Exhaled breath condensate pH assays are not influenced by oral ammonia


Background: Measurement of pH in exhaled breath condensate (EBC) is robust and simple. Acidic source fluid (airway lining fluid) traps bases while volatilising acids, leading to EBC acidification in many lung diseases. Lower airway ammonia is one determinant of airway lining fluid pH, raising the concern that addition of the base ammonia by contamination from the mouth might confound EBC pH assays.

Methods: Three discrete methods were used to limit oral ammonia contamination of EBC collections: endotracheal intubation, oral rinsing, and −40°C condenser temperatures. Separately, ammonia was removed from collected EBC samples by lyophilisation and resuspension. Intraweek and intraday variability of ammonia concentration was determined in 76 subjects, and ammonia and pH from a further 235 samples were graphically compared. Ammonia was assayed spectrophotometrically and pH was assessed after deaeration.

Results: Data from 1091 samples are presented. Ammonia was reduced in EBC by all methods. Endotracheal intubation decreased EBC ammonia from a mean (SD) of 619 (124) µM to 80 (24) µM (p < 0.001, n = 32). Oral rinsing before collection also led to a decline in EBC ammonia from 573 (307) µM to 224 (80) µM (p = 0.016, n = 7). The colder the condensation temperature used, the less ammonia was trapped in the EBC. Lyophilisation removed 99.4 (1.9)% of ammonia. Most importantly, the pH of EBC never decreased after removal of ammonia by any of these methods. Intraweek and intraday coefficients of variation for ammonia were 64 (27)% and 60 (32)% which is substantially more variable than EBC pH assays.

Conclusions: Although ammonia and pH appear to correlate in EBC, the oral ammonia concentration is not an important determinant of EBC pH. No precautions need to be taken to exclude oral ammonia when EBC pH is of interest. The low pH and low ammonia found in EBC from patients with lung diseases appear to be independent effects of volatile compounds arising from the airway.
until assay, except for collections taken at home which were stored initially in home freezers for up to 1 week at approximately $-10^\circ\text{C}$. Assays were performed within 2 months of collection except where noted.

**Measurement of pH**

pH was assayed with a glass microelectrode (Orion, Beverly, MA, USA) after bubbling 200 µl of the sample with argon at 350 ml/min until the pH reading was stable, as previously described.1

**Ammonia assay**

Within 2 months of collection, ammonia concentrations in EBC were determined spectrophotometrically using a diagnostic ammonia assay kit (Diagnostic Chemicals Ltd, PEI, Canada). Our standard curves (ammonium chloride in water) were linear down to 20 µM.

**Lyophilisation and resuspension of EBC**

To remove ammonia from EBC, 1.5 ml aliquots of EBC were lyophilised in 5 ml polypropylene test tubes in a Savant SpeedVac Concentrator (Farmingdale, NY, USA) for 12 hours at +43°C. Immediately thereafter the lyophilates were resuspended to a volume of 1.5 ml with deaerated ultrapure deionised water ($>18$ megaOhms resistivity) adjusted to pH 7.

**Oral rinse**

Samples were collected from seven subjects immediately before and after thorough rinsing of the mouth with three 20 ml aliquots of water. EBC collections were performed at an initial condenser temperature of $-17^\circ\text{C}$ for 10 minutes each. Salivary pH was not measured.

**Altering temperature of EBC collection**

Ten subjects provided consecutive 10 minute EBC collections at the following mean temperatures: $-44^\circ\text{C}$, $-16^\circ\text{C}$, $-6^\circ\text{C}$, and $+13^\circ\text{C}$. The temperature of the condenser was kept relatively stable using a modified cooling sleeve for the RTube that contained a chamber into which chemicals with desired temperatures of phase changes were inserted. The phase change from solid to liquid allowed energy to be absorbed from the condensing breath with minimal change in condenser temperature.

