Prevalence and Genotypic Characteristics of β-Lactamase Negative-Ampicillin-Resistant *Haemophilus influenzae* in a Children's Hospital in Turkey

Türkiye'de Bir Çocuk Hastanesinde β-Laktamaz Negatif Ampisilin Dirençli *Haemophilus influenzae*'ların Prevalansı ve Genotipik Özellikleri

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ABSTRACT Objective: This study's aim is to investigate the serotypes and ampicillin resistance mechanisms in *Haemophilus influenzae* pediatric isolates in several clinical samples. **Material and Methods:** One hundred thirty nine patients' clinic samples were examined. Serotyping using slide agglutination and antimicrobial susceptibilitiy testing using microbroth dilution test methodology were performed. Strains for which ampicillin MICs were ≥0.5 μg/mL were indiscriminately selected, and penicillin binding protein (PBP) profiles were analyzed. The bla_{TEM-1}, bla_{ROB-1} genes and *ftsI* gene mutations were examined by polymerase chain reaction (PCR) and then sequenced. **Results:** The serotypes in 139 serial isolates of *H. influenzae* were detected as serotype b (n=19; 13.7%), serotype a (n=9; 6.4%), serotype d (n=2; 1.4%), serotype f (n=2; 1.4%) and serotype c (n=1; 0.7%), serotype e (n=1; 0.7%). One hundred and five (75.5%) strains were found as nontypeable. Ampicillin, cefaclor, amoxicillin-clavulanic acid and cefotaxime resistance rates were 4.3%, 11.5%, 0%, 0%, respectively. Resistance was not detected in serotype b and e. Ampicillin resistant, beta lactamase positive four isolates (two serotype a and two nontypeable) were positive for bla_{TEM-1}. All strains were negative for blaROB-1. A total of 4 (11.1%) serotype a, 2 (5.5%) serotype b and 30 (83.3%) nontypeable *H. influenzae* strains were positive for low BLNAR. **Conclusion:** Among pediatric isolates nontypeable *H. influenzae* was found to be the most common serotype. Ampicillin resistant, TEM-1 beta lactamase positive strains still involve risk in our country. Significant rate of low BLNAR positive strains in nontypeable *H. influenzae* is considerable. This is the first investigation on the specific gene mutations that encode PBP-3 INT and their connection with TEM-1 in low BLNAR *H. influenzae* strains in Turkey.

Key Words: *Haemophilus influenzae;* ampicillin resistance; drug resistance, bacterial; β-lactamase

ÖZET Amaç: Bu çalışmanın amacı çocuk hastalara ait çeşitli klinik örneklerden izole edilen *Haemophilus influenzae* suşlarında serotip ve ampisilin direnç mekanizmasının araştırılmasıdır. **Gereç ve Yöntemler:** Yüzotuzdokuz hastanın klinik örnekleri çalışıldı. Serotiplendirme lam aglütinasyon ile ve antibiyotik duyarlılık testleri mikrobroth dilüsyon yöntemiyle yapıldı. Ampisilin MİK'i ≥0,5 μg/mL olanlar gelişigüzel seçildi; bu suşların beta laktamaz enzimleri ve penisilin bağlayan protein (PBP) profili incelendi. bla_{TEM-1}, bla_{ROB-1}, genleri ve *ftsI* gen mutasyonları polimeraz zincir tepkimesi (PZT) ile çalışıldı ve sekanslandı. **Bulgular:** Çalışmamızda 139 seri *H. influenzae* suşunun serotipleri; serotip b (n=19; %13,7) , serotip a (n=9; %6,4), serotip d (n=2; %1,4), serotip f (n=2; %1,4), serotip c (n=1; %0,7), serotip e (n=1; %0,7) olarak belirlendi. Yüzbeş suş (%75,5) tiplendirilemeyen olarak saptandı. Ampisilin, sefaklor, amoksisilin-klavulanik asit ve sefotaksim direnç oranları sırasıyla %4,3, %11,5, %0, %0'dır. Serotip b ve e'de direnç saptanmadı. Ampisilin dirençli beta laktamaz pozitif dört suş (2'si serotip a, 2'si tiplendirilemeyen) bla_{TEM-1} pozitif idi; bu suşlarda aynı zamanda PBP-3 INT mutasyonu saptandı. Bütün suşlar blaROB-1 negatifti. Zayıf BLNAR olarak saptanan *H. influenzae* suşlarının 4'ü serotip a (%11,1), 2'si serotip b (%5,5), 30'u tiplendirilemeyen *H. influenzae* (%83,3) idi. **Sonuç:** Çocuk yaş grubu örneklerinde en çok bulunan tiplendirilemeyen *H. influenzae* serotipidir. Ampisilin dirençli, beta laktamazı pozitif suşlar ülkemizde halen risk oluşturmaktadır. Tiplendirilemeyen *H. influenzae*'da saptanan zayıf BLNAR pozitif suşların azımsanmayacak oranı dikkat çekicidir. Bu çalışma Türkiye'de *H. influenzae*'da zayıf BLNAR suşlarında PBP-3-INT spesifik gen mutasyonlarını ve bunların TEM-1 ile ilişkilerini araştıran ilk çalışmadır.

