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RESEARCH ARTICLE

Molecular epidemiology and antimicrobial resistance of *Haemophilus influenzae* isolates from otitis media in Bulgaria

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ABSTRACT

Haemophilus influenzae is one of the main bacteria responsible for otitis media (OM) among children worldwide. We aimed to estimate the distribution of encapsulated and non-capsulated variants (NTHi), biotypes, antibiotic susceptibility, and molecular epidemiology of *H. influenzae* isolates recovered from pediatric OM cases in Bulgaria.

Capsule detection was done by PCR for *bexB* gene, absent in NTHi. All encapsulated strains were subjected to PCR serotyping. MIC susceptibility testing was performed according to the criteria of EUCAST. MLST was conducted for all 71 OM isolates.

The capsule detection and PCR – serotyping disclosed a predominance of NTHi (90.1%) and a few "a", "f", and "c" types. Biotype I was the most widespread (42.3%). β-lactam resistance was found in 35.2% of the isolates. MLST represented heterogenic population structure, whereas the most represented clonal complexes belonged to ST-3, ST-57, ST-105, and ST-1426. 42.3% of the STs showed relatedness to globally represented clones, and 11.3% displayed affiliation to international type 2.

Most of the *H. influenzae* isolates recovered from children with otitis media were non-typable strains from biotype I. The examined population structure was genetically diverse, with a predominance of international type 2 isolates.

KEYWORDS

Haemophilus influenzae, otitis media, clonal structure, antimicrobial resistance

INTRODUCTION

Haemophilus influenzae (*H. influenzae*) is one of the main habitual bacteria responsible for otitis media (OM) in children globally along with *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Streptococcus pyogenes* [1]. OM is a common infection in early childhood and a major cause of clinician visits and antibiotic prescriptions [2]. It varies from uncomplicated OM and acute otitis media with spontaneous rupture of the tympanic membrane to recurrent and chronic cases [2].

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The typeable *H. influenzae* strains possess a polysaccharide capsule encoded by the cap locus, in which Region II genes determine capsule types "a" through "f" and arrange the serotypes [3]. The non-capsulated *H. influenzae* strains are named non-typeable (NTHi) isolates. Among the encapsulated *H. influenzae* types, type b (Hib) is responsible for more than 90% of systemic infections [4]. Hib is part of two vaccines included in the routine immunization program in Bulgaria – a combinatory vaccine against diphtheria, tetanus, pertussis, polio, hepatitis B and Hib, and the pneumococcal polysaccharide protein D-conjugate vaccine (PHiD – CV). In PHiD – CV eight of all ten capsular polysaccharide serotypes from *S. pneumoniae* are conjugated to a nonlipidated cell-surface lipoprotein (protein D) of non-typeable *H. influenzae*. The incidence of Hib decreased significantly after the mass immunization and has resulted in an increase of the prevalence of NTHi or other capsular *H. influenzae* variants [5, 6].

The antimicrobial resistance of *H. influenzae* to ampicillin and other β – lactam antibiotics is generally due to either the production of a β – lactamase (β – lactamase-positive ampicillin-resistance – BLPAR) or the presence of altered PBPs with a lowered affinity for β – lactams in β – lactamase-negative ampicillin-resistant (BLNAR) isolates. A small proportion of strains are β – lactamase-positive amoxicillin-clavulanate-resistant (BLPACR) and possess both mechanisms [7]. All BLPAR *H. influenzae* isolates are positive for nitrocefin hydrolysis and can be differentiated by the presence of $bla\text{-}_{TEM}$ or $bla\text{-}_{ROB\text{-}1}$ genes for TEM-type and ROB-1 plasmid-mediated class β – lactamases [8]. Detection of *fstI* gene recognizes the PBP3 resistance in BLNAR strains [9]. The precise genotyping by multilocus sequence typing (MLST) helps to disclose the successfully circulating genetic lineages.

We aimed to investigate the prevalence of encapsulated and non – capsulated variants, characterize the biotypes and antibiotic susceptibility, and explore the molecular epidemiology of *H. influenzae* isolates recovered from children with OM.

