

Exhaled Breath Condensate pH Assays

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KEYWORDS

• Exhaled breath condensate • Assay • pH • Airway acidification

KEY POINTS

- Exhaled breath condensate (EBC) collection is the only noninvasive method of assessing airway acidity.
- Like all tests, EBC pH is neither perfectly sensitive nor specific for lower airway acidification.
- Acid at any level in the airway can lead to EBC acidification, and an absence of EBC acidification does not exclude the presence of some degree of airway acidification.
- EBC pH assays remain the most successful manner to date to evaluate the role of airway acidification in respiratory disease.

INTRODUCTION

Airway pH is central to the physiologic function and cellular biology of the airway. The causes of airway acidification include (1) hypopharyngeal gastric acid reflux with or without aspiration through the vocal cords, (2) inhalation of acid fog or gas (such as chlorine), and (3) intrinsic airway acidification caused by altered airway pH homeostasis in infectious and inflammatory disease processes.¹ The recognition that relevant airway pH deviations occur in lung diseases is opening doors to new simple and inexpensive therapies.^{2,3} This recognition has resulted partly from the ability to use exhaled breath condensate (EBC) as a window on airway acid-base balance, about which data are otherwise difficult or ethically impossible to obtain.

EBC consists primarily of water with trapped aerosolized droplets from the airway lining fluid, and water-soluble volatile compounds. The pH of EBC is determined primarily by the water-soluble volatile gases^{4–7} and reflects (but does not precisely quantitate) the pH of airway lining fluid. A low pH value in EBC results from the

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enhanced volatilization of acids that occurs from an acidic source; in other words, acidic airway lining fluid. Nonvolatile basic anions become protonated within an acidic airway lining fluid and thereby turn into volatile (exhalable) acids. Formic and acetic acid,^{7,8} and numerous other acids, join the exhaled airstream when the airway is acidic, are captured by the water condensing in the exhaled breath, and lower the pH of the EBC. In contrast, when the airway lining fluid is alkaline, the ionized conjugate bases of these acids are the dominant species. The ionized conjugate bases are not volatile, and therefore not appreciably exhaled.⁶ The association of low airway pH with low EBC pH has been proven empirically with studies of experimental airway acidification in cows.⁹ These straightforward concepts have allowed a simple assay (pH), performed on easily collected samples (EBC),^{4,10} to provide large amounts of data regarding airway acidity from patients, even in the presence of marked acute airway disease.^{11–14}

ADDRESSING THE ARTIFACT OF CARBON DIOXIDE

Most investigators address in some manner the important issue of carbon dioxide (CO₂) affecting the EBC pH. CO₂ is a volatile gas that is a precursor to carbonic acid. CO₂ is not appreciably more volatile from acidic airway lining fluid than from alkaline airway lining fluid. Although CO₂ contributes to the acidity of airway lining fluid, the presence of CO₂ in EBC does not communicate that the airway lining fluid is acidic, because the CO₂ will affect the pH of the EBC regardless of the airway pH. CO₂ leads to substantial EBC acidification if not accounted for in some fashion, and provides noise in the system that limits the ability to identify the smaller effects of the volatile acids, and shrinks the overall effect size of pH measurements in disease by lowering the EBC pH normal range. Gas standardization with either a CO₂-free gas^{4,10,15,16} or a gas that contains a known quantity of CO₂¹⁷ seems wise, and has been commonly adopted.

Gas standardization before EBC pH measurement allows samples to be assayed at any time after collection, and clearly improves assay repeatability and overall reproducibility in patient collections.^{4,10,15–18} Not all CO₂ is removed through gas standardization via exposing (bubbling) the sample with a CO₂-free gas, partly because the standardizing gas is unlikely to be truly CO₂-free. Overall, however, the levels of CO₂ in EBC are brought to similar low levels for all samples during gas standardization, but some minor effect of residual CO₂ remains.