Anahtar Kelimeler: *Haemophilus influenzae;* ilaç direnci,bakteriyel; ampisilin direnci; β-laktamaz

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Haemophilus influenzae infections are important invasive disease in children especially under aged 2 years; but may also occur in adults.[1,2] Emergence of resistance to several antimicrobial agents, especially to β lactam drugs is complicating the therapy in *H. influenzae* infections as aminopenicillins are used extensively in these infections. Two major mechanisms are involved in resistance to β-lactam antibiotics: the enzymatic mechanism which is because of the increased releasing of β-lactamases (commonly TEM-1, rarely ROB-1 enzymes) and besides enzymatic mechanism which is because of the decreased affinity of β-lactams to the suppositious penicillin-binding proteins (PBPs) targets.[1] β-lactamase-negative ampicillin resistant (BLNAR) *H. influenzae* are going to prevalent global and their prevalence varies in different countries.[1,3] Isolates that have an ampicillin MIC ≥ 4 mg/L using CLSI breakpoints and do not produce β-lactamase are defined as BLNAR isolates. Ampicillin non-susceptible isolates with MIC's 2 mg/L are referred to as low BLNAR (L-BLNAR).[4] The BLNAR strains are separated into 3 groups (I, II, and III) by Ubukata et al. considering presence of different amino acid substitions in the transpeptidase area of PBP3. Almost all isolates have aspartic acid at location 350 replaced with asparagine (D350N) and S357N; besides, group 1 strains have R517H, group 2 strains have N526K, and group 3 strains have N526K, M377I, S385T, and L389F. Several group 1 strains additionally have M377I and/or S385T. Part I and II replacements are collectively referred to as low BLNAR, part III is called BLNAR in genetically.[1,5] Moreover, some *H. influenzae* strains have both beta lactamase and altered PBPs mutations. These strains have much higher ampicillin MICs than BLNAR strains. They called beta lactamase positive amoxicillin clavulanic acid resistance (BLPACR) strains.[1]

Some of penicillin binding protein (PBP) mutations in *H. influenzae* strains have been reported from Turkey particularly BLNAR.[6,7]

Spread of BLNAR and low BLNAR *H. influenzae* strains have an significant effect on public sanitary because of these bacteria are frequently account for community acquried infections and they indicate reduced susceptibility both ampicillin and other β-lactam antibiotics, particularly cephalosporins.

On the other hand, the resistance of *H. influenzae* in Turkey is obscure. The aim of the study was to explore both phenotypic and genotypic criteria to determine the currancy of BLNAR strains; amino acid replacement in PBP-3 and their relationship with β-lactam susceptibilities within children in Ankara, Turkey.

MATERIAL AND METHODS

A total of 139 *Haemophilus influenzae* isolated consecutively between July 2008- May 2010 from clinically meaning samples were examined. The samples included sputum (116) bronchoalveolar lavage (17) tracheal aspirate (3), catheter (1), and pus (2). Only one isolate from each patient was studied. All patients were informed about the study and signed informed consent was obtained. The study was approved by the Ethics Committee of Medical Faculty in 10/01/2012 and number 1/6. Every isolates were defined by classical microbiological methods, such as; Gram stain, X and V factors requirements for growth, hemolysis on horse blood agar. Results were situated using Crystal test (Crystal H/N panel Becton Dickinson). The isolates were stored at -80 °C until studious. Capsule serotyping was performed using specific antisera (*Haemophilus influnzae* Antiserum Types a, b, c, d, e, f; Becton Dickinson, Maryland, USA). Antimicrobial susceptibility testing was studied using microbroth dilution test methodology in Haemophilus Test Medium broth with ampicillin, cefaclor, amoxicillin-clavulanic acid and cefotaxime, Clinical Laboratory Standards Institute (CLSI) guidelines were followed in the tests.[8] Tests were repeated three times and the results were assessed together. *H. influenzae* ATCC 49247 and ATCC 49766 were used as quality control. Ampicillin resistant isolates were examined by nitrocefin solutions for investigating β-lactamase enzyme producing.[1] Cultures of both ampicillin-resistant and β-lactamase positive isolates were grown overnight