MATERIALS AND METHODS

Patients and specimen collection

The AOM diagnosis was made by a pediatrician or an otorhinolaryngologist after an otoscopic examination of the patient's eardrum. Otorrhea specimens were obtained by collecting middle ear fluids (MEF) during an episode of spontaneous perforation of the tympanic membrane. Nasopharyngeal swabs were collected from patients without rupture but manifested typical OM symptoms such as ear pain, redness, swelling, fever, and hearing difficulties. A total of 253 OM specimens were collected voluntarily from microbiological laboratories in Sofia, Bulgaria for the period 2018–2021. Demographic data such as the date for hospitalization, clinical presentation, age, and vaccine status were collected from the medical records.

Microbiological identification and biotyping

A conventional diagnostic was performed with an oxidase test, Gram stain, satellite growth, requirements on factors X and V, and Remel Rapid NH biochemical identification tests (Thermo Fisher Scientific, USA). The eight biotypes I – VIII of *H. influenzae* were assigned based on the ability of the isolates to produce indole, urease, and ornithine decarboxylase [10].

Capsule detection and PCR-serotyping

The capsular variants were detected by amplification of the *bexB* gene [11]. All encapsulated strains were subjected to a PCR – serotyping to detect the specific capsule types "a" to "f" with primers designed by Falla et al. [12].

Antimicrobial susceptibility testing and detection of β – lactamase–encoding genes

The susceptibility testing was performed by Antimicrobial susceptibility plate (Sensititre HPB1 Plate, HTM (T3470), Thermo Fisher Scientific, USA). Interpretation was done according to the criteria of EUCAST, 2023 (https://www.eucast.org/clinical_breakpoints). PCR detection of $bla\text{-}_{TEM\text{-}1}$, $bla\text{-}_{ROB\text{-}1}$, and mutated *ftsI* gene was prepared, as described previously [13].

MLST

The phylogenetic relationships among *H. influenzae* isolates were determined by MLST. The internal fragments of the seven housekeeping genes were compared to the MLST database (https://pubmlst.org/organisms/haemophilus-influenzae) to identify the allelic profiles and sequence types (STs). Clusters of related STs were grouped into clonal complexes (CCs) by the use of PHYLOViZ (https://online.phyloviz.net/). CCs were defined at the single-locus variant (SLV), double-locus variant (DLV), or triple-locus variant (TLV) levels. The CCs genetically related to reference MLST clones were named after the international clone name. CCs, which did not show relatedness to global clones were named after the predominant sequence type or, if they were present in equal proportion, it was given the name of the sequence type that occurred earlier in the time.

Pathogenwatch (https://www.sanger.ac.uk/tool/pathogenwatch/) was used for comparing our findings to other pathogen genome assemblies from around the world.

RESULTS

Overall 71 *H.influenzae* isolates (23 MEFs and 48 nasopharyngeal specimens) were recovered from 253 collected OM pediatric specimens. The patient collection was not consecutive throughout the study period. Mixed infection was defined as two pathogens found in the same specimen or different pathogens isolated from specimens obtained from different ears of the same patient during the same visit.

Coinfection was detected in 12 cases with *S.pneumoniae*, *M. catarrhalis*, and *Pseudomonas aeruginosa*.

The specimens were from children aged 10 months to 10 years, most of them younger than five years of age (77.5%).

All children were vaccinated according to the Bulgarian immunization program with both Hib-containing vaccines.

The biotyping represents widespread distribution for biotypes I and II with rates of 42.3% and 22.5%, followed by types III (12.7%), IV (8.4%), V (7.04%), VI (5.6%) and VII (1.4%).

The presence of a capsule was confirmed in seven strains (9.9%) from capsule types "a" ($n = 3$), "f" ($n = 3$) and "c" ($n = 1$). The NTHi prevailed among the studied population (90.1%).

The antimicrobial susceptibility testing disclosed 62.0% resistant strains. Highest resistance rates were observed for trimethoprim-sulfamethoxazole (43.6%). A total of 35.2% were β-lactam resistant isolates. Among them, the BLPAR and BLNAR strains were 52.0% and 36.0%, respectively. Three isolates revealed simultaneously the presence of TEM – 1 β-lactamase and PBP3 mutations (Fig. 1).

The BLPAR strains were more common in the nasopharyngeal specimens (21.3%), where the same mechanism of resistance was represented by 12.5% of the MEF isolates. The PCR amplifications revealed only TEM – 1 β-lactamase. The BLNAR strains were recovered in 16.4% of the otorrhea specimens and 10.4% of the nasopharyngeal swabs. The conducted PCRs for the *ftsI* gene verified the PBP3 mutation mechanisms in all BLNAR isolates.