More recent work from Kullman and Horvath's group¹⁷ in Hungary has raised the possibility that using a known concentration of CO₂ for gas standardizing may improve reproducibility of the assay further. Some loss of effect size between disease and health occurs by so doing, but standardizing on a known concentration of CO₂ may allow for more interlaboratory cooperation. Using CO₂-free gas allows for variable amounts of contaminating CO₂, which, in addition to EBC collection equipment differences, may partly explain why some variability of normal occurs in different laboratories.

It should not be a surprise that the volatile acids trapped in EBC are not as affected by gas standardization procedures as is the CO₂. This finding is simply because CO₂ is a highly volatile gas that is a precursor of an acid, as opposed to being an acid itself, whereas the volatile acids that acidify EBC are trapped by ionization. CO₂ is easy to remove from solution, which likewise removes the carbonic acid that was formed when EBC initially absorbed the CO₂. Thus, the unhelpful effect of exhaled CO₂ on EBC pH is minimized by having the EBC "breathe it back off" during gas standardization.

EBC PH ASSAY VALIDATION AND TECHNICAL ISSUES

The gas standardized EBC pH measurements are substantially reproducible and immune to most potential confounding influences. Compared with other EBC assays,

pH is solidly in the range of available assays. Although EBC is a dilute fluid with low ionic strength, pH probes are available that are designed to function even in distilled water. Thus, EBC pH measurement has benefited from the decades of advances in measurement of this standard chemical property. Technological capabilities are readily available to assess concentrations of hydrogen ions as low as 10 fmol/L (which is equivalent to an alkaline pH of 14) and as high as 1 mol/L (an acid pH of 0). Measurement of pH is routinely performed, even in highly purified water such as that used in steam turbines. Measurement of pH of fluids is a well-understood process with no technical hurdles to overcome.

The authors' center has specifically examined and found that the following factors have no effect on the final deaerated EBC pH value: gender, time of day collected, volume of EBC collected, duration of EBC collection, degree of hyperventilation or hypoventilation of the subject, temperature or duration of sample storage, choice of CO₂-free gas used for deaeration (oxygen vs argon), acute use of albuterol/salbutamol by the patient, or methacholine-induced airway obstruction.^{4,5,10,19} Age does not seem to be a factor, except in the very elderly, who have a substantially higher incidence of low EBC pH.²⁰ Although average and median values between races stay consistent, African Americans have been shown to have a higher prevalence of episodes of low pH.²¹

Ammonia is a volatile base that is readily measurable in EBC. Much EBC ammonia seems to be formed in the upper airways through bacterial metabolism of urea.²² Removal of this oral source of ammonia does not affect EBC pH in healthy adults.⁵ However, reasonable evidence exists to support that the ammonia reduces the sensitivity of EBC pH to airway acidity. Oral ammonia neutralizes CO₂ initially, and also exhaled acids that are relevant to airway acidity, lessening the ability of the exhaled acids to lower EBC pH. As a result, orally collected EBC pH in lung diseases is less likely to be acidic than EBC collected from endotracheally intubated patients. However, in healthy patients, the values of orally versus endotracheally collected samples are similar.⁴ This loss of sensitivity to acidic airways of orally collected EBC samples seems to prevent the EBC pH measurement from being overly sensitive in the primary clinical indication for its measurement (discussed later).

Condenser temperature does seem to affect EBC pH during collection. In healthy subjects (without breath acidification), the authors found no difference in EBC pH at collection temperatures of -56°C to room temperature. However, in subjects with acidic breath, collecting at condenser temperatures low enough to cause sample freezing is found to lead to less capture of exhaled acids (which cannot go into solution into ice, after all). Thus the authors always perform EBC collection at temperatures that do not allow sample freezing during collection.

