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The Comparative Molecular Typing of *Haemophilus Influenzae* Strains Isolated from the Adenoid and Tonsils in Patients Undergoing Adenotonsillectomy

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ABSTRACT

Objective: Microbiota in the upper respiratory tract begins to form immediately after birth. Aerobic and anaerobic flora will form when a healthy child reaches the age of five. Tonsillitis and adenoiditis are the most common upper respiratory tract infectious diseases in childhood and adulthood. Transmission of bacterial infections from tonsils to adenoid tissue (endogenous transmission) can be prevented by usage of suitable antibiotics. At the same time, continuous exogenous infections of each or both sites require adenotonsillectomy surgery. In this study, to understand the role of endogenous transmission of *Haemophilus influenzae* (*H. influenzae*) in adenotonsillectomy, we aimed to investigate the genetic relatedness of *H. influenzae* strains obtained from the tonsil and adenoid of the same patient.

Materials and Methods: Twenty eight patients were included the study for displaying the growth of 56 *H. influenzae* strains (28 isolates per site). We investigated the genetic relatedness of *H. influenzae* strains obtained from the tonsils and adenoids of the patients by using the pulsed-field gel electrophoresis (PFGE) method.

Results: Twenty one isolates were isolated from the tonsils and adenoids of unrelated patients. We excluded nine (32.2%) out of 28 patients' *H. influenzae* isolates from the study, that had identical strains depending on the ≥80% similarity Dice coefficient.

Conclusion: The probability of endogenous transmission between the two sites was very high, which means that some adenotonsillectomy surgery might be avoided and treated with antibiotics.

Keywords: Adenotonsillectomy, pulse field gel electrophoresis, *Haemophilus influenzae*

INTRODUCTION

Microbiota in the upper respiratory tract starts to evolve after birth immediately, aerobic and anaerobic flora form at the age of five. Tonsillitis and adenoiditis are the most common upper respiratory tract infectious diseases in childhood and adulthood. Frequent recurrence and chronicity of tonsil and adenoid infections could cause a high cost and loss of the workforce. In untreated cases, acute tonsillitis and adenoiditis complications may invade adjacent respiratory tract areas, and suppurative complications might develop (1-5). In our previously published study, we reported the distribution of microorganisms and their susceptibilities to different antibiotics in adenoids and tonsils in patients undergoing adenotonsillectomy (6). 21 of the isolates were taken from the tonsils and adenoids of unrelated patients and excluded from the study, whereas we included 28

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patients displaying growth of 56 *Haemophilus influenzae (H. influenzae)* strains (28 isolates per site). Transmission of bacteria between tonsils and adenoid tissue (endogenous transmission) refers to acute infection, and can be treated with suitable antibiotics with no need for adenotonsillectomy. The continuous infection of one or both sites with exogenous *H. influenzae* strains (exogenous transmission) means that these sites become infection foci, and indicates chronic infection and the need for adenotonsillectomy surgery. To understand the role of endogenous transmission of *H. influenzae* in adenotonsillectomy, we aimed to investigate the genetic relatedness of *H. influenzae* strains obtained from the tonsil and adenoid of the same patient and vice versa.

MATERIALS AND METHODS

Patients who were admitted to the Otorhinolaryngology Clinic of the Istanbul University, Istanbul Faculty of Medicine in Istanbul, Turkiye were examined. Patients who met the surgical criteria and had both tonsil and adenoid tissue removed at the same time were included in the study. A total of 100 patients (64 males and 36 females; mean age ± standard deviation, 7.625 ± 3.267 years) who had chronic adenoiditis/tonsillitis and did not receive any antibiotics for the previous two weeks were selected for adenotonsillectomy surgery and enrolled in the study. The ethical approval was obtained from the Istanbul University, Istanbul Faculty of Medicine Clinical Research Ethics Committee (08.03.2011/533).

Bacterial Isolation

The samples were examined in the bacteriology laboratory of Istanbul University, Istanbul Faculty of Medicine, Department of Medical Microbiology. All samples were homogenized with sterile saline (0.85% NaCl) solution and cultured in chocolate agar (Oxoid, England) for bacterial isolation. All cultures were incubated at 35°C in 5% CO_2 atmosphere for at least 24-48 hours. Gram-negative coccobacilli that required factors X and V (BD, USA) when tested on tryptic soy agar (BD, Germany) were identified as *H. influenzae,* stored for studies at -80 °C.

Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) is the accepted "Gold Standard" for bacterial typing (7). Genetic relatedness among tonsil and adenoid isolates of the same patient were investigated by PFGE (8, 9). Concisely, examples embedded in genomic DNA were digested with 20 U SmaI-digested (Takara, Japan) enzyme for two hours at 37°C. A lambda ladder PFGE marker (BioLabs, Ipswich, MA, USA) was used as a molecular measure ladder. An electrophoretic run was performed in 0.5X TBE buffer (90 mM tris, 90 mM boric acid, and 2 mM EDTA) under the following conditions: 6 V/cm^2 for 20 h, pulse times from 5.3 to 34.9s at 14°C, using CHEF DRIII (Bio-Rad Laboratories, Belgium). The gel was smudged with ethidium bromide (1µg/mL) for 30 min, imaged under UV light using a transilluminator, and imaged and stored as a TIFF file (Figure 1). Genetic imprints for separate strains were obtained from the GelCompar II system (version 6.0, Applied Maths, Sint-Martens-Latem, Belgium). They were approximated and analyzed using dendrograms of the band profiles constructed employing the mathematically averaged unweighted double cluster manner. The association of the isolates was determined by the membrane coefficient with a band position tolerance setting of 1-1.5%. If the Dice coefficient was ≥80%, the isolates were identified as the same type (clonal). Tenover criteria were used in the analysis of the bands (8, 9).

Statistical Analysis

Similarity analysis and clustering were calculated by the Dice coefficient method, assumed via an unweighted pairwise group mean relationship (UPGMA) with GelCompar II v.6.0. The isolates showing a similarity coefficient of ≥80% were considered to belong to the same cluster(7).

RESULTS

H. influenzae growth was detected in 86 (86%) patients out of 100 patients. However, only 28 (28%) patients were included in the study since growths in both tonsil and adenoid tissue would be compared.

Nine (32.2%) out of 28 patients' *H. influenzae* isolates had identical strains depending on the ≥80% similarity Dice coefficient. That is why it was thought that the probability of endogenous transmission between the two sites was very high, whereas both sites of the same patient were colonized or infected with 38 unidentical strains in 19 (67.8%) patients. More likely, their source of infection was exogenous (Table 1). Isolates with a Dice band-based resemblance coefficient value of ≥80.0% were thought to belong to the same cluster, which implied a two- to three-fragment dissimilarity in gels with an average of 12 bands. The nine identical strains were as follows: 11A-11T, 34A-34T, 78A-78T, 80A-80T, 90A-90T, 94A-94T, 100A-100T, 111A-111T, 114A-114T; PFGE agarose gel raw data results are given in supplementary data.

DISCUSSION

Adenoid and tonsil tissues are common sites of colonization for *H. influenzae*. Adenotonsillectomy, the surgical removal of both the tonsils and adenoids, is a commonly performed procedure to improve breathing in children with sleep disorders or recurrent infections.

In recent years, comparative molecular typing methods have been developed to differentiate between *H. influenzae* strains isolated from different anatomical sites or patient groups. These methods are based on the analysis of genetic material, such as DNA or RNA, to identify variations in the strains' genetic code.

Tonsillitis and adenoiditis are serious infections caused by different microorganisms. In another study of us, (6), we isolated 14 different pathogenic bacteria in the adenoid and tonsils of patients who underwent adenotonsillectomy and *H. influenzae* strains were the most dominant (31.8%) among

Table 1. PFGE analysis of *H. influenzae* strains between adenoid and tonsil tissue.

Tonsil Tissue	Adenoid Tissue	Genotype
10 T	10 A	Similar
11 T	11 A	Not Similar
20 T	20 A	Not Similar
34 T	34 A	Similar
37 T	37 A	Not Similar
39 T	39 A	Not Similar
41 T	41 A	Not Similar
44 T	44 A	Not Similar
45 T	45 A	Not Similar
56 T	56 A	Not Similar
65 T	65 A	Not Similar
73 T	73 A	Not Similar
78 T	78 A	Similar
80 T	80 A	Similar
81 T	81 A	Not Similar
82 T	82 A	Not Similar
90 T	90 A	Similar
91 T	91 A	Not Similar
94 T	94 A	Similar
95 T	95 A	Not Similar
97 T	97 A	Not Similar
98 T	98 A	Not Similar
100 T	100 A	Similar
102 T	102 A	Not Similar
110 T	110 A	Not Similar
111 T	111 A	Similar
114 T	114 A	Similar
118 T	118 A	Not Similar

