Biomarkers, March–April 2006; 11(2): 118–142

Taylor & Francis
Taylor & Francis Group

Evaluation of methodological and biological influences on the collection and composition of exhaled breath condensate

P. REINHOLD, J. JAEGER, & C. SCHROEDER

Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, Jena, Germany

Abstract
The purpose of this inter-species comparison (calves and pigs) was to identify methodological and biological influences on the collection and composition of exhaled breath condensate (EBC). A total of 352 EBC samples were collected, whilst variables of ventilation were registered in parallel. Partial pressure of carbon dioxide (pCO_2) and pH were analysed in non-degassed EBC samples. The concentration of total protein in EBC was measured colorimetrically. In both species, lung function was evaluated before and after EBC collection. Statistical analyses were performed to study the effect of EBC collection on lung function and to identify the influence of ventilatory variables on the collection and composition of EBC. Collection of EBC did not affect lung function. Despite the volume of EBC collected per unit time being primarily dependent on ventilation per unit time, species-specific conditions during the EBC collection process resulted in different dependences of EBC collection from other variables of ventilation (i.e. maximal airflow during expiration or expired tidal volume kg^{-1} body weight). The concentration of protein ml^{-1} EBC increased with the expired volume per min and with peak expiratory flow. Although the pCO_2 in fresh EBC was significantly negatively dependent on the duration of collection, comparable pHs (5.6 −6.2) were measured in EBC of both calves and pigs. The obtained data may help one standardize EBC collection in different species.

Keywords: *Exhaled breath condensate (EBC), respiratory physiology, pulmonary function, animal models, calves, pigs*

(Received 25 August 2005; accepted 10 January 2006)

Introduction

There is increasing interest in the use of non-invasive biological markers (biomarkers) that can reflect the character and intensity of pathological processes in both humans and animals. Exhaled breath contains a variety of potential biomarkers that can be measured either in the gaseous phase or in the cooled and condensed exhalate (Scheideler et al. 1993, Kharitonov & Barnes 2002, Amann & Smith 2005). Because exhalate is the product of alveolar gas exchange and airway water loss, it most likely contains metabolites from the lungs or substances originating from inflammatory reactions in the airway mucosa. With respect to respiratory physiology and the

Correspondence: P. Reinhold, Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, Naumburger Str. 96a, D-07743 Jena, Germany. Tel: 49-3641-804-269. Fax: 49-3641-804-228. E-mail: petra.reinhold@fli.bund.de

ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.1080/13547500600572764

metabolic functions of the lung, the following gaseous and non-gaseous components could be expected to be present in the exhaled breath:

- Metabolic products released by cells within the respiratory system.
- Metabolic products of other organs reaching the lung via circulation.
- Inhaled substances, e.g. irritants to which the respiratory system is exposed.
- Exhaled toxic or environmental substances entering the body via skin or the enteric route.
- Microorganisms (in the case of respiratory infections).

In accordance with this theoretical assumption, a wide variety of volatile and non-volatile substances have already been detected in the exhaled breath or in exhaled breath condensate (EBC), respectively, of both humans and animals (Scheideler et al. 1993, Phillips et al. 1999a, 2000, Risby et al. 1999, Marczin et al. 2003, Effros et al. 2004, Laakso et al. 2004, Wyse et al. 2004b, Moser et al. 2005, Reinhold et al. 2005).

Because the method of exhaled breath collection is completely non-invasive, repeatable and does not require patient cooperation, exhaled breath analysis is increasingly used in human medicine mainly for a non-invasive assessment of the respiratory system (Montuschi 1999, 2005, Phillips et al. 1999b, Risby & Sehnert 1999, Kharitonov & Barnes 2001, 2002, Mutlu et al. 2001, Wood et al. 2003, Amann & Smith 2005). According to the recommendations of a joint Task Force of the European Respiratory and the American Thoracic Societies, 'exhaled breath condensate' is the preferred term to describe the method of collecting condensate by cooling the exhaled breath (Horváth et al. 2005). To date, EBC collection has been widely described in humans and also in conscious animals including calves (Reinhold et al. 2000), horses (Deaton et al. 2004), cats (Sparkes et al. 2004, Kirschvink et al. 2005) and dogs (Hirt et al. 2003, Wyse et al. 2004a). Concentrations of numerous mediators detectable in EBC (H_2O_2, NO-related products, products of lipid peroxidation, proteins and cytokines, electrolytes, etc.) as well as the pH of EBC are influenced by respiratory diseases and modulated by therapeutic interventions (Kharitonov & Barnes 2001, 2002, Vaughan et al. 2003, Carpagnano et al. 2004, Deaton et al. 2004, Carraro et al. 2005, Montuschi 2005, Ojoo et al. 2005).

Despite these encouraging reports, there are still fundamental problems and pitfalls associated with analysis of samples of EBC (Effros et al. 2004, 2005, Rosias et al. 2004, Harrison & Andersen 2005, Montuschi 2005). Comparison of the results of different studies is often limited by the fact that different collection methods (mainly based on home-made devices) have been used. The use of commercially available devices may help to overcome potential problems arising from the use of different techniques. Thus, the purpose of the study reported here was to evaluate EBC collection in animals (healthy calves and pigs) used as models in order to identify the most important methodological peculiarities of the collection process using the same sampling device that is used most frequently in human medicine (ECoScreen). In the present inter-species comparison, attention was focused on: (1) the effect of EBC collection on lung function, (2) methodological and biological aspects influencing the collection of EBC in spontaneously breathing subjects, and (3) the dependence of EBC composition on conditions of EBC collection.

To the best of our knowledge, this is the first comparative report concerning methodological and biological aspects that may have a significant influence on collection and composition of EBC. The data obtained may help one to clarify the

influence of the collection process on sampling and composition of EBC in different species including human subjects.

Materials and methods

Animals

All calves were crossbred and conventionally reared. They were purchased from different agricultural farms at 2–3 weeks of age and were fed with commercial milk replacers and coarse meal. Water and hay were supplied *ad libitum*.

All pigs were colostrum-fed female (Deutsche Landrasse breed) provided by Charles River (Sulzfeld, Germany). Animals originated from one conventional breeding farm where piglets were reared separately with their mothers. Piglets were brought in the institute at 3–4 weeks of age. They were fed twice a day with the same commercially available feed they were given in the breeding farm. Water was supplied *ad libitum*.

Calves and pigs were housed in the animal house of the Friedrich-Loeffler-Institut, in conformity with the guidelines for animal welfare of the European Community. None of the feed supplied contained antibiotics. Daily observation of both species included appetite (feed intake), rectal temperature, respiratory rate and the existence of diarrhoea or respiratory symptoms (cough, nasal discharge or ocular secretions). After a quarantine period of at least 2 weeks to confirm clinically healthy status, animals were included in the study.

In calves, all experiments were performed in a conscious state; none of the calves was anaesthetized or sedated. Since pigs can rarely be examined consciously, each pig was sedated approximately 15 min before each test (diazepam, 1.5–2.0 mg kg^{-1} body weight, intramuscularly). During either EBC collection or lung function testing, the individual pig was restrained using a canvas sling with openings for the limbs. Although all phases of the study were non-invasive, they still had ethical approval from the Institutional Commission for the Protection of Animals.

Procedures for collection of exhaled breath condensates

The system for collection of EBC samples used was commercially available for human medicine (ECoScreen, Viasys Healthcare, Hoechberg, Germany). A non-rebreathing valve ensures that all the exhaled volume directed in this system is passed through the Teflon-coated condensation chamber of the device. The reported temperature on the condensation surface was -10 to -20°C (given by the manufacturer).

Both calves and pigs were connected to the sampling system using a tightly fitting facemask of an appropriate size in relation to the animal's head (Figure 1). To avoid any contamination of the EBC from undefined environmental conditions, an inspiratory filter (Pall Europe Ltd., Portsmouth, UK) was used for each sampling procedure. Ambient conditions in the animal house during collection were similar to usual room conditions (ambient temperature, $18-22^{\circ}$C; ambient relative humidity, 60–65%).

