RESEARCH NOTE

TEM-1 AND ROB-1 PRESENCE AND ANTIMICROBIAL RESISTANCE IN *HAEMOPHILUS INFLUENZAE* STRAINS, ISTANBUL, TURKEY

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Abstract. Resistance of 235 *Haemophilus influenzae* clinical isolates from Istanbul Medical Faculty Hospital, Turkey were determined against 19 antibiotics by disc diffusion method, and minimum inhibitory concentrations (MICs) of those found resistant to ampicillin, cefuroxim, chloramphenicol and meropenem were measured using E-test. Ampicillin-resistant isolates producing beta-lactamase as demonstrated by a nitrocefin assay were analyzed for the presence of TEM-1 and ROB-1 genes by PCR. Eleven percent of the isolates were resistant to ampicillin (10 μg/ml), of which 73% were beta-lactamase positive and carried TEM-1 gene, but none were positive for ROB-1 gene. All isolates susceptible to amoxicillin-clavulanate (20/10 μg/ml), azithromycin (15 μg/ml), aztreonam (30 μg/ml), cefotaxime (30 μg/ml), ceftriaxone (30 μg/ml), ciprofloxacin (5 μg/ml), levofloxacin (5 μg/ml), and telithromycin (15 μg/ml) but 24%, 15%, 4%, 4%, 2%, 1%, 1%, 0.5%, 0.5% and 0.5% were resistant to trimethoprim-sulfamethoxazole (1.25/23.75 μg/ml), tetracycline (30 μg/ml), cefaclor (30 μg/ml), clarithromycin (15 μg/ml), cefuroxime (30 μg/ml), meropenem (10 μg/ml), chloramphenicol (30 μg/ml), ampicillin-sulbactam (10/10 μg/ml), nalidixic acid (30 μg/ml), and fosfomycin (30 μg/ml), respectively. MIC values of three cefuroxime-resistant isolates was 24, 48 and > 256 μg/ml, respectively; of two meropenem-resistant strains > 256 μg/ml; and of two chloramphenicol- susceptible isolates (by disc diffusion method) 6 μg/ml (considered as intermediate susceptible). Multiple-antibiotics resistance was detected in 15% of the strains, with resistance to 2, 3, 4, 5 and 6 antibiotics in 8.5%, 4%, 2%, 0.5% and 0.5% of the isolates, respectively. By identifying beta-lactamase-negative ampicillin-resistant *H. influenzae*, empirical therapy with beta-lactam/beta-lactamase inhibitor combinations and second generation cephalosporins would be inappropriate for such patients (approximately 3%). Our findings will contribute to the epidemiological and clinical data regarding *H. influenzae* infection in Turkey.

Keywords: *Haemophilus influenzae*, ampicillin resistance, beta-lactamase, ROB-1, TEM-1

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INTRODUCTION

Haemophilus influenzae is the etiological agent of invasive infections, such as meningitis, pneumonia and epiglottidis, and non-invasive infections, such as chronic obstructive lung disease exacerbation, sinusitis, otitis media and conjunctivitis (Srifuengfung *et al*, 2007; Tristram *et al*, 2007). So far ampicillin and amoxicillin has succesfully been used in the empiric therapy of these infections (Srifuengfung *et al*, 2007; Tristram *et al*, 2007; Chotpitayasunondh *et al*, 2012). Ampicillin-resistant bacteria were first reported in 1974 from USA, and resistance rates to antimicrobials, especially to beta-lactams, have been increasing (Srifuengfung *et al*, 2007; Tristram *et al*, 2007; Chotpitayasunondh *et al*, 2012;). Resistance is as a result of beta-lactamase production mediated by plasmids, especially TEM-1 and ROB-1, including *H. infleunzae* (Srifuengfung *et al*, 2007). However, in 1980 beta-lactamase-negative ampicillin-resistant isolates (BLNAR) were reported (Leaves *et al*, 2000; Srifuengfung *et al*, 2007; Tristram *et al*, 2007). This type of resistance is due to decreased affinity of mutated penicillin-binding proteins (PBPs) to beta-lactam antibiotics (Leaves *et al*, 2000). These two types of resistance mechanisms can be found occasionally in the same isolate, known as beta-lactamase-positive amoxicillin clavulanate-resistant (BLPACR) strain (Leaves *et al*, 2000; Tristram *et al*, 2007).

