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# A method of processing nasopharyngeal swabs to enable multiple testing

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## Abstract

**Objective—**To develop a method to perform multiple tests on single nasopharyngeal (NP) swab.

**Study design—**We collected a NP swab on children aged 2–12 years with acute sinusitis and processed it for bacterial culture, viruses, cytokine expression, and 16S ribosomal RNA gene sequencing analysis. During the course of the study, we expand the scope of evaluation to include RNA sequencing, which we accomplished by cutting the tip of the swab.

**Results—**Of the 174 children enrolled, 126 (72.4%) had a positive bacterial culture and 121(69.5%) tested positive for a virus. Cytokine measurement, as judged by the adequate levels of a housekeeping enzyme (GAPDH), appeared successful. From the samples used for 16S ribosomal sequencing we recovered, on average, 16,000 sequences per sample, accounting for a total of 2,646 operational taxonomic units across all samples sequenced. Samples used for RNA sequencing had a mean RNA Integrity number of 6.0. Cutting the tip of the swab did not affect the recovery yield for viruses or bacteria, nor did it affect species richness in microbiome analysis.

**Conclusion—**We describe a minimally invasive sample collection protocol that allows for multiple diagnostic and research investigations in young children.



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Author Contributions

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## INTRODUCTION

A major challenge in clinical and translational research is the collection of multiple biological samples from a single patient; this is especially true in children. Although obtaining a nasopharyngeal (NP) swab is minimally invasive, performance of multiple tests usually requires obtaining multiple specimens. Although some investigators have reported using nasal samples for multiple tests,(1–5) we are not aware of any studies that have used a single NP swab to evaluate viral, bacterial and host parameters.

We present a method of sample collection and processing that uses a single swab for bacterial culture, viral detection using PCR, cytokine measurement by qPCR, RNA sequencing (RNA-Seq), and 16S ribosomal RNA gene sequencing.

## MATERIALS AND METHODS

### Participant eligibility and recruitment

Subjects were children 2–12 years of age (before 12$^{th}$ birthday) who were participants in an ongoing multicenter prospective clinical trial of acute sinusitis (AS, http://www.clinicaltrials.gov/, study identifier: NCT02554383). The diagnosis of AS was made according to stringent clinical criteria, defined *a priori,* consistent with the American Academy of Pediatrics guidelines (6). Eligible subjects had either: (1) persistent upper respiratory tract symptoms, i.e., 11–29 days of cough (not exclusively nocturnal) and/or nasal symptoms (rhinorrhea of any quality) which was not improving; or (2) 6 to 10 days of worsening symptoms (substantial worsening of nasal symptoms and/or fever after a period of improvement). We excluded children who: 1) had AS with severe presentation; 2) had received antimicrobial treatment within 15 days before presentation; 3) had evidence of another infection (i.e., acute otitis media or pneumonia); 4) had underlying immune deficiency, cystic fibrosis, ciliary dyskinesis, or major developmental delay, or 5) had asthma or allergic rhinitis as a main diagnosis for the presenting symptoms. We present data from children enrolled in the parent study that had testing for viruses and cytokines. The University of Pittsburgh Institutional Review Board approved the study and parents provided written consent.

### Specimen collection and processing

We obtained a NP sample from one nostril using a sterile, flexible, thin, flocked swab (ESwab™ -comprises 1mL of liquid Amies and a FLOQswab™-, Copan Diagnostics Inc.) (7,8). The tip of the nose was raised and the swab was introduced gently along the floor of the nasal cavity, passing under the inferior turbinate until the pharyngeal wall was reached. Once the swab was in contact with the pharyngeal wall it was removed gently. The swab was placed in ESwab™ liquid transport media and refrigerated at 2–8°C until transported to the laboratory on ice. On arrival to the microbiology laboratory, the swab was removed and standard bacterial cultures were performed (i.e., streaked plates with the proximal part of the swab). The remaining liquid Amies was divided into four cryovials, two of which contained 250μL of MagMAX™ Lysis/Binding Solution Concentrate (Applied Biosystems). During the course of the study, we decided to expand the scope of evaluation to include RNA-Seq.

We tested three protocols: 1) Partition; 2) Aliquot; and 3) Centrifuge to determine which would yield the necessary quantity and quality of RNA needed for RNA-sequencing.