them. This result prompted us to ask whether this bacterium transferred between two sites (endogenous source), or whether these sites had become a suitable environment for colonizing of *H. influenzae* strains a part from infection of sites (exogenous source). First outcome that was thought,, the treatment with a suitable antibiotic might work, and there would be no need for adenotonsillectomy. But in the second case, the use of antibiotics is a permanent choice, and adenotonsillectomy is evitable. For this purpose, we investigated the genetic proximity of 56 *H. influenzae* strains isolated from tonsils and adenoids belonging to 28 patients. We showed that nine (32.2%) patients' strains were identical (100% similarity), and 19 (67.8%) were not identical. The exogenous source of *H. influenzae* strains in this study was higher compared with the endogenous source. This result indicates that adenotonsillectomy was evitable in most examined patients.

In the study by Choi et al., microorganisms grown in tonsils and saliva samples isolated from pediatric patients who underwent tonsillectomy for the therapy of tonsil hyperplasia were compared and, the growing microorganisms were evaluated by the 16S rRNA sequence analysis method. 24.3% of growing *Haemophilus spp.* were isolated from tonsils and 10.4% from saliva (29 tonsil samples) (10). In our study, we evaluated both the surface and core parts of the tonsils together, and *H. influenzae* was detected in 86% of patients. When *H. influenzae* studies are examined, it has been observed that it also plays a significant role in tonsillar hyperplasia (10-12).

A study in 2019 reported on preschool children aged between three to five years with hypertrophy of the pharyngeal or palatine tonsils. It stated that during this period, behavioral and sleep quality deterioration may occur depending on the enlargement of the tonsils, physical development difficulties such as respiratory tract difficulties, apnea, and snoring development (13). Infections of the upper respiratory system might extend to include the middle ear and cause acute otitis media (AOM), and also extend to the lower respiratory system and cause more serious infections, such as pneumonia. Fuji et al (14) reported a study on AOM in 2021. They conducted their study on children aged 6 to 30 months. They studied samples from 565 healthy individuals and 130 acute otitis patients. *H. influenzae* was detected in 5.9% of healthy visiting and 27% of acute otitis visits. *H. influenzae* was sequestered in 43% of middle ear fluid (MEF) specimens. With the widespread use of the *H. influenzae* type b (Hib) vaccine, the number of cases has decreased. For example, in the USA, the introduction of the Hib vaccine in 1985 dramatically decreased the incidence of invasive Hib disease (14). The Hib vaccine was introduced in Turkiye recently compared with the USA. The Hib vaccine (under a year old) was launched in Turkiye in 2006 by the Turkish National Immunization Program (NIP). Amoxicillin is the antibiotic recommended as the first-line treatment forAOM in the USA and most European countries, including Turkiye. Today, most strains produce beta-lactamase, which inactivates this antibiotic. A beta-lactamase inhibitor such as amoxicillin/clavulanic acid is alternatively used in the treatment of AOM, tonsillitis, and pharyngitis. Chekrouni et al. (15) collected data for twelve years and determined that 4% of bacterial meningitis episodes were caused by *H. influenzae* (an annual incidence of 0.5 patients per 1,000,000). They identified the predisposing factors as otitis and/or sinusitis, with a rate of 49%. Community-acquired pneumonia might be the exogenous source of *H. influenzae*. But this requires more data, and our study was

