**DEPARTMENT OF SURGERY**



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**Tonsil and Adenoid Analysis Study (TAAS)**

**Research Protocol**

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Abstract

Understanding the complexities of the adenotonsillar-associated microbiota, and especially the manner in which they interact with the host innate immune response, is vital for the development of future treatment options. In this study we aim to expand our knowledge of this disease and assist the development of targeted approaches to improving perioperative care and postoperative outcomes.

General Information

Protocol title - Tonsil and Adenoid Analysis Study.

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Locality - Auckland District Health Board.

Academic Institution - The University of Auckland.

Keywords

Tonsil, Adenoid, Microbiome.

Introduction

Commensal bacteria colonize the extensive folds and crypts of the adenoids and tonsils soon after the infant is born (Winther et al. 2009). These microbes subsequently affect the innate immune response of the mucosa, and may be a significant factor in protecting or predisposing the infant to mucosal infection depending upon the composition of the microbial community that is established (Perry & Whyte 1998). Pathogenic bacteria associated with the adenoids and tonsils cause a great deal of morbidity within the paediatric population: adenoidal hyperplasia is associated with otitis media effusion, tonsillar hyperplasia with recurrent tonsillitis and both with obstructive sleep apnea. The 'pathogen reservoir hypothesis' for adenoids has recently been investigated (Nistico et al. 2011; Ren et al. 2013; Stępińska et al. 2014), but not yet definitively established.

Most of current knowledge on the microbiology of the upper airways has been derived from cultivation studies, which reflect only a very small fraction of the bacteria present on a mucosal surface. Modern molecular microbiology techniques are now being employed to determine the microbiota (entire microbial community) on the surface and within the tissue of adenoids (Ren et al. 2013). However, we wish to extend upon previous studies by including greater sample numbers (to encompass inter-personal variations), controls (healthy samples) and by also collecting specimens from the oral and nasal cavity. A recent study examining species-species interactions of the nasal microbial community has generated a lot of interest (Yan et al. 2013). We wish to apply a similar approach in this study, especially with respect to *Staphylococcus* *aureus* and other adenoid-associated microorganisms.

Additionally we aim to augment this study by collecting information on the immune cells in the adenoids, particularly T lymphocyte populations susceptible to the actions of bacterial SAg. *S. aureus* is commonly recovered from adenoid tissue (Emaneini et al. 2011; Ren et al. 2013; Badran et al. 2015; Nourizadeh et al. 2016) and isolates are likely to possess multiple distinct SAg which can perturb T cell function, including skewing of the TCR V repertoire in a characteristic fashion (Lam et al. 2015). TCR V profiles indicative of *S. aureus* SAg mediated skewing have been identified in several studies on polyps from chronic rhinosinusitis patients (Conley, Tripathi, Seiberling, Suh, et al. 2006; Conley, Tripathi, Seiberling, Schleimer, et al. 2006) . We have been studying the role of *S. aureus* SAg in tonsil hyperplasia and have found a surprisingly high prevalence of *S. aureus* within hyperplastic or recurrently infected tonsils. Flow cytometric analysis of the tonsillar TCR V population suggests a TCR skewing highly suggestive of a local response to staphylococcal Sags.

This potential pathogenic role of *S. aureus* in tonsillar hyperplasia is a novel and unexpected finding. It highlights the relative paucity of molecular microbiology data on this condition. It also suggests the possibility that manipulation of the microbiome may offer a new therapeutic avenue in the management of this condition. It is clear that the use of antibiotics do not bring about a lasting resolution of the problems caused by adenotonsillar hyperplasia. We wish to pursue the possibility that altering the microbiome by the administration of probiotics or biocidal agents may reduce the morbidity caused by this condition.

The clinical implications of this work are manifold. Understanding the complexities of the adenoid-associated microbiota, and especially the manner in which they interact with the host innate immune response, is vital for the development of future treatment options. In this study we aim to expand our knowledge of this disease and assist the development of targeted approaches to improving perioperative care and postoperative outcomes.

In summary our proposed research plan is made up of three primary objectives:

1. To identify the members of the adenoids microbiota in disease and healthy individuals.

2. To investigate the 'pathogen reservoir hypothesis' with respect to adenoid- associated microbiota.

3. Examine the effect of *S. aureus* SAgs on T lymphocytes in adenoid tissue.

Methods/Design

One-hundred paediatric patients (age 0-16 years) with diseases, including hypertrophic adenoids, chronic adenoiditis, chronic otitis media and obstructive sleep apnoea who are candidates for adenoidectomy and will attend the department of Otolaryngology Starship Hospital, Auckland are to be included in this study. Other centers to be included in this study (once permissions obtained) include Gillies Hospital (A Southern Cross Hospital), Waitemata District Health Board, and Counties Manukau District Health Board. Patients are not to have received antibiotic therapy for at least two weeks prior to surgery. Patients with genetic syndromes, metabolic disorders, neurologic diseases or congenital malformations will be excluded. Adenoidectomy +/- tonsillectomy is to be performed under general anesthesia. The age, sex, ethnicity, clinical symptoms, and adenoid/choana ratio (A/C) will be obtained for analysis. That is the size of adenoid tissue will be graded as a percentage according to obliteration of the choanae.

