Title: Sinusitis in Children and the Nasopharyngeal Microbiome “My Nose Study”

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ABSTRACT
Acute bacterial sinusitis in children is one of the most common clinical problems presenting to the primary care practitioner. A diagnosis of sinusitis results in over $1.8 billion spent on children < 12 years of age and is responsible for over 20 million antibiotic prescriptions per year in the US. Equally important, overtreatment contributes in large part to the development of antimicrobial resistance which has become one of the most serious obstacles to effective therapy since the pre-antibiotic era. Despite the frequency and importance of acute bacterial sinusitis, its incidence and the identity of the antecedent predisposing viral infections has never been reported. The goals of this study are to determine the burden of acute bacterial sinusitis in children and use state-of-the-art molecular techniques to determine the role of the nasopharyngeal microbiome and antecedent acute viral infections on the development of acute bacterial sinusitis in children.

To accomplish these goals, we propose to conduct a systematic longitudinal study of the nasopharyngeal environment of otherwise healthy children who acquire viral upper respiratory infections (URIs). The specific viral agents that cause URIs in children 49 to 84 months of age will be identified and the nasopharyngeal microbiome will be characterized (regarding sinopathogens and commensal microbiota) using high-resolution, culture-I independent methods as well as conventional microbiologic techniques at baseline and again within 2 to 3 days of onset of the viral URI. Children will be followed prospectively to determine which subjects develop acute bacterial sinusitis and which resolve their URI spontaneously.

It is our overall hypothesis that the nasopharyngeal microbiome is a major factor in the maintenance of normal upper respiratory tract physiology and strongly influences the risk of developing sinusitis and other bacterial infections of the respiratory tract. By identifying certain demographic features (e.g., age, gender, siblings, attendance at daycare, etc), the characteristics of the nasopharyngeal microbiome (diversity, evenness and relative abundance of specific sinopathogens and commensal microbiota) and the antecedent viral infection, we will determine risk factors for the development of acute bacterial sinusitis. Ultimately, this information will help us to focus treatment on those at highest risk to develop acute bacterial sinusitis thereby limiting exposure to antimicrobials. Examining the bacterial diversity of the nasopharyngeal microbiome in health and after viral infection will provide critical insights with regard to several secondary bacterial infections of the respiratory tract in which there is an antecedent viral infection (acute otitis media, acute bacterial pneumonia and acute bacterial sinusitis). Finally, defining the mechanisms by which respiratory viruses cause changes in the nasopharyngeal microbiome will lead to new preventative and therapeutic measures which may be instituted long before the bacterial infection evolves.
**Significance**

**Acute bacterial sinusitis is a common, important and expensive clinical problem.** Acute bacterial sinusitis is one of the most common problems presenting to the primary care practitioner in the ambulatory setting. Sinusitis affects more than 15% of the U.S. population annually and results in over $5.8 billion in direct health care expenditures, of which $1.8 billion are spent on children 12 years of age or younger. Assuming a birth cohort of 4 million/year in the US, and a frequency of viral URI of 5 episodes/year, we estimate that between 3 and 7 million episodes of sinusitis occur each year in children between 4 and 7 years of age. A diagnosis of sinusitis is responsible for over 20 million antibiotic prescriptions per year in the U.S. As importantly, inappropriate treatment contributes in great part to the development of antimicrobial resistance which is a major health care problem in the 21st century.

There are no accurate current estimates of the actual burden of acute bacterial sinusitis in children. Estimates derived from studies of respiratory infections occurring in young children attending a variety of day care arrangements indicate that sinusitis complicates viral URI approximately 6-13% of the time. Recent epidemiologic studies indicate that sinusitis, using contemporary definitions, complicates URI in children 6 to 36 months of age approximately 8% of the time. However, this may be an underestimate since the study was designed to detect cases of acute otitis media, which when detected were treated with antibiotics (thus curtailing the development of acute bacterial sinusitis). Investigations emerging from clinical practice provide estimates that acute bacterial sinusitis is found in 7.3 to 17.3% of children who are visiting their primary care practitioner with respiratory complaints.

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<td>6-13</td>
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<td>Ueda³</td>
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<td>Reval⁴</td>
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<td>Primary care</td>
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</tr>
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</table>

Importantly, no prospective studies have been performed in the modern era (using PCR-based viral diagnostics and post-introduction and widespread use of the pneumococcal conjugate vaccine) to determine the incidence of acute bacterial sinusitis as a complication of viral URI in children over 36 months of age, an age group in which the incidence of acute otitis media is reduced substantially and the incidence of acute bacterial sinusitis peaks.

**The major pathogenic event underlying the development of acute bacterial sinusitis in children is a preceding viral URI.** The URI causes 1) a mucositis, which results in partial or complete obstruction of the sinus ostia through which the paranasal sinuses drain into the nasal cavity, 2) impairment of mucociliary function, and 3) excess mucus secretion. The obstruction of the ostia and negative pressure within the paranasal sinuses favor aspiration of mucus from a heavily colonized nasopharynx into a presumably sterile paranasal sinus. Obstruction of the ostia and impairment of the mucociliary apparatus fosters multiplication of these bacteria and creation of a secondary bacterial infection. Although there are a plethora of species in the nose and nasopharynx which are aspirated into the paranasal sinuses, to date, only three microorganisms commonly cause acute bacterial sinusitis in children, probably reflecting unique properties of adherence between these microorganisms and the respiratory mucosa. Fortunately, most viral URIs resolve spontaneously. The specific determinants of outcome (benign resolution or progression to bacterial complications) of the viral URI are unknown. One important element determining outcome must be the microbiome of the nasopharynx (specifically the presence, relative abundance and overall diversity of sinopathogens and
commensal species; see preliminary data). Importantly, the pathogenesis of many serious bacterial infections of both the upper and lower respiratory tract involve a preceding viral URI followed by a secondary bacterial infection, including, in addition to acute bacterial sinusitis, acute otitis media and most cases of acute bacterial pneumonia in childhood.

It is our overall hypothesis that the nasopharyngeal microbiome is critical to the maintenance of the normal physiology of the upper respiratory tract and strongly influences the risk of developing secondary bacterial infections. This investigation will provide essential insights into the impact of common viral infections on the nasopharyngeal microbiome; the results will lead to studies of the mechanisms of perturbation of the microbiome, which in turn will lead to new preventative and therapeutic strategies for the common bacterial diseases of childhood (affecting both individual and public health).