**The Partition method—**(Figure 1) consisted of cutting off the distal swab tip (~0.5 cm) with clean scissors immediately after collection, placing the tip into a cryovial with 350μL of RLT Plus® (Qiagen) with 350 μL 2-beta mercaptoethanol (Invitrogen), and vortexing the cryovial for 30 seconds, and transporting the sample to the laboratory on ice for storage at −80° C. The proximal part of the swab was placed in ESwab™ liquid Amies and processed as previously described.

**Aliquot method—**The entire NP swab was placed in ESwab™ liquid transport media and transported to the laboratory on ice. Upon arrival to the laboratory, the sample was vortexed and then the swab was removed for streaking the bacterial culture plate as before. However, the liquid medium was split into 5 aliquots instead of 4; to the $5^{th}$ aliquot (which was intended for RNA sequencing) we added 350μL of RLT plus® with 2-BM.

**Centrifuge method—**The entire NP swab was placed in ESwab™ liquid transport media and transported to the laboratory on ice. Upon arrival to the laboratory, the sample vortexed and then the swab was removed for streaking the culture plate as before. However, the specimen medium was then centrifuged at 1000'RPM for 5 minutes, the supernatant removed, and the pellet resuspended in 800μL RLT Plus. The resuspended pellet was then partitioned into 5 aliquots as in the aliquot method.

The Partition, Aliquot, and Centrifuge methods were piloted on 3 subjects; the RNA concentration was highest when using the Partition method (73.1, 21.8, 30.3 ng/μL, respectively). Accordingly, this method was used as our standard operating procedure for the study at all three clinical study sites. Processing samples to allow for RNA sequencing was instituted approximately 10 months after the study began.

## Bacterial Identification

For bacterial culture, (Figure 1) the swabs were used to inoculate three types of media: trypticase soy agar, 5% sheep blood agar, and chocolate agar in the standard fashion. For swabs processed using the partition methods, the distal portion of the cut swab was used to inoculate the plates. Cultures were incubated overnight at 37°C with 5% $CO_2$. If no growth was present after overnight incubation, the culture was reincubated for another 24 hours. Growth of pathogenic bacteria (*S. pneumoniae, H. influenzae, and M. catarrhalis*) was assessed using standard techniques (9) (growth in 0, 1, 2, 3, or 4 quadrants); 3+ and 4+ were considered heavy growth.

## Viral Identification

Nucleic acid extraction was performed with the ABI MagMax96 Express automated instrument and the MagMax Viral Isolation Kit from one of the 4 aliquots prepared from the original sample (see Figure 1). Individual real-time RT-PCR assays for the detection and subtyping of adenovirus, influenza subtypes A/B/C, human metapneumovirus (HMPV), human rhinovirus (HRV), parainfluenza virus (PIV) subtypes 1–4, and respiratory syncytial

virus (RSV) were performed as previously described (10–19). All specimens were tested for RNase P to confirm RNA integrity and monitor for PCR inhibitors. Samples were run in batches on an ABI Step One Plus 96-well real-time PCR instrument. Negative and positive controls were included with each run, including RNA runoff transcripts to generate a quantitation curve. Specimens were considered positive if the Ct value was <40 cycles.

### Cytokine Measurement

Nucleic acid extraction was performed with the ABI MagMax96 Express automated instrument from one of the 4 aliquots prepared from the original sample (see Figure 1). Gene expression of interleukin (IL)-6, IL-10, IL-13, IL-17, IL-25, IL-33, Interferon: alpha; beta; gamma; and lambda and IL-8 was measured using exon-spanning primers and probes according to the manufacturer's instructions (TaqMan™, Applied Biosystems). All values were normalized to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene.

### Bacterial 16S rRNA gene amplicon sequencing

Sequencing was performed at the Environmental Sample Preparation and Sequencing Facility at Argonne National Laboratory. Total nucleic acids were extracted from a frozen specimen without buffer using the DNeasy UltraClean DNA extraction kit (Qiagen) and quantified using the Qubit™ assay (Invitrogen) from one of the 4 aliquots prepared from the original sample (see Figure 1). The 16S rRNA gene V4-V5 region was amplified using a modified 515F-806R primer pair (20). Sequencing of amplicons was completed using the Illumina MiSeq platform to generate 2×250 paired-end reads (17). Sequence reads were demultiplexed with *split_libraries_fastq.py* and operational taxonomic units (OTUs) were picked with *pick_open_reference_otus.py* using default QIIME version 1.9.1(21) parameters against the SILVA database version 128 (22). For rarefaction curves, samples were rarefied to 10,000 sequences per sample with set.seed (04012017) and the observed species richness measure was calculated with the rarecurve vegan function in R. Data analysis was performed in R v3.4.0 using the vegan (v2.4–3)(23) and phyloseq (v1.20.0) packages (24).

