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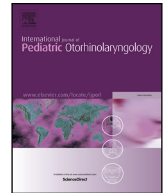

Paired analysis of the microbiota between surface tissue swabs and biopsies from pediatric patients undergoing adenotonsillectomy

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ABSTRACT

Introduction: Culture-independent methods, based on bacterial 16 S rRNA gene sequencing, have been used previously to investigate the adenotonsillar microbiota. However, these studies have focused on a single sampling site (usually a surface swab). We aimed to investigate potential differences in adenotonsillar microbiota according to sampling location, both on and within the adenoids and palatine tonsils.

Methods: Pediatric patients (n = 28, mean age five years) undergoing adenotonsillectomy were recruited for this study. At the time of surgery, a mucosal adenoid surface swab and an adenoid tissue biopsy was collected. Immediately following surgery, the crypts of the right and left tonsils were swabbed, and a surface and core tissue sample from the right tonsil were also collected. Bacterial 16 S rRNA gene-targeted amplicon sequencing was used to determine the bacterial composition of the collected samples.

Results: There was no significant difference in diversity or composition of the adenoid microbiota based on sampling site. However, the Shannon–Wiener and Inverse-Simpson diversity indices differed significantly ($p < 0.05$) between the microbial communities of the three different tonsil sampling sites. There was a higher average relative abundance of members from the genera *Streptococcus*, *Actinobacillus,* and *Neisseria* in the tonsil crypts when compared with surface and core tonsil tissue samples.

Conclusion: Our results indicate that there is variation in bacterial diversity and composition based on sampling sites in the tonsils but not the adenoids. The difference in microbiota between the surface and the tissue may have implications for our understanding of the pathogenesis of recurrent tonsillitis and have treatment implications.

1. Introduction

Pathogenic bacteria associated with the tonsils and adenoids cause much morbidity in the pediatric population [1]. In a national study performed in Sweden, the incidence rate of adenoidectomy was reported as 740/100,000 in children under ten years old [2]. In Denmark, a nationwide cohort study reported a tonsillectomy incidence rate in 2012 of 129.4/100,000 [3]. Hyperplasia of the palatine tonsils is associated with both recurrent tonsillitis and obstructive sleep apnoea, and hyperplasia of the adenoids is associated with otitis media with effusion [4]. Most current knowledge of the microbiology of the tonsils and adenoids has been derived from culture-based studies, which can reflect a small fraction of the bacteria present on the mucosal surface [5]. These bacteria are mostly reported as *Streptococcus* spp., *Haemophilus* spp., and *Staphylococcus aureus* [4,6,7]. However, *Actinomyces* spp. and *Helicobacter pylori* have also been implicated in adenotonsillar disease [8–13].

Culture-independent molecular surveys based on 16 S rRNA gene sequencing are now being employed to determine the microbiota of the tonsils and adenoids [7]. Unlike culture studies, these studies are beginning to reveal complex, diverse, and highly variable bacterial communities in adenotonsillar tissue [7,14,15]. Jensen and colleagues investigated the microbiota of tonsillar crypts and noted a complex microbiota of between 42 and 110 taxa in both children and adults [15]. They observed that *Haemophilus influenza*, *Neisseria* spp., and *Streptococcus pneumoniae* were almost exclusively detected in children. Interestingly, they noted that species which are traditionally associated with acute tonsillitis, such as pyogenic streptococci and *Staphylococcus*

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aureus, were scarce. However, one of the limiting factors of this study is that a single sampling location was selected. It is unknown if the microbiome differs in tonsil and adenoid tissue depending upon the chosen anatomical sampling site.

One study investigated the spatial organization of the microbiota in hyperplastic tonsils and adenoids using a broad range of fluorescent oligonucleotide probes targeted to bacterial 16 S rRNA gene [16]. It was found that *Haemophilus influenzae* mainly infiltrated the tissue, *Bacteroides* and *Streptococcus* were predominantly in fissures, and *Burkholderia, Fusobacteria, and Pseudomonas* were located only within adherent bacterial layers [16]. They also noted that the microbiota at each site was always polymicrobial. These findings indicate that the microenvironment on the surface of the tissue is most likely to be different to that of the deep tissue. Therefore, we would expect to see different microbial communities at various sites, which emphasizes the importance of investigating the microbiota at multiple sampling sites in adenotonsillar tissue. This is supported by Kim et al. who examined the difference in bacterial composition and diversity between middle meatal swabs and tissue in patients undergoing surgery for chronic rhinosinusitis [17]. This study found that while swab and tissue samples revealed similar bacterial diversity, bacterial composition differed significantly between the two sample types. To our knowledge, only one culture independent sequencing-based study has used tonsil and adenoid tissue as opposed to swabs for bacterial community analysis [14]. Broader application of the results of this study is limited, as this was a case study of a single patient [14].

This study aimed to investigate the adenotonsillar microbiota according to sampling location using bacterial 16 S rRNA gene amplicon sequencing. By doing so, it was hoped that a greater understanding of the role of bacteria in the pathogenesis of adenotonsillar hyperplasia might be achieved, and the optimal type of sample to guide antibiotic therapy defined.

