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The bacterial community and local lymphocyte response are markedly different in patients with recurrent tonsillitis compared to obstructive sleep apnoea

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ABSTRACT

Introduction: Obstructive sleep apnea (OSA) is now a more common indication for tonsillectomy than recurrent tonsillitis (RT) [1,2]. Few studies have addressed possible differences in pathogenesis between these two conditions. Children with RT and OSA are often being treated in the community with multiple courses of antibiotics before surgery. Current understanding of the role of bacteria in disorders of the tonsils is mainly based on the culture of tonsil swabs. Swab cultures reflect only a very small fraction of the bacteria present on the mucosal surface and may not represent the bacterial communities within the tonsil crypts [3,4]. This study aimed to evaluate the local lymphocyte response and associations with bacterial community composition using molecular techniques of the tonsils removed from children for RT or OSA.

Method: The palatine tonsils were removed by extracapsular dissection from 24 patients with age range one to ten years, 14 of whom had RT and 10 had OSA. The fixed tonsil tissues were evaluated for bacteria by Gram-staining and presence of connective tissue by safranin staining. B lymphocytes and T lymphocytes were also measured immunohistochemically. Finally, previously published bacterial community data for this cohort were reassessed for associations with RT and OSA, and with the observed lymphocyte patterns.

Results: In tonsils from patients with RT, large micro-colonies of bacteria were observed in the tonsil crypts, and a large number of B and T lymphocytes were noted immediately adjacent to the tonsil crypt itself. In marked contrast, the tonsils from patients with OSA had no bacteria identified, and no significant skewing of lymphocytes based on site (such as follicles or crypts). We observed that the majority of lymphocytes surrounding the bacterial micro-colonies were B lymphocytes with a mean ratio of 109:55 (B lymphocytes: T lymphocytes). Bacterial community diversity was not different between the two cohorts; however, there were significant differences in bacterial community composition. Children with RT had a higher relative abundance of members from the genera *Parvimonas*, *Prevotella,* and *Treponema.* While children with OSA had a higher relative abundance of *Haemophilus*, and *Capnocytophaga*.

Conclusion: These results demonstrate significant differences in the local lymphocyte response and bacterial community composition in tonsil tissue between RT and OSA patients. It suggests that the response to antibiotics used in the treatment of these two conditions may be different. Furthermore, the presence of lymphocytes in RT within the tonsil crypt outside the tonsil epithelium is a unique observation of the location of these cells.

1. Introduction

Before the introduction of antibiotics in the 1950's tonsillectomy for recurrent tonsillitis (RT) was one of the most commonly performed operations worldwide [5–7]. There was a subsequent decrease in the number of tonsillectomies being performed [1,2] until the 1970's when the first cases of obstructive sleep apnea (OSA) were identified in children [8]. Following this observation tonsillectomy rates have been rising, with a doubling of the rate being reported in Sweden over the last several decades [1]. Adenotonsillar (AT) hyperplasia resulting in

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upper airway obstruction and OSA is now the most common indication for tonsillectomy in children [1,9].

There is currently no consensus about the underlying etiology of OSA related AT hyperplasia. It has been observed that T lymphocytes are more highly proliferative in the tonsils of children with OSA when compared with RT and this is said to promote AT hyperplasia [10,11]. It has also been observed that upregulation of cysteinyl leukotrienes, an inflammatory lipid mediator, may promote AT hyperplasia in children with OSA [12–15]. These findings do not suggest an underlying pathology, or why this condition may be more prevalent than it once was.

While there is an established link between pathogenic bacteria and infective AT hyperplasia, very little is known about the role of microbes in the pathogenesis of OSA related AT hyperplasia [3,4]. It has been suggested that early exposure to the respiratory syncytial virus (RSV) may increase AT proliferation through up-regulation of nerve growth factor (NGF)-neurokinin 1 (NK1) receptor-dependent pathways [16]. This theory has not been definitively established. Another group has excluded *Actinomyces* as a causative pathogen in OSA related AT hyperplasia [17]. Only one study thus far has compared the microbiome of tonsillar crypts between OSA and RT patients using molecular techniques [18]. A complex microbiota consisting of between 42 and 110 taxa in both children and adults was found. A core microbiome of 12 abundant genera was present in all samples regardless of whether patients had OSA or RT [18]. Variation in microbial communities do not currently explain the differences in clinical presentation.

In this study, we performed a histological analysis of the palatine tonsils surgically removed from participants with RT and OSA. Previously published bacterial microbiota data for this cohort collected at the same intra-operative time point were also re-examined for associations with RT and OSA, and in light of the lymphocyte findings [51]. The aim was to identify any demographic, clinical, histological, and microbial differences that may exist between these two groups of participants.

