International Journal of Medical Microbiology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

Carriage of antibiotic-resistant *Haemophilus influenzae* strains in children undergoing adenotonsillectomy

Elżbieta A. Trafny[a,*], Olga Olszewska-Sosińska[b], Małgorzata Antos-Bielska[a], Krystyna Kozłowska[a], Małgorzata Stępińska[a], Magdalena Lau-Dworak[c], Beata Zielnik-Jurkiewicz[b]

[a] *Department of Microbiology, Military Institute of Hygiene and Epidemiology, Kozielska 4, 01-163 Warsaw, Poland*
[b] *Department of Otolaryngology, Children's Hospital, Niekłańska 4/24, 03-924 Warsaw, Poland*
[c] *Department of Laboratory Diagnostics, Children's Hospital, Niekłańska 4/24, 03-924 Warsaw, Poland*

ARTICLE INFO

Article history:
Received 4 July 2013
Received in revised form 17 February 2014
Accepted 24 March 2014

Keywords:
Chronic adenoiditis/tonsillitis
Hypertrophy of adenoid/tonsils
Persistent genotypes
Antibiotic susceptibility

ABSTRACT

Haemophilus influenzae is one of the major pathogenic bacteria in upper respiratory tract of children. In this study, the presence of various *H. influenzae* genotypes were followed-up for at least 13 weeks, starting from one week before surgery. Forty-one children with chronic adenoid hypertrophy were prospectively enrolled to the study. The consecutive swabs of adenoid and tonsils, two before adenotonsillectomy and two after the surgery together with homogenates of adenotonsillar tissues and lysates of the $CD14^+$ cells fraction were acquired from 34 children undergoing adenotonsillectomy. Up to ten isolates from each patient at each collection period were genotyped using a PFGE method and their capsular type and antibiotic susceptibility was determined. Of the 1001 isolates examined, we identified 325 isolates grouped into 16 persistent genotypes, which colonized throats for more than seven weeks and were not eliminated by the surgery. The other 506 isolates grouped into 48 transient genotypes that had been eliminated by the surgery. The resistance to ampicillin were found in 23.8% of the transient strains, and 4.7% of the newly acquired strains following the surgical intervention. In contrast, none of the persistent strains were resistant to ampicillin; however, these strains showed apparently higher level of resistance to co-trimoxazole when compared to transient strains. The transient and persistent strains did not significantly differ in bacterial viability in the biofilms formed in vitro. Some of the strains were identified in two or three different patients and were considered as the strains circulating in the region between 2010 and 2012.

Introduction

Haemophilus influenzae still remains the main bacterial etiologic agents of upper respiratory tract infection (UTI) together with *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *Staphylococcus aureus*. As a consequence of the introduction of anti-Hib (anti-*H. influenazae* type b) vaccination, the majority of the isolates colonizing the respiratory tract are nontypeable strains (NTHi) (Agrawal and Murphy, 2011). Recently, the appearance of capsulated strains of *H. influenzae* of type e and f (Hif and Hie) and a slow steady increase in their incidence have been reported in children with UTI (de Carvalho et al., 2011; Ladhani et al., 2012). Nasopharynx, which is densely populated by diverse strains of commensal and pathogenic bacteria, is recognized as a reservoir of the pathogens causing UTI and ear infections, among them otitis media and sinusitis (Torretta et al., 2013). The common manifestations of infection are adenotonsillar hypertrophy and recurrent tonsillitis. The close relationship between recurrent tonsillitis and hypertrophy has not been yet elucidated (Biermans et al., 2009). However, in both phenomena the role of biofilm, an organized multi-species community of bacteria has been indicated (Hoa et al., 2010). The biofilm structures cover the mucosal surface of adenoids of children with recurrent acute otitis media. Nevertheless, only scarce biofilm structures have been observed on the mucosa of children with obstructive sleep apnea. These findings may indicate that mucosal biofilms serve as bacterial reservoirs (Zuliani et al., 2009).

Recently, another mechanism of bacterial persistence within the nasopharynx has been postulated – an intracellular survival of common UTI's pathogens in epithelial cells and macrophages of adenoids (Hotomi et al., 2010; Morey et al., 2011). Such

* Corresponding author. Tel.: +48 22 685 32 06; fax: +48 22 838 10 69.
E-mail addresses: e.trafny@wihe.waw.pl, trafny.elzbieta@gmail.com (E..A. Trafny).

http://dx.doi.org/10.1016/j.ijmm.2014.03.004
1438-4221/

intracellular persistence would allow the bacteria to avoid host immune defences as well as antibacterial action of antibiotics (Clementi and Murphy, 2011). The effect of adenotonsillectomy on the oropharyngeal flora has already been studied in details in large randomized controlled trial (Le et al., 2007). The results showed that the prevalence of *H. influenzae* decreased from 40% to 24 and 26% after 3 and 12 months after surgery, respectively. At the same periods of time, no isolates of *Staphylococcus aureus* and *Streptococcus pyogenes* were cultured from this group of children.

Colonization of the nasopharynx is a dynamic process, with high turnover of the colonizing *H. influenzae* isolates in healthy children (Farjo et al., 2004, LaCross et al., 2008). The carriage of *H. influenzae* may be influenced by living conditions and age (Dabernat et al., 2003). The investigation of homogeneity of microflora of nasopharynx before and after the surgery intervention is important in understanding the aetiology of middle ear and paranasal sinuses disease. The carriage of *H. influenzae* strains as well as their antibiotic susceptibility before and after adenotonsillectomy has not yet been characterized. In the previous study on the dynamics of the colonization and persistence of *H. influenzae* in the throat of children only the isolates from swabs were compared by genotyping (Otsuka et al., 2012). We expanded the scope of research by introducing the analysis of restriction patterns of *H. influenzae* strains isolated from the homogenates of the excised adenoid and tonsils tissue as well as from the macrophage/monocytes from lymphoid nasopharyngeal tissues.