Invasive *H. influenzae* can lead to life threatening infections and patients can recover only as the result of efficient antibiotics therapy (Chotpitayasunondh *et al*, 2012). However, in recent years, an increase in resistance of invasive *H. influenzae* to antibiotics, especially to ampicillin, has led to difficulties in the treatment of these severe diseases (Chotpitayasunondh *et al*, 2012). Identification of antibiotic resistance strains will guide appropriate therapy of these infections.

In this study, prevalence of antibiotic resistance and beta-lactamase production, and identification of beta-lactamase types by molecular analysis in *H. influenzae* isolates were determined.

MATERIALS AND METHODS

Sample collection

H. influenzae isolates (n = 235) were collected between May 2009-June 2011 from 202 out-patients and 29 in-patients attending Istanbul Medical Faculty Hospital, Turkey, of whom 108 (47%) were males and 123 (53%) were females. Seventy-six samples were from patients with cystic fibrosis. The sources of the isolates were as follows: respiratory tract, 123 (52%); sputum, 88 (37%); deep pharyngeal (from cystic fibrosis patients), 10 (4%); bronchoalveolar lavage fluid, 6 (2%); tracheal aspirate, 3 (1%); nasal, 2 (1%); blood, 1 (0.5%); vaginal/urethral secretion, 1 (0.5%); and cerebrospinal fluid, 1 (0.5%). Identification of *Haemophilus* spp was carried out by conventional methods, such as Gram staining, X and V factor requirements, and catalase and oxidase activities (Garcia and Isenberg, 2007).

Antimicrobial susceptibility assay

Antimicrobial susceptibility of the *H. influenzae* isolates were carried out using the disc diffusion method according to Clinical and Laboratory Standards Institute; Nineteenth Informational Supplement (CLIS, 2009). The antibiotics tested were: ampicillin (AMP, 10 μg/ml), amoxicillin-clavulanic acid (AMC, 20/10 μg/ml), ampicillin-sulbactam (SAM, 10/10 μg/ml), azithromycin (AZM, 15 μg/ml),

aztreonam (ATM, 30 μg/ml), cefaclor (CEC, 30 μg/ml), cefotaxime (CTX, 30 μg/ml), ceftriaxone (CRO, 30 μg/ml), cefuroxime (CXM, 30 μg/ml), chloramphenicol (C, 30 μg/ml), ciprofloxacin (CIP, 5 μg/ml), clarithromycin (CLR, 15 μg/ml), fosfomycin (FOT, 30 μg/ml), levofloxacin (LEV, 5 μg/ml), meropenem (MEM, 10 μg/ml), nalidixic acid (NA, 30 μg/ml), trimetoprim-sulfamethoxazole (SXT, 1.25/23.75 μg/ml), telithromycin (TEL, 15 μg/ml), and tetracycline (TE, 30 μg/ml). Whenever isolates were found to be resistant to AMP, CXM, C and MEM, minimum inhibitory concentrations (MICs) were determined by the E-test (AB Biodisk, Solna, Sweden). Beta-lactamase production was assayed by a nitrocefin test (Oxoid, Hamshire, England). *H. influenzae* ATCC 49247 and ATCC 49766 were used as control strains.