The β – lactamase positive strains were 36.6% and all of them were resistant to ampicillin, amoxicillin, and piperacillin. Sixteen (22.5%) of the isolates were non-susceptible to amoxicillin-clavulanic acid. A bigger part of these strains (68.8%) showed MIC $\leq$2 mg L^{-1} for amoxicillin-clavulanic acid and was interpreted as susceptible, increased exposure (I) strains with β-lactamase resistance only. The BLPAR strains with high MIC levels of ampicillin (4–8 mg L^{-1}) were 7.0%.

The BLPACR strains that harbored both beta-lactamase and PBP3 mutations, and revealed high MIC values above 4 mg L^{-1} for amoxicillin-clavulanic acid and >2 mg L^{-1} for cefixime were recovered from nasopharyngeal specimens.

Equal proportions of 12.7% were detected for resistant (MIC >1 mg L^{-1}) and susceptible, increased exposure strains to cefuroxime.

Resistance to ceftriaxone with MIC range (0.5 – 2.0 mg L^{-1}) was found in single cases (4.2%).

The population structure of the studied OM isolates was heterogenic. MLST disclosed 18CCs ($n = 49$), most of them comprised two – three closely related isolates. Four CCs were better represented: ST-3 complex, ST-57 complex, ST-105 complex, and ST-1426 complex. Except for CC334, CC832, and CC1898, all other CCs showed an exact match to reference clones in the MLST database ($n = 15$) or were genetically related to these clones on different levels: SLVs ($n = 13$), DLVs ($n = 12$), and TLVs ($n = 5$). The singletons were 31.0%, and around half of them (45.5%) belonged to known international genotypes (Fig. 2).

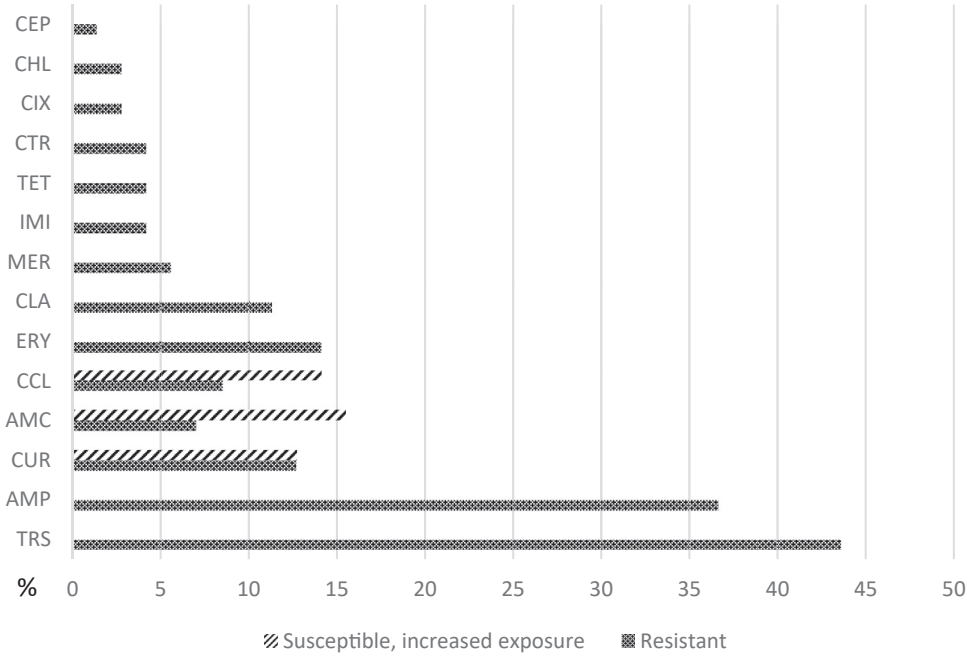

Fig. 1. Antimicrobial non-susceptibility of *H. influenzae* OM isolates among Bulgarian children aged 10 months to 10 years
Legend: AMP – Ampicillin, AMC – Amoxicillin/Clavulanic Acid 2:1 ratio, CTR – Ceftriaxone, CHL – Chloramphenicol, CLA – Clarithromycin, TRS – Trimethroprim/Sulfamethoxazole, ERY – Erythromycin, CCL – Cefaclor, CEP – Cefepime, CIX – Cefixime, CUR – Cefuroxime, IMI – Imipenem, MER – Meropenem, TET – Tetracycline. The interpretation was done according to the criteria and breakpoints of EUCAST, 2023