at 37ºC in horse blood agar and bacterial suspensions prepared in 250 ml 0.1 M phosfate buffered saline. Bacterial suspensions were disintegrated by sonification (six times for 15s each at 20Hz (phospholyser Vibra Cell 300; Bioblock, Illrich, France) and were centrifugated (15000xg, 15s, 4 ºC). Supernatant which contains enzymes was submitted to isoelectric focusing with a mini IEF 111 apparatus (Bio-Rad, USA) with a polyacrylamide gel containing a gradient invented ampholytes with an area of 3 to 10 (Bio-Rad, USA). The focused β-lactamases were identified by overlaying the gel with 1mM nitrocefin (Albiochem) in a 50 mM phosphate buffer (pH:7.0). The pI values were determined and compared to known standards β-lactamases enzymes, SHV-1 (pI:7.6), OXA-14 (pI: 6.2) and TEM-1 (pI: 5.4). β-lactamase specific activities of TEM-1 positive isolates were determined as defined previously.[9] One unit of enzyme efficiency was determined as the efficiency which hydrolysed 1nmol of cephalotin per min per mg of protein. The total protein content was measured with the Bio Rad DC Protein assay kit. Genomic DNA's from isolates with ampicillin MICs ≥0.5 μg/mL were extracted at 95 °C for 10 min and sedimenting the wreck for 10 min at 12 000 x g.[10.] DNAs were stored at -80 ºC until studied. For every reaction 2 μg of genomic DNA from *H. influenzae* was used. The master mixture was formed of 1x Buffer (from DNA Taq polymerase (Fermentas, Lithuania), 1.5 mM $MgCl_2$, 0.8 mM dNTPs, 50 pmol primers each and 1.5U of Taq polymerase. Amplification was perfect after a 5 min denaturation at 95°C by 30 cycles of 30s at 95 °C, 30s at 60.5°C and 45s at 72 °C. PCR products were run on a 1% agarose gel at a constant 12 V/cm and pictured using an imaging system (Gene Image SCI, Image analysis system). The PCR amplifications were made with TEM-1, ROB-1, PBP-3-INT and PBP-3 BLN primers in ampicillin resistant (MIC ≥0.5 μg/mL) strains. For detection of beta lactamase genes (bla_{TEM-1}, bla_{ROB-1}) TEM-1 and ROB-1 primers were used. PBP-3-INT primer was used to amplify a potion of *ftsI* gene containing the Lys-526 amino acid substition and PBP-3 BLN primer to amplify a part of *ftsI* gene having Thr-385 aminoacid substitution and Lys-526 substitution. The primers were as follows; TEM-1 F(5'-TTGCCGGGAAGCTAGAGTAA-3') and R(5'-GCGCATCTAAGATTTGAACG-3'), ROB-1 F(5'-CTAATCCGCAGCCTGCTAGT-3') and R(5'-ACAACGCCTTGAAAGTGGAC-3'), PBP-3-INT F(5'-GATACTACGTCCTTTAAATTAAGCG-3') and R(5'-CCCGCAGTAAATGCCACATATTTC-3'), PBP-3-BLN F(5'-GTCACACCACGGTTACTTGAA-3') and R(5'-CCCGCAGTAAATGCCACATATTTC-3').[11] The sequence tests were done after outcome purification using High Pure PCR Product Purification Kit (Roche Diagnostics, Basel, Switzerland). The sequencing was studied at Iontek Inc. (Istanbul, Turkey). Sequence data were read and edited by Chromas pro (http://www. ncbi.nlm.nih.gov).

STATISTICAL ANALYSIS

Clustal W2, a freely available software for multiple sequence alignment at EBI (http://www.ebi.ac.uk/) was used for analysis. SPSS software programme 17.0 (IBM Corporation, NY, USA) was used for statistical analysis. Statistical calculations were performed with chi square test and a p value of <0.05 was considered significant.

RESULTS

The most frequent strains were nontypeable (n=105; 75.5%) among 139 *H. influenzae* isolates, followed by serotype b (n=19, 13.7%), serotype a (n=9; 6.4%), serotype d (n=2; 1.4%), serotype f (n=2;1.4%) and serotype c (n=1; 0.7%) and serotype e (n=1; 0.7%). Out of 139 *H. influenzae* 6 (4.3%) were resistant to ampicillin. Ampicillin resistance was observed in four nontypeable *H. influenzae* and two *H. influenzae* type a strains. All resistance strains were isolated from sputum samples. Sixteen (11.5%) isolates were resistant to cefaclor and resistance to cefotaxime was not observed. Four of the isolates were β-lactamase positive, ampicillin and cefaclor resistant but decreased susceptibility to amoxicillin clavulanic acid (0.5 to 1 μg/mL). The serotypes, antibiotic resistance rates and MIC_{50}, MIC_{90}, MIC ranges of 139 isolates are shown in Table 1. By using bla_{TEM-1} and bla_{ROB-1} specific primers, PCR fragment was acquired from genomic DNA from four β-lactamase positive, ampi-

TABLE 1: Resistance rates to antibiotics in *H. influenzae* serotypes and MIC ranges, MIC_{50}, MIC_{90}.