The following variables of ventilation were recorded for each animal during collection of EBC using an electronic spirometer (ECoVent, Viasys Healthcare), which was designed to be adapted to the expiratory part of the ECoScreen device (Figure 1B):

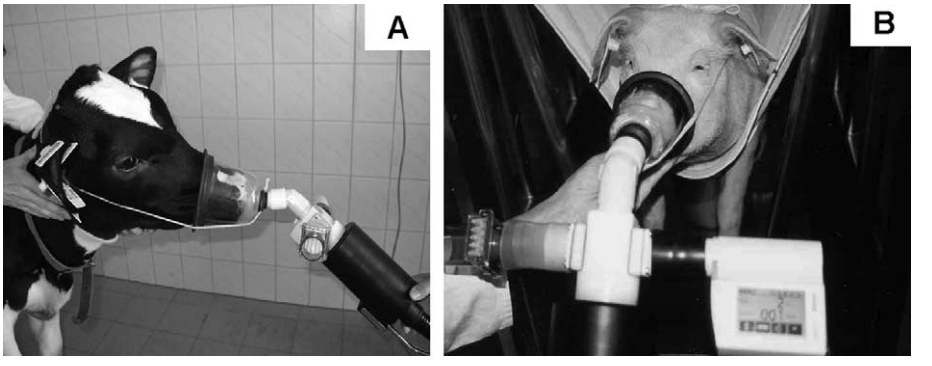

Figure 1. Collection of exhaled breath condensate in a conscious calf (A) and a sedated pig (B). The electronic spirometer 'ECoVent' can be seen attached to the expiratory port of the system on the right used to collect EBC from pigs.

- Expiratory tidal volume (V_{tex}).
- Respiratory rate (RR).
- Expiratory minute ventilation ($V_E = V_{tex} \star RR$).
- Maximal airflow during spontaneous expiration (V'_{Emax}).

Expiratory tidal volume per kg body weight ($V_{tex}\ kg^{-1}$) was calculated using the body weight measured before each EBC collection. The total expired volume was registered for each single sampling period per animal ($V_{total} = V_E \star$ sampling duration; min).

Protocol to evaluate the effect of the EBC sampling procedure on lung function

To evaluate the possible effects of the EBC sampling procedure on ventilatory parameters and respiratory mechanics, a lung function test was performed immediately before and after each EBC collection in clinically healthy calves ($n = 48$) and pigs ($n = 32$). For lung function testing, the impulse oscillometry system (IOS) was used in both calves and pigs. This technique was previously validated for calves and pigs (Reinhold et al. 1996, 1998a,b, Klein & Reinhold 2001, Klein et al. 2003).

Each lung function test consisted of three consecutive IOS measurements (the duration of each single measurement was 60 s, while three test impulses were generated per 1 s). The sampling rate was set at 200 Hz (the period between two sampling points of 5 ms) selecting 32 sampling points after each impulse. The mean results of the three consecutive measurements per animal and time point were used for further analysis.

The following variables of ventilation and respiratory mechanics were compared before and after collection of EBC:

- Respiratory rate (RR).
- Tidal volume (V_t); averaged between inspiration and expiration.
- Volume of minute ventilation ($V_{min} = V_t \star RR$); averaged between inspiration and expiration.
- Tidal volume and minute volume both related per kg body weight ($V_t\ kg^{-1}$; $V_{min}\ kg^{-1}$).
- Respiratory resistance at 3, 5, 10, 15 and 20 Hz ($R_{3Hz}, \ldots, R_{20Hz}$) for calves, and at 5, 10, 15 and 20 Hz ($R_{5Hz}, \ldots, R_{20Hz}$) for pigs, respectively.

- Respiratory reactance at 3, 5, 10, 15 and 20 Hz ($X_{3Hz}, \ldots, X_{20Hz}$) for calves, and at 5, 10, 15 and 20 Hz ($X_{5Hz}, \ldots, X_{20Hz}$) for pigs, respectively.
- Resistance of proximal airways (R_{prox}) and resistance of distal airways (R_{dist}), both derived from a model-based calculation implemented in IOS software, as described (Reinhold et al. 1998a, Klein et al. 2003).

Within the group of calves included in this part of the study (aged 31–77 days with body weights ranging from 52 to 94 kg), the time necessary for the collection of 5.5 ± 0.9 ml EBC (mean ± SD) per animal varied between 15 and 33 min (mean ± SD, 23 ± 4 min). Consequently, this was the time difference between the two lung function tests before and after EBC collection. For both EBC collection and lung function testing, conscious calves were standing in a normal position.

The pigs used in this part of the study (aged 2–4 months and weighing between 15 and 50 kg) were sedated and fixed in the canvas sling as described (Klein & Reinhold 2001) during both collection of EBC and lung function testing. The duration of individual collection of EBC varied between 30 and 40 min. Thus, the averaged period between the lung function test before and those after EBC sampling was 36 ± 4 min (mean ± SD).

Protocol to evaluate the influence of ventilation on quantity and composition of EBC

To obtain a large range in variables of ventilation, multiple EBC collections were performed at defined periods in two group (12 animals per group per species) while animals were rapidly growing. Consequently, airflow and volumes of ventilation increased physiologically within each subject over time.

Each calf underwent 14 EBC collections at constant time intervals. At first collection of EBC, animals were aged 2–4 weeks and had a body weight of 48 ± 6 kg (mean ± SD). The last collection of EBC was performed when calves were aged 6–7 months (body weight 150 ± 13 kg). Due to this study design, the obtained sample size of $n = 168$ was balanced for the influence of the individual animal.

Five EBC collections were performed in each of the 12 pigs (once per month and animal). Body weights in pigs varied between 11 ± 1 kg (first collection of EBC, aged 1 month) and 70 ± 8 kg (last collection of EBC, aged 5 months). Because of four missing values caused by non-cooperativeness of individual pigs at four different time points, a sample size of $n = 56$ was included in further analysis for this species.

During each collection process, variables of ventilation were registered in each animal using the electronic spirometer ECoVent as described previously. In addition, individual body weight and rectal temperature were measured before starting each EBC collection. Sampling procedures affected by artefacts (such as coughing or a non-regular pattern of breathing) were eliminated from further analysis and the sampling was repeated.

Immediately after finishing one collection process, the quantity of EBC obtained (ml) was measured. Taking the sampling duration (collection time, min) and the total expired volume (V_{total}, litres) for each EBC collection into account, the volume of EBC condensed/100 litres exhaled breath was calculated for each animal.

Immediately after finishing the collection process, EBC samples were stored at −80°C. The concentration of protein was analysed in 157 EBC samples obtained from calves and in all 56 samples obtained from pigs within 6 months after collection.

Total protein was measured colorimetrically using Pierce Micro BCA(TM) Reagent Kit (Rockford, IL, USA) in duplicate. The sensitivity of this method was ≥ 0.5 μg ml^{-1}. In addition to the measured concentration of total protein (μg ml^{-1} EBC), the quantity of total protein related to 100 litres exhaled breath was calculated (μg/100 litres exhaled breath).

Statistical analyses were used to clarify whether variables of ventilation (interactions with body weight and/or rectal temperature were taken into account) significantly influenced the quantity (ml EBC obtained either per min or per 100 litres exhaled breath) and the composition (concentration of total protein either ml^{-1} EBC or per 100 litres exhaled breath) of EBC.

Protocol to evaluate pH and partial pressure of carbon dioxide (pCO_2) in EBC

In clinically healthy young calves (body weight 61 ± 5 kg) and pigs (15 ± 5 kg), EBC was collected from each animal ($n = 24$ in each species) in order to compare physiological values of pH and pCO_2 in EBC between species. In this part of the study, the collection period was determined by the time necessary to collect 3–5 ml EBC.

Measurements of pH and pCO_2 in EBCs were made immediately after finishing the collection process (without de-aeration) and were repeated after 1 h of storage in closed Eppendorf tubes at room temperature (18–22°C) using a commercially available blood-gas analyser (ABL 605, Radiometer, Copenhagen, Denmark). In this system, pH measurements were based on the glass-electrode G707 using the Hg_2Cl_2 (calomel) electrode K606 as a reference electrode. All values were corrected for the actual body temperature of the individual animal measured immediately before each EBC collection.