Patients with palatine tonsil hypertrophy who are undergoing adenotonsillectomy will have both their adenoid tonsils and palatine tonsils removed. These will be collected so that a comparison can be made between the microbiome of the palatine tonsils and adenoid tonsils in the same patient. Palatine tonsils will be graded by the surgeon using the widely accepted Brodsky Scale (Brodsky 1989).

Prior to tissue being excised by the surgeon, a surface swab will be taken from the adenoid tonsil pad, and from the right and left palatine tonsil. In addition, normal controls (swabs from 25 paediatric patients having general anaesthetics for conditions not affecting the upper airways) will be obtained from all of the aforementioned sites. We wish to explore the causes of variation between the sites by using multivariate analyses such as PERMANOVA.

Inclusion criteria for control patients include:

* Age between 3 and 16 years.
* Undergoing otolaryngology surgery for non-infectious pathology.
* Patients selected following study participant recruitment to represent age, ethnicity, and gender of participant population group.

Exclusion criteria for control patients include:

* Patients with genetic syndromes, metabolic disorders, neurologic diseases or congenital malformations will be excluded.
* Patients with anatomical abnormality of the upper aerodigestive tract.
* Patients who have had antibiotics within the 2 weeks prior to surgery.
* Previous adenoidectomy, tonsillectomy, or grommet insertion.

Adenoid tonsil tissue will be removed with forceps or a curette and placed in a sterile container “fresh.” Palatine tonsils will be removed and placed in a sterile container (right and left seperate) “fresh.” These will be stored on ice and immediately collected at the end of the operation and transported to the laboratory where they will be fixed immediately.

A 5ml blood sample from each patient involved in this study will be taken at the time of general anaesthetic when the patient is having an intravenous line sited for anaesthetic.

Once in the laboratory the swab samples will be immediately frozen at -20oC and stored until genomic DNA is extracted. The bacterial diversity of the samples will be measured using next-generation sequencing technologies (explained in more detail below).

The first step toward describing the microbiome is to extract DNA from collected swabs or tissue. This will be conducted using a bead-beating approach that we have previously applied to sinus swab samples (Biswas et al. 2015). Extracted DNA of sufficient quantity and quality will then be subjected to PCR amplification of the bacterial 16S ribosomal RNA (16S rRNA) genes. These genes occur in all bacteria, and their analysis by sequencing or related approaches is a cornerstone of contemporary microbial ecology (Amann et al. 1995; Tringe & Hugenholtz 2008). We will employ Illumina MiSeq sequencing, one of the so-called next-generation sequencing approaches, to sequence bacterial 16S rRNA genes, using sample preparation, bioinformatics and statistics protocols that are well established in our laboratory (Webster et al. 2010; Taylor et al. 2013; Biswas et al. 2014; Schmitt et al. 2012; Simister et al. 2012). This sequencing approach yields thousands to tens of thousands of sequences per sample, enabling us to identify both the abundant and rare members of the microbiome and to survey large numbers of patients (Kuczynski et al. 2010).

A portion of freshly excised adenoid tissue will be prepared for flow cytometry using an established method to quantify the major T and B cell subsets and assess TCR V skewing using the IOTest® Beta Mark TCR V Repertoire Kit (Beckman Coulter). Patient blood samples will be assessed in parallel as a comparator for systemic *versus* localized TCR V skewing. Skewing will be defined as a percentage of Vβ-expression >2 SD of that seen in normal blood (control values supplied with the kit), as described previously , and successfully employed in our laboratory for TCR subset analysis of tonsil tissue. Excess adenoid tissue will be preserved for assessment of bacterial diversity as outlined above, histology, localization of bacteria by fluorescent *in situ* hybridisation (FISH) and cultivation of bacteria.

Assessment of bacterial growth or inhibition involves standard microbiological assays that can be readily established in our laboratory. We routinely use mouse models of *S. aureus* infection, including intranasal colonisation, which can be adapted for *in vivo* competition studies.

Safety Considerations

The safety of research participants is foremost. This study does not put patients at risk given that participation requires no additional risk to the standard of care. All patients will be informed completely of what participation involves and can withdraw or decline to participate at any time. If any adverse events are noted then these will be recorded and addressed on a case-by-case basis.

Dissemination of Results and Publication Policy

Results will be disseminated to the medical literature in the form of publications and presentations at medical conferences. We will also disseminate information to participants if requested. We would disseminate information to the ministry of health if we thought findings would implicate policy-making decisions.

Ethics

The study will only commence once approved by the northern regional ethics committee.

Expected Study Outcomes

We expect this study to contribute to the advancement of knowledge by increasing our understanding of the complexities of the adenotonsillar-associated microbiota, and especially the manner in which they interact with the host innate immune response. This knowledge will allow us to move forward in the development of more effective treatment regimes. If we can reduce the burden of adenotonsillitis even by a minimal amount, this will have large impacts on the population.

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