

Molecular characterization and antibiotic susceptibility of *Haemophilus influenzae* clinical isolates

Hüseyin Kılıç¹, Selcan Akyol², Ömür Mustafa Parkan¹, Gökçen Dinç¹, Hafize Sav³, Gonca Aydemir¹

¹Department of Medical Microbiology, Erciyes University Medical Faculty, Kayseri, Turkey;

²Department of Medical Microbiology, Istanbul University Cerrahpaşa Medical Faculty, Istanbul, Turkey;

³Department of Medical Microbiology, Kayseri Training and Research Hospital, Kayseri, Turkey

SUMMARY

Haemophilus influenzae can cause invasive and severe infections in both adults and children such as otitis media, sinusitis, pneumonia, meningitis and bacteremia. The emerging antibiotic resistance in recent years against ampicillin and several other antibiotics among strains of *H. influenzae* gives cause for serious concern. Here we investigate β -lactamase (BL) activity in clinical isolates of *H. influenzae*, profile their resistance to antibiotics, and characterize the clonal relationship of the isolates. Antibiotic susceptibilities of 92 clinical isolates of *H. influenzae* (March 2011 - May 2012) were determined using the disk diffusion method according to the Clinical & Laboratory Standards Institute (CLSI), and BL activity was detected using the nitrocefin disk method. The Rep-PCR method was used to characterize clonality of the isolates. All strains were found to be susceptible to levofloxacin and cefotaxime. Four isolates out of 92 (4.3%) were found resistant to ampicillin, one isolate (1.1%) was resistant to amoxicillin/clavulanic acid, 21 isolates (22.8%) were resistant to trimethoprim-sulfamethoxazole (SXT), and three isolates (3.3%) showed BL activity. One strain was BL-negative but resistant to ampicillin. The three isolates with BL activity and four isolates with resistance to ampicillin did not have a clonal relationship. Three distinct clones [clone A (with subclones A1 and A2), clone B, and clone C] were identified among the SXT-resistant strains. Most of the *H. influenzae* isolates in this study were susceptible to the antibiotics while SXT resistance was relatively more prevalent, which suggests that significant obstacles in the therapeutic use of antibiotics against *H. influenzae* strains are not expected in our region.

Keywords: *Haemophilus influenzae*, rep-pcr, beta-lactamase.

INTRODUCTION

Haemophilus influenzae is a Gram-negative opportunistic bacterium and is a part of the commensal microbiota of the upper respiratory tract of many healthy people [1]. *H. influenzae* can cause invasive and severe infections in both adults and children such as meningitis, bacteremia, otitis media, sinusitis, and pneumonia [2, 3].

Patients infected with *H. influenzae* are treated with antibiotics such as ampicillin, second and third generation cephalosporins, and quinolones [4]. The first β -lactamase-positive ampicillin resistant (BLPAR) case was reported in 1974, rapidly spread thereafter [5]. Epidemiological studies indicated that 8% to 30% of isolates in Europe and North America, and up to 50% of isolates in East Asia were resistant to ampicillin [6, 7]. The β -lactamase (BL) enzymes, TEM-1 or ROB-1, are responsible for resistance against ampicillin and can be active on all β -lactam antibiotics [8]. Another

Corresponding author

Ömür Mustafa Parkan

E-mail: omurparkan@hotmail.com

er mechanism responsible for antibiotic resistance in these isolates is due to mutations in penicillin binding proteins (PBPs). Mutations in the penicillin binding protein 3 (PBP3) leading to emergence of BL-negative ampicillin-resistant (BLNAR) isolates were first characterized in 1980s [9]. The BLNARs are now more frequently isolated in both Europe and Asia [4]. This increasing trend of antibiotic resistance among clinical isolates of *H. influenzae* has raised the importance of screening clinical isolates using *in vitro* susceptibility tests. Here, we investigate BL activity in clinical isolates of *H. influenzae*, profile their resistance to antibiotics, and characterize clonal relationship of the isolates.

■ MATERIALS AND METHODS

Bacterial strains and characterizations

Ninety-two *H. influenzae* strains isolated from clinical specimens collected during March 2011-May 2012 were included in the study. The isolates were identified in terms of colony morphology, Gram staining, their requirements for X and V growth factors (X, V and XV factor disks; Oxoid, United Kingdom), and biochemical reactions by using API NH panel (bioMérieux, France). The identified isolates were then further confirmed using polyvalent *Haemophilus influenzae* antiserum (Difco™; Becton Dickinson, Ireland).