Although there is common agreement regarding the importance of the preceding viral infection as the major predisposing mechanism in children with acute bacterial sinusitis, there has never been a longitudinal study of the viral antecedent infections in children or adults with sinusitis. In the landmark study that established the microbiology of acute bacterial sinusitis in children in the US, virus identification, which was performed upon clinical presentation, yielded virus isolates in only 13% of cases. This low yield is not surprising because children in general were cultured more than 10 days after their illness began and diagnostic techniques for virus identification were insensitive and limited to culture. Precise knowledge of the viral antecedents to acute bacterial sinusitis might be useful to both direct the development of new antiviral agents and promote the development of particular viral vaccines. Furthermore, understanding the mechanisms of virus-induced inflammation and changes in nasopharyngeal physiology (which result in alteration of the nasopharyngeal microbiome and acute bacterial sinusitis) may also identify novel preventive or therapeutic strategies. Although it is presumed that nasopharyngeal colonization with the common bacterial sinopathogens *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*, must precede the development of acute infection with one or more of these agents, the dynamics of this colonization, particularly following perturbation of the native microbiome by viral infection, have not been studied in the modern era. More information is necessary to understand the determinants of 1) nasopharyngeal colonization with bacterial sinopathogens and the relationship between the composition of the native microbiota and pathogen abundance, 2) the frequency, severity and identity of the antecedent viral illness, and 3) the incidence of acute bacterial sinusitis after viral URI.

The nasopharynx is one of the most densely colonized areas in the human body. Conventional culture techniques allow the identification of the well-recognized sinopathogens (*S. pneumoniae*, *H. influenzae*, and *M. catarrhalis*), *Streptococcus pyogenes*, *Staphylococcus aureus* and several species of the commensal microbiota including *Neisseria* species and alpha hemolytic streptococci. Conventional cultivation techniques have shown that age, season, attendance at day care, socio-economic status, viral co-infection, feeding, and use of antimicrobials can all affect bacterial colonization. Resident nasopharyngeal viridans group streptococci can antagonize colonization by other streptococci including *S. pyogenes* and *S. pneumoniae*. Alpha hemolytic streptococci have also been shown to inhibit both *H. influenzae* and *M. catarrhalis*.
There has long been an appreciation that local native microbiota within and upon the human body represent an important host defense mechanism. Recent interest has focused on using new molecular techniques for the first time to accurately define microbial communities of the gastrointestinal tract, oral cavity, airway and skin. The microbial diversity of the human intestine has received the most study. Many factors influence the composition of and relationships between the bacterial species of the intestinal microbiome. These include host genotype (which influences immune regulation and the availability of specific microbial attachment sites), diet (affecting the presence of specific bacteria and their metabolic activities) and the action of antimicrobial compounds such as bacteriocins (produced by specific bacterial species) which may provide protection against microbial pathogens which have been shown to play a key role in the dysregulated immune response of patients with inflammatory bowel disease.12 Understanding the composition of the intestinal microbiota has led to successful use of probiotic therapy in some forms of inflammatory bowel disease.13 Using a culture-independent approach (16S rRNA PhyloChip), it has recently been demonstrated that probiotic therapy elicits a global change in the composition of the gut microbiota, underscoring the fact that high abundance of a single beneficial bacterial species can lead to substantial community restructuring in the human host, even in an assemblage as diverse as the gastrointestinal tract.14

Although critical to the pathogenesis of the most important illnesses in children (both local and systemic) little is known about the microbiome of the nasopharynx in health or disease. Investigation of the inhabitants of the nasopharynx using conventional microbiologic techniques provides incomplete information; corresponding studies of the gastrointestinal tract indicate that more than 90% of colonic microbes are not detected by culture-based techniques.12 The advent and current availability of culture-independent microbial profiling approaches such as high-throughput sequencing or the 16S rRNA PhyloChip provide much needed tools to define the diversity of the nasopharyngeal microbiome. The PhyloChip is a DNA-based microarray that permits identification of approximately 8,500 types of bacteria or taxa (defined as groups of bacteria that exhibit at least 97% sequence identity at the 16S rRNA sequence level) in a single parallel assay. Compared with species-specific qPCR approaches or laboratory culture, this tool, due to its ability to detect the presence and relative abundance of 8,500 taxa in parallel, provides substantially improved ability to detect species present in the human microbiome. Compared with next-generation sequencing which, depending on the depth of sequencing performed, may only provide information on the most highly abundant species present, the PhyloChip can detect rare members of the community as efficiently as the dominant species, essentially providing a cross-sectional profile of community composition. This latter ability is key to understanding the etiology of idiopathic diseases, including chronic rhinosinusitis (CRS), which, as we demonstrate in preliminary data, is highly dependent on the composition of the microbiota. Thus, by determining the presence, absence and relative abundance of approximately 8,500 taxa under standardized conditions in each sample, the PhyloChip represents an economical approach for providing a high-resolution, cross-sectional profile of the community present. Further, as we demonstrate in preliminary data, due to our ability to normalize the large datasets generated by this method, it is possible to apply robust statistical tools to characterize microbiota and identify lead candidates for disease etiology. We have used these features to characterize the nasopharyngeal microbiota in adult populations,15 as well as to define a novel etiology for adult CRS (Abreu et al, in review; see preliminary data p.8). Hence, application of this tool to investigate relationships between the composition of the nasopharyngeal bacterial community and the risk of developing sinusitis provides an outstanding opportunity to address questions of fundamental clinical importance. Which bacteria comprise the normal nasopharyngeal microbiome in pediatric patients, and which individual host characteristics and environmental exposures influence these communities? Do infections with specific respiratory viruses alter the composition of the nasopharyngeal microbiome thereby impacting bacterial community reassembly and promoting sinusitis? Are there salutary elements of the nasopharyngeal microbiome that can prevent the progression of URI to acute bacterial sinusitis? Description of the nasopharyngeal microbiome in healthy children is a first step to testing the therapeutic potential of manipulating the nasopharyngeal microbiome in viral URI by either inhibiting pathogenic bacteria or promoting colonization by protective species.

Finally, antimicrobial resistance has become one of the most serious obstacles to formerly effective therapy since the pre-antibiotic era. This clinical problem, confined in the past to infections within the hospital environment, now confronts the practitioner in almost all instances in which antimicrobial agents are
indicated in the ambulatory setting (except the streptococcal sore throat). Whether treating acute bacterial sinusitis, acute otitis media, pneumonia, bronchitis or superficial skin infections, the problem of antimicrobial resistance is a major complicating feature. This issue confounds the clinician in two ways: it increases the cost and complexity of designing therapies and increases substantially the chances of clinical failure.

