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Haemophilus influenzae and *Haemophilus parainfluenzae* occurrence in the ear effusion in pediatric patients prone to recurrent respiratory tract infections (RRTI) and with otitis media with effusion (OME)

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ABSTRACT

Introduction. *Haemophilus influenzae* and *Haemophilus parainfluenzae* are known as human-restricted respiratory microbiota representatives. The aim of the present paper was to assay haemophili prevalence in middle ear effusion specimens in pediatric patients with otitis media with effusion (OME).

Methods. A total of 86 ear effusion specimens (from the left and right ear independently) were collected from 43 pediatric patients with OME. For comparison, 58 nasopharyngeal specimens were taken from 58 pediatric patients prone to recurrent respiratory tract infections (RRTI). Isolation and identification of haemophili biotypes and antimicrobial susceptibility was accomplished by standard microbiological methods. The cell surface hydrophobicity (CSH) of isolates was assayed by the method of aggregation in ammonium sulfate (SAT).

Results. Haemophili were isolated in 25.6% (11/43) of all OME patients: in 5/43 (11.6%) – *H. influenzae* (biotypes II, III), in 5/43 (11.6%) – *H. parainfluenzae*, in 1/43 (2.3%) – both species were found. Haemophili-positive nasopharyngeal specimen was found in 27/58 (46.6%) RRTI patients: in 19/58 (32.8%) – *H. influenzae*, in 8/58 (13.8%) – *H. parainfluenzae*. About 90% of all haemophili isolates were characterised by extreme to strong CSH. Antimicrobial resistance occurred mainly among *H. parainfluenzae* (80%) and to a much lower percentage among *H. influenzae* (33.3%) isolates. The obtained data suggest that both *H. influenzae* and *H. parainfluenzae* can be involved in pathology of OME in pediatric patients. The high cell surface hydrophobicity can affect on the haemophili prevalence and ear colonization, and induces predisposition to the presence of these bacteria as a biofilm that serves as a virulence factor with great importance for the survival of these opportunistic bacteria and their persistence in the ear environment.

INTRODUCTION

Middle ear diseases in childhood, such as acute otitisshort duration of illness and an acute beginning. OME is media (AOM) or otitis media with effusion (OME; bluethe clinical term for the presence of fluid in the middle ear drum, glue ear) are a very common set of childhoodbehind the intact eardrum and is characterized by a non-diseases, and they have been studied extensively within thepurulent effusion that may be either mucoid (thick) or last decades [1-3]. AOM is connected with an inflammationserous (thin) [4]. It is an inflammation of the middle ear of the middle ear, especially of the tympanic cavity, withwithout signs or symptoms of acute ear infection, pending beyond its intact eardrum and without systemic or otoscopic symptoms. An OME is the catchall term if the tympanic effusion persists for more than 3 months. Near chronic

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rhinosinusitis is a common disease of childhood, occurring most often between six months and four years of age. It is one of the most common causes of hearing loss in this age group.

According to literature [2,5-8], approximately 80-90% of all children have at least one episode of AOM or OME before school age. The disease is characterized by the presence of fluid in the tympanic cavity and progressive hearing loss. Effusion in the middle ear is often accompanied by upper respiratory tract infections and then is maintained only for a few days. In the course of chronic OME, affecting up to 75% of all children, effusion persists to 6 months and its spontaneous regression is much more rare. It is assumed that every tenth child in preschool and one in six school children has periodic or permanent hearing loss associated with OME [5,6,9]. The wide range and steady increase in the incidence of OME in the last fifteen years has forced multilateral and targeted prevention campaigns, as well as a search for optimal methods of treatment.

The pathogenesis of the disease is not clearly explained and is probably multifactorial. The development of recurrent and chronic otitis is affected by several host factors, such as immunological disorders, recurrent upper respiratory tract infections, allergies, genetic predisposition, being male and also by environmental factors. The carriage of pathogenic microorganisms within upper airways in children during the first years of life depends on their immune system condition, as well as on various epidemiologic and socioeconomic factors [9,10]. *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* have commonly been identified as the bacterial pathogens in the majority of reports from acute otitis media (AOM) patients [11,12].