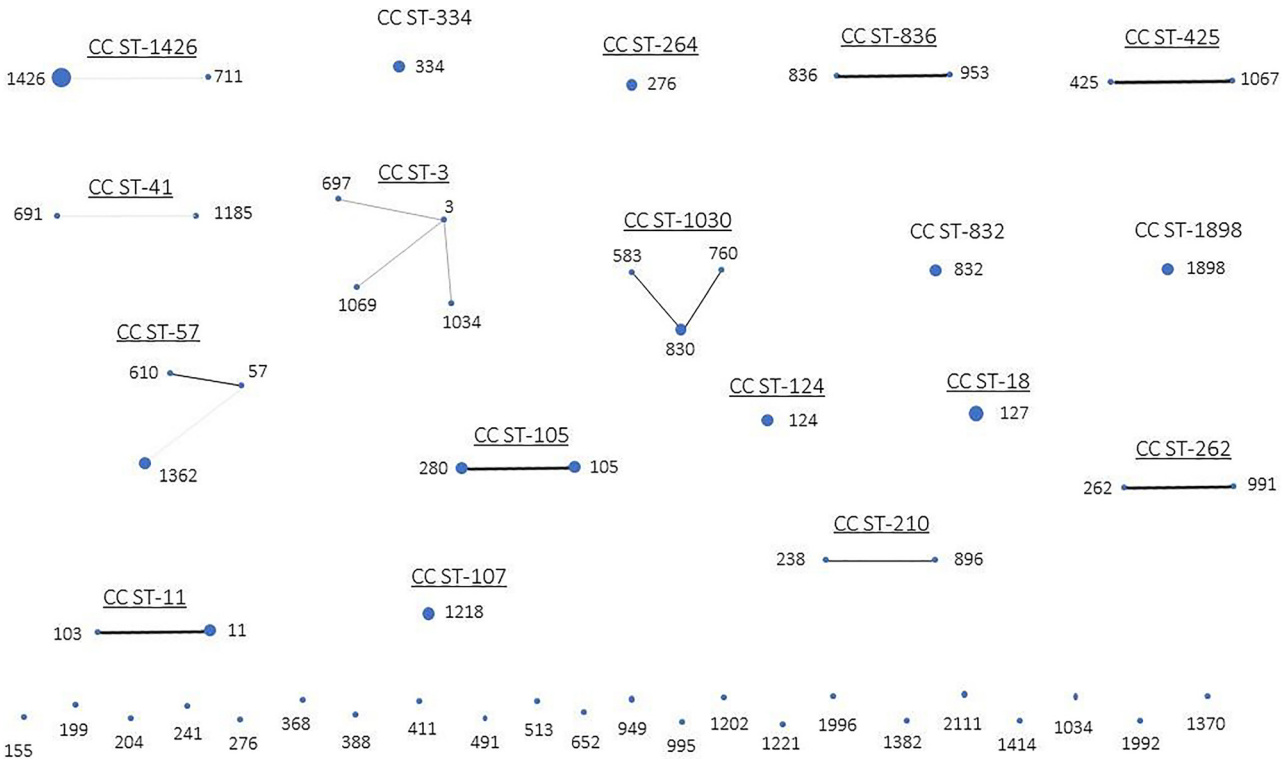

Fig. 2 Population structure of 71 *H. influenzae* isolates recovered from children with otitis media in Bulgaria
Legend: CC – clonal complex; ST – sequence type; SLV- single locus variant; DLV – double locus variant; TLV – triple locus variant. The bold and grey lines indicate the SLVs and DLVs, respectively. The thinnest light grey lines show the TLVs. The underlined names of the CCs represent known reference CCs in the MLST database and were named after these international clones. CCs, unrelated to known global clones, were named with the predominant sequence type or, if they were present in equal proportion, it was given the name of the ST that occurred earlier in the time

Comparing our results from the sequence-based typing to the Pathogenwatch database, we disclosed 42.3% of STs genetically related to international clones successfully spread in different geographic areas, listed in Table 1. International types 1 (8.5%) and 2 (11.3%) stand out among our isolates, followed by type 4 (5.6%).

DISCUSSION

Otitis media is a multifactorial disease, from uncomplicated OM to more complex recurrent and chronic cases [14]. The patients in our study reported a first visit due to otitis-related complaints. We have no information for recurrent OM later in the time.