Statistical analyses

To determine whether datasets were normally distributed, the standardized skewness and standardized kurtosis were calculated for each data variable. Values for skewness and/or kurtosis outside the range -2 to $+2$ indicate significant departures from normality. Normally distributed data are given as mean $\pm$ standard deviation (SD), while non-normally distributed data are always presented as median and range.

To compare means of normally distributed data, a Student *t*-test for paired observations (paired *t*-test) was used for paired data (i.e. a different time point within one group) while the unpaired *t*-test was used to compare two unpaired samples (i.e. differences between two groups at one time point). The Mann–Whitney–Wilcoxon (*W*) test was used to compare data with unknown or non-normal distribution. Coefficients of linear correlation and equations of linear regression were identified, using the linear model of regression analysis. Rank correlations among variables were analysed by use of Spearman rank correlation coefficients, a procedure that uses the ranks of the data rather than the actual data.

For statistical significance, confidence levels are given with the results. Since $p < 0.05$, there is a statistically significant difference at the 95% confidence level.

Results

Effects of the EBC sampling procedure on lung function

Variables of ventilation and respiratory mechanics were measured in both non-sedated calves ($n=48$) and sedated pigs ($n=32$), and in each animal before and after collection of EBC. For both species, results of ventilatory variables (RR, V_t, V_t kg^{-1}, V_{min}, V_{min} kg^{-1}) as well as changes in resistance of proximal and distal airways are shown in Table I, while respiratory impedance data (given as spectral respiratory resistance and spectral respiratory reactance) are shown in Figure 2.

In calves, the period for EBC collection (i.e. the period between the two lung function tests in each animal) lasted 23 ± 4 min (mean$\pm$SD). After EBC collection, the respiratory rate was significantly decreased, while V_t and V_t kg^{-1} remained unchanged. Consequently, both V_{min} and V_{min} kg^{-1} were significantly decreased after finishing the EBC sampling procedure. Respiratory impedance after EBC collection was characterized by a significant decrease in respiratory resistance (R) at 3 and 5 Hz and a significant increase in respiratory reactance (X) between 3 and 10 Hz (Figure 2). Separating the resistance of proximal and distal airways, both R_{prox} and R_{dist} decreased significantly after collection of EBC (Table I).

In sedated pigs, the period between the two lung function tests before and after EBC collection was 36 ± 4 min (mean$\pm$SD). In contrast to conscious calves, a significant increase was seen in both minute ventilation (V_{min}) and minute volume related to body weight (V_{min} kg^{-1}) after the sampling of EBC. This increase in ventilation per time was only caused by an increase in tidal volume (V_t, V_t kg^{-1}),

Table I. Variables of ventilation and resistance of proximal and distal airways before and after collection of exhaled breath condensate (EBC).

			Before collection of EBC[#]		After collection of EBC[#]		
	Variable	Unit	Mean	SD	Mean	SD	Paired t-test
Calves ($n=48$)	RR	breaths min^{-1}	26.0	4.7	25.0	5.0	$p\leq0.05$
	V_t	ml	793.3	128.3	786.9	124.2	n.s.
	V_t kg^{-1}	ml kg^{-1}	11.0	1.8	10.9	1.6	n.s.
	V_{min}	litres	20.3	2.9	19.3	2.9	$p\leq0.01$
	V_{min} kg^{-1}	ml kg^{-1}	281.3	43.0	267.7	38.9	$p\leq0.01$
	R_{prox}	kPa/(l s^{-1})	0.289	0.088	0.257	0.082	$p\leq0.01$
	R_{dist}	kPa/(l s^{-1})	0.211	0.098	0.169	0.107	$p\leq0.05$
Pigs ($n=32$)	RR	breaths min^{-1}	26.9	5.7	26.4	5.4	n.s.
	V_t	ml	270.8	99.1	310.8	116.2	$p\leq0.001$
	V_t kg^{-1}	ml kg^{-1}	7.7	1.6	8.8	2.2	$p\leq0.001$
	V_{min}	litres	7.0	2.1	7.9	2.7	$p\leq0.01$
	V_{min} kg^{-1}	ml kg^{-1}	202.0	48.6	229.2	62.5	$p\leq0.01$
	R_{prox}	kPa/(l s^{-1})	0.213	0.065	0.246	0.113	n.s.
	R_{dist}	kPa/(l s^{-1})	0.214	0.105	0.302	0.181	$p\leq0.01$

[#]Period (mean$\pm$SD) between the two lung function tests before and after collection of EBC: in calves, 23 ± 4 min; in pigs, 36 ± 4 min.

RR, respiratory rate; V_t, tidal volume (averaged between inspiration and expiration); V_t kg^{-1}, tidal volume per kg body weight; V_{min}, min volume of ventilation (averaged between inspiration and expiration); V_{min} kg^{-1}, minute volume per kg body weight; R_{prox}, resistance of proximal airways; R_{dist}, resistance of distal airways; SD, standard deviation; p, probability; n.s., no significant difference at the 95% confidence level.

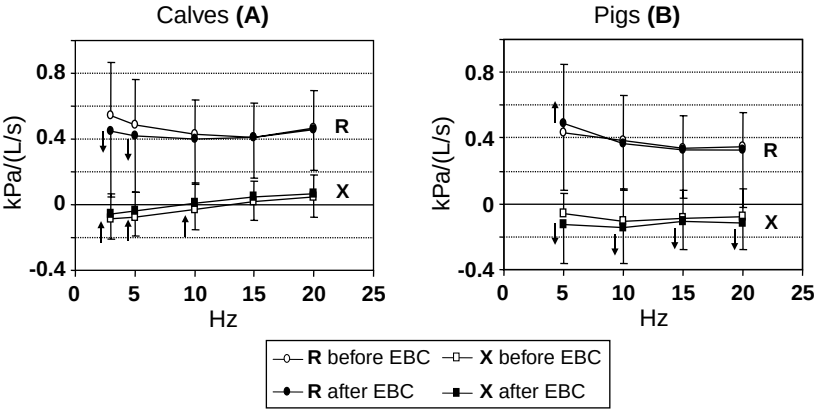

Figure 2. Respiratory resistance (R) and respiratory reactance (X) before and after collection of EBC. Data are medians and ranges within 3 – 20 Hz in calves ($n = 48$) and within 5–20 Hz in pigs ($n = 32$). Periods between lung function tests before and after EBC collection were 23 ± 4 min in calves and 36 ± 4 min in pigs (mean $\pm$ SD). ↑↓, Significant increases or decreases, respectively ($p \leq 0.05$).

whereas the respiratory frequency did not change significantly (Table I). Respiratory resistance increased at a frequency of 5 Hz, while respiratory reactance decreased significantly between 5 and 20 Hz after the collection of EBC (Figure 2). After EBC collection, the resistance of proximal airways remained statistically unchanged, whereas the resistance of distal airways was significantly increased (Table I).

Influence of ventilation pattern on the quantity of EBC

EBC samples were collected in both species, i.e. calves and pigs, starting in each species within the first month of life. Methodological limitations did not allow collection of EBC in calves with body weights higher than approximately 175 kg and in pigs heavier than approximately 80 kg. Consequently, the oldest animals included in the study were calves aged 6–7 months and pigs aged 5 months, respectively.

Because of marked differences in age and body weight and due to different individual patterns of breathing, wide variations of ventilatory variables were seen within both species during the EBC collection procedures (Table II). Significant rank correlations between parameters of ventilation, body weight and rectal temperature are given in Tables III and IV for calves and pigs, respectively.

The average time necessary for one collection of EBC was significantly shorter in calves than in pigs (18 ± 5 min versus 35 ± 15 min (mean $\pm$ SD); $p \leq 0.001$). During this time, comparable amounts of total exhaled volumes were passed trough the sampling system (200–400 litres per calf; 150–440 litres per pig) leading to a total volume of 5 ± 1 ml EBC in calves and 4 ± 1 ml EBC in pigs (mean $\pm$ SD). In relation to time, the EBC volume obtained per min was significantly higher in calves than in pigs (0.29 ± 0.08 ml EBC min^{-1} in calves versus 0.14 ± 0.07 ml EBC min^{-1} in pigs (mean $\pm$ SD); $p \leq 0.001$). In relation to exhaled volume, significantly less EBC was collected from calves than from pigs (1.35 ± 0.41 ml EBC/100 litres in calves versus 1.59 ± 0.34 ml EBC/100 litres in pigs (mean $\pm$ SD); $p \leq 0.001$).