	Serotypes MIC (Mg/mL)										
	a	b	c	d	e	f	Nontypeable	Total			
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)			
Antibiotics	**9 (100)**	**19 (100)**	**1 (100)**	**2 (100)**	**1 (100)**	**2 (100)**	**105 (100)**	**139 (100)**	**MIC50**	**MIC90**	**MIC range**
Ampicillin*	2 (22.2)	-	-	-	-	-	4 (3.8)	6 (4.3)	<0.125	1	<0.125->64
AMC	-	-	-	-	-	-	-	-	<0.125	<0.125	<0.125-1
Cefaclor*	3 (33.3)	-	-	-	-	-	13 (12.3)	16 (11.5)	4	16	<0.125- 32
Cefotaxime	-	-	-	-	-	-	-	-	<0.125	<0.125	<0.125-0.5

*Intermediate resistant strains are included as resistant

- no strains

AMC: amoxicillin/clavulanic acid.

cillin resistant strains. Direct sequencing of the PCR graduate for TEM-1 display 100% identity with bla_{TEM-1}. These results together with the pI data suggested that four strains produced TEM-1. (GeneBank accession number JQ231264). TEM-1 *H. influenzae* have been found in two serotype a and two nontypeable *Haemophilus influenzae*. The MIC of these isolates were 2 μg/mL for ampicillin and <0.125 μg/mL for amoxicillin clavulanic acid, respectively. Two of ampicillin intermediate resistance (accepted as resistant) isolates were β-lactamase negative, bla_{TEM-1} negative, and one of which was also is cefaclor resistant. All strains were negative for bla_{ROB-1}. On the other hand, by using PBP-3-INT specific primers, PCR fragment was obtained from genomic DNA of β-lactamase negative 36 isolates (MIC ≥0.5 μg/mL for ampicillin, MIC <0.125 μg/mL for amoxicillin clavulanic acid), and also bla_{TEM-1} positive four isolates. Direct sequencing of the PCR product for PBP-3-INT revealed 100% identitiy with PBP-3-INT that had a Lys-526 or His-517 amino acid substitution in *ftsI* encoding PBP- 3. Ampicillin intermediate, β-lactamase negative, TEM-1 negative two isolates which accepted as ampicillin resistant were also positive for PBP-3-INT. Although ampicillin resistant, isolates were not resistant to amoxicillin-clavulanic acid, we detected mutations in the *ftsI* gene and the presence of β-lactamase genes with molecular methods. Low BLNAR *H. influenzae* have been found in 4 (11.1%) serotype a, 2 (5.5%) serotype b and 30 (83.3%) nontypeable *H. influenzae* strains. Totally low BLNAR isolates including bla_{TEM-1} positive ones were 6 (15%) serotype a, 2 (5%) serotype b and 32 (80%) nontypeable *H. influenzae*. When PBP-3-BLN specific primers were used, none of the β-lactamase negative (ampicillin MIC ≥0.5 μg/mL) or ampicillin resistant β-lactamase positive isolates were not positive by PCR. Analysis of both amplicon sequences of TEM-1 and PBP-3-INT positive four isolates showed that they were identical to wild types by sequencing/multiple sequence alignment.

DISCUSSION

Nasopharingeal carriage of *H. influenzae* type b (Hib) strains is reduced because of type b protein-conjugated capsular polysaccharide vaccines. In chief diseases caused by *H. influenzae* are juvenility pneumonia, meningitis, and bacteremia, because of type b, and acute otitis media, acute sinusitis, and acute exacerbations of chronic bronchitis, because of untypeable strains.[12]

In our study, we evaluated results of resistance to ampicillin among *H. influenzae* strains isolated from children with special emphasis on co-existing enzymatic and non-enzymatic resistance.

According to our results among 139 *H. influenzae* strains, 6 (4.3%) were resistant to ampicillin. Ampicillin resistance was higher in *H.influenzae* type a (22.2%) and nontypeable *H. influenzae* (3.8%) strains.

