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## Non-Invasive Sample Collection for Respiratory Virus Testing by Multiplex PCR

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### Abstract

**Background**—Identifying respiratory pathogens within populations is difficult because invasive sample collection, such as with nasopharyngeal aspirate (NPA), is generally required. PCR technology could allow for non-invasive sampling methods.

**Objectives**—Evaluate the utility of non-invasive sample collection using anterior nare swabs and facial tissues for respiratory virus detection by multiplex PCR.

**Study Design**—Children aged 1 month – 17 years evaluated in a pediatric emergency department for respiratory symptoms had a swab, facial tissue, and NPA sample collected. All samples were tested for respiratory viruses by multiplex PCR. Viral detection rates were calculated for each collection method. Sensitivity and specificity of swabs and facial tissues were calculated using NPA as the gold standard.

**Results**—285 samples from 95 children were evaluated (92 swab-NPA pairs, 91 facial tissue-NPA pairs). 91% of NPA, 82% of swab, and 77% of tissue samples were positive for  $\geq 1$  virus. Respiratory syncytial virus (RSV) and human rhinovirus (HRV) were most common. Overall, swabs were positive for 74% of virus infections, and facial tissues were positive for 58%. Sensitivity ranged from 17–94% for swabs and 33–84% for tissues. Sensitivity was highest for

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**Ethical approval:** The Institutional Review Boards of Intermountain Healthcare and the University of Utah approved this study. Parents of the study subjects provided informed consent for sampling and diagnostic testing and children older than 7 years provided assent.

**Competing Interests:** LM, MR, CH and MP are employees of Idaho Technology, Inc. AJB, MAA, JAD and CLB collaborate with Idaho Technology, Inc., on several NIH and CDC-funded projects (see funding above).

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RSV (94% swabs and 84% tissues). Specificity was  $\geq 95\%$  for all viruses except HRV for both collection methods.

**Conclusions**—Sensitivity of anterior nares swabs and facial tissues in the detection of respiratory viruses by multiplex PCR varied by virus type. Given its simplicity and specificity, non-invasive sampling for PCR testing may be useful for conducting epidemiologic or surveillance studies in settings where invasive testing is impractical or not feasible.

### Keywords

respiratory virus; viral diagnostics; molecular diagnostics; pediatrics; upper respiratory infection; polymerase chain reaction

## Background

Respiratory tract infections are the most common reason for medical visits and hospital admissions during childhood.<sup>1</sup> Influenza virus and respiratory syncytial virus (RSV) are frequently implicated, however many other viruses have recently been recognized as important.<sup>2–3</sup>

Diagnostic testing for respiratory viruses is usually performed in hospital settings. Testing serves several functions, including facilitating patient cohorting,<sup>4</sup> initiation of antiviral therapy,<sup>5</sup> containment of nosocomial outbreaks,<sup>6</sup> and decreased use of ancillary testing<sup>7–8</sup> and antibiotics.<sup>8–10</sup> In most cases, respiratory samples are collected by invasive methods such as nasopharyngeal swab or nasopharyngeal aspirate (NPA) and diagnostics are performed using immunofluorescence and/or culture-based methods.<sup>11</sup> Increased sensitivity has been demonstrated with the polymerase chain reaction (PCR),<sup>12–13</sup> and the use of PCR diagnostics in hospitals has increased.<sup>11</sup>

Diagnostic testing for respiratory viruses is also important for surveillance and epidemiologic studies performed in both hospital and community settings. Throughout the year, multiple respiratory viruses co-circulate and can cause similar clinical syndromes that cannot be distinguished without diagnostic testing.<sup>14–15</sup> Recent studies of non-pharmaceutical interventions to prevent the spread of influenza have highlighted the need for large-scale, community-based epidemiologic data, including specific laboratory-confirmed diagnosis.<sup>16</sup> PCR-based methods have enabled investigators to perform family-based studies of viral prevalence in the community.<sup>17–21</sup>

Invasive respiratory sample collection methods for respiratory testing have significant limitations for community studies.<sup>22</sup> The performance of a nasopharyngeal aspirate or swab test is stressful, leading to reluctance among children and parents to participate in studies, particularly if repeated testing is required. Based on the higher sensitivity of PCR, we hypothesized that respiratory viruses could be detected from samples collected by non-invasive methods that might be more acceptable to children such as anterior nares swabs or facial tissues. While other studies have investigated the use of anterior nares swabs to collect samples for the detection of respiratory viruses,<sup>23–28</sup> studies investigating the use of facial tissues have not been published.