Statistically significant relationships between different variables of ventilation and the quantity of EBC either collected per unit time (EBC, ml min^{-1}) or per unit

Table II. Body weight, rectal temperature, variables of ventilation and parameters of exhaled breath condensate (EBC) collection obtained in different species.

		Calves ($n=168$)[a]		Pigs ($n=56$)[b]	
	Unit	Minimum	Maximum	Minimum	Maximum
Body weight (bw)	kg	39.5	173.4	8.3	82.4
Rectal temperature	°C	37.8	39.7	37.6	39.9
Respiratory rate (RR)	breaths min^{-1}	16	48	14	59
Expiratory tidal volume (V_{tex})	litres	0.293	1.888	0.133	0.841
V_{tex} per kg body weight (V_{tex} kg^{-1})	ml kg^{-1}	5.9	12.1	5.2	26.3
Expiratory volume per min (V_E)	litres	6.7	40.0	2.3	19.2
Maximal airflow during expiration (V'_{Emax})	l s^{-1}	0.20	1.70	0.15	1.13
Collection time for one EBC sample	min	10	35	13	66
Volume of collected EBC (total)	ml	1.1	8.0	2.1	7.6
Volume of EBC obtained per min	ml min^{-1}	0.079	0.540	0.035	0.253
Volume of EBC/100 litres exhaled breath	ml/100 litres	0.28	2.37	0.92	2.38

[a]Twelve calves, each examined at 14 time points over 6 −7 months.
[b]Twelve pigs, each examined at five time points over 5 months (four missing values).

exhaled volume (EBC, ml/100 litres exhaled breath) are given in Table V, as determined by use of linear regression analysis. Figure 3 shows that the quantity of EBC collected per unit time was positively correlated to the exhaled volume per unit time (i.e. expiratory minute volume, V_E) in both species. However, the coefficient of correlation between EBC collected per min and V_E was stronger in pigs than in calves (Table V).

In calves, the quantity of EBC collected per min was also positively correlated to expiratory tidal volume (V_{tex}) and maximal expiratory flow (V'_{Emax}), while no significant correlation was found with respiratory rate (RR). In contrast, the quantity of EBC/100 litres exhalation was negatively correlated to V_E, V_{tex}, V'_{Emax} and RR. No significant influence of the expired tidal volume per kg body weight (V_{tex} kg^{-1}) was found on EBC collection in calves. In pigs, both the quantity of EBC collected per min and those collected/100 litres exhaled volume were positively correlated to V'_{Emax} but negatively to V_{tex} kg^{-1}. Additionally, positive correlations were found between the quantity of EBC collected per min and V_{tex} or RR, respectively. At the 95% or higher confidence level, there was no statistically significant relationship between EBC collected/100 litres exhalation and V_E, V_{tex} or RR. The most striking influences of ventilatory variables on EBC collection between calves and pigs are shown in Figures 4 and 5.

Influence of ventilation pattern on total protein in EBC

In 98.7% (155/157) of EBC samples collected in calves and in 89.3% (50/56) of those collected in pigs, a concentration of total protein higher than 0.5 μg ml^{-1} (i.e. higher than the detection limit) was measurable. As shown in Figure 6, values were not normally distributed. In general, protein concentrations less than 35 μg ml^{-1} EBC or 25 μg/100 litres exhaled breath were observed in both species, but one outlier value of total protein ($>$ 75 μg ml^{-1} or 95 μg/100 litres exhaled breath) was observed in each species that was not related to any obvious clinical signs. The concentration of total

Table III. Coefficients of Spearman rank correlation (r_{SP}) between variables of ventilation, body weight and rectal temperature in calves ($n = 168$)[a].

	Respiratory rate (RR)	Expiratory tidal volume (V_{tex})	V_{tex} per kg body weight (V_{tex} kg^{-1})	Expiratory volume per min (V_E)	Maximum expiratory airflow (V'_{Emax})	Body weight (bw)	Rectal temperature
Respiratory rate (RR)		$r_{SP} = -0.36$ ($p < 0.0001$)	$r_{SP} = -0.39$ ($p < 0.0001$)	$r_{SP} = 0.33$ ($p < 0.0001$)	$r_{SP} = 0.26$ ($p = 0.0009$)	$r_{SP} = -0.21$ ($p = 0.0056$)	$r_{SP} = 0.22$ ($p = 0.0047$)
Expiratory tidal volume (V_{tex})	$r_{SP} = -0.36$ ($p < 0.0001$)		n.s.	$r_{SP} = 0.73$ ($p < 0.0001$)	$r_{SP} = 0.70$ ($p < 0.0001$)	$r_{SP} = 0.91$ ($p < 0.0001$)	$r_{SP} = -0.47$ ($p < 0.0001$)
V_{tex} per kg body weight (V_{tex} kg^{-1})	$r_{SP} = -0.39$ ($p < 0.0001$)	n.s.		$r_{SP} = -0.24$ ($p = 0.0022$)	$r_{SP} = -0.32$ ($p < 0.0001$)	$r_{SP} = -0.36$ ($p < 0.0001$)	$r_{SP} = 0.18$ ($p = 0.0199$)
Expiratory volume per min (V_E)	$r_{SP} = 0.33$ ($p < 0.0001$)	$r_{SP} = 0.73$ ($p < 0.0001$)	$r_{SP} = -0.24$ ($p = 0.0022$)		$r_{SP} = 0.88$ ($p < 0.0001$)	$r_{SP} = 0.74$ ($p < 0.0001$)	$r_{SP} = -0.34$ ($p < 0.0001$)
Maximum expiratory airflow (V'_{Emax})	$r_{SP} = 0.26$ ($p = 0.0009$)	$r_{SP} = 0.70$ ($p < 0.0001$)	$r_{SP} = -0.32$ ($p < 0.0001$)	$r_{SP} = 0.88$ ($p < 0.0001$)		$r_{SP} = 0.69$ ($p < 0.0001$)	$r_{SP} = -0.32$ ($p < 0.0001$)
Body weight (bw)	$r_{SP} = -0.21$ ($p = 0.0056$)	$r_{SP} = 0.91$ ($p < 0.0001$)	$r_{SP} = -0.36$ ($p < 0.0001$)	$r_{SP} = 0.74$ ($p < 0.0001$)	$r_{SP} = 0.69$ ($p < 0.0001$)		$r_{SP} = -0.47$ ($p < 0.0001$)
Rectal temperature	$r_{SP} = 0.22$ ($p = 0.0047$)	$r_{SP} = -0.47$ ($p < 0.0001$)	$r_{SP} = 0.18$ ($p = 0.0199$)	$r_{SP} = -0.34$ ($p < 0.0001$)	$r_{SP} = -0.32$ ($p < 0.0001$)	$r_{SP} = -0.47$ ($p < 0.0001$)	

n.s., No significant correlation at the 95% confidence level; p, probability.
[a]Twelve calves, each examined at 14 time points, over 6–7 months.

Table IV. Coefficients of Spearman rank correlation (r_{SP}) between variables of ventilation, body weight and rectal temperature in pigs ($n = 56$)[b].