An additional effect on pH arises during sample storage while frozen. The authors have noted that, after freezing a stored sample with low pH, if the sample container is opened before thawing, the measured pH of the sample is higher than the initial measurement. These observations are consistent with sublimation from the solid EBC of relevant volatile acids, a conclusion supported by the ability to prevent this artifact from occurring by allowing the sample to thaw and shaking the container before opening it. The authors strongly recommend this approach.

EBC pH can be affected by duration of gas standardization and temperature during measurement. Although earlier investigators recommended a bubbling time of 7 to 12 minutes to achieve CO₂ standardization,^{4,15-17} more recent studies show stabilization occurring in as few as 1 to 5 minutes.^{18,23} Recent studies have also shown that sample temperature may cause artifact in EBC pH²⁴ consistent with temperature's well-known effects on pH. This finding suggests that in some circumstances, more careful control for sample temperature may be desirable.

A final technical comment regarding EBC pH measurement seems warranted. Not all pH probes are appropriate for measurement in the low ionic strength matrix of EBC. Certain technologies simply will not function. The authors have found that Ross-type pH probes are uniformly effective, and standard glass probes with a substantial leak of internal fluid are generally ineffective. Before selecting a probe, the authors confirm that it calibrates in low ionic strength pH buffers identically to the standard pH buffers, and that addition of ionic strength adjusters/enhancers to samples of EBC does not affect the pH readings provided by the probe. These two tests help assure that the probe is not adversely functioning in the low ionic strength EBC.

NORMAL EBC PH VALUES

Normal values of gas-standardized (CO₂-free) EBC pH have been reported from multiple investigators and range between 7.5 and 8.1.^{4,10,11,16,19,21,25-33} In the authors' normative database from 404 subjects, the mean pH was 7.83 and the median pH was 8.0.¹⁹ The bell curve data distribution cuts off at a pH of approximately 7.4, with a scattering of values below that level that are clearly not part of the normal distribution. EBC pH values below 7.4 occur in 6% of this population. These values likely represent subjects with airway acidification that may be transiently symptomatic or asymptomatic, and may result from proximal acid reflux or from temporary innate immune responses against viral infections. Based on these data and data from more than 10,000 samples collected or analyzed in the laboratory, the authors consider pH values less than 7.4 to be abnormal.

There are potential confounders of the EBC pH levels. The authors have found that substantial intake of ethyl alcohol to the point of inebriation may modestly lower EBC pH. This finding may reflect alcohol's metabolism to acetic acid, which when exhaled can then alter EBC pH. It also may reflect alcohol-induced augmentation of gastroesophageal reflux to the laryngeal level.²⁵ Additionally, recent smoking, oral ingestion of food or beverages, and particularly ingestion of fluids^{6,34,35} with volatile acids (vinegar) within 20 minutes of EBC collection may cause substantial artifact, with a lower EBC pH.^{6,34,35} The authors' protocol for providing an EBC sample for pH assay includes that nothing except water should be ingested for 30 minutes before collection.

ANATOMIC SOURCE OF THE DETERMINANTS OF EBC PH

It is reasonable to assume that any significant acidification of the airway lining fluid that is exposed to the exhaled airstream will add acid to EBC. In patients who are endotracheally intubated, pharyngeal contribution will be nil. As part of the airway, the oropharynx likely can contribute to EBC pH when the sample is collected orally,¹⁵ but it does not in general have a strong effect. In keeping with this, salivary pH and EBC pH do not correlate,⁴ and pH of EBC samples collected through oral breathing are the same as those collected subsequently from the endotracheal tube after intubation in patients undergoing elective surgery.⁴ Therefore, intentional profound experimental acidification of the pharynx can lead to temporary EBC acidification.²⁵ This finding reflects the ability of EBC pH assays to identify the presence of a source fluid, at any airway level, sufficiently acidic to protonate certain bases to their volatile conjugate acids.