### Objectives

Our objective was to evaluate the performance of non-invasive sample collection using anterior nares swabs and facial tissues for respiratory viral detection by multiplex PCR.

## Study Design

### Protection of Human Subjects

The Institutional Review Boards of Intermountain Healthcare and the University of Utah approved this study. Parents of study subjects provided informed consent for sampling and testing. Children older than 7 years provided assent.

### Setting

Primary Children's Medical Center (PCMC; Salt Lake City, UT) is a 252 bed tertiary-care children's hospital. Samples were collected from infants and children aged 1 month through 17 years undergoing evaluation for respiratory illness in the Emergency Department (ED). The study was conducted during 2 winter respiratory seasons (12/2008–3/2009 and 11/2009–4/2010).

### Participant enrollment and sample collection

English-speaking families with non-critically-ill children evaluated with respiratory viral testing by NPA as part of their routine care were eligible. After consent was obtained, the trained research assistant, participant or parent used a nylon-flocked swab (Copan Diagnostics, Inc., 1.5 cm tip and 12 cm shaft) to collect a specimen from the participant's anterior nares. When the parent or participant elected to collect the specimens, the research assistant gave brief, standardized instructions about how to use the anterior nares swab and/or facial tissue. After sample collection, the research assistant placed the swab in a 3 mL cryovial for transport. Next, the participant blew his or her nose or had his or her nose wiped into a white 2-ply facial tissue (Kirkland brand, without scent, lotion or antimicrobial additives). The research assistant then selected an approximately 1 cm<sup>2</sup> piece of facial tissue that appeared to be soiled with nasal secretions near the center of where the tissue was applied to the nares. The research assistant used gloved hands to tear that portion of tissue and insert it into a 3 mL transport cryovial. No transport medium was used with swabs or facial tissues, and validation studies showed no degradation of RNA with storage of up to 7 days at 4°C (data not shown). Samples were stored in the PCMC Microbiology Laboratory at 4°C and transported in a standard cooler to Idaho Technology, Inc. (ITI) within 3 days.

NPA samples were collected by a nurse or respiratory therapist according to ED protocols. This sample was transported to the PCMC Microbiology Laboratory where an aliquot was obtained during processing and was stored at -70° C within 24 hours of receipt in the laboratory. Each month, these aliquots were transferred to ITI in a cooler with dry ice. Upon arrival, the swab, facial tissue, and NPA samples were stored at -80° C until PCR-testing was conducted between 1 and 6 months after collection.

### Multiplex PCR testing

NPA, anterior nares swab, and facial tissue samples were tested for respiratory viruses using ITI's FilmArray® Respiratory Panel. This FDA approved multiplex PCR panel includes assays for adenovirus (AV), influenza A, influenza B, parainfluenza virus types 1–4 (PIV 1–4), respiratory syncytial virus (RSV), human metapneumovirus (hMPV), human rhinovirus (HRV), and 2 coronaviruses (CV, includes NL-63 and HKU-1)<sup>29</sup>. For this study, a research version of the pouch was used that also contains assays for CV 229E and CV OC-43.

Prior to introduction into the FilmArray, specimens were mixed with a denaturing buffer. For both facial tissue and anterior nares swab samples this involved adding buffer to the cryovial containing the facial tissue or swab. Cryovials were then vortexed and the mixture was aspirated into a syringe. Samples were injected into the FilmArray sample container ("pouch") using a blunt tipped cannula.