**Study of reproducibility**

Seventy-six subjects performed 10 minute EBC collections in their homes before breakfast each morning for seven consecutive days and an additional three collections on the seventh day (before lunch, dinner and bedtime). Initial temperatures of the condenser were between $-4^\circ\text{C}$ and $-17^\circ\text{C}$ (standard temperatures of home freezers). Ammonia and pH were assayed in all samples in batches.

**Statistical analysis**

Matched samples of EBC for each study were compared with paired $t$ tests. Multiple comparisons were analysed by one way ANOVA using the Tukey post hoc test. For the study of reproducibility, intraday and intraweek coefficients of variation were calculated for each subject and the means of these results presented. Results are generally presented as mean (SD). Median and range are given where appropriate, and full range is given in addition to mean (SD) when adding that information more clearly communicates the data. Correlations of pH to ammonia concentration and to logarithmically transformed ammonia concentration were performed.

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**Table 1** Characteristics of study subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Sex (M/F)</th>
<th>Age range (years)</th>
<th>Mean age (years)</th>
</tr>
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<tbody>
<tr>
<td>Elective endotracheal intubation volunteers</td>
<td>13/19</td>
<td>29–72</td>
<td>53</td>
</tr>
<tr>
<td>Healthy volunteers providing EBC orally</td>
<td>5/5</td>
<td>19–44</td>
<td>31</td>
</tr>
<tr>
<td>Study of ammonia reproducibility</td>
<td>26/50</td>
<td>18–48</td>
<td>22</td>
</tr>
</tbody>
</table>

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Figure 1  Ammonia concentration in exhaled breath condensate (EBC) during storage. A total of 15 EBC samples were assayed before and after storage at $-80^\circ\text{C}$ (10 samples were frozen for 2 months [closed circles] and five samples for 2 years [open circles]). The plot shows the initial assay and the after storage assay. No differences were identified ($r^2=0.96$, $p<0.001$).

Figure 2  Contribution by the upper and lower airway to EBC ammonia concentrations. Subjects performed oral EBC collections immediately before endotracheal intubation, followed by EBC collection while intubated. EBC collected while intubated had approximately 13% of the ammonia concentration of the matched oral sample (80 [24] µM × 619 (124) µM; *$p<0.001$, n = 32). There was no difference in EBC pH.
using Pearson’s product moment. Linear regression was used to compare ammonia assays before and after storage. p values of <0.05 were considered statistically significant.

RESULTS
Reproducibility of ammonia levels in EBC
A total of 760 samples were collected and assayed from 76 subjects. These samples were collected in their own homes unsupervised. The mean intraweek coefficient of variation for EBC ammonia was 64.3 (27.4)% with a full range of intrasubject CVs from 9.6% to 184.5%, and the mean in-traday coefficient of variation was 60.4 (32.7)% (full range 1–140.5%). The values of the standard deviations were proportional to the means, and the CVs were therefore similar regardless of the mean measurement. In a subgroup comparison there was no difference in the mean (SD) concentration of ammonia in EBC samples collected unsupervised by healthy subjects in their own homes (481 (281) µM, n = 86) and samples collected from healthy controls in the more controlled circumstances of the laboratory (532 (307) µM, n = 48, p = 0.48). Storage of samples at −80°C had no effect on the ammonia concentration in 10 samples assayed twice 2 months apart, nor in five samples assayed 2 years apart (fig 1).

Effect of elimination of oral ammonia by endotracheal intubation on pH of EBC
The mean ammonia concentration in EBC collected orally before intubation was 619 (124) µM which was significantly greater than when the mouth was bypassed by the endotracheal tube (80.2 (24) µM; p<0.001, n = 32). The mean (SD) pH of the EBC in oral collections was 7.9 (0.23) and in the subsequent intubated samples was 7.8 (0.28), (p = NS, fig 2).8

Effect of oral rinsing on EBC ammonia and pH
The mean (SD) ammonia concentration in EBC collected from seven subjects before oral rinsing was 573 (307) µM and the pH was 8.0 (0.2). Immediately after oral rinsing the ammonia concentration declined to 224 (80) µM (p = 0.016; n = 7). Despite the reduction in ammonia, the pH of the EBC remained unchanged at 8.0 (0.1), p = NS (fig 3).