Antimicrobial susceptibility testing

Each isolate was tested for BL activity by the nitrocefin disk method (BBL™; Becton Dickinson, USA). Isolates causing pink to red colors when reacted with nitrocefin disk were assumed to be BL positive (*Staphylococcus aureus* ATCC 29213 used as positive control and *H. influenzae* ATCC 10211 as negative control). Susceptibility tests were performed according to Clinical and Laboratory

Standards Institute (CLSI) standards (disk diffusion method) using *Haemophilus* Test Medium (Oxoid, United Kingdom). Briefly, bacterial suspensions (McFarland 0.5) from cultures grown for 24-hrs were prepared and spread onto the plates in sterile environment and disks of ampicillin (10 µg), amoxicillin/clavulanic acid (30 µg), cefotaxime (30 µg), levofloxacin (5 µg), trimethoprim/sulfamethoxazole (SXT) (1.25/23.75 µg), erythromycin (15 µg), doxycycline (30 µg), cefazolin (30 µg) were quickly placed. The plates were then placed in incubator (5% CO₂, 18 hrs, 35°C). The CLSI standards (M 100-S21) were used to interpret the results.

Genotyping

Rep-PCR was used to determine genetic relationship of the resistant strains of *H. influenzae* (DiversiLab, bioMérieux, France). Once the pure cultures of the strains were obtained the genotyping was performed in four steps including manual genomic DNA extraction, rep-PCR on thermocycler using finger-printing kits, profiling of DNA bands using microfluidic based bioanalyzer, and rapid interpretation using internet-based software. Dendrogram of similarity of the isolates and gel-like images were generated using DiversiLab software (version 3.4).

■ RESULTS

The 92 clinically significant *H. influenzae* strains were isolated from clinical specimens comprising 50 sputum (54.3%), 16 bronchoalveolar lavage fluid (17.4%), 16 endotracheal aspirates (17.4%), 4 blood cultures (4.3%), three ear swabs (3.3%), one cerebrospinal fluid (1.1%), one eye swab (1.1%), and one nasotracheal aspirate (1.1%). BL activity was detected in only 3 (3.3%) isolates. One strain was found to be BL-negative but ampicillin-re-

Table 1 - Antibiotic resistance profiles of *H. influenzae* isolates in this study.

| Antibiotic | Susceptible | Intermediate | Resistant |
|-------------------------------|-------------|--------------|------------|
| Ampicillin | 88 (95.7%) | - | 4 (4.3%) |
| Amoxicillin/clavulanic acid | 91 (98.9%) | - | 1 (1.1%) |
| Cefotaxime | 92 (100%) | - | - |
| Levofloxacin | 92 (100%) | - | - |
| Trimethoprim/sulfamethoxazole | 69 (75%) | 2 (2.2%) | 21 (22.8%) |

Figure 1 - Clonal relationship of *H. influenzae* strains with BL activity.

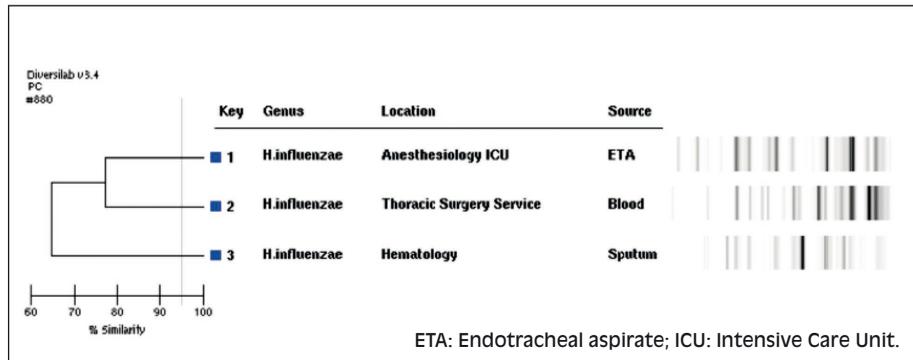


Figure 2 - Clonal relationship of SXT-resistant *H. influenzae* strains.