Respiratory microbiota may be the reservoir of opportunistic pathogens with high (e.g. *H. influenzae*) or low (e.g. *H. parainfluenzae*) pathogenicity [10,13,14]. Both these species types can be responsible for the acute and recurrent or chronic forms of respiratory infections, especially in young children [15-20].

The type of diseases caused by *H. influenzae* has changed considerably in recent years; both *encapsulated* and non-typeable *H. influenzae* (NTHi) remains the most common pathogens to cause respiratory infections (e.g. acute respiratory infection, pneumonia, otitis media), or invasive infections (e.g. bacteremia, meningitis) [17]. *H. influenzae* was the most frequently associated bacterium for patients with chronic OME, recurrent AOM and AOM with treatment failure [12,21-23]. Additionally, Cardines *et al*. [24] identified a case of meningitis due to *H. parainfluenzae* in an adult with a 3-day history of acute otitis media.

The aim of our study was to ascertain *H. influenzae* and/or *H. parainfluenzae* prevalence in a group of pediatric patients diagnosed with chronic otitis media with effusion (OME) and underwent myringotomy, as well as to detect within this patient group, those prone to recurrent respiratory tract infections (RRTI) and without OME. Additionally, the antimicrobial sensitivity and cell surface hydrophobicity preferring to biofilm formation of selected haemophili isolates were assessed.

MATERIAL AND METHODS

Forty-three children aged from 2 to 6 years old (Female – 16, Male – 27), diagnosed with chronic otitis media with effusion (OME) were enrolled to the study. These OME patients were qualified for myringotomy – the surgical incision of the tympanic membrane with simultaneous removal of pathologic secretions. Studies were made during routine hospital procedures using to evaluate of OME, and swabs embodiment from the upper airways were not included.

None of the patients had active rhinosinusitis at the time of aspiration, but had antimicrobial therapy during thirty days before surgery. In the course of treatment, with a surgical microscope, a myringotomy incision was made in the tympanic membrane. Under direct visualization, the effusion was aspirated through sterile polyethylene tubing attached to a disposable middle-ear fluid collector. The physicians who were in charge of the patients were responsible for the diagnostic procedures and all decisions regarding treatment and follow-up. The study was approved by the Ethical Committee of Medical University of Lublin (No. KE-0254/75/2011). A total of 86 middle-ear effusions (two samples from each single patient – from right and left ear independently) were collected intra-operatively for microbiology. The collecting of the effusion was done after cleaning and disinfection of the external auditory canal and tympanic membrane with 70% solution of alcohol; then the tympanic membrane was cut it in the posterolateral quadrant, using an operating microscope. Material was collected under pressure using a sterile disposable Polymed Mucus Extractor sampling set (Poly Pedicure Ltd.). Next, the collected specimens were transported to the Department of Pharmaceutical Microbiology, Medical University of Lublin, where further tests were carried out.

In some of the work making up this study, a group of 58 of pediatric patients aged from 3 to 6 years old (Female – 22, Male – 36) prone to recurrent respiratory tract infections (RRTI, ≥3 episodes/year) and without OME was also included. A total of 58 nasopharyngeal specimens were taken from RRTI-patients who did not have active rhinosinusitis at the time of study, or had not been admitted to hospital for at least three months or had antimicrobial treatment at least thirty days before study.

All collected specimens taken from the nasopharynx and ear effusion were immediately placed onto the selective Haemophilus chocolate agar (BioMerieux, France) in the direction of haemophili, and then cultured in the appropriate atmosphere with an increased CO_2 concentration for 24-48 hrs at 35°C. After incubation, the growth of bacteria in the form of individual colonies or from abundant to a very abundant number of colonies on chocolate agar was observed. Haemophili rods with the same morphology of colonies growing on chocolate agar were treated as the same morphotype and identified on the basis of routine diagnostic methods (macroscopic, microscopic or biochemical assays). The growth requirement, biochemical characteristics, cell surface hydrophobicity (CSH) and antimicrobials sensitivity were assessed for the tested bacteria.