Nontypeable *H. influenzae* was the most predominant pathogen responsible for 90.1% of the otitis media cases in our study. Following the implementation of Hib in our routine immunization program, it was expected, the leading role of NTHi in OM. The emergence of NTHi was declared in other studies and was consistently reported as the most prevalent *H. influenzae* that caused invasive and non-invasive diseases [6, 15]. Other capsular types were already commented as causes for *H. influenzae* diseases, but in our investigations, we identified just a few capsule types – a, c, f.

Biotype I was the most prevalent, and we may assume is correlated with the site of OM *H. influenzae* infection.

The BLPAR strains were more common in our study. TEM – 1 β-lactamase has confirmed its widespread distribution in Bulgaria as well as in other countries in the world, whereas ROB 1 – type is reported to be responsible for single BLPAR cases [7, 8].

We have registered single cases of resistance to third-generation cephalosporins. The highest resistance among the second-generation cephalosporins was found with cefuroxime. Reasons for this can be given both to the wide use of this antibiotic in our country and to some molecular mechanisms. Increasing resistance to cefuroxime in *H. influenzae* strains with non – enzymatic mechanisms of beta – lactamase resistance is also associated with mutations in the *ftsI* gene [9].

In some studies, the genotypic characterization of BLNAR strains was performed by sequencing and analysis of amino acid substitutions in the *ftsI* gene, which showed a classification of BLNAR strains into three groups (groups I to III) [16]. Based on the resistance level, BLNAR can be divided into two groups with high and low resistance. In our research, common strains were low-level resistant BLNAR, which are characterized as being groups I and II and are widely distributed worldwide [17]. Several studies have shown that the group with a high level of resistance belongs to genotypic group III, which is commonly found in Asia [18].

Table 1. Genotypic and phenotypic characteristics of *H. influenzae* isolates among children with Otitis media in Bulgaria

Clonal complex	ST[a]	Specimen[b]	Age	Resistance profile[c]	β-lactam resistance – mechanism[d]	Biotype	Capsule typing[e]	International genotype[f]
ST-3	3	Nph	3y9mo	–		I	NTHi	45
complex	697	Nph	5y6mo	TRS		I	NTHi	4
	1069	Nph	5y8mo	–		I	NTHi	3
	1034	MEF	4y9mo	AMP, CCL, CUR, TET, TRS	BLNAR (PBP3 mechanism)	I	NTHi	124
ST-11 complex	11	Nph	4y3mo	AMP, AMC (I), CCL (I), CUR (I)	BLPAR (beta lactamase only)	I	NTHi	66
	11	MEF	10y	–		II	NTHi	66
	103	Nph	3y6mo	AMP, AMC (I), ERY, CLA	BLPAR (beta lactamase only)	I	NTHi	92
ST-18 complex	127	Nph	3y6mo	AMP	BLNAR (PBP3 mechanism)	I	NTHI	1
	127	Nph	3y11mo	TRS		IV	c-type	1
	127	Nph	3y1mo	TRS		IV	c-type	1
ST-41 complex	691	Nph	4y	AMP, AMC (I), CUR (I), MER	BLPAR (beta lactamase only)	II	NTHi	–
	1185	MEF	4y11mo	–		I	NTHi	–
ST-57	57	Nph	5y2mo	TRS, CLA		II	NTHi	121
complex	610	NpH	4y9mo	AMP, AMC, CUR (I), MER, CHL	BLPAR (beta lactamase only)	I	NTHi	–
	1362	Nph	5y8mo	TRS		I	NTHi	–
	1362	Nph	6y	–		I	NTHi	–
ST-105 complex	105	MEF	4y1mo	AMP, CUR, TRS	BLNAR (PBP3 mechanism)	I	NTHi	15
	105	MEF	4y	–		II	NTHi	15
	280	Nph	4y2mo	AMP, AMC (I), CCL (I), CUR (I), TRS	BLPAR (beta lactamase only)	I	NTHi	1
	280	Nph	3y11mo	AMP, AMC (I)	BLPAR (beta lactamase only)	II	NTHi	1
ST-107	1218	Nph	2y4mo	TRS		IV	NTHi	10
complex	1218	Nph	5y2mo	AMP, TRS	BLNAR (PBP3 mechanism)	IV	NTHi	10
ST-124 complex	124	Nph	3y4mo	AMP, CUR, CTR, TRS	BLNAR (PBP3 mechanism)	II	NTHi	223
	124	Nph	3y5mo	–		I	a-type	223
ST-210	238	Nph	1y3mo	ERY, CLA		V	NTHi	1
complex	896	Nph	4y2mo	TET, TRS		VI	NTHi	–
ST-262	262	MEF	1y9mo	–		IV	NTHi	2
complex	991	MEF	1y10mo	–		I	NTHi	–
ST-264	276	Nph	4y10mo	TRS		V	NTHi	2
complex	276	MEF	10mo	AMP, CUR,	BLNAR (PBP3 mechanism)	VII	NTHi	2
ST-425	425	MEF	2y10mo	TET, CHL, TRS		III	NTHi	13
complex	1067	MEF	4mo	TRS		III	NTHi	–
ST-836 complex	836	MEF	2y	AMP, AMC, CCL (I), CUR (I), TRS	BLPAR (beta lactamase only)	III	NTHi	50
	953	MEF	9y	TRS		I	NTHi	–
ST-1030	583	Nph	4y	TRS		II	NTHi	–
complex	760	Nph	2y6mo	AMP, AMC (I)	BLPAR (beta lactamase only)	I	NTHi	–
	830	Nph	3y1mo	–		I	NTHi	–
	830	Nph	5y1mo	–		VI	NTHi	–
ST-1426	711	MEF					NTHi	–
complex	1426	Nph	3y	AMP, AMC, CCL, CUR, CTR, MER, TRS	BLPACR (beta lactamase + PBP3)	I	NTHi	2
	1426	Nph	2y11mo	–		III	NTHi	2
	1426	Nph	4y1mo	–		II	NTHi	2
	1426	MEF	2y	TRS		II	NTHi	2