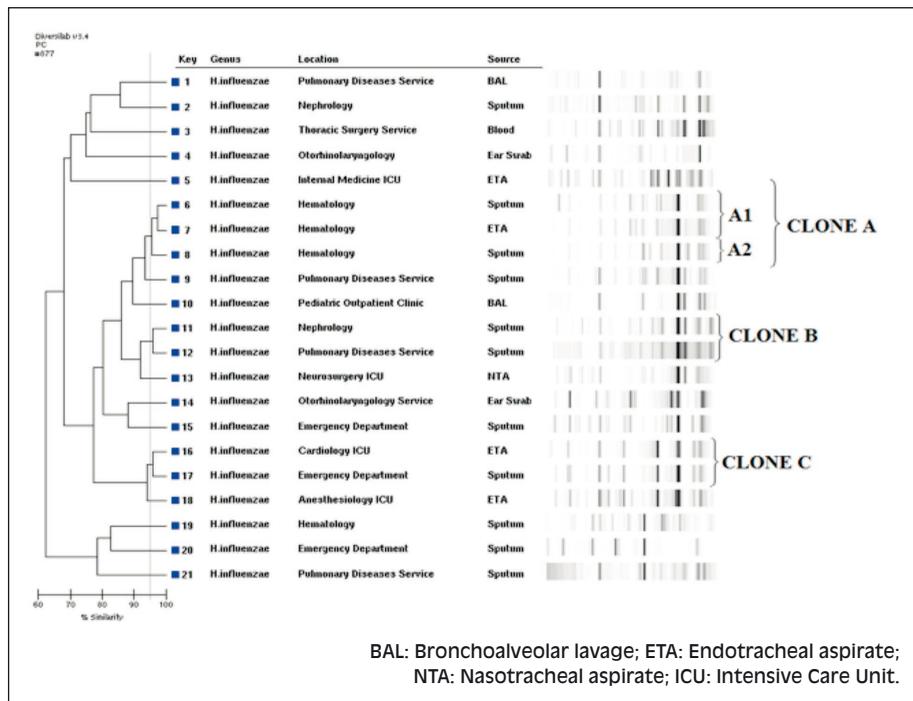
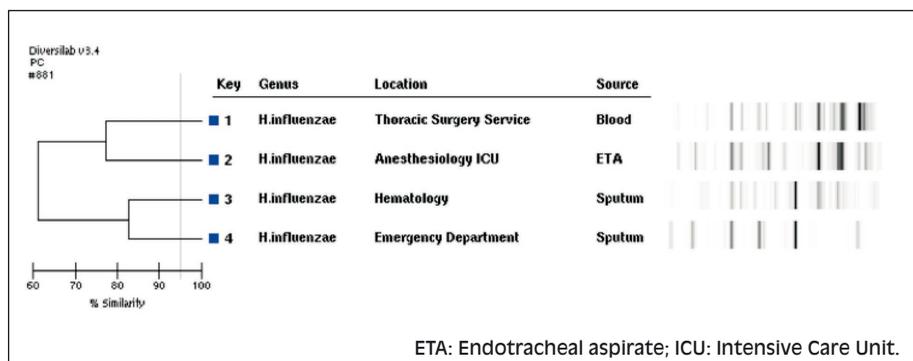


Figure 3 - Clonal relationship of ampicillin-resistant strains.



sistant. The resistance profiles of the strains are shown in Table 1. When the BL-positive strains were analyzed, all three strains were found to be genotypically distinct from each other (Figure 1). Among the 21 SXT-resistant strains found, three main clones were identified (A, B, C). The clone A, isolated from the haematology department, consists of two sub-clones (A1 and A2) as shown in Figure 2. However, ampicillin-resistant strains were found to be clonally different from each other as shown in Figure 3.

■ DISCUSSION

Frequency of *H. influenzae* infections and resistance profiles vary among countries [10,11]. Besides these regional differences, failures in treatments are ascribed to BL activity acquired by the strains of *H. influenzae*. Indeed, reports indicate that BL activity frequency in the United States of America is 36.4%, 27.6% in Brazil, and 9.5% in Greece, pointing to significant regional differences [12-14]. Uncu et al. reported 3.2% BL activity in Turkey; likewise, Gönüllü et al., 5.7%, Berkiten et al. reported 3% BL activity, which are in line with our results in this study with 3.3% BL activity [15-18]. Similarities of the fingerprints of BL-positive strains were less than 95%, suggesting lack of clonal relationship among these strains.

