |
| Ceftriaxone (30 µg)                         | 556 (100)             | -            | -          |
| Cefuroxime (30 µg)                          | 548 (98)              | 2 (< 1)      | 6 (1)      |
| Ciprofloxacin (5 µg)                        | 551 (99)              | -            | 5 (1)      |
| Chloramphenicol (30 µg)                     | 479 (86)              | -            | 77 (14)    |
| Tetracycline (30 µg)                        | 418 (75)              | 30 (5.5)     | 108 (19.5) |
| Trimethoprim-sulphamethoxazole <sup>b</sup> | 288 (52)              | 4 (1)        | 264 (47)   |

<sup>a</sup>20/10 µg; <sup>b</sup>1.25/23.75 µg.

## DISCUSSION

This study confirms a previous report that *H. influenzae* is common in pediatric patients and that respiratory tract was the predominant specimen source (Barbosa *et al*, 2011). Globally, β-lactamase positivity of *H. influenzae* is 15%, but varies greatly by country [eg, < 5% in several countries to 35.8% in China (comparable to this study), 52.4% in Korea and 67.9% in Taiwan] (Farrell *et al*, 2005; Bae *et al*, 2010; Luo *et al*, 2012).

BLNAR prevalence of 9.6% was reported in Europe (Fluit *et al*, 2005) and as high as 22.2% in Japan (Hasegawa *et al*, 2006). This suggests that distribution of BLNAR varies from region to region around the world. BLNAR prevalence in this study declined from 4.4% reported in the 2007-2011 study conducted at Siriraj Hospital for 638 *H. influenzae* clinical isolates (Srifuengfung *et al*, 2016). A recent multicenter study of acute otitis media patients in Thailand indicated a 5% prevalence of BLNAR (Intakorn *et al*, 2014). An

18.2% prevalence of BLNAR was reported in a university teaching hospital in north-eastern Thailand (Lulitanond *et al*, 2012). Reasons for these variations in prevalence have not been explored. Nonetheless, finding of BLNAR should be of concern to clinicians regarding appropriate treatment of these patients (Eldere *et al*, 2014).

BLNAR isolates are divided into 3 groups: Group I containing in PBP3 M377, S385, L389, H517 and N526; Group II with M377, S385, L389, R517 and K526; and Group III with I377, T385, F389, R517, and K526 (Ubukata *et al*, 2001). Group I and II isolates have intermediate ampicillin resistance (0.5-2 µg/ml) and group III demonstrates a higher level of ampicillin resistance (1-16 µg/ml) (Hasegawa *et al*, 2003). Srifuengfung *et al* (2016) reported BLNAR group II in Thailand, as was found in the current study (data not shown).

Our antibiogram profiles of *H. influenzae* were similar to those reported from a worldwide collection of *H. influenzae* isolates ( $n = 8,523$ ) from the Alexander

Project (Tristram *et al*, 2007) and from a study in China (Luo *et al*, 2012). The observation that ampicillin MIC values of  $\beta$ -lactamase-producing *H. influenzae* isolates were in general higher than those of BLNAR was in agreement with other studies in  $\beta$ -lactamase (TEM-1)-producing *H. influenzae* isolates (Bae *et al*, 2010; Barbosa *et al*, 2011), and with those of ampicillin MIC values of BLNAR isolates (Barbosa *et al*, 2011; Dabernat *et al*, 2012). It is well-documented that  $\beta$ -lactamase TEM-1 is the main mechanism for ampicillin resistance in *H. influenzae* (Tristram *et al*, 2007; Luo *et al*, 2012).

In conclusion, surveillance of prevalence of  $\beta$ -lactamase-producing *H. influenzae* and BLNAR, as well as the current status of antimicrobial susceptibility, should be continued to be conducted to evaluate current trends. Of note, BLNAR could transfer its mutant *ftsI* to  $\beta$ -lactamase-negative ampicillin-susceptible isolates via horizontal gene transfer, resulting in a rapid increase BLNAR prevalence (Takahata *et al*, 2007). Thus careful surveillance of BLNAR and studies of genetic properties will help to treat and control this infection.

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#### CONFLICT OF INTERESTS

The authors declare no conflict of interests pertaining to any aspect of this study.

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