Although the mouth can contribute to the acidity of EBC, it also contributes to alkalization of EBC through ammonia (NH₃), which is a volatile base produced in high concentrations in the mouth and lungs of many people and often is the highest-concentration substance measurable in EBC.⁵ Initial speculation about NH₃ controlling EBC pH was forwarded,²² prompting studies to collect the empiric evidence

that has allowed for an improved understanding of the role of NH_3 . Several interesting observations have been made regarding oral ammonia. First, removing the potential oral ammonia contribution to EBC through bypassing the mouth with endotracheal intubation does not affect the EBC pH in healthy subjects.⁴ Second, removing ammonia (likely along with other volatiles) from EBC through lyophilization and resuspension does not affect EBC pH.⁵ Third, it is uncommon to have a low EBC pH value without a low NH_3 level in EBC; however, it is common to find low NH_3 levels with normal EBC pH levels.⁵ These observations led to the current understanding that high oral production of NH_3 can blunt the EBC pH signal of volatile acids coming from the lungs, and thereby decrease the sensitivity of orally collected EBC pH assays to identify lung acidification. However, when the acids exhaled from the airway are sufficient to overcome the modest neutralizing effect of oral NH_3 , a low EBC pH signal represents low airway lining fluid pH. An additional, albeit less confident notion is that minor differences in EBC pH values between groups within the pH range that is considered normal are of uncertain meaning.

Extensive data have now been collected from intubated humans showing that EBC pH is low in disease states.¹² Recently, a study performed in 12 patients showed that an endobronchially intubated healthy lung produces an EBC that is alkaline, whereas when the contralateral tuberculosis-infected lung destined for excision is included in the EBC sampling, the values are acidic.¹³ These data provide invasive measurements necessary to help prove that acidification of the lower airways does occur and, as one would expect, is heterogeneous, with pH low in the sick portions of the lung.

RESEARCH AND CLINICAL UTILITY OF EBC PH MEASUREMENTS

EBC pH is now perhaps the most commonly performed EBC assay, probably for two reasons. Foremost is that investigators realize that airway pH deviation is a particularly relevant pathologic process that affects almost every other aspect of lung disease that may interest them. One cannot ignore such a core component of how the airways and lungs function. Secondly, the pH assay is easy to perform.

The relevance of EBC pH and what it reflects—airway pH—is important to reiterate. Airway acidification is not just a biomarker of inflammation. Rather, airway acidification seems to be a central pathologic process in its own right that results in multiple broadly important adverse effects. Airway acidity leads to inflammation (neutrophilic and eosinophilic), bronchospasm, bronchial hyperreactivity, ciliary dysfunction, epithelial dysfunction, augmented oxidative damage, abnormal fluid transport, inhibition of transport of cationic drugs such as albuterol, and alteration of cellular death pathways, including inhibition of apoptosis.¹ Airway pH is entirely relevant to almost any project undertaken by an airway researcher. The authors now believe that cell and molecular biology research into airway and lung diseases risks yielding substantially incorrect conclusions if the researchers fail to take into account the disease-associated deviations in pH from normal that are likely present. How can one ascertain what normal neutrophil apoptosis rate is in patients with chronic obstructive pulmonary disease (COPD) when the cells on the bench are only incubated at neutral pH, yet the airway pH in disease may be pronouncedly acidic?

Unlike most other assays in EBC, no value of pH exists that is “undetectable.” However, this is misleading; pH measurement represents a net proton concentration signal resulting from amounts and ratios of multiple acids and bases within the EBC. A “normal” EBC pH may mean that no volatile acids are present (which can therefore be interpreted as “undetectable”), or it may mean that volatile bases from an alkaline mouth or alkaline proximal airway have neutralized the volatile acids arising from an

acidic area in the distal airway (thereby suggesting that EBC pH has relevant limitations to its sensitivity, similar to other EBC assays). A low EBC pH can confidently be said to result from a low airway lining fluid pH at some level. However a normal EBC pH does not exclude the presence of airway acidification, unless little NH_3 is present in the EBC also.