Materials and methods

Patients

Forty-one children undergoing adenotonsillectomy because of upper airway obstruction: recurrent tonsillitis/adenoiditis and adenotonsillar hypertrophy were prospectively enrolled to the study. Inclusion criteria for this study were age between 4 and 6 years, and selection for adenotonsillectomy was due to clinical symptoms. Exclusion criteria were allergy, and smoking in the child environment. The 34 patients from whom *H. influenzae* was isolated from swabs before adenotonsillectomy or/and from homogenates of lymphoid tissues were selected as study participants. Other seven patients from whom *H. influenzae* was isolated only from follow-up swabs after the surgery were excluded from the study. No antibiotics had been administered to the patients within two weeks before the intervention. The throat swabs and tissue specimens (fragments of the excised adenoid and tonsils) were collected from January 2011 to June 2012. The median age of children was 5 years (quartiles, 4, 6) and 41.2% of them were girls. Informal consent was obtained from the parents. Relevant information regarding the participant, and their immunization status was obtained. None of the children were acutely ill. Eighteen children were vaccinated against Hib, and thirteen of them were vaccinated against *S. pneumoniae*. The medical ethics committee of Regional Medical Chamber in Warsaw approved the study protocols. The fragments of the excised tissues underwent histopathological analysis to confirm that the inflammatory process takes place in the throat.

Throat swabs collection

Paired pharyngeal swabs were collected from each participant. The swabs were obtained by wiping the mucosal surfaces with a sterile cotton swabs (van der Veen et al., 2006a). Swabs were collected from: (1) the adenoid and palatine tonsils one week before the operation in outpatient clinics; (2) the adenoid and palatine tonsils upon anaesthesia during operation; (3) the mucosal surfaces of nasopharynx and the palatine tonsils at 6–8 (swab I after surgery) and 12–20 weeks (swab II after surgery) after the intervention during two follow-up visits in outpatient clinics. The swabs of two patients were taken two weeks before the surgical intervention as they waited for the improvement of the parameters of blood and urine. Swabs of right and left tonsils were taken with the same cotton swab. Contact of the swabs with the oral cavity mucosa was carefully avoided. The throat swabs were transported in Stuart transport medium to the hospital microbiological laboratory and processed the same day. The excised fragments of the adenoids and palatine tonsils were transported to the microbiological laboratory at MIHE within two hours after the surgery in DMEM medium (DMEM with GlutaMax™-I, GIBCO, Invitrogen) at 4 °C. The isolates of *H. influenzae* were cultured on a chocolate agar plate (bioMerieux) for 48 h at 37 °C in 5% CO_2. The other bacterial species taken with swabs were cultured on a Columbia Agar with 5% sheep blood (bioMerieux) for 48 h at 37 °C in ambient air. The plates were examined and the colonies with morphology characteristic of *H. influenzae* were recultured separately in new plates and incubated at the same conditions. From the primary agar plate up to five presumptive *H. influenzae* colonies were isolated. This means that for one swabbing occasion up to ten colonies of *H. influenzae* per patient were replated for further identification. The isolates were identified by determining the NAD solution (factor V) and hemin (factor X) dependence, and by using VITEK 2 system (bioMérieux). The phenotypic identification was confirmed by duplex PCR as described below.

Adenoid and tonsils tissue homogenization and CD14⁺-cells enrichment

Briefly, the excised tissues were carefully washed several times with sterile glass pearls (4 mm diameter, Roth) to remove the biofilm bacteria from the tissue fragments. The fragments were then digested with collagenase A (Roche) and passed by the Falcon™ Cell Strainers, a nylon mesh with pore size of 100 μm (BD Biosciences Discovery Labware), and a needle (20G). The Dynabeads M-450 CD14 (DYNAL, Norway) was used according to Forsgren et al. (1994) to separate $CD14^+$ cells (monocyte/macrophages) from the tissue homogenates. The extracellular bacteria were efficiently killed with a cocktail of gentamycin and polymyxin B (both at a final concentration of 100 μg/ml). The control plates of chocolate agar and Columbia agar were streaked each time with the supernatant after several washing of the cells with a Dynal magnet (Dynal, Norway). The cells harvested by centrifugation were then lysed and the lysates were streaked on the appropriate media. The intracellular *H. influenzae* isolates were subjected to PCR-based identification and serotyping, as well as antimicrobial susceptibility of the isolates was determined.

PCR analysis

The four sets of primers were used in this study. To distinguish *H. influenzae* from *Haemophilus haemolyticus* the primers targeting at the *p6* gene, encoding a membrane protein characteristic of *H. influenzae* was used according to Ueyama et al. (1995). The second primer pair was used to find out the presence of the IgA protease (*iga*) gene in the genomes of *H. influenzae* strains (Vitovski et al., 2002). The fragments of both genes were amplified together in a duplex reaction as follows: 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, followed by 72 °C for 10 min. According to Binks et al. (2012), the presence of the amplification products in the gel after electrophoresis generally confirms the presence of *H. influenzae* isolate; however, we are aware that the eventual presence of *H. haemolyticus* cannot be completely excluded.

The capsular typing of *H. influenzae* isolates was performed using duplex PCR method with two sets of primers: the first used to

Please cite this article in press as: Trafny, E..A., et al., Carriage of antibiotic-resistant *Haemophilus influenzae* strains in children undergoing adenotonsillectomy. Int. J. Med. Microbiol. (2014), http://dx.doi.org/10.1016/j.ijmm.2014.03.004