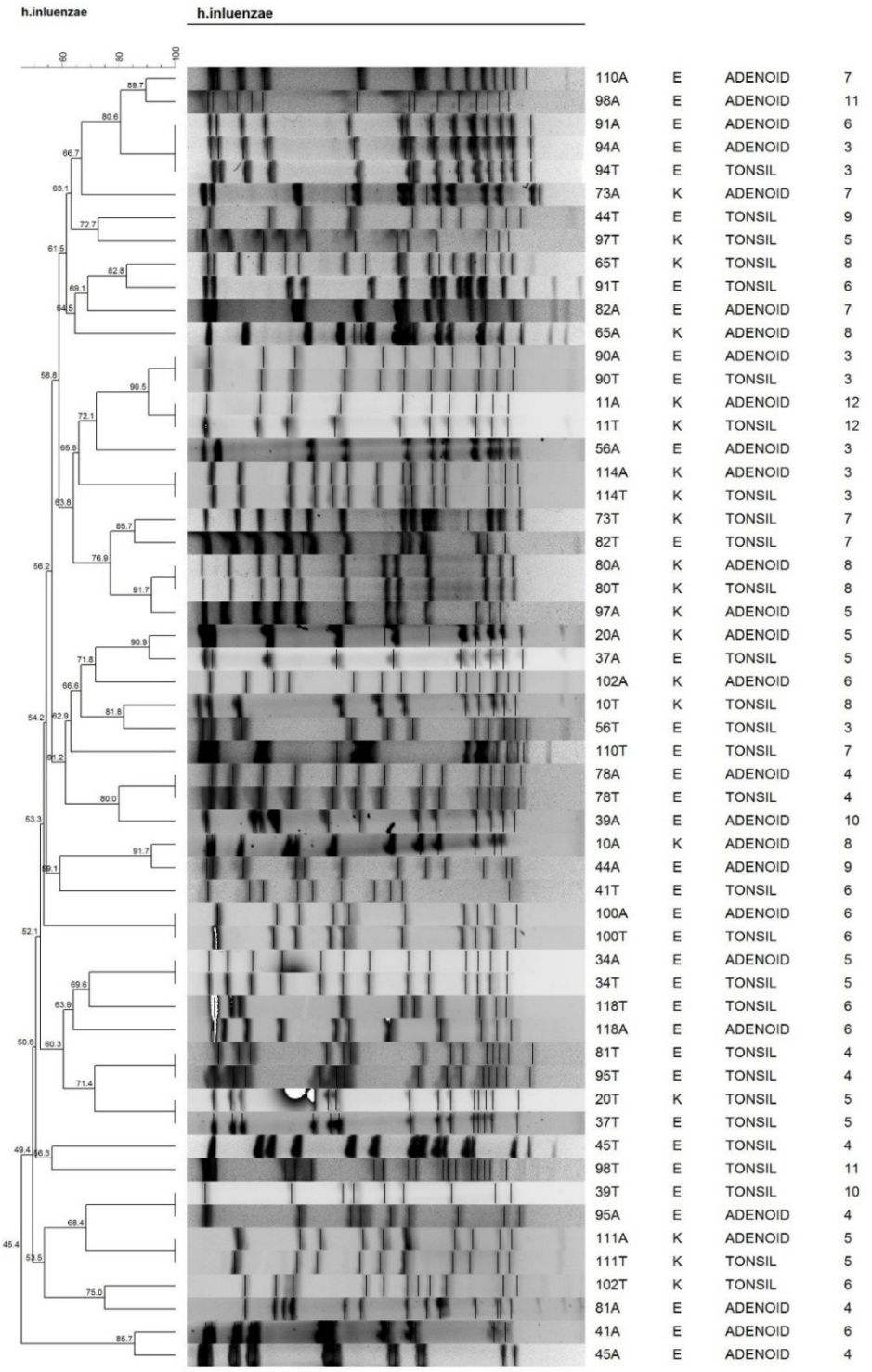

Figure 1. Dendrogram of *H. influenzae* by PFGE analysis by SmaI digestion. The membrane coefficients are shown above the dendrogram. Isolates with ≥80% on the dendrogram are considered highly genetically related.

not conducted to include *H. influenzae*-related pneumonia in hospitalized patients (15). When we investigated the studies in Turkiye (16), the microflora on the tonsillar surface, nucleus, and posterior surface have been compared. Only bacterial isolation techniques were used in this study, and 28% of *H. influenzae* strains were isolated (13). Unal et al. (14) compared the superficial and deep tonsillar flora, and the *H. influenzae* strain was found at a rate of 12% (17).

Our study compared *H. influenzae* strains isolated from adenoid tissue and tonsils, typified by conventional methods. However, when we discussed the previous studies, we could not find any comparative study of these two tissues. Hadi et al. (18) compared tonsil and adenoid tissue as superficial tissue cultures. On the other hand, we compared direct tissue cultures from both regions. A similar result was obtained in our study, which detected *H. influenzae* as a reproduction (15).

CONCLUSION

The endogenous transmission in this study was 32.2%, which means that some adenotonsillectomy surgery could be avoided and treated with antibiotics. Tonsillitis and adenoiditis are most frequent childhood diseases. Some children in this age group experience various physical and cognitive problems. We aimed to shed light on the relationship between these two close tissues. Also, we wanted to be a pioneer in studies to be carried out on this subject.

Ethics Committee Approval: The ethical approval was obtained from the Istanbul University, Istanbul Faculty of Medicine Clinical Research Ethics Committee (08.03.2011/533).

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Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Data: PFGE agarose gel raw data results.