Undoubtedly some published results have suffered from unrecognized deficiencies of pH probes, effects of freeze-sublimation that artifactually raises EBC pH, and excessively cold condenser temperatures. Most likely more technological validations remain to be undertaken, but the field has advanced well, and application of this assay to provide outcome measures in large clinical studies and to assist in clinical patient management is now a reasonable consideration.

Continuous EBC pH Monitoring in Intubated Patients

A system was developed by Respiratory Research, Inc. (a University of Virginia faculty-owned company of which the corresponding author is a director) and the United States Air Force that allows for measurement of EBC pH continuously. Known as the ALFA monitor, this system condenses breath from the expiratory port of mechanical ventilators, performs gas standardization at two levels using hospital wall oxygen supplies, and measures, displays, and records the EBC pH in digital and graphical formats. Because of the time delay for condensation and processing, the result is a second-to-second recording of an EBC pH moving average representing EBC production from the lungs during the previous 5 to 10 minutes. To date, the authors have performed continuous pH condensimetry on more than 250 humans, ranging from neonates to the elderly, for as long as 70 days uninterrupted. With the oropharynx eliminated as a source of volatile acids, this system allows for selective measurement of lung acidity. In the absence of oral NH_3 contribution, the EBC pH becomes a higher-sensitivity assay for airway acidity.

Diseases for which the authors have continuous pH condensimetry anecdotal data revealing lung acidification include acute asthma, cystic fibrosis, COPD, infection with respiratory syncytial virus, acute respiratory disease syndrome (ARDS), and bronchopulmonary dysplasia. In these disease processes, pH changes gradually over hours to days. An EBC pH greater than 7.4 is commonly associated with relative lung health, and pH levels associated with disease fall within the 4.0 to 6.0 range,¹² similar to those found in oral EBC collections from healthy and ill patients, respectively.¹⁰ In unpublished data, the authors have observed frequent gradual decline in EBC pH during general anesthesia, which is currently of uncertain significance. Additionally, they have observed drastic pH fluctuations after witnessed aspiration events (**Fig. 1**).

This technology is functioning for research to study time course of pH changes in the airways. Currently, knowing that the airway lining fluid pH is abnormally low may be only prognostically valuable, because no approved therapeutic is clinically available for neutralizing acidic airway pH. But airway pH modifying therapies are being tested,³ and when available will create a desire to know what the airway pH is in a given patient so that these airway pH-modifying therapies can be used appropriately.

Assessing for Acid Reflux-Induced Respiratory Symptoms

Initially, the authors and others considered that acid reflux could confound EBC pH assays and prevent the gaining of insights into airway pH. However, with time, they have realized that the airways can acidify intrinsically or extrinsically, with the extrinsic pathways being inhalation of acid gases and aspiration of acid reflux. When considering factors that confound, acid reflux is at the top of the list, because it confounds or complicates every respiratory illness. Acid reflux is perhaps the most diagnostically

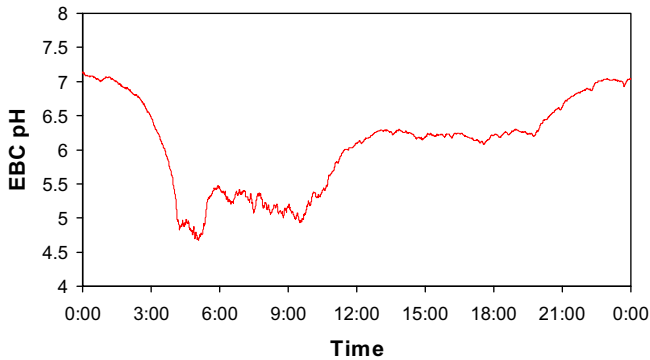


Fig. 1. Twenty-four-hour ALFA monitor continuous breath pH condensimetry tracing of a neonatal patient. Note the sudden pH decrease followed by slow recovery to baseline over the subsequent 12 hours. The drop corresponded with a sudden increase in respiratory support needs accompanied by suctioning of formula from the endotracheal tube.

frustrating entity in pulmonary medicine, because (1) the diagnostics are invasive and not designed or validated for use in respiratory system indications (eg, esophageal pH probes, which use normal reference ranges relevant to esophageal disease but irrelevant to aspiration disease) and (2) it is so common.