By identifying demographic characteristics of the study population of healthy children, the antecedent viruses that cause common respiratory infections, and the constituents of the nasopharyngeal microbiome in health and infection, we will determine risk factors for the development of acute bacterial sinusitis in children. Ultimately, this information may suggest alternatives to antimicrobial agents in both prevention and treatment, thereby decreasing antimicrobial resistance and thus providing a huge benefit to the public health. Importantly, what we learn with regard to control and reduction and/or treatment of acute bacterial sinusitis may provide mechanistic insights to those same issues for children with acute otitis media and acute bacterial pneumonia.

**Approach**

1. **Overview.** This will be a prospective, longitudinal cohort study designed to include all symptomatic episodes of URI occurring in children between the ages of 49 and 84 months for a period of one year to determine relationships between bacterial colonization of the nasopharynx, acute viral URIs, and acute bacterial sinusitis. To accomplish this goal we will enroll approximately 355 children, ages 49 to 84 months, over a 40 month period (~9 children/month); with an expected attrition rate of 15% this will yield ~300 children. Nasal washes (for virus identification) and nasopharyngeal swabs (for nasopharyngeal bacterial culture and microbiota profiling) will be obtained at entry, and surveillance of both sites will be performed on four other occasions (February, April, September and December) throughout the one-year study. In addition, when acute upper respiratory symptoms develop, we will obtain nasal washes and nasopharyngeal swabs during the acute illness and again 1-2 weeks later during the period of spontaneous recovery or when the patient is diagnosed to have acute sinusitis. These samples will be obtained to identify infecting viruses and also to characterize the changes in bacterial microbiota associated with an acute URI and recovery versus an acute URI followed by a secondary bacterial sinus infection. The bacterial assemblages will be characterized using both standard bacteriologic techniques (which will allow semi-quantitation of recognized sinopathogens) and a high-density microarray (16S rRNA PhyloChip) in parallel with high-throughput 454-pyrosequencing (to validate array findings) to comprehensively describe the nasopharyngeal microbiome. Respiratory viruses will be identified from nasal washes using a molecular diagnostic system that can detect all common respiratory viruses (Respiratory MultiCode Assay, EraGen Biosciences).

**Aim 1:** To determine the incidence of acute bacterial sinusitis in children ≥4 through <8 years of age (which is the peak age incidence of sinusitis) as a complication of an antecedent viral URI.

a) We will enroll and follow eligible children between ≥4 through <8 years of age prospectively for one year. Using high-throughput multiplex PCR-based viral diagnostics, the identities of antecedent viral infections will be determined for each symptomatic URI, and during four surveillance periods. We hypothesize that rhinovirus will be the most common viral URI to predispose to acute bacterial sinusitis. This information can potentially direct the development of antiviral medication or viral vaccines.

b) Using stringent clinical definitions for acute bacterial sinusitis, we will determine the incidence of sinusitis as a complication of viral URI. This has not been previously determined
and we hypothesize that sinusitis will complicate viral URI in 8% of cases in these young children.

**Aim 2:** To define relationships between the nasopharyngeal microbiome, viral illnesses, and progression to clinical sinusitis.

Nasopharyngeal samples and a pilot number of stool samples obtained before, during and after viral respiratory infections will be analyzed for bacterial species using array-based diagnostics (PhyloChip) and conventional semi-quantitative microbiologic techniques in two groups of children:

a) those with viral URIs that resolve spontaneously and

b) those whose URI progresses to acute bacterial sinusitis.

We hypothesize that the presence and density of sinopathogens and inhibitory commensal microbiota, together with modifications caused by infection with common respiratory viruses, will influence the likelihood of progression to acute bacterial sinusitis. Knowledge of these predictive elements will direct preventive strategies and treatment decisions.

**The primary objectives of the study are as follows:**

1. To determine a) the incidence of acute bacterial sinusitis in children ≥ 4 through < 8 years of age as a complication of an antecedent viral URI, b) if certain viruses are more likely than others to precipitate an episode of bacterial sinusitis and c) what percentage of sinus infections are, in fact, preceded by viral infection.

2. To compare children who develop acute bacterial sinusitis to children who recover spontaneously with regard to the characteristics of the nasopharyngeal microbiome (both gross features of community composition e.g. diversity, as well as the relative abundance of specific taxa). These comparisons will include samples obtained during the most recent surveillance visit, samples obtained during the acute URI and samples obtained when either a sinus infection has been confirmed or the subject has recovered spontaneously.

The secondary objective of the stool sample sub-studies are:

1. To determine the relationship, if any, between changes that occur in the nasopharyngeal microbiome and the fecal microbiome in the sub-cohort of subjects, and in the index subject and household family members, over time and with respiratory infections.

2. **Timeline for conduct of study:** There was a 3 month run-in period for IRB approval and training of personnel. The goal is to enroll nine patients per month (over 40 months). Each patient will be followed for 12 months; final follow-up will be completed at month 55. Enrollment began in February, 2012 and is ongoing. To date, 242 subjects have been enrolled. The average enrolled per month is approximately 6.5.

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3. **Eligibility and enrollment:**
Main Study: Children will be eligible to join this study if they are between ≥ 4 through < 8 years of age and are otherwise healthy (without upper respiratory symptoms to obtain baseline nose samples). Exclusion criteria include underlying conditions which would predispose them to the development of sinusitis including congenital or acquired immunodeficiencies, craniofacial abnormalities, cystic fibrosis, allergic rhinitis or a previous episode of chronic sinusitis. Children will be recruited from four large pediatric practices. Informed consent will be obtained from a parent or legal guardian. Enrollment is ongoing and may extend further than the initial proposed timeline.

Health Link IT will be asked to run a monthly list of potential subjects who are 4-7 years of age and have a clinic appointment for a well-child clinic visit at 20 S Park Pediatrics, University Station, East Towne or West Towne Pediatrics. A letter of introduction will be signed by the primary pediatrician and mailed to each family. The research nurse will put a reminder note on the door after the child is roomed at the clinic to remind the pediatrician that this is a potential subject for the study (20 S Park and West Towne). The reminder note will have inclusion/exclusion criteria and a brief synopsis of study participation.

During the visit, the clinic staff or pediatrician will ask the family if they are interested in learning more about the study (all sites). If yes, the study nurse will be summoned at the end of the clinic visit to discuss the study with the family (20 S Park, West Towne, and University Station). The handout or flyer will be available to clinic staff to give to families. East Towne families will be directed to contact the research staff directly. If families inquire the reception or nursing staff about the study the study nurse will be on site to answer any questions or clinic staff can direct the family to call the research cell phone.