For the primarily identification of isolated morphotypes, growth factors requirements were evaluated on TSA (Tripticasein Soy Lab-Agar, Biocorp, Poland) medium. Diagnostic discs (Oxoid, England) with hemin (X factor), nicotinamide adenine dinucleotide (V factor), and both X and V factors were used. Next, biochemical identification of Gram-negative isolates was carried out using the API NH microtest (bioMérieux, France). Haemophili isolates were assigned to I – VIII biotypes on the basis of indole production, urease activity and ornithine decarboxylase activity.

Cell surface hydrophobicity (CSH) assay. Herein, spontaneous aggregation of bacterial cells was carried out in 0.85% NaCl. A positive test result was recorded when the microorganisms remained clumped, and a negative test result was assigned when a smooth turbid suspension formed after gentle vortexing. The auto-aggregation assay was done by dispensing 20 µl of a bacterial suspension, resuspended in 20 µl of the phosphate-buffered saline (PBS) on a glass slide. Auto-aggregation of bacteria was observed against a spotlight by manually rotating the droplet for up to 1 min at room temperature. Bacterial suspensions that remained turbid and homogeneous were considered non-auto-aggregative, while bacterial suspensions presenting clumped cells were considered auto-aggregative [15].

For quantifying the hydrophobic cell surface properties of haemophili isolates, the salt aggregation test (SAT) according to [18] was used. Twofold serial dilutions of $(NH_4)_2SO_4$ (ammonium sulfate, Avantor Performance Materials, Poland) in PBS ranging from 0 to 3.2 M [final concentration], and bacterial cell suspensions from HAEM agar-grown cultures resuspended in PBS (with very high density in about 9-10 McFarland standard) were prepared. Twenty-microliter aliquots of each ammonium sulfate solution were subsequently placed on glass slides and mixed thoroughly with 20 µl of bacterial suspensions for visible aggregation by "salting out". Test results were interpreted taking into account the lowest concentration of ammonium sulfate, wherein the tested strains underwent aggregation (SAT value). The surface hydrophobity of bacteria was classified as follows, according to SAT value: (i) PBS alone – extremely hydrophobic surface, (ii) <0.4 M – very strong hydrophobic surface, (iii) 0.4 M-1.0 M – strong hydrophobic surface, (iv) 1.2 M-1.6 M – hydrophobic surface, (v) ≥1,8 M – hydrophilic surface.

Antimicrobial sensitivity assay. Antibiotic sensitivities of haemophili isolates were determined by the disc diffusion method, by means of Haemophilus Test Medium (HTM, Oxoid, England) according to the Clinical Laboratory Standards Institute (CLSI) recommendation for *Haemophilus* species [19]. Direct colony suspensions standardized to 0.5 McFarland (~10^8 CFU/ml) were prepared using the colonies from an overnight HAEM agar incubation at 35°C in the atmosphere with about 5% CO_2. *H. influenzae* ATCC10211 was used to verify the growth promotion properties of HTM. Different discs with antimicrobial agents (BD BBL, Becton Dickinson and Company, USA), namely ampicillin, amoxicillin-clavulanic acid, ampicillin-sulbactam, cefazoline, cefuroxime, cefotaxime, ceftazidime, imipenem, aztreoname, azithromycin, amikacin, tetracycline, trimethoprim/sulfamethoxazole, ciprofloxacin, gentamycin were used. Isolates resistant to ampicillin were screened for beta-lactamase production by applying the Pen test (API NH, bioMerieux, France) and nitrocefin by means of the chromogenic cephalosporin method (Cefinase disks, BD BBL, Becton Dickinson and Company, USA).