The FilmArray is a closed system that combines nucleic acid extraction from clinical specimens, nested multiplex PCR, and post PCR analysis.<sup>29</sup> After introduction into the FilmArray pouch, cells and viruses within the sample are lysed using ceramic beads. Nucleic acids are extracted using magnetic beads, and used as the template for the first-stage multiplex PCR. After a limited number of first-stage amplification cycles, the amplification products are diluted with buffer and moved into an array of 1 microliter wells in which a second-stage singleplex nested PCR is performed. Assays within both stages of the nested PCR target specific respiratory pathogens. Amplification products generated in the second stage PCR are detected by their specific melting profiles using LCGreen®, Idaho Technology's proprietary melting dye. Due to the closed nature of the FilmArray pouch container, PCR contamination risk is low; however, negative control runs were included.

### Statistical Analysis

For each sample-type, the proportion of samples that were positive for a virus was calculated. Then the proportion of positive samples was compared between children age 2 years or younger, children 3 through 4 years, and children 5 years and older using the Cochran-Armitage trend test. Next, the detection rate was determined for each virus by sample-type. All influenza, PIV, and CV types were grouped for analyses. For each virus, the detection rate was calculated by dividing the number of positive tests per sample-type by the total number of positive tests for that virus.

Overall test accuracy was calculated by comparing results of PCR testing on paired anterior nares swab-NPA samples and paired facial tissue-NPA samples. For anterior nares swab or facial tissue-NPA pairs, a complete match was identified when each virus detected by NPA was also detected by its paired swab or facial tissue or both the NPA and its paired swab or facial tissue were negative for all viruses. The sensitivity and specificity of anterior nares swab and facial tissue were calculated for each virus using the paired swab-NPA and tissue-NPA data. NPA was used as the gold standard. For each virus, a test positive was identified when the swab or facial tissue sample was positive for the virus, and a true positive was identified when the paired NPA sample was also positive for that virus. Sensitivity was calculated as test positives divided by true positives. For specificity, the PCR testing was considered negative if it did not identify a particular virus, even if it identified a different virus. Specificity was calculated as all test negatives (swab or facial tissue) divided by all true negatives (NPA) for each virus. Ninety-five percent confidence intervals were calculated.

### Results

Ninety-five children participated in the study. Median age was 1 year (range 1 month – 15 years). The majority (77%) were 2 years or younger; 10% were 5 years or older. In total, 285 samples were collected. At least one virus was identified by PCR in 91% (84/95) of NPA, 82% (75/92) of anterior nares swab, and 77% (70/91) of facial tissue samples (Table 1). More than one virus was detected in 39% (37/95) of NPA, 23% (21/92) of anterior nares swab and 20% (18/91) of facial tissue samples. While the proportion of positive samples among older children appeared to be slightly higher than the proportion of positive samples among younger children for anterior nares swab and facial tissue sample-types (Table 1), these differences were not statistically significant.

RSV and HRV were the most frequently detected viruses (Table 2). For most viruses, NPA had the highest detection rate, although anterior nares swabs had detection rates equal to NPA for influenza viruses and RSV, and higher for CV. Over 50% of cases of influenza, CV, HRV, PIV, RSV and hMPV were detected by anterior nares swab. Detection rates for facial tissue samples were lower than those of NPA and swabs for most viruses, but higher than

swabs for adenoviruses (AV). Over 50% of cases of influenza, PIV, RSV and hMPV were detected in facial tissue samples.

Paired data were available for 92 anterior nare swab-NPA pairs and 91 facial tissue-NPA pairs. Anterior nare swab results completely matched NPA results in 50% (46/92). Facial tissue results completely matched NPA results in 27% (34/91). Test accuracy was also calculated using data from the 49 participants whose NPA samples were positive for only a single virus. In this analysis, anterior nare swab results completely matched NPA results in 63% (31/49), and facial tissue results completely matched NPA results in 53% (26/49).

Table 3 shows the sensitivity and specificity of anterior nare swabs and facial tissues when compared to NPA as the gold standard for each virus. Anterior nare swabs showed high sensitivity ( $\geq 80\%$ ) for influenza, hMPV and RSV. Facial tissues also showed high sensitivity for RSV (84%). Specificity of both non-invasive sample-types was  $\geq 95\%$  for all viruses except HRV.