	Respiratory rate (RR)	Expiratory tidal volume (V_{tex})	V_{tex} per kg body weight (V_{tex} kg^{-1})	Expiratory volume per min (V_E)	Maximum expiratory airflow (V'_{Emax})	Body weight (bw)	Rectal temperature
Respiratory rate (RR)		n.s.	$r_{SP} = -0.61$ ($p < 0.0001$)	$r_{SP} = 0.46$ ($p < 0.0009$)	$r_{SP} = 0.50$ ($p < 0.0003$)	$r_{SP} = 0.39$ ($p = 0.0050$)	$r_{SP} = 0.29$ ($p = 0.0352$)
Expiratory tidal volume (V_{tex})	n.s.		n.s.	$r_{SP} = 0.84$ ($p < 0.0001$)	$r_{SP} = 0.68$ ($p < 0.0001$)	$r_{SP} = 0.78$ ($p < 0.0001$)	n.s.
V_{tex} per kg body weight (V_{tex} kg^{-1})	$r_{SP} = -0.61$ ($p < 0.0001$)	n.s.		$r_{SP} = -0.54$ ($p = 0.0001$)	$r_{SP} = -0.63$ ($p < 0.0001$)	$r_{SP} = -0.79$ ($p < 0.0001$)	n.s.
Expiratory volume per min (V_E)	$r_{SP} = 0.46$ ($p < 0.0009$)	$r_{SP} = 0.84$ ($p < 0.0001$)	$r_{SP} = -0.54$ ($p = 0.0001$)		$r_{SP} = 0.85$ ($p < 0.0001$)	$r_{SP} = 0.86$ ($p < 0.0001$)	n.s.
Maximum expiratory airflow (V'_{Emax})	$r_{SP} = 0.50$ ($p < 0.0003$)	$r_{SP} = 0.68$ ($p < 0.0001$)	$r_{SP} = -0.63$ ($p < 0.0001$)	$r_{SP} = 0.85$ ($p < 0.0001$)		$r_{SP} = 0.81$ ($p < 0.0001$)	n.s.
Body weight (bw)	$r_{SP} = 0.39$ ($p = 0.0050$)	$r_{SP} = 0.78$ ($p < 0.0001$)	$r_{SP} = -0.79$ ($p < 0.0001$)	$r_{SP} = 0.86$ ($p < 0.0001$)	$r_{SP} = 0.81$ ($p < 0.0001$)		n.s.
Rectal temperature	$r_{SP} = 0.29$ ($p = 0.0352$)	n.s.	n.s.	n.s.	n.s.	n.s.	

n.s., No significant correlation at the 95% confidence level; p, probability.
[b]Twelve pigs, each examined at five time points, over 5 months (four missing values).

Table V. Linear regression between variables of ventilation and the quantity of exhaled breath condensate (EBC) collected.

Equations of linear regression	r	R^2 (%)	p
Calves ($n=168$)[a]:			
EBC (ml min^{-1}) $=0.16666+0.00541598\ V_E$ (litres)	0.46	21.19	<0.0001
EBC (ml min^{-1}) $=0.20999+0.10019\ V_{tex}$ (litres)	0.34	11.44	<0.0001
EBC (ml min^{-1}) $=0.213865+0.0781477\ V'_{Emax}$ (l s^{-1})	0.33	10.87	<0.0001
EBC (ml/100 litres) $=2.31133-0.0405061\ V_E$ (litres)	−0.72	52.29	<0.0001
EBC (ml/100 litres) $=2.06278-0.861479\ V_{tex}$ (litres)	−0.59	35.07	<0.0001
EBC (ml/100 litres) $=2.21156-0.866722\ V'_{Emax}$ (l s^{-1})	−0.73	52.61	<0.0001
EBC (ml/100 litres) $=1.72597-0.0128614$ RR (breaths min^{-1})	−0.21	4.61	0.0052
Pigs ($n=56$)[b]:			
EBC (ml min^{-1}) $=0.000769295+0.0161229\ V_E$ (litres)	0.92	84.1	<0.0001
EBC (ml min^{-1}) $=0.0261689+0.313966\ V_{tex}$ (litres)	0.70	48.35	<0.0001
EBC (ml min^{-1}) $=0.0204795+0.264641\ V'_{Emax}$ (l s^{-1})	0.88	76.96	<0.0001
EBC (ml min^{-1}) $=0.0284192+0.00457466$ RR (breaths min^{-1})	0.47	22.23	0.0002
EBC (ml min^{-1}) $=0.231057-0.00749263\ V_{tex}$ kg^{-1} (ml kg^{-1})	−0.64	41.03	<0.0001
EBC (ml/100 litres) $=1.33475+0.598738\ V'_{Emax}$ (l s^{-1})	0.41	16.77	0.0019
EBC (ml/100 litres) $=2.04186-0.0362525\ V_{tex}$ kg^{-1} (ml kg^{-1})	−0.63	39.42	<0.0001

r, Coefficient of linear correlation; R^2, coefficient of determination; p, probability; V'_{Emax}, maximal airflow during spontaneous expiration; RR, respiratory rate; V_E, expiratory volume per min; V_{tex}, expiratory tidal volume; V_{tex} kg^{-1}, expiratory tidal volume per kg body weight.
[a]Twelve calves, each examined at 14 time points over 6–7 months.
[b]Twelve pigs, each examined at five time points over 5 months (four missing values).

protein (either expressed ml^{-1} EBC or/100 litres exhaled breath) was significantly higher in pigs compared with calves (Figure 6).

Coefficients of Spearman rank correlation (r_{SP}) between the concentration of total protein in EBC and either variables of ventilation or variables of EBC collection are summarized in Table VI for calves and pigs (taking a probability level of 90% ($p \leq 0.10$) into account). In both species, the concentration of total protein measured ml^{-1} EBC increased with the expired volume per min (V_E) and with the maximal airflow during expiration (V'_{Emax}), but decreased with an increasing volume of EBC collected

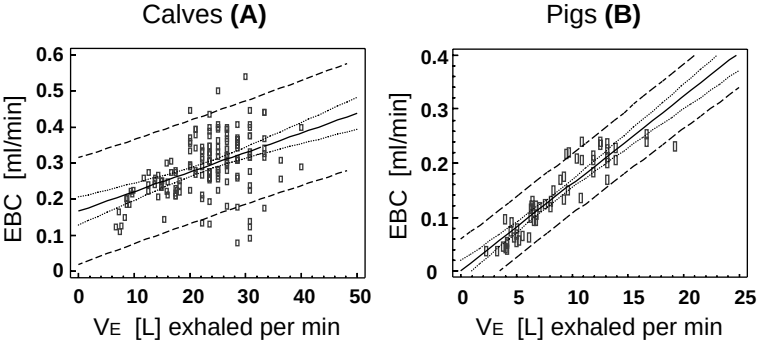

Figure 3. Linear correlation between expired volume per min and the quantity of EBC collected per min: ——, line of linear regression; ·····, 95% confidence limits for the regression line; – –, 95% prediction limits for new observations. Equations and coefficients of linear regression are given in Table V.

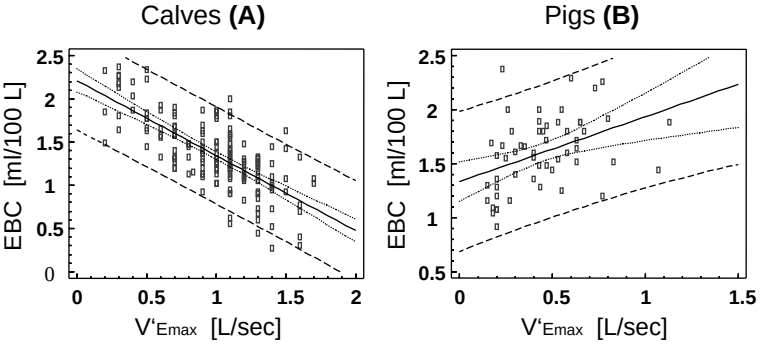

Figure 4. Linear correlation between maximal airflow during spontaneous expiration and the quantity of EBC collected/100 litres exhaled breath: ——, line of linear regression; ·····, 95% confidence limits for the regression line; – –, 95% prediction limits for new observations. Equations and coefficients of linear regression are given in Table V.

from 100 litres exhaled breath. The concentration of protein calculated/100 litres exhaled breath, however, increased significantly with the volume of EBC collected from 100 litres exhaled breath, but was negatively correlated to the expiratory tidal volume (V_{tex} in calves, V_{tex} kg^{-1} in pigs).

Evaluation of pH and pCO_2 in EBC

The period necessary to collect 3–5 ml EBC was 22 ± 4 min in calves and 48 ± 12 min in pigs (each mean $\pm$ SD). Both pH and pCO_2 were measured in EBC (1) immediately after finishing the collection process and (2) after storage of EBC in a closed tube at room temperature for 1 h.