PCR detection of TEM-1 and ROB-1 genes

Genomic DNA extraction of ampicillin-resistant strains grown on chocolate agar for 24 hours was carried out using High Pure PCR Template Preparation kit (Roche, Mannhein, Germany). The presence of TEM-1 and ROB-1 gene was investigated using primer pairs TEM-1 (5'ACCAGTCACAGAAAAGCATC3' and 5'TTATCCGCCTCCATCCAGTC3') (amplicon of 327 bp) and ROB-1 (5'GCGCCTGTGCAACAATCA3' and 5'CAAATTCGCCAAAGTCTGTTGA3') (338 bp), respectively (Sanbongi *et al*, 2006). PCR reaction was carried out in a total volume of 50 μl containing 5 μl of 1X reaction buffer, 3 μl of $MgCl_2$ (25 mM), 4 μl of dNTP mix (5 mM), 3 μl of primers (50 μM) and 0.2 μl of *Taq* DNA polymerase (5 U/μl). Thermocycling (conducted in Takara TP 600 thermocycler, Tokyo, Japan) were as follows: 98°C for 2 minutes; then 30 cycle at 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 3 minutes; with a final step of 72°C for 10 minutes. A 10 μl aliquot of the PCR reaction solution was electrophoresed at 90 V for 1 hour in 1.5% agarose gel, which then was stained with ethidium bromide and amplicons visualized under UV light.

Statistical analysis

This was performed using SPSS 11.5-Windows program (SPSS, Chicago, IL). Chi-square test was used to assess differences among gender, type of clinical sample and antibiotic resistance, with $p < 0.05$ considered as being statistically significant.

RESULTS

Of the 235 *H. influenzae* clinical isolates, 19 (8%) were beta-lactamase positive, and TEM-1 gene was detected in all beta-lactamase-positive and AMP-resistant strains but ROB-1 gene was not present (Table 1). Seven (3%) of the isolates were BLNAR type. Multiple antibiotic-resistance (at least to two antibiotics) was observed in the AMP-resistant strains.

Fifty-seven (24%), 36 (15%) and 26 (11%) of the isolates were resistant to SXT, TE and AMP, respectively (Table 2). Three isolates were resistant to CXM with MIC of 24, 48 and 256 μg/ml, respectively. MIC of MEM for two resistant strains was 256 μg/ml, but both were intermediate susceptible to C with MIC of 6 μg/ml. Multiple antibiotic resistance was observed in 36 (16%) of the isolates, of which 20 (8.5%) were resistant to two antibiotics, 9 (4%) to three antibiotics, 5 (2%) were resistant to four antibiotics, and 1 (0.5%) each to five and six antibiotics. There is no statistically significant difference between prevalence of antibiotics resistance and gender or type of clinical sample.

DISCUSSION

Ampicillin resistance of *H. influenzae* is frequently the result of beta-lactamase

Table 1
AMP minimum inhibitory concentrations (MIC), beta-lactamase positivity, beta-lactamase types and antibiotic resistance patterns of AMP-resistant *H. influenzae* clinical strains from Istanbul Medical Faculty Hospital, Turkey.

Strain no.	AMP MIC (μg/ml)	Beta-lactamase	TEM-1	ROB-1	Antibiotic resistance pattern
1	>256	+	+	-	AMP, CLR, NA, SXT, TE
2	>256	+	+	-	AMP, SXT
3	96	+	+	-	AMP, CEC, SXT, TE
4	64	-	-	-	AMP, TE
5	>256	+	+	-	AMP, SXT, TE
6	>256	+	+	-	AMP, SXT, TE
7	96	+	+	-	AMP, SXT, TE
8	>256	+	+	-	AMP, CXM, SXT, TE,
9	>256	-	-	-	AMP, SXT
10	>256	+	+	-	AMP, CEC, CXM, MEM, SXT, TE
11	64	-	-	-	AMP, CLR
12	>256	+	+	-	AMP, SAM, SXT
13	>256	+	+	-	AMP, CEC, SXT, TE
14	>256	-	-	-	AMP, SXT
15	>256	+	+	-	AMP, CEC, FOT, SXT,
16	>256	+	+	-	AMP, CEC
17	>256	-	-	-	AMP, SXT
18	>256	+	+	-	AMP, CEC
19	>256	-	-	-	AMP, CLR, SXT
20	>256	+	+	-	AMP, SXT
21	>256	+	+	-	AMP, CEC, TE
22	>256	+	+	-	AMP, CLR
23	>256	+	+	-	AMP, CLR, SXT
24	6	+	+	-	AMP, SXT
25	4	-	-	-	AMP, SXT
26	>256	+	+	-	AMP, SXT