## Discussion

Our data demonstrate that non-invasive methods for collecting respiratory samples can be used to identify respiratory viruses with multiplex PCR testing. Detection rates were  $>50\%$  for most viruses and  $>80\%$  for RSV. Using NPA as the gold standard, the sensitivity of non-invasive methods ranged from 17%-93% for individual viruses. As with other studies of viral detection, AV had a low detection rate.<sup>30-31</sup> In general, anterior nare swabs had higher detection rates and higher sensitivity than facial tissues. For both sample types, specificity was  $\geq 95\%$  for all viruses except HRV. These data suggest that, while further investigation is required, non-invasive collection of respiratory samples with viral testing by multiplex PCR may be useful for conducting surveillance or epidemiology studies in community settings where invasive testing is impractical or not feasible.

Other studies have investigated the use of anterior nare swabs for the detection of respiratory viruses using antigen detection, viral culture, and PCR.<sup>23-28</sup> Sample collection from the anterior nares with flocked swabs has been shown to be comparable to collection by NPA,<sup>23, 25</sup> although some studies have reported difficulties in the detection of RSV with swabs.<sup>24, 26</sup> This included one study in which detection was by culture<sup>26</sup> and one by PCR.<sup>24</sup> In the culture-based study, the lability of RSV was hypothesized as a factor decreasing the sensitivity of swabs. In our study, the sensitivity of both swabs (94%) and facial tissue samples (84%) were highest for RSV.

To our knowledge no other studies have looked at the use of facial tissue as a sample collection method for the detection of respiratory viruses in children. Facial tissues are inexpensive, readily available, and in our experience, children generally accept them without significant distress. This method could be particularly beneficial for use in population surveillance, allowing for self-collection by large numbers of individuals quickly in the setting of a public health emergency. In studies where large groups or repeated sampling is involved, facial tissue might be a more practical and acceptable method for obtaining specimens. Sensitivity of facial tissues was lower than that of swabs, and they are not an adequate sample type for individual diagnostics. However, their specificity for viral detection may make them appropriate for community surveillance as the detection of positives likely indicates the pathogen is in the population.

In this study the sensitivity of each sample collection method varied by virus type. While the number of positive specimens for many viruses was too low for comparisons, we could compare RSV and HRV. Sensitivity for the detection of RSV was  $>80\%$  for both anterior nare swabs and facial tissues while for HRV it was  $< 60\%$ . The reasons for this are unclear,

but could be due to virus or symptom-related factors that would require further investigation<sup>32–33</sup>.

High specificity for detection of viruses is crucial for surveillance, as it provides confidence that a detected virus is circulating in a population. Some current methods for influenza surveillance are not virus-specific and are performed, for example, in schools through absenteeism data and through sentinel clinics by influenza-like illness (ILI) reporting. Absenteeism can be due to a number of factors unrelated to influenza,<sup>34–35</sup> and the specificity of ILI for culture-proven influenza has been shown to range from 35 to 71%.<sup>15, 36–38</sup> Use of non-invasive sampling methods combined with molecular detection could offer the potential to broaden population-based testing with the possibility of a significant improvement in data. In other studies of infectious pathogens, non-invasive collection methods combined with molecular detection has improved epidemiologic understanding.<sup>39–41</sup> The cost of molecular testing could be considered prohibitive for its use in surveillance settings. However, sample strategies that involve pooling for testing may result in significant savings and further studies of this are warranted.