### RNA-Seq

RNA extraction was performed using the RNeasy Plus Mini kit (Qiagen) following the manufacturer's protocol from sample prepared from the tip of the swab (see Figure 1). RNA purity was assessed using Qubit 2.0 fluorometer and Agilent TapeStation 2200 for RNA quantity and quality. Host RNA sequencing libraries were generated using Illumina TruSeq RNA Access, which converts total RNA into template molecules, followed by sequence-specific capture of coding RNA (25). The cDNA libraries were quantified using Qubit 2.0 fluorometer and quality examined using Agilent Tapestation 2200.The cDNA libraries were pooled at a final concentration 2.0pM. Cluster generation and paired-read, dual-indexed 75bp sequencing was performed on Illumina NextSeq 500.

### Statistical method

For categorical data we used the Chi-square test to compare distributions. To compare mean values between groups we used paired t-test and analysis of variance (ANOVA).

## RESULTS

We enrolled 174 patients with AS, with a mean (standard deviation [SD]) age of 5.6 (2.7) years. A total of 95 patients were enrolled before the Partition protocol was instituted. [Table 1]. Because of cost restrictions we performed RNA-Seq on only 25 children.

### Bacterial cultures, viral detection, and cytokine measurement

A total of 126/174 (72.4%) children had a positive bacterial culture. There were no statistical differences between yield of bacterial cultures before and after the Partition protocol was initiated [Table 1]. A total of 121/174 (69.5%) children tested positive for a virus [Table 1]. All samples showed excellent RNP amplification with a mean (SD) cycle threshold (Ct) values of 23.6 (2.4) with no significant difference before and after the partition protocol was initiated [Table 1]. Cytokine expression was performed on specimens from 169 children [mean (SD) GAPDH Ct =24.4 (3.9)]; no significant differences in cytokine expression was occured after the Partition protocol was initiated [Table 1].

### Bacterial 16S rRNA gene sequencing

We processed 163 samples for 16S rRNA gene amplicon sequencing. We were able to recover on average >16,000 sequences per sample, accounting for a total of 2,646 OTUs. When rarefied to10,000 sequences per sample in order to capture most of the samples (n=100) and normalize depth of coverage, rarefaction curves indicated that the NP swabs and sequencing depth adequately sampled microbial species richness [Fig. S1A]. As expected, when more sequences were analyzed per sample, the lines start to plateau [Fig. S1A]. This indicates that even though more sequences were analyzed, few new species were detected. Furthermore, no significant difference (p=0.55) was observed in species richness after the partition protocol was adopted [Fig. S1B and Table 1].

### RNA-Seq

A total of 25 samples were sequenced with an average of 63 million paired-reads per sample. All 25 RNA samples were within the expected purity ratio of ≥1.8. The mean (SD) RNA yield was 772.3 (939.4) ng. All RNA samples used in this study had an RNA Integrity number of ≥6.0. On average 93% of reads had a quality score of 30 of higher. Samples were mapped to the Hg19 Human genome build with an average of 97% mapping rate [Table S1].

## DISCUSSION

We report here a method for the evaluation of host gene expression, cytokine levels, viruses, bacteria, and microbiome in the upper airway using a single nasopharyngeal swab. A limiting factor in studies to date, has been reliance on nasal wash, (1–3), or nasal brushing/curretage (26–28) to obtain samples. Because NP swabs are relatively noninvasive (compared to nasal wash or nasal curettage which are traditionally used), we are hopeful that the method described here may enhance facilitate collection of samples needed for research studies in a more non-invasive fashion.

Although other non-invasive sample collection methods for respiratory testing, such as anterior nares swabs, nose blowing (29), and nasosorption (30), can be used to collect sample, the sensitivity of these methods for viral detection is low(29). Furthermore, because of the small amount of columnar nasal epithelial cells in the sample, the amount of RNA is also likely to be suboptimal.