2. Materials and methods

2.1. Patient information

Twenty-eight participants undergoing adenotonsillectomy were recruited for this study and adenoid and tonsil samples were collected from 24 patients each. Participants were required to be under the age of 15 years and to have taken no antibiotics for at least eight weeks before surgery. Mean patient age was five years and 15 were male. Written consent was obtained from the parent or legal guardian of each participant and ethical approval from the New Zealand Health and Disability Ethics Committee (16/STH/53) was obtained for this study.

2.2. Sample collection

Adenotonsillectomy was performed under general anesthetic by a pediatric ORL surgeon. Intravenous antibiotics were not administered at induction. Following induction, tonsillectomy was performed with either bipolar diathermy or coblation diathermy (Smith & Nephew, London, United Kingdom). Each tonsil was removed and placed immediately into individual sterile pots. Before adenoidectomy and under mirror visualization, a sample of adenoid was removed with biopsy forceps and put into a sterile container. These samples were stored on ice and taken to the laboratory within 1 h.

It is not practical to obtain an adequate tonsil crypt swab in a patient before tonsillectomy without contamination. To accurately compare the tonsil crypt with surface and core tissue, swabs were taken deep within the tonsil crypts immediately following tonsillectomy. Pairs of sterile rayon-tipped swabs (Copan, #170KS01) were used to swab the crypts of each palatine tonsil and the mucosal surface of the adenoid. Using sterile techniques, the right tonsil and adenoid tissue were dissected as follows: a sample of medial wall tonsil surface mucosa was excised using a sterile blade, then the tonsil and adenoid samples were cut in half with a sterile blade and then a fresh sterile blade was then used to excise a core tissue sample, with care taken to ensure the sample did not include any surface mucosa. Immediately following collection, the tip of each swab and the individual tissue samples were removed aseptically and placed in a sterile 1.5 mL Eppendorf tube containing RNAlater®. All tubes were labelled and stored at −20 °C until further analysis.

2.3. DNA extraction

Two replicate swabs from each sample site were thawed on ice and placed together into a sterile Lysing Matrix E tube (MP Biomedicals, Australia). Each tissue sample was also thawed on ice and put into a sterile Lysing Matrix E tube (MP Biomedicals, Australia). Genomic DNA was extracted from the swab and tissue samples using the AllPrep DNA/RNA Isolation Kit (Qiagen) following the manufacturer's instructions and eluted in 30 μL of DNase-free water. Cells were ruptured using a Qiagen TissueLyser II at 25 m/s for 2 × 40 s. The quality and quantity of genomic DNA were measured on a Nanodrop 3300 fluorospectrometer.

2.4. Bacterial 16 S rRNA gene sequencing

The V3-V4 region of the bacterial 16 S rRNA gene was amplified from the extracted genomic DNA using primers 341 F and 803 R [18]. Sample preparation for amplicon sequencing was as described previously [19], with some minor modifications. In brief, the aforementioned 16 S rRNA gene-targeting primers, complete with Nextera DNA library Prep Kit adaptors, were used in equimolar concentrations (0.2 μM) together with dNTPs (0.2 μM), HotStar PCR buffer (x1), $MgCl_2$ (2 mM), 0.5U HotStar DNA polymerase (Qiagen, Germany) and PCR-certified water to a final volume of 25 μL. PCR amplification was performed in an Applied Biosystems Thermal Cycler for 35 cycles. Negative PCR controls (without the addition of genomic DNA) were included for all PCR reactions, with no detectable PCR product. Amplified products were purified using Agencourt AMPure beads (Beckman Coulter Inc. United States) and quantified using Qubit dsDNA High-Sensitivity (Life Technologies, United States). Equimolar concentrations of prepared amplicons for 144 samples in total (including adenoid swab and tissue samples for 24 patients (48 samples in total), and right tonsil swab, surface tissue, and core tissue, and left tonsil swab samples for 24 patients (96 samples in total)) were submitted to the sequencing provider (Auckland Genomics Ltd., Auckland, New Zealand) for library preparation and sequencing on the Illumina MiSeq platform (2 *x* 300 bp paired-end reads).

2.5. Bioinformatic analyses

Processing of obtained sequence reads was carried out as described previously [19]. Briefly, sequences were merged and quality filtered in USEARCH [20,21]. Sequences were clustered using the UCLUST algorithm into operational taxonomic units (OTUs) based on 97% sequence similarity [22]. OTUs were taxonomically assigned in QIIME [23] using the SILVA database (v. 128) [24]. Samples were rarefied to an even sequencing depth of 2000 reads per sample for further analysis. Beta-diversity distance matrices (based on weighted and unweighted UniFrac [25]) were calculated in QIIME.

2.6. Statistics

Diversity indices (including Shannon–Wiener index and Inverse-Simpson index) and rarefaction curves were calculated for all samples from OTU tables using QIIME [23], and then values were formally compared using Student's *t*-test and the Kruskal-Wallis test. The Adonis function in Calypso [26] was used to analyze the impact of sampling site on the multi-species community structure of adenoid samples. An

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