SHORT COMMUNICATION

Persistence of non-typeable Haemophilus Influenzae in the pharynx of children with adenotonsillar hypertrophy after treatment with azithromycin


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Abstract

This study was performed in children with adenotonsillar hypertrophy to evaluate the effect of azithromycin (AZT) on the presence of NTHi in monocyte/macrophages (CD14+ cells) of adenoids/tonsils and the persistence of NTHi after adenotonsillectomy. A total of 36 pediatric patients participated in the study: 20 children were treated with AZT before adenotonsillectomy, and 16 children did not receive the antibiotic prior to surgery. NTHi were identified by culture and PCR in swabs and tissue samples. NTHi was detected in the lysates of CD14+ cells by fluorescence in situ hybridization (FISH) and by culture. The molecular typing was used to cluster NTHi isolates from each child. The intracellular NTHi was found in 10 (62.5%) untreated patients and was identified in three (15%) azithromycin-treated patients (P = 0.003). The proportion of the persistent NTHi strains was similar in both groups. AZT treatment followed by adenotonsillectomy did not completely eliminate NTHi from pharynges; however, it significantly reduced the risk of carriage of Haemophilus influenzae inside the CD14+ cells.

Keywords: NTHi; adenoid; macrophages; azithromycin; children

The occurrence of NTHi in nasopharyngeal swabs of children is not a permanent phenomenon (Bogaert et al. 2011). The failure in detection of NTHi in nasopharyngeal swabs do not necessarily indicate the absence of the pathogen because the bacteria may be present in biofilms or intracellularly (Morey et al. 2011; Stępińska et al. 2014; Nazzari et al. 2015). In spite of the targeted therapy with antibacterials, NTHi infections can recur and become chronic.

NTHi strains display a clinical sensitivity to AZT (Clinical and Laboratory Standards Institute, 2007). The antibiotic penetrates into host cells, impacts biofilm formation on mucous surfaces and, therefore, its use in children with adenotonsillar...
hypertrophy and NTHi in nasopharynx seems to be highly reasonable (Retsema et al. 1987). Macrolides are not the first-line antibiotics in NTHi acute infections but their use in chronic upper and lower respiratory tract diseases was demonstrated to be beneficial (Thomas et al. 2008; Chen et al. 2013). The aim of this study was to evaluate the effect of AZT and adenotonsillectomy on the persistence of NTHi in the pharynx of children with adenotonsillar hypertrophy. The impact of AZT on presence of intracutaneous NTHi in CD14+ cells of adenoids and tonsils was also investigated.

A prospective, controlled, interventional and non-randomized study included 36 children with adenotonsillar hypertrophy. All the children had symptoms of nasal obstruction lasting at least one year, the indications of obstructive sleep apnea and more than five upper respiratory tract infections in the past 6 months. The nasal fibroscopy showed adenoid tissues filling a little more than half the size of choanae. The tonsils extended beyond the tonsillar pillars or to midline in all patients. Any child received azithromycin in 6 months prior to surgery. Exclusion criteria were age 2–10 years and presence of NTHi in nasopharyngeal swabs performed seven days before surgery. Exclusion criteria based on medical history were allergy and passive smoking. AZT was administered in dose 20 mg kg\(^{-1}\)/24 h for 3 days to 20 children of age 2–10 (mean age 4.95 ± 1.96; 14 boys, 6 girls). The reference group (untreated patients) consisted of 16 children in age 3–8 (mean age 5.44 ± 0.96; 7 boys, 9 girls) who did not receive AZT.

Two swabs (tonsils and adenoid) probes separately were collected from each child in the following scheme: (i) 1 week before the surgery in outpatient clinics; (ii) upon anesthesia during surgery; (iii) at 6–8 weeks; (iv) 12–20 weeks after the surgery. The colonies with morphology characteristic of Haemophilus influenzae were cultured on a chocolate agar plates for 48 h at 37°C in 5% CO\(_2\). From the primary agar plate, up to five presumptive H. influenzae colonies were isolated (therefore, 10 presumptive colonies per child at each sampling event were analyzed). The tissues samples (1–3 g) excised during the surgery (within 1 h after the surgery) were homogenized, and the homogenates were plated on solid media and incubated according the growth requirements of throat microorganisms. The CD14+ cells (monocyte/macrophages) were isolated from the tissue homogenates by the immunomagnetic separation with Dynabeads M-450 CD14 (DYNAL, Norway), and the intracellular H. influenzae were detected by a fluorescence in situ hybridization (FISH) technique in the lysates of CD14+ cells according to the methods described by Forsgren et al. (1994) and Stepieńska et al. (2014). The extracellular bacteria were killed for 2 h with gentamicin and polymyxin B (100 μg ml\(^{-1}\) each). The CD14+ cells were then washed three times and, in each experiment, the supernatant was seeded on Columbia agar plates in order to verify the effectiveness of killing the extracellular bacteria. Haemophilus influenzae was isolated from the CD14+ cells, the tissue samples and swabs using common microbiological protocols and identified with PCR, as it has been described previously (Trafny et al. 2014). Serotyping of H. influenzae isolated was done with PCR according to Falla et al. (1994) and Satola et al. (2007). Only isolates confirmed as NTHI were investigated in this study. All NTHI isolates were genotyped by pulsed field gel electrophoresis (PFGE) after digestion with 40 U of Smal endonuclease (Trafny et al. 2014). The isolates were considered clonal when their macrorestriction patterns did not differ more than three bands among each other and were obtained from the same child. The susceptibility to AZT was determined by E-tests (AB bioMerieux, Solna, Sweden). All the strains with the MIC values ≤ 4 μg ml\(^{-1}\) of AZT were considered as susceptible.