Table VII summarizes results for pH and partial pressure of pCO_2 measured in EBC samples of both species. In fresh EBC, pH measured immediately after collection was normally distributed, varying between 5.6 and 6.2, and was not significantly different between species. In contrast, pCO_2 in fresh EBC was significantly lower in pigs than in calves. Including both species in a linear regression

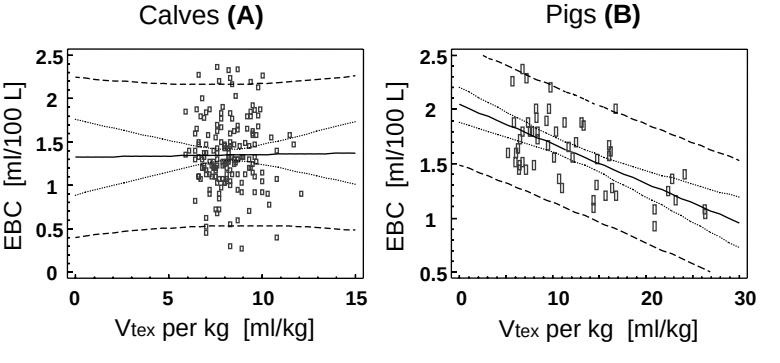

Figure 5. Linear correlation between tidal volume expired kg^{-1} body weight and the quantity of EBC collected/100 litres exhaled breath: ——, line of linear regression; ·····, 95% confidence limits for the regression line; – –, 95% prediction limits for new observations. Equations and coefficients of linear regression are given in Table V.

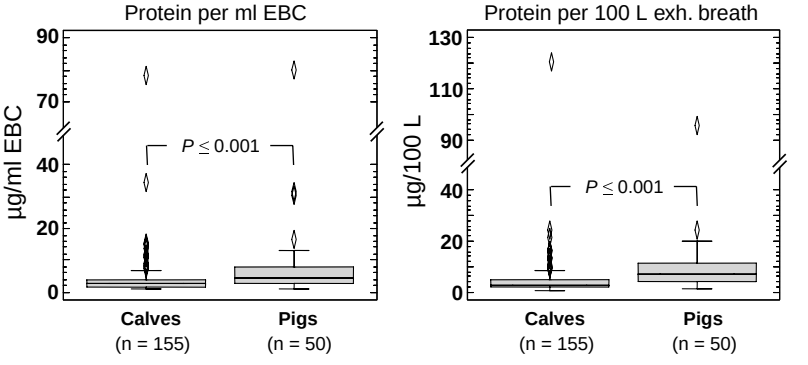

Figure 6. Concentration of total protein in exhaled breath condensate (measured ml^{-1} EBC and calculated/100 litres exhaled breath) in clinically healthy calves and pigs. $p \leq 0.001$ indicates a significant difference between species (Mann–Whitney–Wilcoxon test) that was found taking the outlier value within each species into account or not. Results are based on 12 calves (14 EBC collections per animal) and 12 pigs (five EBC collections per animal). Values below the detection limit (<0.5 µg ml^{-1}) were not taken into account.

Table VI. Coefficients of Spearman rank correlation (r_{SP}) between the concentration of total protein in exhaled breath condensate (EBC) and variables of ventilation or variables of EBC collection, respectively, in calves and in pigs.

	Calves ($n=154$*)		Pigs ($n=49$*)	
	Total protein (µg ml^{-1} EBC)	Total protein (µg/100 litres)	Total protein (µg ml^{-1} EBC)	Total protein (µg/100 litres)
Respiratory rate (RR)	n.s.	n.s.	n.s.	$r_{SP}=0.27$ ($p=0.06$)
Expiratory tidal volume (V_{tex}, litres)	$r_{SP}=0.20$ ($p \leq 0.01$)	$r_{SP}=-0.19$ ($p<0.05$)	n.s.	n.s.
V_{tex} per kg body weight (V_{tex} kg^{-1}, ml kg^{-1})	n.s.	n.s.	n.s.	$r_{SP}=-0.26$ ($p=0.07$)
Expiratory volume per min (V_E, litres)	$r_{SP}=0.26$ ($p \leq 0.001$)	n.s.	$r_{SP}=0.30$ ($p \leq 0.05$)	n.s.
Maximum expiratory airflow (V'_{Emax}, l s^{-1})	$r_{SP}=0.27$ ($p \leq 0.001$)	n.s.	$r_{SP}=0.34$ ($p \leq 0.05$)	n.s.
Collection time for one EBC sample (min)	$r_{SP}=-0.26$ ($p \leq 0.001$)	n.s.	n.s.	n.s.
Volume of EBC obtained per min (ml min^{-1})	n.s.	$r_{SP}=0.18$ ($p \leq 0.05$)	n.s.	n.s.
Volume of EBC obtained/ 100 litres exhaled breath (ml/100 litres)	$r_{SP}=-0.23$ ($p \leq 0.01$)	$r_{SP}=0.30$ ($p \leq 0.001$)	$r_{SP}=-0.53$ ($p \leq 0.001$)	$r_{SP}=0.25$ ($p=0.10$)
	$r_{SP}=0.82$ ($p \leq 0.001$)		$r_{SP}=0.87$ ($p \leq 0.001$)	

*Results are based on 12 calves (14 EBC collections per animal) and 12 pigs (five EBC collections per animal). Outlier values (>50 µg ml^{-1} EBC or >90 µg/100 litres exhaled breath, respectively) and values below the detection limit (<0.5 µg ml^{-1}) were not taken into account for correlation analysis.
n.s., No significant correlation at the 90% confidence level; p, probability.

Table VII. Partial pressure of carbon dioxide (pCO_2) and pH measured in exhaled breath condensate (EBC) samples of clinically healthy calves and pigs.

			Immediately after collection of EBC		After 1-h storage (closed tube, room temperature)		
	Variable	Unit	Mean	SD	Mean	SD	Paired t-test*
Calves$^{\#}$ ($n=24$)	pH		5.87	0.16	6.08	0.14	$p \leq 0.001$
	pCO_2	kPa	5.38	2.16	3.34	1.23	$p \leq 0.001$
Pigs$^{\#}$ ($n=24$)	pH		5.83	0.15	5.98	0.15	$p \leq 0.001$
	pCO_2	kPa	2.83	1.12	1.78	0.64	$p \leq 0.001$
Unpaired t-test$^{\$}$	pH		n.s.		$p=0.025$		
	pCO_2		$p \leq 0.001$		$p \leq 0.001$		

$^{\#}$Period (mean $\pm$ SD) for collection of EBC: in calves, 22 ± 4 min; in pigs, 48 ± 12 min.
*Paired t-test (to identify significant differences between time points within each species; within line).
$^{\$}$Unpaired t-test (to identify significant differences between species at each time point; within column).
n.s., No significant difference at the 95% confidence level; p, probability.

model, a significant negative linear correlation was found between the duration of EBC sampling in min and the pCO_2 measured immediately after collection (coefficient of linear correlation: $r=-0.55$, $p \leq 0.001$, $n=48$).

After a storage time of 1 h, pH increased by 0.20 in calves and by 0.16 in pigs, and this increase was significantly correlated with the decrease in pCO_2 in both species (Figure 7).

Discussion

The collection of EBC is a simple and non-invasive approach currently used as a research and diagnostic tool to assess lung diseases or specific types of airway inflammation by means of potential biomarkers that derive from the lower respiratory

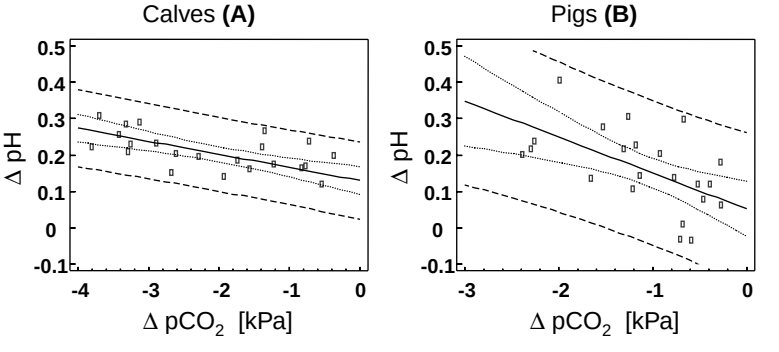

Figure 7. Linear correlation between the increase in pH and the decrease in pCO_2. EBC samples were obtained from clinically healthy calves and pigs. Both, pH and pCO_2 were measured in fresh EBC as well as one hour after storage. Differences (Δ) in pH and pCO_2 caused by storage were negatively correlated in calves ($r=-0.71$; $R^2=50.22\%$; $p<0.001$; $n=24$) and in pigs ($r=-0.60$; $R^2=35.99\%$; $p<0.01$; $n=24$), according to the following equations of linear regression: $\Delta pH = 0.1299 - 0.0359\ \Delta pCO_2$ (kPa) for calves and $\Delta pH = 0.0519 - 0.0985\ \Delta pCO_2$ (kPa) for pigs: ——, line of linear regression; ·····, 95% confidence limits for the regression line; – –, 95% prediction limits for new observations.