AMP, ampicillin (10 μg/ml); CEC, cefaclor (30 μg/ml); CXM, cefuroxime (30 μg/ml); CLR, clarithromycin (15 μg/ml); FOT, fosfomycin (30 μg/ml); MEM, meropenem (10 μg/ml); NA, nalidixic acid (30 μg/ml); SXT, trimetoprim-sulfamethoxazole (1.25/23.75 μg/ml); TE, tetracycline (30 μg/ml).

production (Cerquetti *et al*, 2004). The prevalence of this type of resistance varies according to the geographical region, ranging from 3% to 65% (Farrell *et al*, 2005; Tristram *et al*, 2007). The prevalence in this study (11%) is in the lower range. Some 73% of these ampicillin-resistant *H. influenzae* isolates produced beta-lactamase, a frequency consistent with that reported in European countries, such as the Czech Republic, Portugal, Sweden and Switzerland, and in South America (Brazil and Colombia), but lower than in Canada, England, France, India, Ireland, Israel, South Korea and Taiwan (Critchley *et al*, 2005; Farrell *et al*, 2005; Fluit *et al*, 2005; Jain *et al*, 2006; Jansen *et al*, 2006). However, in Japan the majority

Table 2
Antibiotic susceptibility of 235 *H. influenzae* clinical isolates collected from Istanbul Medical Faculty Hospital, Turkey.

Antibiotic	Sensitivity, *n* (%)			
	S	I	R	Total resistant[a]
AMC	235 (100)	0	0	0
AMP	209 (89)	1 (0.5)	25 (10.5)	26 (11)
SAM	234 (99.5)	0	1 (0.5)	1 (0.5)
AZM	235 (100)	0	0	0 (0)
ATM	235 (100)	0	0	0
CEC	225 (96)	5 (2)	5 (2)	10 (4)
CTX	235 (100)	0	0	0
CRO	235 (100)	0	0	0
CXM	231(98)	1 (0.5)	3 (1)	4 (2)
C	233 (99)	2 (1)	0	2 (1)
CIP	235 (100)	0	0	0
CLR	227 (96.5)	0	8 (3.5)	8 (3.5)
FOT	234 (99.5)	0	1 (0.5)	1 (0.5)
LEV	235 (100)	0	0	0
MEM	233 (99)	0	2 (1)	2 (1)
NA	234 (99.5)	0	1 (0.5)	1 (0.5)
SXT	178 (76)	0	57 (24)	57 (24)
TEL	235 (100)	0	0	0
TE	199 (84.5)	25 (10.5)	11 (5)	36 (15)

S, sensitive; I, intermediate susceptible; R, resistant; [a]Sum of intermediate susceptible and resistant isolates. AMC, amoxicillin-clavulanic acid (20/10 μg/ml); AMP, ampicillin (10 μg/ml); SAM, ampicillin-sulbactam (10/10 μg/ml); AZM, azithromycin (15 μg/ml); ATM, aztreonam (30 μg/ml); CEC, cefaclor 30 μg/ml); CTX, cefotaxime (30 μg/ml); CRO, ceftriaxone (30 μg/ml); CXM, cefuroxime (30 μg/ml); C, chloramphenicol (30 μg/ml); CIP, ciprofloxacin (5 μg/ml); CLR, clarithromycin (15 μg/ml); FOT, fosfomycin (30 μg/ml); LEV, levofloxacin (5 μg/ml); MEM, meropenem (10 μg/ml); NA, nalidixic acid (30 μg/ml); SXT, trimethoprim-sulfamethoxazole (1.25/23.75 μg/ml); TEL, telithromycin (15 μg/ml); TE, tetracycline (30 μg/ml).

of ampicillin-resistant *H. influenzae* strains were BLNAR isolates (Hasegawa *et al*, 2003; Sunakawa and Farrell, 2007). The increased prevalence of BLNAR strains in Japan might be due to differences in antibiotic prescription practices compared to other countries.