Statistical analysis

Data processing and analysis were achieved through Stat-Soft. Inc Statistica 2010 for Windows. Frequencies of colonization and isolation of bacteria in the follow-up were compared with those at ear effusion by means of Fisher's test. Relative risk (RR) and their 95% confidence intervals (CIs) were calculated. A p value ≤ 0.05 was considered as statistically significant.

RESULTS

The frequency of patient colonization

As shown in Table 1, haemophili were found in 11/43 (25.6%) pediatric patients with OME in the ear effusion specimens: in 5/43 (11.6%) patients with *H. influenzae* alone and in 5/43 (11.6%) patients with only *H. parainfluenzae*, while in 1/43 (2.3%) patients, *H. influenzae* together with *H. parainfluenzae* were found. In comparison, haemophili-positive nasopharyngeal specimen were discovered in 27/58 (46.6%) of all RRTI-patients: 19/58 (32.8%) of all patients were colonized by *H. influenzae* and 8/58 (13.8%) were colonized by *H. parainfluenzae*, in 1/49 (2%), non-identified *Haemophilus* spp. was collected.

Table 1. The prevalence of haemophili-positive specimens in the middle ear-effusion taken from pediatric patients with otitis media effusion (OME) who had undergone myringotomy, and in the nasopharyngeal swabs taken from pediatric patients prone to recurrent respiratory tract infections, but without OME

Group of pediatric patients	*Haemophilus influenzae*	*Haemophilus parainfluenzae*	*Haemophilus influenzae* together with *Haemophilus parainfluenzae*	Total number of colonized patients
RRTI patients (n=58)	19 (32.8)	8 (13.8)	0 (0)	27 (46.6)
OME patients (n=43)	5 (11.6)	5 (11.6)	1 (2.3)	11 (27.9)
p value	0.0177	1.000	nd	0.0387
RR	1.563	1.186	nd	1.444
95%CI	1.156-2.113	0.4169-3.375	nd	1.046-1.994

nd – not determined

Statistically significant differences (p=0.0387) were observed in the number of pediatric patients with OME colonized by haemophili in the ear effusion (11/43, 27.9%), as compared to RRTI-patients colonized by these bacteria in the nasopharynx (27/58, 46.6%). Similarly, statistically significant differences were seen in the number of patients colonized by *H. influenzae* (6/43, 13.95% vs. 19/58, 32.8%; p = 0.0177), but not by *H. parainfluenzae* (p = 1.000). There were no differences between the frequency of haemophili-positive ear effusion specimens taken from Male and Female (8/27, 30% vs. 3/21, 14.3%, p > 0.1).

Haemophili isolates characteristics

Haemophili phenotypes were differentiated based on observable properties in the growth morphology (e.g. the shape and size of the colony, smooth or rough surface, texture, colony elevation), on a set of biochemical reactions (according to API NH results) and antimicrobial susceptibility results.

As was shown in Figure 1, in 17/86 (19.8%) of all haemophili-positive ear-effusion specimens, a total of 19 haemophili isolates were identified: 9/19 (47.4%) as *H. influenzae* (designated as two biotypes: II – 3 isolates and III – 6 isolates) and 10/19 (52.6%) as *H. parainfluenzae* (designated as biotypes: I – 3 isolates, II – 5 isolates, III – 1 isolate, and V – 1 isolate).