**Optional Stool Collection Sub-Studies**
Because the gastrointestinal and respiratory tracts share a common beginning in the mouth and throat (nasopharynx), there is interest in determining whether there are similarities of the microbiome of the NP and feces and if changes that occur at one site are also found in the other. There is also interest in determining whether there are similarities/differences in the microbiome of household members.

Sub-Study 1
Subjects will be invited to participate in an optional sub-study for which we would be collecting a stool sample at every encounter that a nasopharyngeal swab is obtained. We will enroll the first 25 subjects who agree to be in the sub-study. Subjects will be invited during an enrollment visit. At each visit subjects will be assessed for acute diarrhea (4 or more loose stools per day) and antibiotic exposure in the last 4 weeks. If either of these conditions is present, we will not obtain a stool sample.

Sub-Study 2
The second sub-study will invite subjects who enroll in the stool collection sub-study to collect stool samples of household members. We will enroll siblings ages 8-17 years and parents/guardians. We will enroll a convenience sample of whoever is available during the visit and willing to participate. At additional visits, if other family members are present during the visit, we will invite them to enroll one time at a subsequent visit. We will enroll the first 20 family members who agree to the collection of stool samples in household member in addition to the subject obtainment of stool samples. Exclusion criteria for sub-study enrollment is gastrointestinal (GI) malformations, recent GI surgery, or the presence of an ostomy. At each visit, participants in the sub-study 2 will be assessed for acute diarrhea (4 or more loose stools per day) and antibiotic exposure in the last 4 weeks. If either of these conditions is present, we will not obtain a stool sample.

**4. Study procedures**
**Table 3 Study Procedure Flow Chart**

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<th>Visits:</th>
<th>Entry</th>
<th>Surveillance</th>
<th>Acute URI</th>
<th>Sinusitis</th>
<th>Recovery</th>
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a) Entry visit. Table 3 shows procedures according to visit. At enrollment, two specimens will be obtained from subjects by the study nurse: a nasal sample obtained by blowing the nose into a plastic bag after saline instillation (for identification of baseline viral infection in asymptomatic subjects) and a nasopharyngeal swab for evaluation by both culture-independent approaches and conventional bacterial culture. These specimens will establish the background rate and nature of viruses in the community and the specific bacterial colonization status of individual subjects. Data forms will be completed including demographic information, number of siblings, number of individuals in the household, whether the child shares a bedroom with a sibling, attendance at day care, presence of pets, history of allergic disease in child and first degree relatives, recent antibiotic use (< 30 days), vaccination status for pneumococcal, haemophilus, and influenza vaccines, and smoking in the house. Calendars will be distributed to parents for a 12 month period. Parents will be asked to record on their calendars daily whether their child has any respiratory symptoms, i.e., nasal discharge, nasal stuffiness and/or cough.

Subjects participating in the stool sample collection part of the study will be given instructions and a stool collection kit to take home, collect the sample, and bring to the research staff at the next visit (within 2 days) or the research staff will pick up during a home visit.

b) Surveillance samples. Four visits will be scheduled during the year (February, April, September, and December) to determine the seasonal prevalence of respiratory viruses and microbiota in healthy children. During this visit an interval history will be reviewed and specimens obtained of nasal mucus for virus identification (by nasal wash) and a nasopharyngeal swab. The distal half of the swab will be subjected to DNA extraction for subsequent PhyloChip (and sequence analysis). The remainder will be processed by conventional microbiologic culture methods. The nasal and nasopharyngeal samples will provide information regarding virus identification and bacterial colonization when the subject is healthy. The most recent surveillance data will be used for comparison to data generated during an acute respiratory illness. A letter will be mailed to families to inform them of upcoming clinic walk-in times for the surveillance visits.

Subjects participating in the stool sample collection part of the study will be given instructions and a stool collection kit to take home, collect the sample, and bring to the research staff at the next visit (within 2 days) or the research staff will pick up during a home visit.

c) Acute respiratory illness. Follow-up and classification of episodes: Parents who enroll their children will be instructed to call the study nurse at the first sign of a viral URI. An illness will be defined as at least 48 consecutive hours of respiratory symptoms including nasal congestion, nasal discharge or cough (with or without fever). Subjects will be seen on day 3 to 4 of their illness. A physical examination, including vital signs, will be performed and recorded focusing on the ears, nose, throat and chest to detect signs and symptoms of an existing bacterial infection (otitis media, severe onset sinusitis or pneumonia). A nasal wash specimen (by blow technique) and a nasopharyngeal swab will be obtained, the former to identify infecting respiratory viruses and the latter to characterize the colonization status of subjects for the usual sinus pathogens (S. pneumoniae, H. influenzae, M. catarrhalis), S. pyogenes and for nasopharyngeal microbiota profiling by conventional microbiologic methods and the PhyloChip respectively. Each episode of viral URI will be tracked until day 15 after onset by telephone contact on days 7, 10 and 15. Each episode will be classified as either:

i. uncomplicated viral URI -- symptoms of nasal congestion, obstruction, sore throat and cough are definitely improving by 10 days or have resolved completely.
ii. **sinusitis – persistent symptoms** – respiratory symptoms (including nasal discharge or cough or both) have lasted more than 10 days and are not improving by parental report\(^\text{\textsuperscript{16}}\)

iii. **sinusitis – severe symptoms** – combination of purulent (thick, colored and opaque) nasal discharge plus temperature > 39\(^\circ\) C for at least 72 hours\(^\text{\textsuperscript{16}}\)

iv. **sinusitis – worsening symptoms** – sudden worsening of a viral URI after apparent improvement usually beyond the 6\(^{th}\) day of illness. The worsening is characterized by new onset of fever or recurrent fever if fever had been present at the outset or worsening respiratory symptoms (either increased cough or nasal discharge or both).\(^\text{\textsuperscript{17}}\)

These characteristic clinical presentations that are most likely associated with a bacterial rather than viral infection were adopted and endorsed by a consensus panel in 2003, jointly established by 5 national societies (the American Academy of Allergy, Asthma and Immunology, the American Academy of Otolaryngic Allergy, the American Academy of Otolaryngology – Head and Neck Surgery, The American College of Allergy, Asthma, and Immunology, and the American Rhinologic Society).\(^\text{\textsuperscript{17}}\) These clinical definitions for acute sinusitis have now been adopted by the American Academy of Pediatrics and the Infectious Disease Society of America and will appear in both guidelines, which are currently in press. (The PI is an author of both guidelines). In four clinical studies it has been shown that by using these stringent criteria, sinusitis is diagnosed in only 7-8% of respiratory infections presenting to the practitioner who cares for children.\(^\text{\textsuperscript{3,4,6,7}}\)

If a child meets criteria for the definition of sinusitis an additional nasal wash and nasopharyngeal swab will be obtained to identify virus and perform both PhyloChip and conventional culture at that time. A nasal wash and nasopharyngeal swab will also be obtained from children who have recovered spontaneously 10 -14 days after symptoms began. These “recovery” samples will provide information about changes in bacterial community composition that occur following perturbation by viral infection, and will serve as an important comparator to samples obtained during acute sinus infections. Families may choose to have their child seen by the primary pediatrician and if sinusitis is diagnosed the pediatrician may treat with an appropriate antibiotic (standard of care visit). Clinical progress will be tracked until the patient experiences resolution of symptoms.