All NTHI strains and genotypes were classified as follows: the persistent (in the swabs collected before or during surgery and after surgery), the transient (present before or during surgery and absent after surgery) and the newly acquired (after surgery) (Trafny et al. 2014). Such classification of isolates was used in order to observe whether azithromycin treatment followed by surgery might reduce the number of persistent NTHI clones in throat of children with adenoid hypertrophy.

Statistical comparisons were carried out with Chi-square test (Yates’ chi-squared) (Statistica 9). Hypothesis testing for level of statistical significance was done using 95% confidence interval and P-value < 0.05. The odds ratio and its 95% confidence interval was calculated from 2 × 2 tables according to Altman (1991).

In this study, the available patients with adenotonsillar hypertrophy were divided in two groups: the group of 20 children, which were treated with AZT before adenotonsillectomy (the azithromycin-treated patients), and a reference group of 16 children that were not treated with the AZT prior to surgery (the untreated patients). This study was performed in the same time period for both groups, i.e. between September 2011 and May 2013. The detection of the intracellular bacteria in the lymphoid tissues was done by FISH and by culture for all the patients. The detailed characteristics of NTHI genotypes of 14 children from the reference group has already been presented elsewhere (Trafny et al. 2014).

Overall, 891 NTHI isolates were collected from all the specimens analyzed in this work. Among them, 411 isolates came from the azithromycin-treated patients (20.6 isolates per patient) and 480 were obtained from the untreated patients (30 isolates per patient).

The total number of genotypes was 46 in the azithromycin-treated patients and 33 in the untreated patients. The number (percentage) of the persistent strains was 5 (10.9) in the patients treated with AZT and 5 (15.2) in the untreated patients \(P = 0.825\). There were 28 (60.9) and 20 (60.6) \(P = 0.981\) transient strains in the azithromycin-treated and the untreated patients, respectively. Total 13 (28.3) and 6 (18.2) \(P = 0.443\) of newly acquired strains occurred in the clinical specimens in the patients treated with AZT and in the untreated patients, respectively. There were no significant differences in the occurrence of these different genotypes between these two groups of the patients.

The detailed characteristics of NTHI isolates obtained from the patients treated with AZT before the surgery is presented in Table 1. The persistent strains of H. influenzae in these patients were not resistant to AZT. Only from one patient (No 78), the same strain of a genotype P78 A was isolated from the lymphoid tissue, CD14+ cells and from the swabs taken at least 6 weeks after surgery. The isolates of this strain were also susceptible to the macrolide. Among four children from whom the persistent strains were obtained, only one patient (No 108) carried the resistant NTHI isolates in the tissue homogenates. Nine isolates of the genotype P18A were identified in the patient’s specimens and two of them display the MIC values equal to 8 μg ml\(^{-1}\). The intracellular H. influenzae was detected by neither of the methods in CD14+ cells of lymphoid tissues of this patient. The NTHI resistant to AZT were also recognized in two patients in the azithromycin-treated patients and these strains were classified as transient in our study. Therefore, it could not be concluded from these results that the ability of NTHI strain to survive in lymphoid tissues of the throat was directly associated with resistance to AZT.
Administration of AZT followed by adenotonsillectomy did not completely eliminate NTHi from the throat, since similar number of persistent H. influenzae strains survived in nasopharynx of both groups of the patients. This phenomenon occurred despite the sensitivity of the bacteria to AZT. To our best knowledge, this is the first study describing the carriage of NTHi in throat of children that were treated with AZT prior to adenotonsillectomy. We also recognized that the antibiotic significantly reduced the risk of carriage of H. influenzae inside the CD14+ cells of lymphoid tissue when compared to the patients untreated with AZT. The patients who did not received AZT treatment followed by adenotonsillectomy had 9.4 times more often intracellular bacteria in their adenoids and tonsils than patients who received the macrolide (odds ratio = 9.44; P = 0.006).

Only few previous studies have found that AZT had the bactericidal effect in vitro against viable NTHi and this was observed for human bronchial epithelial cells and HEp-2 cells (Sekiya et al. 2008; Hotomi et al. 2010). In vitro study of Euba at al. (2015) showed that macrolides present the potential to eradicate intracellular susceptible and resistant NTHi. So far, therapeutic effect of AZT in NTHi infections has been confirmed only in an animal model (Girard et al. 2005). To our knowledge, this is the first report of AZT effect on the intracellular NTHi in clinical conditions.

In conclusion, this study showed similar prevalence of NTHi isolates and similar proportion of the persistent strains in nasopharynx of children with adenotonsillar hypertrophy, independently from AZT treatment. However, AZT reduced the risk of carrying the intracellular NTHi in CD14+ cells of adenoids and tonsils in the pharynx of children with adenotonsillar hypertrophy who underwent adenotonsillectomy. The patients in this study were not randomized; therefore, one can bear in mind that the observations reported here should be confirmed on much larger and randomized group of patients. However, even information gathered during observation of small group of patients may provide some insights into the mechanisms of persistence of H. influenzae strains in the throat of children.

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Conflict of interest. None declared.

REFERENCES