(continued)

Table 1. Continued

Clonal complex	ST[a]	Specimen[b]	Age	Resistance profile[c]	β-lactam resistance – mechanism[d]	Biotype	Capsule typing[e]	International genotype[f]
ST-334	334	Nph	6y	TRS		I	NTHi	–
complex	334	MEF	8y	TRS		II	NTHi	–
ST-832	832	Nph	2y5mo	–		II	NTHi	–
complex	832	Nph	3y6mo	TRS		III	NTHi	–
ST-1898	1898	MEF	3y8mo	–		II	NTHi	–
complex	1898	MEF	3y	–		III	NTHi	–

Legend:
[a]ST – Sequence Type
[b]Specimen: Nph – nasopharynx, MEF – middle ear fluid
[c]Antimicrobial agents: AMP – Ampicillin, AMC – Amoxicillin/Clavulanic Acid 2:1 ratio, CTR – Ceftriaxone, CHL – Chloramphenicol, CLA – Clarithromycin, TRS – Trimethroprim/Sulfamethoxazole, ERY – Erythromycin, CCL – Cefaclor, CUR – Cefuroxime, IMI – Impenem, MER – Meropenem, TET – Tetracycline; (I) – susceptible, increased exposure
[d]β-lactam resistance – mechanisms: BLPAR – β-lactamase-positive ampicillin-resistance; BLNAR – β-lactamase-negative ampicillin-resistance with a presence of PBP3 mutations; BLPACR – β-lactamase-positive amoxicillin-clavulanate-resistance (β-lactamase and PBP3 mutations)
[e]NTHi – non-typeable *H. influenzae*
[f]International genotype according to "Pathogenwatch" database

The molecular epidemiology of the Bulgarian *H. influenzae* OM isolates disclosed 56.3% globally distributed STs in our study. More widespread were CC1426, CC105, and CC1030 represented by susceptible, BLPAR, or BLPACR strains, and we did not observe a correlation between the antimicrobial susceptibility and the STs. The predominant NTHi strains in our findings were genetically widely variable, while other investigations showed that the serotypeable strains are more clonal [19].

A comparison of the successfully circulating STs in our geographic area and the global data set (Pathogenwatch) revealed 11.3% of STs that belong to international type 2, found mainly in Italy, Poland, and the USA. International type 1, disseminated mostly in the USA, Brazil, and Sweden, covered 8.5% of our isolates. Type 4, reported mainly in Australia, was also found in our country (5.6%).

CONCLUSIONS

That is the first study describing the molecular epidemiology of *H. influenzae* in our country. It represented a genetic heterogeneity of the examined population and has provided information about the circulating genetic lineages in our geographic area, where almost all of them were non-typeable strains from biotype 1.

Conflict of interest: The authors declare no conflict of interest.

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