Study personnel will send a weekly text to the parent to determine whether there any URI symptoms have occurred in the last 7 days. The text message will read, “Please call the research staff if your child has had a cough, runny nose, or stuffy nose in the last 7 days. (608) 228-4940. Do not respond to this text.” A weekly phone call can take the place of the text message based on parent preference. All subjects included in the study receive their primary and emergency care through the University of Wisconsin Hospital and Clinics. Urgent or immediate care clinics and an “after hours” clinic use the same electronic medical record as does the primary care provider. Thus review of the medical record will permit identification of any episode of sinusitis, URI or other illness for which medical care was sought. Families who are non-responsive after 2 months of follow-up phone calls will be mailed a letter attempting to reconnect with the family and to ascertain whether they wish to continue in the study. A follow-up phone call after 1 week of the mailed letter will be made if we have not heard back from the family.

The family will be mailed a quarterly newsletter that will contain information on colds/sinusitis and an activity for the child. It will also contain a message to call the research staff whenever upper respiratory symptoms are present.

The identity of the viral infection and characteristics of the bacterial colonization of children with an uncomplicated viral URI will be compared to baseline and compared to children whose URI progresses to acute bacterial sinusitis.

Subjects participating in the stool sample collection part of the study will be given instructions and a stool collection kit to take home, collect the sample, and bring to the research staff at the next visit (within 2 days) or the research staff will pick up during a home visit.

d) **Study methods – Stool Collection Sub-studies**
Participant will be instructed to stick a paper collection device onto the toilet seat, and void stool into the paper collection device. Participant will then place the stool sample into a sample cup provided using a scoop (also provided), and write the date and time on cup. The remaining stool and paper collection device can then be flushed down the toilet. First bowel movement of the day will be collected, placed in a biohazard bag and refrigerated, at least one day before the next visit. Research staff will transport the samples to the pediatric lab and placed in a -80 freezer. Research staff will batch ship these to UCSF for analysis.

**Study methods – Visits**
Home visits will be offered for enrollment, acute, recovery, and surveillance. At enrollment, if the family does not have time to enroll after the well-child visit or if the child is upset from having vaccinations, the family can choose to return to the clinic at an arranged time or we will offer a home visit. For acute, recovery, and surveillance families will have the option of coming to the clinic or a home visit. This will ensure completeness of our data.

**Study methods - Culture-independent 16S rRNA PhyloChip profiling of nasopharyngeal and stool samples.**
Nasopharyngeal swab and stool samples collected in this study will be shipped on dry ice to Dr. Lynch’s laboratory at University of California San Francisco. Samples will be thawed on ice prior to centrifugation at 13,000 g at 4 °C for 10 minutes. DNA and RNA will be extracted using a beat-beating/AllPrep kit protocol that we have optimized for nucleic acid extraction from both Gram positive and negative species. DNA will be quantified using a Qubit fluorimeter and the 16S rRNA gene will be amplified using the non-degenerate universal primers (27F and 1492R) across a gradient of annealing temperatures and processed for PhyloChip analysis as previously described. To validate array findings, 454-pyrosequencing of the V3-V4 region amplified from the same DNA template will be performed in parallel according to the method of Knight and colleagues. In a recent study of 4 pediatric patient stool samples profiled in parallel by what is considered relatively deep 454 pyrosequencing (20,000 reads per sample) and PhyloChip, we demonstrated that at the genus level, 3 of the samples exhibited 100% concordance while the 4th exhibited 96% concordance, i.e., organisms detected by sequencing were also detected by the PhyloChip. However it should be noted that in all samples, the PhyloChip detected 75% more taxa than these sequencing efforts, representing less abundant members of the estimated 1 x 10^{14} organisms present in the gastrointestinal microbiota (Lynch et al, unpublished data).

e) **Study methods - processing and interpreting the PhyloChip data.** Detection and quantification criteria for each taxon detected by the PhyloChip will be conducted as described previously using a positive fraction threshold of ≥ 0.90. Statistical analyses using log-transformed fluorescence intensities will be performed in the R environment (www.R-project.org), using the ecological community analysis package vegan [version 1.16-1; (129)] to calculate metrics of bacterial community composition, including richness (number of bacterial types present), evenness (relative distribution of species in a community) and diversity (index based on both richness and evenness metrics). Bray-Curtis dissimilarity measures of ecological distance will be calculated before performing non-metric multi-dimensional scaling, a nonparametric ordination method that maps community relatedness based on species presence and abundance. This approach will be used to assess variability in bacterial community structure (particularly to examine if a distinct community composition is associated with differential sinusitis outcomes). Between-group differences (e.g. sinusitis vs no-sinusitis) in gross community metrics (e.g. richness, evenness and diversity) as well as taxon abundance will be determined by two-tailed t-testing or ANOVA and adjusted for false discovery using q-values, as we have recently described. Taxa exhibiting p- and q-values < 0.05, will be considered statistically significant. Correlation analysis of taxon abundance against that of key sinonasal pathogens (both bacterial and viral) will be performed using the multtest package available as part of the Bioconductor suite of analysis programs to determine relationships between these species and relative abundance of all other taxa detected. All data will be maintained on a secure server in the Department of Medicine at UCSF, only accessible to those individuals involved in the study. Sequence data generated by 454-pyrosequencing will be used to validate 16S rRNA PhyloChip findings as we have previously described.

f) **Study methods – Conventional bacteriology of nasopharyngeal swabs.** Half of the nasopharyngeal swab will be submitted for routine bacterial cultures to the CLIA certified microbiology laboratory of the University of Wisconsin Hospital and Clinics. The plates will be interpreted semi-quantitatively. Isolates of S.
pneumoniae will be identified by using the optochin disk susceptibility test (Taxo P, Becton Dickinson Microbiology Systems); S. pneumoniae isolates will not be serotyped. M. catarrhalis will be identified by the API QuadFerm assay (bioMerieux, Inc., Hazelwood, MO, USA), and NT- H. influenzae by the Haemophilus ID Quad Plate with Growth Factors (Becton Dickinson Microbiology Systems).