Effect of very low temperatures on EBC ammonia and pH
Ten subjects provided back to back 10 minute EBC collections at the following temperatures: +13°C, −6°C, −16°C, and −44°C in no set order. The mean ammonia concentration in the EBC depended on collection temperature (697 (442) µM at +13°C, 534 (473) µM at −6°C, 451 (218) µM at −16°C, and 140.2 (99.4) µM at −44°C; fig 4). By ANOVA, these ammonia concentrations were significantly different (p = 0.002) between collections at +13°C and −44°C and between −6°C and −44°C. Despite the decreased concentration of ammonia at the lowest temperatures, the pH of the EBC remained identical irrespective of the temperature of collections (pH = 7.9 (0.2), 7.9 (0.3), 7.9 (0.3), 7.8 (0.3) respectively; p = NS), as previously reported.8

Effect of lyophilisation and resuspension on EBC ammonia
The median ammonia concentration before lyophilisation of the sample was 628 µM (range 100–1760 µM, n = 10) and the pH = 7.9. Lyophilisation was very successful at purging ammonia from the EBC. When the lyophilate was resuspended in deionised water, ammonia was undetectable (<20 µM) in 9/10 samples. Resuspension of lyophilate in pH 7 adjusted deaerated deionised water was performed before repeated pH measurement. The pH of this reconstituted EBC was 8.1 (p = 0.023, n = 10), higher than the original sample despite complete removal of ammonia (fig 5).

Correlation of ammonia and pH in EBC samples
Data from an additional 235 samples collected during the course of various investigations into EBC were used to compare ammonia and pH in EBC without regard for any underlying disease. Figure 6 shows that the ammonia concentrations in EBC were low when the pH was low. There was a weak but significant correlation between log transformed ammonia and pH (correlation coefficient = 0.52, p<0.001).

DISCUSSION
Exhaled breath sampling for assessment of airway disease is increasing in utility and application. The biochemistry of the
Figure 5  Effect of removal of all ammonia from EBC by lyophilisation on pH of the sample. EBC samples underwent lyophilisation and resuspension in deionised water (pH = 7). Ammonia was essentially completely removed by this process (n = 10). The pH rose slightly in the reconstituted samples despite removal of the base (*p < 0.023).

Figure 6  Low levels of ammonia in EBC are necessary but not sufficient for low EBC pH to be present. Low ammonia levels occur both in low and high pH samples.
EBC ammonia levels. These technical issues are not a concern for EBC pH assays. As a result, the reproducibility of EBC ammonia measurements compares poorly with the excellent reproducibility of EBC pH, which is less than 5% for intra-day and intraweek sampling and 0.5% for back to back collections.

Based on our data, we expect that neither delay in performing the ammonia assay nor collecting the sample at home instead of in the laboratory would adversely influence the reproducibility of the ammonia concentrations. Our extensive data reveal that oral ammonia is not an important determinant of EBC pH. As a corollary, oral ammonia does not adversely affect or seem to interfere with EBC pH assays. No special efforts are necessary to exclude oral ammonia when the pH of EBC is of interest. The pH of EBC has become an important characteristic that is enhancing our understanding of airway biochemistry during disease states. Because of their simplicity, ammonia assays may cautiously be used to complement pH assays in large studies, especially when subjects are intubated. The search for the specific acids that are released from the airway and lung during various diseases continues. Although the identification of the acids that cause EBC acidification may lead to recognition of previously unconsidered pathways of airway and lung disease development, the simple notion that the airway pH deviates in diseases in itself has substantial implications for airway pathophysiology.

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Conflict of interest: JV and JH are minority shareholders of Respiratory Research Inc, the company that manufactures the exhaled breath condensate collection equipment used in this study.

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