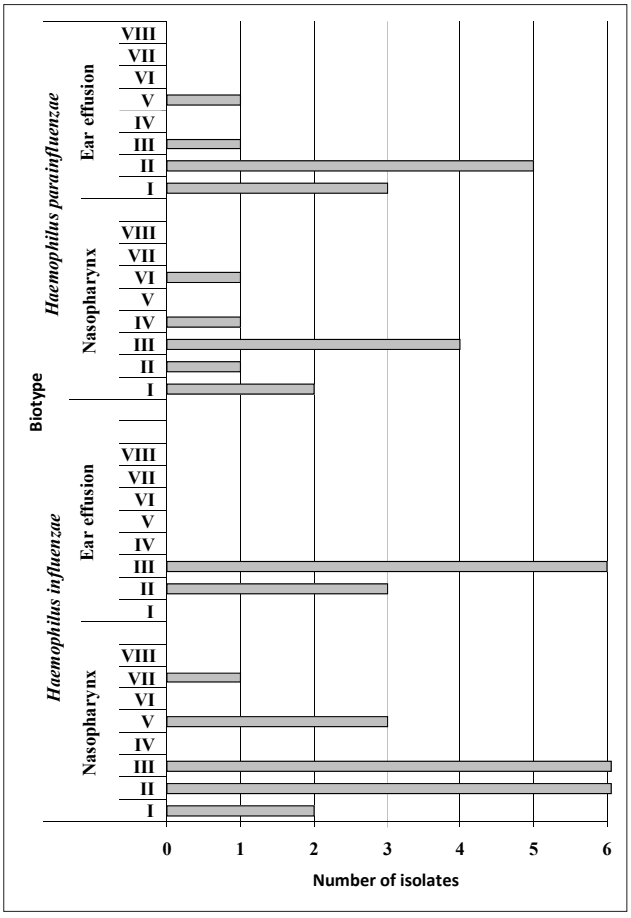

Figure 1. *Haemophilus influenzae* and *Haemophilus parainfluenzae* biotypes occurrence in the middle ear effusion specimens taken from pediatric patients with otitis media with effusion (OME) who qualified for myringotomy, and from the nasopharynx of RRTI patients

Among a total of 19 haemophili isolates (9 – *H. influenzae* and 10 – *H. parainfluenzae*) selected in the ear effusion specimens of OME patients, 11/19 (57.9%) isolates were detected as resistant to antimicrobials (Table 2). This resistance occurred mainly among isolates of *H. parainfluenzae* (8/10, 80%) and in much lower percentage among isolates of *H. influenzae* (3/9, 33.3%). As was detected, 6/11 (54.5%) isolates were ampicillin-resistant and beta-lactamase (penicillinase) positive, including 3/11 (27.3%) *H. influenzae* and 3/10 (27.3%) *H. parainfluenzae* strains.

The same biotypes, together with the same antimicrobials sensitivity profiles of *H. influenzae* and *H. parainfluenzae,* were found in 3/11 (27.3%) and 1/11 (9.1%) of all patients with colonized OME, respectively, both in the left and right ears (Table 2). In 1/11 (9.1%) of all patients, *H. parainfluenzae* isolates independently selected from the left and right ear had the same biotype (I), but they differed in the antimicrobials susceptibility.

Table 2. Haemophili characteristics in the ear effusion specimens taken from patients with otitis media with effusion and who qualified for myringotomy

No. of patient	Ear with haemophili isolation	Species	Biotype	Cell surface hydrophobicity	Resistance profile
11	Left	*Haemophilus parainfluenzae*	II	extremely hydrophobic	Am (Pen_{pos}) SxtTe
	Right	*Haemophilus parainfluenzae*	II	extremely hydrophobic	Am (Pen_{pos}) SxtTe
12	Left	*Haemophilus parainfluenzae*	III	very strong hydrophobic	SxtCz
	Right	*Haemophilus parainfluenzae*	V	very strong hydrophobic	Cz
14	Left	*Haemophilus parainfluenzae*	II	extremely hydrophobic	Sxt
		Haemophilus influenzae	III	extremely hydrophobic	-
	Right	ng	-	-	-
15	Left	ng	-	-	-
	Right	*Haemophilus parainfluenzae*	II	extremely hydrophobic	-
16	Left	*Haemophilus parainfluenzae*	I	extremely hydrophobic	-
	Right	*Haemophilus parainfluenzae*	I	extremely hydrophobic	CzTe
17	Left	ng	-	-	-
	Right	*Haemophilus parainfluenzae*	I	extremely hydrophobic	Am (Pen_{pos}) SxtTe
		Haemophilus parainfluenzae	II	extremely hydrophobic	SxtTe
21	Left	ng	-	-	-
	Right	*Haemophilus influenzae*	II	extremely hydrophobic	Am (Pen_{pos})
24	Left	*Haemophilus influenzae*	III	strong hydrophobic	-
	Right	*Haemophilus influenzae*	III	strong hydrophobic	-
32	Left	*Haemophilus influenzae*	II	strong hydrophobic	Am(Pen_{pos})
	Right	*Haemophilus influenzae*	II	strong hydrophobic	Am(Pen_{pos})
39	Left	*Haemophilus influenzae*	III	extremely hydrophobic	
	Right	ng	-	-	-
43	Left	*Haemophilus influenzae*	III	hydrophilic	-
	Right	*Haemophilus influenzae*	III	hydrophilic	-