g) Study Methods-Virus Identification The Respiratory Multicode Assay (RMA) is a high throughput and sensitive multiplex PCR based on unique chemistry (Multicode, EraGen Biosciences) , and was developed by researchers at the University of Wisconsin-Madison and EraGen BioSciences (Madison WI). This state-of-the-art assay detects the following viruses: human rhinoviruses-A, B, and C, enteroviruses, coronaviruses (including OC43, 229, NL63, and SARS), adenoviruses B, C, and E, influenza A and B, parainfluenza viruses I-IV, respiratory syncytial virus A and B, metapneumovirus, and bocavirus. The assay has been validated against standard viral diagnostics. Furthermore, rhinoviruses are expected to cause the majority of URIs in the proposed clinical study and the RMA assay has been proven to detect all 100 rhinovirus serotypes as well as the newly discovered C species. Primers have been refined in accordance with sequence information from over 1000 clinical isolates.

h) Preliminary Data:

i. Bacterial etiology of acute sinusitis in children and response to antimicrobial agents. In a series of studies done between 1981 and 2009, Wald and colleagues have provided meticulous data outlining the three major causes of acute bacterial sinusitis in children (S. pneumoniae, H. influenzae and M. catarrhalis) using the gold standard of sinus aspiration. Using stringent clinical definitions of acute bacterial sinusitis, systematic study comparing antimicrobial to placebo has established the benefit of antimicrobials in enhancing clinical cures, reducing clinical failures and shortening the course of the clinical illness.

ii. A novel etiology for Chronic Rhinosinusitis (CRS) identified using the 16S rRNA PhyloChip. In addition to our recent PhyloChip-based study which was the first to demonstrate distinct, niche-specific bacterial communities in paired nasopharyngeal and oropharyngeal samples of healthy adult subjects, we have also examined the sinus microbiota of patients with CRS. Maxillary sinus samples (7 CRS and 7 healthy individuals) were profiled using PhyloChip. Comparative analysis of gross metrics of bacterial community composition
detected in both CRS patients with, and healthy subjects without, sinonasal symptoms. Thus, the mere detection of a suspected or known pathogen at a given niche does not automatically imply pathogenic activity and suggests that the composition of the microbiota at that site may play a large role in defining the activity of community members.

To identify specific taxa that characterized healthy and, more specifically CRS sinus microbiota, we determined which taxa exhibited significantly higher or relative abundance in patients with CRS compared to healthy subject groups. Following correction for false discovery (p ≤ 0.05, q ≤ 0.05), a total of 1,482 taxa were detected in significantly lower relative abundance in the sinuses of patients with CRS compared to healthy subjects, underscoring the extent of native microbiota loss from CRS sinus mucosal surfaces. In stark comparison, only a single taxon with the representative species, *Corynebacterium tuberculostearicum* exhibited a significant increase in abundance in patients with CRS (p ≤ 0.03, q ≤ 0.003). Figure 4 underscores the clinical significance of these findings, we examined the microbiota data to identify those species that correlated with Sino-Nasal Outcome Score 20 (SNOT-20) symptom severity scores. The SNOT-20 is quality-of-life instrument for sinonasal conditions. A large group of 228 taxa were significantly (p < 0.05) correlated with lower SNOT-20 scores (indicative of healthy sinuses). In contrast, the relative abundance of only two taxa was positively correlated with increased symptom severity; both belonged to the Corynebacteriaceae, and the taxon most positively correlated with symptom severity was again represented by *C. tuberculostearicum*.

To confirm our findings and demonstrate that a combination of both depletion of the microbiota and outgrowth of *C. tuberculostearicum* were responsible for the pathology in CRS we developed a novel model of sinusitis. In mice characterized by antibiotic-induced depletion of the sinus microbiota and nasal inoculation with *C. tuberculostearicum* (ATCC #35694). Four groups of 5 mice per group (antibiotic treated or untreated with or without inoculation of *C. tuberculostearicum*) as shown in figure 4 were studied. Histological analysis (H&E staining) of the maxillary sinuses of these animals demonstrated airspace obstruction only in mice exposed to a combination of both antibiotic and *C. tuberculostearicum* (Fig. 4A). Subsequent periodic acid-Schiff (PAS) staining specifically for mucin, demonstrated that mice in this group also exhibited evidence of goblet cell hyperplasia and hypersecretion of mucus (Fig. 4B). Compared to all other groups, animals exposed to both antibiotics and *C. tuberculostearicum* exhibited profound goblet cell hyperplasia (Fig. 4D), confirming that the combined conditions of depleted sinus microbiota and *C. tuberculostearicum* inoculation represent key factors defining sinus mucosal health. Data generated in our murine studies suggested that the primary source of mucin secretion originated from goblet cells of the surface epithelium. We therefore examined MUC5A expression levels in our healthy subjects and patients with CRS and confirmed that CRS patients exhibited significantly increased MUC5A expression, compared to healthy subjects (Fig 4D), corroborating our murine model findings. Finally, we hypothesized that profiling cervical lymph node cytokine production of these mice would provide information on immune responses relevant to patients with CRS. Of the cytokines profiled, only IL-2 exhibited an increase in secretion across treatment groups; mice exposed to a combination of antibiotics and *C. tuberculostearicum* exhibited the greatest increase in lymph node IL-2 expression compared to control animals (data not shown). We subsequently profiled IL-2 expression in our patients with CRS and healthy subjects and demonstrated significantly increased expression in the patients with CRS (p < 0.008). Hence by using the PhyloChip to produce standardized microbiota profiles for comparative statistical analyses, we identified and subsequently confirmed in a mammalian model, a novel etiology for CRS that is dependent both on the abundance of a specific *Corynebacterium* species and the composition of the sinus microbiota. These data underscore the ability of PhyloChip to profile bacterial communities and the utility of correlative statistics using these data to identify features of these assemblages responsible for disease (Abreu et al, in review).

### i) Data Analysis

**Aim 1** - To assess the effects of virus type on progression to acute bacterial sinusitis, the type and number of background asymptomatic viruses, the season, and the interaction of season and background asymptomatic viruses and demographic variables, we will fit a generalized linear model to the URI episode data (1500 observations on 300 children, 0 = non progressing, 1 = progressing) using child as the main sampling unit and assuming a binomial distribution for the data. It is anticipated that the average number of URI episodes per child will be 5 per year. This model accounts for the potential correlation among the multiple episodes in each child. For predictors which take values of yes or no (such as virus type), we will have 80% power to detect the
difference between the percent of a particular virus type equal to 30% for the acute bacterial sinusitis group and 45% for the control group. We will test that rhinovirus is the most common viral URI to predispose to acute bacterial sinusitis using a Chi-squared test for goodness of fit. If rhinovirus accounts for 40% of the URIs leading to sinusitis and the second most common leads to 20% or less then we will have 80% power to conclude that rhinovirus is the most common viral antecedent to acute bacterial sinusitis.