There are several limitations to our study. The primary limitation was sample size. While 95 participants provided samples, the numbers of each virus detected were small. Our study was not designed to determine the non-inferiority of swabs or tissues for individual diagnostics when compared to NPA. The age range of our participants was biased toward children younger than 2 years who may have had higher respiratory viral loads than older children; therefore, our reported virus detection rates may be higher than would be found for older children. In addition, the young age of our participants may have made it more difficult to evaluate sample collection by facial tissue, as small children cannot blow their noses to adequately expel nasal secretions. Only one brand of facial tissues was tested, and it is possible that others will not perform in the same manner. The recovery of viruses from the facial tissue samples, in particular, may have been affected by the lack of precision in sample collection and transfer to cryovials; however, we believe our results are still useful as our methods reflect what is likely to occur during community epidemiologic studies where this sample-type might be used. Finally, all children tested were in the ED and were thought to be sufficiently ill that viral testing was ordered. These children may have higher respiratory viral loads making detection easier. In surveillance of healthy children who may have low viral loads, non-invasive testing may not perform as well.

Overall, both anterior nare swabs and facial tissues are promising tools for non-invasive diagnostic testing for respiratory viruses in certain circumstances. While not appropriate for patient care, the use of non-invasive respiratory sampling may prove valuable for epidemiologic or surveillance studies in community settings where respiratory diagnostics have been limited.

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## Abbreviations used in this manuscript

**NPA**            nasopharyngeal aspirate

<b>PCR</b>	polymerase chain reaction
<b>RSV</b>	respiratory syncytial virus
<b>PCMC</b>	Primary Children's Medical Center
<b>ED</b>	emergency department
<b>ITI</b>	Idaho Technology, Inc
<b>DFA</b>	direct fluorescent antibody
<b>AV</b>	adenovirus
<b>PIV</b>	parainfluenza virus
<b>hMPV</b>	human metapneumovirus
<b>HRV</b>	human rhinovirus
<b>CV</b>	coronavirus
<b>IV</b>	influenza virus
<b>ILI</b>	influenza-like-illness

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**Table 1**

Proportion of positive samples (one or more viruses detected) by sample type and age group.

Sample Type	Proportion of Positive Samples by Age Group			
	All age groups	2 years or younger	3 though 4 years	5 years or older
Nasopharyngeal aspirate	91% (86/95)	91% (67/74)	92% (11/12)	89% (8/9)
Anterior nare swab	82% (75/92)	79% (58/73)	91% (10/11)	87% (7/8)
Facial tissue	77% (70/91)	75% (54/72)	82% (9/11)	87% (7/8)

**Table 2**

Virus detection rate by collection method [number detected (% of total)].

Sample Type	Virus						
	AV	CV	IV	HRV	hMPV	P1V	RSV
Nasopharyngeal aspirate	6 (100)	8 (73)	6 (86)	38 (81)	14 (100)	6 (86)	31 (91)
Swab	1 (17)	9 (82)	6 (86)	30 (64)	12 (86)	4 (57)	31 (91)
Facial tissue	2 (33)	4 (36)	4 (57)	22 (47)	9 (64)	4 (57)	28 (82)
<b>Total Number of Positives</b>	6	11	7	47	14	7	34

AV = adenovirus, CV = coronaviruses, IV = influenza A and B, HRV = human rhinovirus, hMPV = human metapneumovirus, P1V = parainfluenza viruses, RSV = respiratory syncytial virus

**Table 3**

Sensitivity and specificity by sample and virus type (nasopharyngeal aspirate is “gold standard”).

Pathogen	Anterior Nasal Swab		Facial Tissue	
	Sensitivity (95% CI)	Specificity (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
Adenovirus	17% (0.4–64)	100% (96–100)	33% (4–78)	100% (96–100)
Coronaviruses	75% (35–97)	96% (90–99)	56% (21–86)	100% (96–100)
Influenza A and B	83% (36–100)	99% (94–100)	50% (12–88)	99% (94–100)
Rhinovirus	59% (42–74)	87% (75–95)	50% (33–67)	94% (84–99)
hMPV	80% (52–96)	100% (95–100)	64% (35–87)	100% (95–100)
Parainfluenza viruses	50% (12–88)	99% (94–100)	50% (12–88)	99% (94–100)
RSV	94% (79–99)	97% (89–100)	84% (67–94)	97% (89–100)

hMPV = human metapneumovirus, RSV = respiratory syncytial virus