tract. With further technical developments, EBC may possibly help to diagnose specific diseases by means of a pattern of biomarkers generating a 'fingerprint' characteristic of a certain disease (Rahman & Kelly 2003). At present, however, comparison and interpretation of data on this rapidly growing field is mainly hampered by the lack of standardization of the collection process itself and the lack of specific high-sensitivity assays to analyse EBC components (Rosias et al. 2004, Harrison & Andersen 2005). Consequently, several methodological issues need to be addressed and require thorough validation before this technique can be used clinically (Horváth et al. 2005, Montuschi 2005).

Especially in subjects who cannot cooperate or perform breathing manoeuvres (i.e. neonates, children, non-cooperative adults, animals), EBC collection cannot be standardized by an actively influenced pattern of breathing. Consequently, the dependence of the EBC collection process on methodological and biological aspects should be evaluated in order to take those influences into account for interpretation of EBC-related results. Using large animals ranging in body weight between 8 and 173 kg (i.e. a range in body weight comparable with humans ranging from small children to heavy adults) and a commercially available collection system developed for human medicine, the inter-species comparison presented here is one step towards providing information concerning the methodological and biological influences on the collection and composition of EBC in non-cooperative subjects.

Effects of the EBC sampling procedure on lung function

In the literature, collection of EBC has frequently been described as a safe and non-invasive method to obtain samples from airways. No data in any species have been reported concerning possible consequences of the EBC collection process on lung function. To the best of our knowledge, this is the first study aimed at evaluating changes in parameters of ventilation and respiratory mechanics after EBC collection in different species and related to different sampling conditions.

In calves, EBC samples were collected in conscious non-sedated animals in physiological body position (standing) with a collection period lasting approximately between 15 and 30 min. No deterioration of lung function was seen after collection of EBC. While parameters of ventilation (i.e. tidal volume and respiratory rate) did not change significantly, parameters of respiratory mechanics actually improved after EBC collection. The decrease in respiratory resistance at 3 and 5 Hz (R_{3Hz}, R_{5Hz}), and the increase in respiratory reactance between 3 and 10 Hz ($X_{3Hz}, \ldots, X_{10Hz}$) indicate lower resistive loads of breathing and/or less capacitance (i.e. improved compliance) of the respiratory system (Figure 2A). In addition, both resistance of proximal airways and resistance of distal airways decreased after the collection process. One should not interpret these results as an improvement in lung function caused by EBC collection. It is more likely the calves became more adapted to the procedure (room, mask and other ambient conditions) over time leading to a calming and more relaxed tonus of all muscles, including those of the abdomen and thorax. Because the principle of impulse oscillometry is based on external test signals applied to the respiratory system under investigation, mechanical properties of the thoracic wall are included in respiratory impedance measurements (Reinhold et al. 1998a, Klein et al. 2003).

In pigs, collection of EBC was only possible while the individual animal was sedated and restrained. Furthermore, the collection period was longer than in calves lasting

30–40 min. After EBC collection, significant changes in lung function were noticed (Figure 2B) characterized by a significant increase in respiratory resistance at 5 Hz and a significant decrease in respiratory reactance at all frequencies (5–20 Hz). These findings represent higher resistive loads and less compliance (i.e. more capacitance) localized especially in the peripheral respiratory system and likely including the thoracic wall. Resistance of distal airways increased significantly while resistance of proximal airways did not change. These changes in respiratory mechanics do not indicate changes in the upper airways due to EBC sampling, but could be interpreted as a significant effect of thorax compression and/or lung compression over time caused by the canvas sling used for fixation. This would also be related to the body weight of each pig. Tidal volume was significantly increased after collection of EBC leading to increased ventilation per time, whilst respiratory rate remained unchanged. Increased ventilation indicates a higher work of breathing either to overcome higher resistive loads or to compensate a mismatched ventilation–perfusion ratio in the lung. The latter can likely be regarded as an effect of the pig's non-physiological body position in the sling, while perfusion of the lung is influenced by gravitation, but ventilation in ventral lung regions is limited by compression over time. Furthermore, a decreased effect of sedation must be taken into account between the two lung function tests before and after collection of EBC. As shown in a previous study in pigs, diazepam not only has a relaxing effect on muscles, but also reduces tidal volume and respiratory rate (Klein & Reinhold 2001). As a reduced effect of diazepam over the period necessary for EBC collection ($>$ 30 min) should occur, an increased muscle tonus and increases in ventilation per time could be expected after collection of EBC compared with previous baseline values.

In conclusion, restraint of pigs in a canvas sling to facilitate EBC collection resulted in changes in lung function that were likely related to sedation and/or fixation itself, rather than to EBC collection procedure per se. In non-sedated subjects in a normal body position during collection of EBC (such as standing calves in this study), no deterioration of lung function would be anticipated as a direct result of the collection process.

Standardization of the EBC collection process

In the present study, a commercially available sampling device that is widely used in humans was applied to healthy calves and pigs. Although the methodology of collecting EBC in spontaneously breathing animals is comparable with that used in humans when using the same technique, methodological differences exist in nose breathing of the animals and in using a face mask (instead of a mouthpiece) to adapt the collection system to the animal's head. Latzin et al. (2003), however, compared condensate collection via nasal and oral exhalation in 11 healthy adults and found no difference between either collection methods for the amount of condensate collected. Nevertheless, the equipment dead space in the collection systems will be greater in animals wearing a mask than in humans, where there is direct connection to the mouth or nasal passages. Consequently, the equipment dead space should be kept as low as possible to avoid significant carbon dioxide rebreathing and also to minimize condensation or deposition of aerosol on the mask inner surface.

To standardize the collection process, it is important first to clarify the dependence of EBC collection on variables of ventilation and/or the pattern of breathing.

Effect of minute ventilation. Due to body weights varying between 8 and 173 kg and because of significant correlations between body weight and expiratory tidal volume (V_{tex}), expiratory volume per min (V_E) or expiratory airflow (V'_{Emax}), respectively, wide ranges of volumes and airflow were measured in calves and pigs. As documented herein, the volume of EBC collected per unit time (min) increases with an increased expired volume over time (V_E) in both species. Since a higher minute ventilation corresponds with higher tidal volumes and higher airflows (Tables III and IV), positive correlations were also seen between the quantity of EBC collected per min and expiratory tidal volume or expiratory airflow in both calves and pigs (Table V).

The finding that EBC volume collected per min was linearly related to the volume exhaled per min is in agreement with previous results documented for calves and humans (Reinhold et al. 2000, Gessner et al. 2001, McCafferty et al. 2004). Based on this knowledge, the sample collection time could be determined for each subject, taking the expired volume per min into consideration. In healthy subjects under thermo-neutral conditions, resting minute ventilation depends mainly on body weight (but is also influenced by oxygen consumption and carbon dioxide production) (Tables III and IV). Therefore, in younger subjects of individual species, a longer period of collection is required to obtain the same volume of EBC as in adult subjects (the same is true when comparing smaller species with larger ones). The duration of EBC sampling, however, might influence some components of EBC, as shown for pCO_2 in this study. Furthermore, less stable or highly reactive species or compounds may degrade or react during prolonged collection periods.