In general, over 90% of ampicillin resistance of *H. influenzae* are associated with TEM-1 and about 5% with ROB-1 (Scriver *et al*, 1994; Karlowsky *et al*, 2000; Farrell *et al*, 2005; Tristram *et al*, 2007). In our study, all ampicillin-resistant beta-lactamase-producing *H. influenzae* isolates carried TEM-1 gene. When all of the isolates are taken into consideration, prevalence of TEM-1 beta-lactamase positivity and BLNAR was 8% and 3%, respectively. In a multi-center study, among 82 *H. influenzae* strains isolated in Turkey between 2004-2005, beta-lactamase positivity is 2% and BLNAR isolates constitutes 11%,

but the presence of TEM-1 and ROB-1 was not investigated (Jansen *et al*, 2006). The differences in beta-lactamase positivity and BLNAR prevalence between the latter and the present study may be due to geographical differences in the use of ampicillin. In another multi-center study in which Turkey was included, beta-lactamase positivity in Turkish isolates is 5.4% and TEM-1, but no ROB-1, type was detected in all beta-lactamase producing strains, but when all strains are considered, beta-lactamase positivity rises to 15%, with TEM-1 and ROB-1 frequency >90% and 4.6%, respectively (Farrell *et al*, 2005). According to geographic region, the presence of ROB-1 can be different; in some countries, such as Mexico, ROB-1 frequency can reach as high as 30%.

Resistance to cephalosporins is variable throughout the world; cefaclor resistance was reported at 88.3% in some countries (Morrissey *et al*, 2008). In Turkey, cefaclor resistance has beeen determined to be 3-4%, but no resistance to third generation cephalosporins (Gur *et al*, 2002; Sener *et al*, 2002). However, low levels of resistance to third generation cephalosporins have appeared in other regions of the world (Hoban *et al*, 2001; Morrissey *et al*, 2008).

As regards resistance in Turkey to antibiotics other than penicillins and cephalosporins, over 20% resistance to cotrimoxazole and tetracycline have been reported (Sener *et al*, 2007), in agreement with the current study. Cotrimoxazole resistance reaching as high as 50%-60% can be present worldwide (Turnak *et al*, 2001). Tetracycline resistance in Turkey is generally higher than other countries (Jansen *et al*, 2006; Sener *et al*, 2007; Morrissey *et al*, 2008). In other countries macrolide resistance can vary from 1.1% to over 20% (Brown and Rybak, 2004), and in Turkey this reaches 7% (Budak and Gur, 2003). Resistance to chloramphenicol and quinolone in Turkey are very low, 0-6% and 0-0.8%, respectively (Budak and Gur, 2003; Brown and Rybak, 2004; Sener *et al*, 2007). Although high resistance level (57.7%) to carbapenems has been reported in Japan, resistance is generally at a low level (0-1%) worldwide including Turkey (Critchley *et al*, 2007; Gomi *et al*, 2007; Srifuengfung *et al*, 2007; Morrissey *et al*, 2008).

Similar to other gram-negative bacteria, multi-antibiotics-resistant *Haemophilus* strains have been detected, with levels as high as 15%-20% (Levy *et al*, 1993), similar to our results. In our study, it was found that multiple resistance includes various combinations; most often co-trimoxazole, ampicillin and tetracycline. It was found that multi-resistance was most often associated with resistance to two antibiotics (8.5%). As ampicillin is the mainstay in the therapy of *H. influenzae* infections, it is important to follow up on the current status of the ampicillin resistance in Turkey. For definitive detection, the presence of beta-lactamase gene(s) should be determined by molecular methods. In our study, the nitrocefin test was positive in all beta-lactamase producing isolates (which carried TEM-1 type beta-lactamase gene). These results show that nitrocefin test still is a useful and convenient in our country. By identifying BLNAR phenotype, it was shown also that beta-lactamase inhibitor combinations and second generation cephalosporins were not appropriate in the empiric therapy of 3% of patients from whom ampicillin-resistant strains were isolated.

In conclusion, in Turkey where empiric therapy is usually prefered, there should be continuing investigations into antibiotics resistance, multiple-drug resistance phenotypes and beta-lactamase

types, as well as more extensive epidemiological studies.

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