Another mechanism leading to ampicillin resistance is mutations in PBPs [4]. In general, the BL-negative ampicillin-resistant (BLNAR) strains are detected in low prevalence (0.04%-2.5%) [10]. However, per some reports the prevalence of the BLNAR strains are higher in Japan and Spain [18-21]. Interestingly, the prevalence of the BLNAR strains in Spain in 1996-1997 was 13.5% but a decade later (2006-2007) this rate dropped to 0.7%. This decrease was possibly because of increased awareness in prescribing antibiotics and spread of susceptible strains [21].

There are several reports worldwide on antibiotic susceptibility of *H. influenzae* strains. Park et al. reported that of 123 *H. influenzae* strains, 26.2% were BL-negative ampicillin-susceptible (BLNAS), 9% BL-positive ampicillin-resistant (BLPAR), 24.6% BL-positive amoxicillin/clavulanic acid-resistant (BLPACR), and 40.2% BL-negative ampicillin-resistant (BLNAR) [21]. In a study conducted in Japan, Hasegawa et al. reported these ratios as

follows: 29.1% BLNAS, 15.4% BLPAR, 10.9% BLPACR, and 30.6% BLNAR [19]. In this study, only one amoxicillin/clavulanic acid-resistant strain and four ampicillin-resistant strains were identified, of which three strains were determined to be BLNAR, one strain was BLPAR. On the other hand, epidemiology of the present study is different from that described by Park et al., who investigated *H. influenzae* carriage in the nasopharynx of 360 children, and Hasegawa et al., who evaluated strains isolated from patients suffering from meningitis.

Although recent studies observed decreased susceptibility against amoxicillin/clavulanic acid, the prevalence of these strains is quite low [23, 24]. While the amoxicillin/clavulanic acid resistance was reported 1.6% in Saudi Arabia [25], and 0.7% in the United States, an upward trend of amoxicillin/clavulanic acid resistance in Japan was reported in years 1999-2008 and all BLPACR strains (23 strains) were reported to be clonally identical [24, 26]. Here, we identified only one amoxicillin/clavulanic acid-resistant strain.

Resistance against cephalosporins is mainly due to PBP alteration [4]. In a survey in Thailand, cefotaxime susceptibility was found to be 100% and cefuroxime susceptibility was 96.6% [27]. A Korean study on 582 healthy children indicated that 52.1% of the isolated strains showed cefaclor resistance [28]. Again, in another Korean study 46% of the strains isolated from respiratory tract of patients were resistant to cefaclor [29].

In general, resistance to SXT among *H. influenzae* isolates is relatively higher. Resistance to SXT arises from overproduction of structurally altered dihydrofolate reductase [30]. In a multi-center Chinese study from 2000 to 2002, susceptibilities to SXT of the isolates from Shanghai, Guangzhou and Beijing were found to be 47%, 54%, 35%, respectively [31]. In Malaysia, Mohd-Zain et al. reported that resistance to SXT ranked third (26.5%) in prevalence after ampicillin and tetracycline [32]. In contrast, in a surveillance study carried out in Cuba during 1990-2002, resistance to SXT was reported 51.3% [33]. In our current study the resistance rate was 23% among the *H. influenzae* isolates.

Resistance to quinolones is rarely observed and mutations in DNA gyrase and topoisomerase IV are responsible for emerging resistance [4]. The lack of strains resistant to levofloxacin found in this study is in line with other findings. Indeed,

a survey on antimicrobial susceptibility of respiratory pathogens conducted in Brazil, during the period 2001-2002, found no *H. influenzae* strains resistant to levofloxacin [34]. Likewise, also Ho et al., in 2004, during a 7-month period, from a population of 1,978 children recovered a total of 563 *H. influenzae* strains. Only 5 (0.9%) of them had MICs values equal to 0.125 µg/ml [35].

In conclusion, resistant isolates of *H. influenzae* vary temporally by country and even regions within countries. Here, we demonstrated that significant resistance to antibiotics in central Turkey was not found. We conclude that low prevalence of ampicillin resistance and high susceptibility to other antibiotics except SXT suggests that significant obstacles in therapeutic use of antibiotics against *H. influenzae* strains are not expected.

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Conflict of interest. The authors have no conflicts of interest to disclose.

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