Abbreviations: ng – no growth; Am – ampicillin; Cz – cefazoline; Sxt – trimethoprim/sulfametoksazol; Te – tetracycline; Pen_{pos} – penicillinase-positive

In 27/58 (46.6%) of all nasopharyngeal specimens taken in RRTI patients, 29 haemophili isolates were selected (Figure 1). Thus, 20/29 (69%) isolates were classified as *H. influenzae* (biotypes I, II, III, V and VII) and 9/29 (31%) as *H. parainfluenzae* (biotypes I, II, III, IV and VI). 1/20 (5%) *H. influenzae* and 2/9 (22.2%) *H. parainfluenzae* isolates were beta-lactamase-positive.

As was evaluated on the basis of SAT value (Table 2), a total of 17/19 (89.5%) haemophili isolates in OME patients were characterized by their hydrophobic cell surfaces: these were all the selected *H. parainfluenzae* isolates (10/10, 100%) and a majority of *H. influenzae* isolates – 7/9 (77.8%).

Broken down, 11/19 (57.9%) of all isolates were characterized by extremely hydrophobic cell surface (*H. influenzae* – 3, *H. parainfluenzae* – 8 isolates), 2/19 (10.5%) isolates – by very strong hydrophobic cell surface (only *H. parainfluenzae* – 2 isolates), 4/19 (21.1%) isolates – by strong hydrophobic cell surface (only *H. influenzae*), and 2/19 (10.5%) isolates – by hydrophilic cell surface (only *H. influenzae*).

DISCUSSION

Our study indicates that about 28% of all OME pediatric patients were colonized by haemophili in the ear effusion, either by *H. influenzae* and *H. parainfluenzae*. On the basis of our results, we suggest that both *H. influenzae* and *H. parainfluenzae* are etiological agents of OME disease in young children. Accordingly, their high cell surface hydrophobicity predisposes to the presence of these bacteria in biofilm. This can be a pathogenic factor of great importance for the survival of these opportunistic bacteria and their persistence in the ear environment.

Although respiratory viruses have an important role in the pathogenesis of OME [25], the isolation of bacteria from ear effusion specimens has long been the gold standard for the etiologic diagnosis of the causative pathogens for acute otitis media. Our work reveals that only approximately 50-60% of all diagnostic samples were culture-positive for any of the major causative pathogens [21-23,26]. According to Klein *et al*. [27], although bacterial pathogens have been isolated in the majority of cases, cultures have still yielded negative results for pathogenic bacteria in about 12%–35% of all cases.