EBC pH offers a noninvasive assessment of airway pH that can be performed repeatedly. For example, transient acidification of the breath associated with chronic cough seems to be an excellent indicator that the cough will respond to acid blockade therapy with proton pump inhibitors (PPIs). The authors' studies have shown that patients whose cough symptoms decreased with PPI use invariably had transient breath acidification, and those whose cough failed to respond had no breath acidification (**Fig. 2**). These findings provide a novel method to determine which patients with asthma or COPD have confounding acid reflux without resorting to expensive and indefinite (and commonly indeterminate) PPI empiric trials.

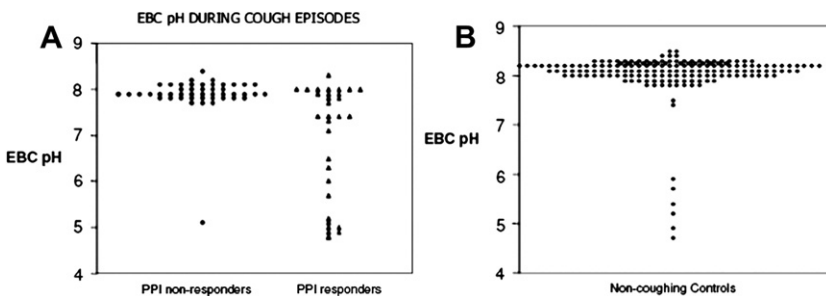


Fig. 2. EBC pH values (A) from patients whose chronic cough did and did not respond to proton pump inhibition (PPI) and (B) from noncoughing controls. Note that multiple samples were collected from each patient, with all data plotted as individual samples. Approximately 50% of the EBC samples from PPI-responsive coughers had low pH values. Note that all patients whose symptoms responded had at least one episode of breath acidification during four to eight breath collections collected at the time of cough. (*Adapted from* Hunt J, Yu Y, Burns J, et al. Identification of acid reflux cough using serial assays of exhaled breath condensate pH. *Cough* 2006;2:3; with permission.)

Studies of Lung Diseases in Spontaneously Breathing Subjects

The initial purpose for which the authors developed the EBC pH assay was to open a window onto lung chemistry, with pH a centrally involved characteristic. The results of their work and of others using the EBC pH assay has provided the strongest support to date that the airways become acidified in numerous respiratory disorders. The EBC pH assay, although not perfect, has undergone validation and vetting internationally. It is also the assay that has the most published technical validation data supporting the technique.¹⁵ The authors currently believe that EBC pH decline is most relevant in the settings of acute asthma exacerbations, COPD, and acute lung injury and ARDS (representing intrinsic airway acidification), and for assessing gastric acid aspiration in the settings of chronic cough, asthma, COPD, lung transplant rejection, pulmonary fibrosis, vocal cord dysfunction, and exercise-induced dyspnea/bronchospasm. This purpose may be the most immediately valuable from a patient point of view, because therapies exist to help reflux-induced symptoms if a diagnosis is made.

SUMMARY

EBC is the only convenient, noninvasive, ethically acceptable method of assessing airway acidity that is currently available, especially for repeated sampling in acutely ill patients. Like all tests, EBC pH is neither perfectly sensitive nor specific for lower airway acidification. Many of the validation issues have been adequately addressed. The data must be interpreted cautiously, because acid at any level in the airway can lead to EBC acidification, and an absence of EBC acidification does not exclude the presence of some degree of airway acidification. EBC pH measurements remain the most successful method to evaluate the role of airway acidification in respiratory disease.

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