2. Methods

2.1. Participant information

Twenty-four participants undergoing tonsillectomy were recruited for this study. Recruited participants were under the age of 15 years and had taken no antibiotics for at least eight weeks prior to surgery. Ten of the participants had a surgical indication of OSA alone, and the remaining 14 participants had RT. Written consent was obtained from the 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.

The following demographic data were collected: age, sex, BMI, and ethnicity. Clinical data included the indication for surgery, tonsil and adenoid grades [19], the presence of ear disease, medical comorbidities, antibiotics prescribed in the community, and throat swab for culture before admission. Outcome data included postoperative visits to the general practitioner (GP), 30-day readmission rate, and associated complications.

Participant demographics and clinical characteristics were summarised using descriptive statistics. Univariate analysis was used to assess potential factors that were associated with differences in participants with OSA versus those with RT. *Chi*-square tests were performed to assess categorical variables, and the Student's T-test was conducted to evaluate continuous variables. A two-tailed *p*-value less than 0.05 was regarded as statistically significant. IBM® SPSS® version 24 software was used for all statistical analyses.

2.2. Sample collection and microbiome analyses

Tonsillectomy was performed under general anesthetic by a single pediatric ENT surgeon. Intravenous antibiotics were not administered at induction. Following induction, tonsillectomy was performed with either bipolar diathermy or coblation (Smith & Nephew, London, United Kingdom). The left and right tonsil were removed and placed immediately into separate sterile pots. These samples were stored on ice and taken to the laboratory within one hour.

Pairs of sterile rayon-tipped swabs (Copan, #170KS01) were used to swab the crypts of the left palatine tonsil. The right tonsil was dissected for use in another study investigating the microbiome community composition at various sites in the human palatine tonsil [51]. The focus of the previous study was intra-patient microbiota variation based on sampling sites across the heterogeneous patient group, and so associations with RT and OSA specifically were not previously assessed. The results from the right tonsils of these participants have not been used in this study. Immediately after swabbing, the tip of each swab was 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.

Genomic DNA was extracted from paired swabs, PCR amplification of the V3-V4 region of the bacterial 16 S rRNA gene was undertaken, and sequencing of the purified amplicons on the Illumina platform was performed, and raw sequences were processed using USEARCH, UCLUST and QIIME [20–23], as previously described [24].

2.3. Histological analysis

Following swab collection, the left palatine tonsil from each patient was fixed in formalin and set in paraffin wax. Each palatine tonsil was sectioned in the coronal plane at 250 μm intervals, with five adjacent 5 μm thick sections being cut at each point. This resulted in approximately 150 sections per tonsil. Initially, a Gram stain was performed on each coronal section of the 24 tonsils, followed by a counterstain with safranin to identify Gram-negative bacteria. All sections were screened at ×40 magnification on a Leica DMR upright microscope looking for the presence or absence of bacteria in each section.

A subset of ten participants (five from the RT group and five from the OSA group) were selected at random, and tonsil sections from these patients were subject to further histological analysis. Each adjacent coronal section from these ten patients was subjected to heat-induced epitope retrieval (2100 retriever, PickCell Laboratories, Amsterdam, The Netherlands) in citrate buffer pH 6.0. This was followed by a peroxidase block for 5 min and a protein block for 5 min. A CD3 rabbit polyclonal antibody (Cell Marque, Rocklin USA; 1:600) was then applied to each section for 1 h. Sections were then covered in a *Novalink* polymer (Leica Biosystems Newcastle upon Tyne, UK) for 30 min, followed by DAB staining for 10 min. A CD20 antibody (clone L26, Leica Biosystems Newcastle upon Tyne, UK 1:200) was then applied for 1 h, followed by post-primary block for 30 min, *Novalink* polymer for 30 min, and then DAB with nickel ammonium sulfate. A Gram stain was then performed followed by a counterstain with safranin to identify Gram-negative bacteria.

These processes resulted in CD3 (T lymphocytes) staining brown and CD20 (B lymphocytes) staining black. Connective tissue was seen as light pink and epithelium as grey (Fig. 1). Adjacent sections were stained with hematoxylin and eosin to assess the cellular response. All sections were imaged with the Metasystems V-slide scanner which provided us with high-resolution images for digital analysis at 80× magnification. Image J software with a cell counting function was used to determine the ratio of T lymphocytes to B lymphocytes in tonsil crypt areas immediately adjacent to bacterial microcolonies. This was achieved by taking three still images at 100× magnification on a Leica DMR upright microscope of three separate microcolonies from each patient with recurrent tonsillitis and comparing the ratio of brown T lymphocytes from the black B lymphocytes. The size of each image was standardized at 2560 × 1920 pixels (2.15 micrometers/pixel).