The specimens obtained at baseline and during four other surveillance periods (approximately 1500 - 1800 specimens) will allow us to determine the changes due to season in background level of asymptomatic viral infection and the diversity of viruses. We will use robust multinomial regression using the R package multinomRob that accounts for over-dispersion due to the clustering of the URI episodes within children. This method of modeling multinomial data will allow us to assess the effect of season and the demographic variables on asymptomatic background viruses.

To estimate the incidence of acute bacterial sinusitis as a complication of URI we will use a random effects generalized linear model assuming binomial distribution. We expect a total of 1500-1800 episodes of URI among the children and hypothesize that ABS will complicate 8% of these cases resulting in approximately 120 cases of acute bacterial sinusitis. If the true percent of viral URIs progressing to acute bacterial sinusitis is 8% then 1500 cases will give us 80% power to estimate the incidence with a 95% confidence interval no longer than 2.9%. If we were to observe exactly the 8% incidence the 95% confidence interval would be (6.68%, 9.49%).

**Aim 2** - The episodes of acute bacterial sinusitis and uncomplicated URI chosen for PhyloChip analysis will be from two groups. The “acute bacterial sinusitis group” will consist of the first episode of acute bacterial sinusitis occurring among 50 randomly selected children who have one or more episodes of acute bacterial sinusitis. Only the first episode will be eligible to avoid data clustered within child. To avoid running all the PhyloChip analysis at the end of the study we will select the children in groups of 10 evenly spaced over the 52-months of enrollment and follow-up. An additional 50 matched control cases of uncomplicated URI will be selected (10 at a time) from the group of children who have not yet had an episode of acute bacterial sinusitis. The controls will be chosen so that their uncomplicated URI closely matches the case of acute bacterial sinusitis in calendar time. If a control child later develops acute bacterial sinusitis, the control child will become eligible to be chosen for the acute bacterial sinusitis group. This process will result in a few extra PhyloChips being run but is necessary as the processing of PhyloChips is time consuming and cannot be delayed until the end of the study. In addition, the surveillance sample most closely preceding the episode of acute bacterial sinusitis and uncomplicated URI will also be analyzed using the PhyloChip.

Regression analyses will be used to assess relationships between progression to acute bacterial sinusitis, demographic variables (including age, gender, attendance at daycare, family or personal history of asthma, pets, etc.), season and gross metrics of bacterial community composition (richness, evenness, and diversity indices) as well as the relative abundance of all taxa detected (including sinopathogens), to determine if these variables are related to specific features of the nasopharyngeal microbiota. A term will be included to account for the paired structure of the data. For each of the continuous predictors, a difference in the mean values between the acute bacterial sinusitis group and the control group equal to 50% of the variability of that predictor will give us greater than 80% power to find a significant effect of the predictor. For predictors which take values of yes or no (such as presence of a sinopathogen), we will have 80% power to detect the difference between the percent of sinopathogens present equal to 55% for the acute bacterial sinusitis group and 30% for the control group.

PhyloChip outcomes including richness, evenness and diversity indices of the nasopharyngeal microbiome sample obtained during each of the 50 episodes of acute bacterial sinusitis will be compared to those obtained during the most recent surveillance period by using paired t-tests. Differences in specific taxon abundance in these two samples will be determined by paired t-test and adjusted for false discovery.
Correlation analysis of the abundance of each individual taxon against that of key sinonasal pathogens (both bacterial and viral) will also be performed to determine relationships between these species and relative abundance of all other taxa detected. Hierarchical cluster analysis will be used to display similarity of microbiota among nasopharyngeal samples.

The pilot stool sample size is based on the base sample size of 12 per group when no prior information is available (Julious, 2005). We propose to double that number to ensure enough samples over the seasonality of the one year enrollment. This feasibility is based on our average enrollment of 7 subjects per month and if 20% join the sub-study (1-2 per month), enrollment would take about 12 months including family members. **Innovation** This study will address numerous gaps in our knowledge of secondary bacterial infections (acute bacterial sinusitis, and also acute otitis media and bacterial pneumonia) of viral URI. First, due to limitations in previously available viral diagnostics and a lack of stringent clinical criteria for acute bacterial sinusitis, there have been no previous studies to determine the incidence of acute bacterial sinusitis as a complication of viral URI. We propose to collect specimens quarterly during periods of health, and then multiple times during the course of an illness in order to gather a unique data set to define the relationship between viral URI and bacterial sinusitis. The high-throughput multiplex PCR-based viral diagnostics are ideal for identification of respiratory viruses in the over 5000 specimens to be collected. Second, no studies have been conducted to identify specific viral antecedents of acute bacterial sinusitis in children. Third, despite the immense clinical importance of respiratory infections in children, the nasopharyngeal microbiome of the child, whether in health, after exposure to common respiratory viruses, or during acute sinusitis, is virtually unknown. Using culture-independent approaches will allow us to better understand the ecology of the microbiota of the nasopharynx and how these relationships are altered during viral infection or bacterial sinusitis. In fact, to date there have been no previous studies of the nasopharyngeal microbiome in children.

In addition to the major knowledge gaps that we have identified in the preceding paragraph, this unique data set will allow us to address other outstanding questions of clinical importance including:

a) Are children who experience closely spaced viral URIs more likely than those who experience infrequent URIs to develop acute bacterial sinusitis?
b) What proportion of children with a first episode of acute bacterial sinusitis experience multiple episodes of sinusitis in one year?
c) What proportion of episodes of acute bacterial sinusitis actually represent sequential episodes of very closely spaced viral URIs? (virus identified during episode of sinusitis is different from virus identified during the beginning of the respiratory illness)
d) What proportion of episodes of acute “severe onset” bacterial sinusitis is caused by viral infections?
e) Are children with asthma more likely than children without asthma to progress to acute bacterial infection after an antecedent acute viral URI?

**Existing Challenges**

a) **Collection of representative samples of nasal mucus and nasopharyngeal specimens repeatedly from young subjects.** To encourage families to report all respiratory illnesses, even mild URI, $25 will be provided for each illness visit. To maintain participation in the study, the method of acquiring respiratory samples must be acceptable to young children and not uncomfortable yet retain sensitivity and be useful for processing by culture-independent approaches. Various methods were considered but the following were deemed the best on the basis of past experience and review of the literature.

