Published in final edited form as:
J Virol Methods. 2010 January ; 163(1): 144–146. doi:10.1016/j.jviromet.2009.08.019.

Exhaled breath condensate appears to be an unsuitable specimen type for the detection of influenza viruses with nucleic acid-based methods

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Abstract

Exhaled breath condensate is an airway-derived specimen type that has shown significant promise in the diagnosis of asthma, cancer, and other disorders. The presence of human genomic DNA in this sample type has been proven, but there have been no reports on its utility for the detection of respiratory pathogens. The suitability of exhaled breath condensate for the detection of influenza virus was investigated, as an indication of its potential as a specimen type for respiratory pathogen discovery work. Matched exhaled condensates and nasopharyngeal swabs were collected from 18 adult volunteers. Eleven cases were positive for influenza A virus, and one was positive for influenza B virus. All swab samples tested positive in real-time amplification assays, but only one exhaled condensate, an influenza A positive sample with a very high viral load, tested positive in the real-time RT-PCR assay. Most of the positive nasopharyngeal swab samples inoculated for virus culture also tested positive, whereas influenza virus was not grown from any of the exhaled condensate specimens. It was concluded that influenza viruses are not readily detectable with culture or nucleic acid-based techniques in this sample type, and that exhaled breath condensate may not be suitable for respiratory pathogen investigations with molecular methods.

Keywords

Exhaled breath condensate; Influenza; Respiratory virus

Exhaled breath is an airway-derived specimen type that has been recognized recently to show significant promise in the diagnosis of asthma, cancer, and other disorders. The gas

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Conflict of interest
None.

phase includes volatile compounds (*e.g.*, alkanes, alcohols, and aldehydes), and the aqueous phase entails water-soluble analytes (*e.g.*, hydrogen peroxide and glutathione). The aqueous phase is collected by cooling the exhaled breath of the subject in a handheld or portable condensor, while he or she is seated and breathing quietly. There is a strong precedent for chemical analysis of exhaled breath for volatile markers of oxidative stress and chronic inflammation in the lung (Mutlu et al., 2001; Paredi et al., 2002; Phillips et al., 2003; Carpagnano et al., 2003; Risby, 2003).

While larger molecules are less likely to be aerosolized and suspended in exhaled breath, there are recent reports of mutated DNA detectable in exhaled breath condensate (Gessner et al., 2004; Carpagnano et al., 2005). The presence of genomic DNA in exhaled condensate in replicate samples has been confirmed in a previous study (Han et al., 2006). The presence of genomic DNA in this sample type has been verified in over 40 subjects, including those following caustic bisulfite deaminating treatment for DNA methylation analysis (S.D.S., unpublished observations). The exhaled DNA is not cell-associated.

There have been no reports, however, on the utility of this type of specimen for the detection of respiratory pathogens, and exhaled breath condensate is of particular interest as a potential sample type for deep-lung respiratory pathogen discovery work. In this preliminary study, therefore, its suitability for the detection of influenza virus was investigated.

Matched samples of exhaled breath condensates and nasopharyngeal swabs were collected from adult volunteer subjects presenting to the Albany Medical Center Emergency Department during the 2006–2007 and 2007–2008 influenza seasons, with symptoms of influenza-like-illness, defined as fever >38.5 °C and myalgias or headache, along with either respiratory complaints or other non-localizing symptoms. Exclusion criteria included known bronchiectasis or chronic suppurative disease, tuberculosis, immunodeficiency, sufficient hypoxia to warrant face-mask supplementation or ventilation, or intubation/mechanical ventilation, as well as known bleeding diatheses (hemophilia or severe platelet disorders). The study was approved by the Institutional Review Board of the Albany Medical Center, and informed consent was obtained from all volunteers prior to participation.

The exhaled breath condensate samples were collected with a disposable, handheld, commercially available device, RTube® (Respiratory Research, Charlottesville, VA). The subject performs normal tidal (quiet) breathing, with the mouthpiece in the mouth, exactly as for a common nebulizer mouthpiece. The device offers neither significant airflow resistance nor discomfort, and sufficient liquid volume (>1 ml) of condensate is obtained for DNA analysis within 10 min of normal tidal breathing. Nasopharyngeal swabs were collected by standard procedures, and the swab placed in a vial of sterile viral transport medium immediately after collection.

Samples were refrigerated and transported within 48 h to the Virus Reference and Surveillance Laboratory at the Wadsworth Center, New York State Department of Health, for analysis. Specimens were tested for influenza A and B viruses by both classical and molecular methods. Conventional culture was performed on primary rhesus monkey kidney cells (Diagnostic Hybrids, Athens, OH). In-house developed real-time RT-PCR assays that target the matrix and NS genes of influenza A and B, respectively, were performed following easyMAG extraction (BioMerieux, Durham, NC). The molecular testing methods include an internal control amplification test that verifies extraction efficiency and the absence of PCR inhibitors in all samples. All methods used are in routine use at the Wadsworth Center for seasonal influenza surveillance.