Haemophili, mainly non-typeable *H. influenzae* (NTHi), is a leading causative pathogen responsible for acute otitis media [21-23]. The presence of *H. parainfluenzae* in the respiratory tract microbiota composition also may have a pathogenic role [28]. Although *H. parainfluenzae* has not been considered of much clinical importance in humans, the case describe by Cardines *et al*. [24] confirms that it can be regarded as an opportunistic pathogen able to cause life-threatening infections, not only in children, but also in adults, especially when underlying conditions are present. Cardines *et al*. [24] supposed that the same *H. parainfluenzae* isolate was responsible for both meningitis and acute otitis media, although they did not prove this hypothesis since no culture of the middle ear fluid was performed. In our opinion, the near pathogenic role of these bacteria can cause other negative results due to their presence, such as co-colonization by other microorganisms. The causative pathogens frequently populate the nasopharynx and bring infection to the middle ear via the Eustachian tubes [29]. It is recognized that middle-ear disorders are related to the presence of typical pathogens such as *Streptococcus pneumoniae*, *H. influenzae*, *Moraxella catarrhalis* or *Staphylococcus aureus* – all of which commonly colonized the mucous membranes of the upper airways [11,13,30].

Some differences were demonstrated in the distribution of haemophili biotypes and number of antimicrobial resistant isolates. According to our results, compared with only two *H. influenzae* biotypes (II and III) detected in the ear effusion specimens in OME patients, a greater diversity of biotypes (mainly II and III, and rarely I, V or VII biotype) of this species was observed in the nasopharyngeal specimens in RRTI patients. These differences were lower among *H. parainfluenzae* biotypes selected in the ear effusion specimens (mainly I and II, and rarely III and V) and in the nasopharyngeal specimens (mainly I and III, and rarely II, IV, VI) in these two groups of children. All beta-lactamase producing *H. influenzae* isolates were classified as biotype II and were ampicillin-resistant, while *H. parainfluenzae* isolates were classified as biotypes I and II – with resistance against ampicillin, trimethoprim/sulfamethoxazole and tetracycline.

Other authors have reported the oropharyngeal prevalence of mainly I and II biotypes of *H. parainfluenzae* compared to the low frequency of the other biotypes of this species (31; 32; 33; 34). For example, the *H. parainfluenzae* isolate classified as biotype I was found associated with isolates from healthy carriers in a previous report [14]. As found by other authors [31,34,35], biotypes I and II constituted most of *H. parainfluenzae* isolates in patients with other respiratory diseases such as chronic bronchitis or cystic fibrosis.

High cell surface hydrophobicity was shown for isolated haemophili strains through use of the SAT method. The adherence capacity of bacteria to host cells depends on the bacterial surface properties such as hydrophobicity [36,37]. A more hydrophobic nature of cell surface usually may be regarded as a most relevant parameter for the maximum capability of biofilm formation of microbes [38]. Medical biofilms are involved in a number of chronic infections, including otitis media with effusion (OME) and chronic rhinosinusitis (CRS), which are common pediatric infectious diseases. Many features of otitis media support biofilm ear infection. This is formed by bacteria growing in slime-enclosed aggregates and provides an explanation for the lack of success of antibiotic therapy, the presence of negative bacterial cultures, and persistence of bacteria in the middle-ear space between acute episodes and local inflammatory responses to persistent bacteria [39]. The ability to form a biofilm by opportunistic coagulase-negative staphylococci strains emphasizes the pathogenic character of these strains in some cases of otitis media with effusion. More detailed studies on the ability to form biofilm by haemophili rods isolated from patients with OME will continue in the future. The high ability to grow in biofilm form especially of *H. parainfluenzae* and at low degree of *H. influenzae* was shown during our studies in pediatric patients with recurrent respiratory infections undergoing adenoidectomy [40], as well as in adult patients with sarcoidosis [41]. Bacteria in biofilms are generally well protected against environmental compounds, antimicrobials and the host immune system, and this is the reason why they are extremely difficult to eradicate [42-45].

CONCLUSIONS

The obtained data suggest the role of *H. parainfluenzae* and *H. influenzae* in OME pathology in pediatric patients. In these, bacterial high cell surface hydrophobicity can predispose to ear colonization and the growth in biofilm form, and they are favored as an OME etiological factor.

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