**Collection of nasal mucus.** Study subjects will collect samples of their own nasal mucus using a “nose-blowing” technique. The “nasal blow” procedure will be taught to the study participants at the first study visit. Briefly, participants spray saline into one nostril, occlude the other one, and then blow the nose into a “baggie”. The procedure is repeated on the other side. Viral transport medium is added to the baggie which is then sealed and placed into a container in the freezer. To model effects of storage conditions on detection of rhinoviruses (HRV), we conducted preliminary experiments in which samples of low-dose HRV (10^2 particles per sample) were stored in plastic self-locking bags in the saline/gelatin mix at either room temperature, 4°C, or -20°C. Specimens at all temperatures did not lose signal in our PCR-based diagnostic assays for at least 5
weeks (which was the duration of the test). These techniques have been used extensively in our previous pediatric studies and are extremely well tolerated when used for routine sampling.29

**Collection of nasopharyngeal specimens for conventional culture and microarray analysis.**

Nasopharyngeal swab samples for assessment of the nasopharyngeal microbiome and standard bacterial cultures will be collected at enrollment, during surveillance visits and at the first and a subsequent visit of each URI episode using flocked swabs (Copan Diagnostics, Inc. Murrieta California). The soft strands of nylon on the swab result in tremendously improved patient comfort and efficiency in specimen collection. The velvet brush-like texture collects liquid by capillarity and rapidly and efficiently dislodges cells, providing an optimal approach to sampling for this study. First, the swab is inserted into the nostril along the floor of the nasal cavity until the posterior wall is reached. The swab is held in place for 2-3 seconds while rotating gently. The swab (cut by sterile scissors) will be placed immediately into 2 ml of RNALater in preparation for PhyloChip processing. Tubes will be stored at 4°C overnight prior to storage at -80°C.

We have extracted DNA from 8 nasopharyngeal swab samples; total DNA concentrations ranged from ~650 ng to 2.3 µg. To determine the relative ratio of microbial and mammalian DNA retrieved, we also performed 16S rRNA and β-actin q-PCR analysis of all 8 samples. A ratio of approximately 1:100 bacterial to mammalian DNA was observed across the samples, which is very similar to what we have previously observed in bronchial brush samples of asthmatic patients. Finally, to determine whether sufficient bacterial DNA was present in these samples to produce sufficient 16S rRNA amplicon to analyze by PhyloChip, we performed 12 PCR reactions across a gradient of annealing temperatures using 30 ng of total DNA as template, as we do routinely for all samples to be analyzed by array. The lowest concentration of pooled 16S rRNA product was 732 ng, suggesting that all samples could be analyzed by PhyloChip at a standard concentration of 500 ng. In previous studies we have successfully profiled communities using as little as 100 ng of amplified PCR product per sample. These data show that nasopharyngeal swab samples provide sufficient material for bacterial community profiling by the 16S rRNA PhyloChip.

**b) Overcoming variability of the nasopharyngeal microbiome.** Characteristics of nasopharyngeal colonization vary according to a variety of factors including attendance at day care, season, age, recent illness, socioeconomic status, etc. To overcome this variability and still have a valuable baseline specimens that will permit comparison with an illness specimen, we will obtain surveillance specimens at entry and 4 other times. The specimens obtained closest in time preceding the episode of URI will be used as the baseline comparator.

**c) Overcoming frequency of asymptomatic colonization with respiratory viruses.** To interpret relationships between respiratory viruses and clinical illness, it is important to consider that respiratory viruses are detected in children without respiratory symptoms. The frequency of asymptomatic infection varies with age: viruses are detected in ~15% of healthy school-aged children and up to 31% of healthy infants.29,30 We will perform surveillance viral diagnostics on nasal wash samples at entry and 4 other occasions to estimate the seasonal frequency of asymptomatic infections for our study population; this information will be used as a reference point in analyses of viral infections during periods of illness.

**d) Definition of Sinusitis:** The 3 clinical presentations of sinusitis detailed in C.3.c above are widely accepted by 5 national societies: the American Academy of Allergy, Asthma and Immunology, the American Academy of Otolaryngic Allergy, the American Academy of Otolaryngology – Head and Neck Surgery, the American College of Allergy, Asthma, and Immunology, and the American Rhinologic Society.17 The American Academy of Pediatrics and the Infectious Diseases Society of America are including these presentations in their new guidelines (in press and PI is an author). In 4 clinical studies it has been shown that by using these stringent criteria, sinusitis is diagnosed in only 7-8% of respiratory infections presenting to the pediatrician.3,4,6,7

**e) Feasibility of enrollment, follow-up and Phylochip analysis.** Both the principal investigator and the co-investigators have conducted several longitudinal studies over several years of large numbers of participants (daycare, asthma, birth cohort)2,29,30. Subjects were recruited from the same practice that is participating in this study. The use of experienced coordinators and tested strategies for retention has led to excellent rates of
participation and retention in these studies. For example, the COAST cohort had 90% retention from prenatal enrollment to age 6 years\textsuperscript{30} and the 1-year RhinoGen study (which includes recurrent sampling of nasal secretions, 400 participants) has an 85% rate of retention and full participation with study procedures (unpublished data). In addition, serial nasopharyngeal cultures as proposed in this study were performed by Revai et al with only 10% attrition.\textsuperscript{31} The practice associated with the University of Wisconsin Hospital and Clinics, has an eligible population of nearly 2500 children; we do not anticipate any difficulty in recruiting patients to this study. We estimate that children will have an average of 5 infections/year and 4 surveillance visits when asymptomatic in addition to the entry visit. The study visits and procedures are designed to minimize participant burden and discomfort. Dr. Lynch has extensive expertise in translational microbiome studies\textsuperscript{15,32-35} and PhyloChip analyses of a variety of clinical samples, including those from adult patients with CRS (see preliminary data). Her group has acted as beta-testers for the G2 16S rRNA PhyloChip and helped develop and optimize the sample processing and statistical approaches described in 3d and e. The study investigators have the extensive experience and expertise to successfully conduct this study.

**Future Uses**

We will be storing all data collected from the study and the material leftover from the nasal samples for potential future infectious disease research such as sinusitis. We also will be maintaining the master key linking the samples to identifiable information. The data will be maintained indefinitely on the Department of Pediatrics password-protected server and the master key will be stored in a separate folder on the server. The nasal samples will be maintained indefinitely in a -80 freezer located in the H4/4 pediatric lab space. Subjects may withdraw the data and samples by contacting the research staff.