A total of 19 volunteers were recruited during the study: 11 subjects during the first season and 8 during the second. The most common symptoms besides fever were dry cough,

myalgia, and shaking chills. Additionally, extreme fatigue and headache were reported by more than half of the patients, and arthralgia by 8. For one case in the first season, a nasopharyngeal swab could not be collected; therefore, matched samples were available for 18 cases. As shown in Table 1, 12 of these patients tested positive for influenza: 6 in the 2006–2007 season (5 for influenza A, 1 for influenza B) and 6 during the 2007–2008 season (all for influenza A). All of these cases were detected by positive results on the nasopharyngeal swab, but only one, an influenza A case during the first season, was detected by a positive result on the exhaled breath condensate. The internal control amplification assay confirmed efficient extraction and the absence of amplification inhibition in all samples. While the real-time assays for influenza that were used in this study are not quantitative, relative viral load in the specimens can be gauged from the Ct values obtained in the assay. When these were reviewed (Table 2), the only exhaled condensate specimen that tested positive, had a Ct value of 37.1, indicative of a very low viral load. Conversely, the nasopharyngeal swab from that patient tested positive with a Ct value of 17.2, indicative of a very high viral load, several log higher than that in the exhaled condensate.

Culture testing was discontinued in the second season, since no virus had been isolated from condensate samples during the first season. This simplification allowed the entire exhaled breath condensate sample to be made available for molecular testing, and the extraction procedure was also altered in an attempt to increase the nucleic acid yield. Previously, 350 μl of condensate had been extracted into 110 μl of eluate, the same procedure as that routinely used for the nasopharyngeal swab samples. In the second season, the extraction procedure for exhaled breath condensates was altered such that 1 ml was extracted into 55 μl of eluate. Furthermore, a small amount of human embryonic lung cell suspension (Media Glassware and Tissue Culture Services, Wadsworth Center) was spiked into each sample immediately before extraction to provide carrier nucleic acid, in an additional attempt to enhance the extraction of any viral nucleic acid that potentially might have been present. The internal control amplification assay confirmed in all cases that these modifications did not cause either PCR inhibition or loss of nucleic acid due to cell nuclease degradation prior to extraction. However, as can be seen from the results, none of these modifications resulted in the detection of influenza virus in the condensate samples.

In previous studies, exhaled breath condensate has proven to be a promising specimen type in the investigation of numerous lung disorders, including for the DNA sequence analysis of lung cancers. Here it was investigated as a potential novel specimen type for the study of infections with respiratory pathogens, using influenza testing with culture and molecular methods as the model. In this preliminary study of patients with known influenza disease however, a several-fold lower sensitivity of detection of this virus was observed in exhaled condensates, than that observed for the more routinely collected specimen type of nasopharyngeal swabs, when tested with either conventional culture or real-time RT-PCR. Control assays with quantitated exogenous spiked material verified that these differences were not caused by the presence of amplification inhibitors in the condensates. It was conjectured that the single condensate sample that was influenza positive by molecular testing had come from a patient with a more pneumonia-like syndrome. However, a review of the symptoms revealed that this was not the case. It was therefore concluded that influenza viruses are not routinely detectable with nucleic acid-based methods in this sample type, and that exhaled breath condensates may not be suitable for respiratory pathogen investigations using these techniques. Whether the detection of other molecules such as proteins, or small molecules that may be markers of infection, would be more successful, remains to be investigated.

Acknowledgments

The authors wish to thank the staff of the Virus Reference and Surveillance Laboratory at the Wadsworth Center for performing conventional virus culture.

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Table 1

Specimens received and results of influenza testing.

	Specimens received		Influenza-positive	
	NPS[a]	EBC[b]	NPS[a]	EBC[b]
2006–2007	10	11	6	1
2007–2008	8	8	6	0
Total	18	19	12	1

[a] Nasopharyngeal swab.

[b] Exhaled breath condensate.

Table 2

Test results for influenza-positive nasopharyngeal swabs and exhaled breath condensates.

Case #	Influenza type	Ct value on real-time RT-PCR		NPS culture result[a]
		NPS[b]	EBC[c]	
02	A	21.5	Neg	Pos
03[d]	A	17.2	37.1	Pos
08	A	29.5	Neg	Pos
09	A	32.2	Neg	Neg
10	B	24.6	Neg	Pos
11	A	26.5	Neg	Pos
12	A	27.6	Neg	ND
14	A	21.4	Neg	ND
15	A	19.7	Neg	ND
16	A	26.6	Neg	ND
17	A	30.1	Neg	ND
19	A	22.2	Neg	ND

[a] Culture was performed on specimens during first season only.

[b] Nasopharyngeal swab.

[c] Exhaled breath condensate.

[d] Shaded row indicates the only case where both nasopharyngeal swab and exhaled breath condensate specimens tested influenza-positive.