Ampicillin resistant, beta lactamase positive, TEM-1 positive strains are still includes hazard in our country. In four of ampicillin-resistant isolates that production of β-lactamase, IEF suggested that this enzyme has a pI of 5.4. PCR results and se-

quencing confirmed it to be a TEM-1 enzyme that reported as the most frequent β-lactamase found in ampicillin-resistant *H. influenzae* in the world.[13-15] In this study, ampicillin MICs were ≥0.5μg/mL in 36 isolates and IEF and PCR results showed that it was not due to a β-lactamase and later confirmed by sequencing that they were low BLNAR according to Ubukata classification. On the other hand, four ampicillin-resistant and β-lactamase positive isolates had reduced amoxicillin clavulanic acid susceptibility (0.5 to 1 μg/mL) isolates that TEM-1 positive and were low- BLNAR. Similiar to our study, Kubota et al. found 9 of 24 β-lactamase positive isolates had reduced susceptibility but were not resistance to amoxicillin clavulanic acid using phenotypic screening.[16] In our study, two of ampicillin intermediate (accepted as resistant) strains were low BLNAR according to Witherden et al. and Ubukata et al.[4,5] They were intermediate resistant to ampicillin but not amoxicillin clavulanic acid. The strains with an MIC of 2.0 μg/ml which between the CLSI susceptible and resistant breakpoints are commended as intermediate, but they are inclusive of the BLNAR because they are not susceptible.[1,17] Reduced susceptibility to ampicillin brought about *fts*I gene using PCR and sequencing mutations both β-lactamase positive and negative.[18] By detecting private mutations in the *ftsI* gene, using PCR and sequencing strains can be classified as low BLNAR or BLNAR according to *ftsI* gene mutations and related PBP-3 replacement. Low BLNAR strains are infrequently isolated, and their rates are increasing all of the world.[18-20] Occasionally low BLNAR strains will be classified as resistant because recurrent MIC determinations can be expected to be different by a size of positive or negative a fold concentration.[21]

In pediatric isolates nontypeable *H. influenzae* was the predominant seroytpe. Encapsulated type b strains can occasionally have low β-lactams susceptibility, but most BLNAR strains are noncapsulated.[2,18,22] Similarity, in our study low BLNAR *H. influenzae* have been found in 4 type a, 2 type b and 30 nontypeable *H. influenzae* strains.

Incidence of BLNAR *H. influenzae* strains has been reported in Japan 50%, Spain 19.2%, Poland and United Kingdom 20%.[1,16,18,21] In Japan β-lactam resistance caused by *H. influenzae* PBP- 3 mutations have been reported by several investigators.[5,15,18] Conversely, in our country BLNAR incidence is as low as 1.6%[(7)]. Among pediatric isolates that we studied, there were not BLNAR strains. However we have detected low BLNAR isolates among pediatric strains. Low BLNAR are important because they may affect the succes of emprical therapy of community acquried respiratory tract infections. However, plenty of clinical laboratories only test for β-lactamase production in *H. influenzae* in Turkey, and it may be hard to identify the occurance of a β-lactam resistant strain due to PBP modifications.[22]

The findings of this study showed that among pediatric specimens from respiratory tract the nontypeable serotypes were most commonly isolated *H. influenzae*. Resistance rates to cefaclor is quite high particularly in nontypeable *H. influenzae* strains. A high level rates of low BLNAR isolates were found among pediatric specimens. Emergence and possible spread of these isolates is important and should be considered for resistance detection. This is the first report of low BLNAR *H. influenzae* from Turkey. Although low- BLNAR strains occur common and are badly detected with ampicillin-related MIC screening tests and CLSI breakpoints, their clinical importance marks obscure. It is also essential to judge modify in laboratory procedures to contain PCR or any other methods that let identification of low BLNAR isolates, and to re-evaluted the explication of susceptibility tests in order to reduce the risk of treatment unsucceed.[23] On the other hand; this is the first study on the specific gene mutation that encodes PBP-3-INT and their relationship with β-lactam susceptibility in low BLNAR strains in Turkey. Presence of both resitance mechanisms low BLNAR by mutation by bla_{TEM-1} encoded beta lactamase is also emerging in Turkey. We would like to emphsize the importance of continuing surveillance studies as essential tools to define trends in antimicrobial resistance of *H. influenzae.*

In conclusion, we describe a collection of clinical *H. influenzae* isolates with reduced suscepti-

bility to β-lactam antibiotics due not only to TEM-1 β-lactamase production but also novel resistance mechanisms; PBP-3-INT positivity. This is the first description of PBP-3-INT and their relationship with β-lactam susceptibility in low BLNAR *H. influenzae* strains in Turkey.

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