Mid-turbinate sampling is a well-established technique for the assessment with similar sensitivity as NP swabs for most viral, bacterial pathogens (7,16,31,32). Accordingly, if future research supports it, the protocol described here could potentially be performed using a mid-turbinate swab, further reducing the invasiveness of the sampling procedure.

In conclusion, we describe a reproducible, minimally-invasive sample collection protocol that allows for multiple testing in young children.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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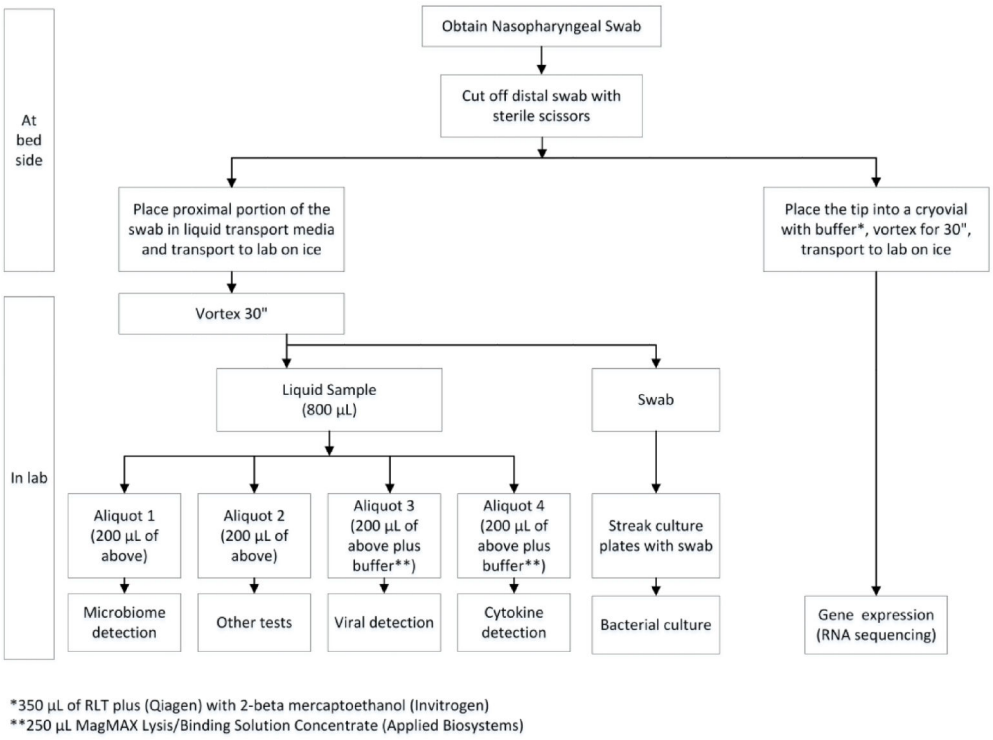


**Figure 1.**
Sample processing after partition protocol was instituted

**Table 1.**

Yield before and after Partition Protocol was instituted

| | Before swab partitioning[a] (N=95) | After swab partitioning[a] (N=79) | p value |
|---|---|---|---|
| Bacterial pathogen detected on culture, no. (%) | 68 (71.6) | 58 (73.4) | *0.92* |
| Virus detected by polymerase chain reaction, no. (%) | 65 (68.4) | 56 (70.9) | *0.85* |
| RNP[b], cycle threshold, mean (sd), n | 23.9 (2.4), 95 | 23.3 (2.5), 79 | *0.11* |
| GAPDH[c] level in samples used for cytokine analysis, mean (sd), n | 23.7 (3.3), 94 | 24.9 (4.4), 75 | *0.05* |
| Total RNA (nanograms), mean (sd), n | N/A | 772.3 (939.4), 25 | N/A |
| Species richness on16s ribosomal sequencing, mean (sd), n | 33.6 (26.5), 48 | 30.9 (16.8), 52 | 0.55 |

N/A: Not applicable

[a]Tested positive/number of patients tested (%)

[b]RNP: ribonucleoprotein

[c]GAPDH: glyceraldehyde 3-phosphate, cycle threshold

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