Effect of expiratory airflow. If factors other than exhaled volume can influence the collection of EBC significantly, it cannot be assumed that identical exhaled volumes will lead to identical volumes of EBC (despite the same device being used for collection). As shown in this study, results expressed in ml EBC obtained/100 litres exhaled volume, were significantly lower in calves (mean 1.4 ml/100 litres) compared with pigs (mean 1.6 ml/100 litres), and were very differently influenced by ventilatory variables when comparing both species. While increasing expiratory flow rates were positively correlated to the volume of EBC obtained/100 litres expiration in pigs, higher expiratory flow rates in calves led to decreasing volumes of EBC in relation to exhaled volume. These data indicate methodological differences rather than biological ones. In calves weighing up to 173 kg and breathing with maximal expiratory flow rates up to 1.7 l s^{-1}, the efficacy of EBC collection would be predicted to decrease with increasing flow rates leading to less EBC volume/100 litres exhaled volume with increasing V'_{Emax} values (Figure 4A). This result indicates that the collection device became increasingly inefficient at increasing expiratory flow rates. The latter finding supports data relating to a decrease in the condenser efficiency with an increase in minute ventilation reported in ten adult humans using the same collection device (McCafferty et al. 2004). However, using the collection system in subjects with maximal expiratory flow rates less than 1.2 l s^{-1} (pigs), higher volumes of EBC per unit time or per unit exhaled volume were obtained with higher airflows (Figure 4B), indicating that the condenser did not reach a limitation in efficiency under these conditions.

Effect of tidal volume. Other variables that influenced the collection of EBC were the absolute expiratory tidal volume (V_{tex}) and V_{tex} related kg^{-1} body weight (expressed

in ml kg^{-1}). As expected, increasing expiratory tidal volumes (related to increasing age and consequently to increasing volume of minute ventilation) resulted in increased quantities of EBC collected per time (expressed in ml min^{-1}) in both species (Table V). The quantity of EBC collected/100 litres exhaled breath, however, decreased significantly with increasing V_{tex} in calves (Table V). In contrast, no significant relationship between V_{tex} and the quantity of EBC collected/100 litres exhaled breath was seen in pigs. Both findings are in disagreement with data reported in humans, where an increase in tidal volume resulted in increased volumes of both exhaled water and EBC l^{-1} respired (McCafferty et al. 2004). The main reason for these conflicting observations might be that the influence of tidal volume was examined at a fixed minute volume (i.e. with breathing manoeuvres) in the human study, while increases in V_{tex} were correlated to increases in minute ventilation (V_E) in spontaneously breathing animals examined in this study.

Effect of dead space. While the tidal volume related per kg body weight (V_{tex} kg^{-1}) had no significant influence on EBC collection in calves, a clear negative correlation existed between EBC collection and V_{tex} kg^{-1} in pigs. Both EBC collected per unit time (ml min^{-1}) and expressed as percentage of the total volume exhaled (EBC ml/100 litres) were negatively correlated with V_{tex} kg^{-1}. In other words, deeper breaths led to relatively smaller EBC volumes and vice versa. The fact that V_{tex} kg^{-1} decreased with increasing body weight in pigs might be an effect of the fixation in a sling. Due to increasing body weights, the thorax and abdomen might be more compressed while hanging in the sling, leading to a reduced depth of inhalation (and consequently to a decreasing V_{tex} kg^{-1}). However, lower tidal volumes kg^{-1} body weight indicate higher dead space ventilation (V_d) while higher tidal volumes kg^{-1} are related to a higher percentage of alveolar ventilation. Since decreasing expiratory tidal volumes kg^{-1} were correlated to higher volumes of EBC, one must assume that a higher dead space ventilation (i.e. a higher V_d/V_t ratio) is correlated to a higher EBC volume. However, this implies that the higher EBC volume derives mainly from conducting airways rather than from peripheral ones. Different origins of EBC, however, might have effects on the composition of EBC. This aspect has not reported in literature and needs to be examined in further studies.

In conclusion, despite the volume expired per min (V_E) being the most important factor affecting the collection of EBC, expiratory airflow rates, and the ratio between dead space volume and tidal volume may also influence EBC collection and the origin of EBC. Consequently, care should be taken to standardize the collection procedure as far as possible. Because species-specific conditions for EBC collection influence the values obtained, the collection process should be standardized at least within each species in order to minimize unknown or variable factors that result from the collection process but may influence the composition of EBC. In order to allow comparison of different studies, not only the conditions of collection (temperature, duration of condensation, etc.), but also the breathing pattern during EBC collection should be reported.

Influence of ventilation pattern on the composition of EBC

In comparison with blood serum or broncho-alveolar lavage fluid, EBC is a highly diluted sample consisting of more than 99.9% condensed water vapour. In a

concentration of less than 0.1 μl ml^{-1}, EBC contains aerosol particles in which several biomolecules have been measured (Effros et al. 2004, 2005, Montuschi 2005). It is likely that parts of the airway surface liquid (ASL, a synonym for epithelial lining fluid, ELF) become aerosolized during turbulent airflow, so the content of droplets or aerosols in the condensate reflects the composition of ASL (Kharitonov & Barnes 2002).

There are different reasons that one cannot assume droplet formation to be related in a constant manner to the production of water vapour. Distribution of respiratory droplets is variable with droplet size ranging from the lower limits of detection (0.3 μm) to approximately 8.0 μm and a large inter-subject variability between healthy volunteers even in physiological conditions (Papineni & Rosenthal 1997). In the same study, the quantity of droplets produced was ranked as coughing > mouth breathing > nose breathing, although this ranking was not consistent for particles greater than 1.0 μm. However, neither the different sources of aerosols (mouth or nose, larynx, pharynx, trachea, bronchi, alveoli) nor the proportional contribution of these potential sources has yet been sufficiently studied for EBC collection (Kharitonov & Barnes 2002). In addition, both the concentration of different substances in the ASL and the ratio of the volume of aerosols to the volume of the condensate may vary between individuals even in physiological conditions (Effros et al. 2004).

In the present study, the concentration of total protein was assessed to study both the absolute concentration of the aerosol fraction (EBC ml^{-1} as well as per 100 litres exhaled breath) and its dependence on the ventilatory pattern. In both species, the concentration of total protein varied within a large range (from 'undetectable' to >75 μg ml^{-1} EBC or >95 μg/100 litres exhaled breath) without showing a normal distribution. Median concentration was significantly lower in calves (2.6 μg ml^{-1} EBC or 3.0 μg/100 litres exhaled breath) compared with pigs (4.3 μg ml^{-1} EBC or 6.6 μg/100 litres exhaled breath). Both median concentrations and large ranges of non-normal distributed data as observed in both species are in full agreement to protein concentrations reported for healthy human volunteers ranging from zero to 20.0 μg ml^{-1} EBC with one outlier of 107.7 μg ml^{-1} EBC (Scheideler et al. 1993; $n = 10$) and averages of 2.3 ± 0.3 μg ml^{-1} (Dwyer 2004; $n = 31$) or 6.0 ± 3.4 μg ml^{-1} (McCafferty et al. 2004; $n = 10$).

While McCafferty et al. could not identify any significant influence of the ventilatory pattern (i.e. tidal or minute volume of ventilation) on the protein concentration in EBC of ten healthy individuals, data from the present study based on higher sample sizes ($n = 154$ in calves, $n = 49$ in pigs) indicate significant correlations between variables of ventilation and the concentration of total protein in EBC. In both species, the protein concentration EBC ml^{-1} increased with higher expiratory airflows (V'_{Emax}) and with higher expired volumes (V_E) per min (Table VI). Maximal airflow and expired volumes per min were strongly correlated to each other (Tables III and IV) in this study, and increases in both can be expected to induce more turbulence in airflow leading to an increase in aerosol generation. Furthermore, the concentration of total protein increased with decreasing volumes of EBC obtained/100 litres exhaled breath indicating that the aerosol fraction was higher in less diluted samples. Eliminating the dilution factor by using 100 litres exhaled breath as the reference scale (instead of EBC ml), the amount of total protein (μl/100 litres) and the volume of EBC (ml/100 litres) were slightly positively correlated.

In summary, there are different possibilities to explain the high variability and the presence of outliers in the protein concentration of EBC. First, the number or size of respiratory droplets produced and exhaled per unit time can be augmented by increased airway secretions, cough and other undefined processes (Effros et al. 2004). Such influences on aerosol generation could not be evaluated in this study but may explain the single outlier value within each species. Second, the breathing pattern (especially more turbulence due to higher airflow rates) has to be regarded as a determinant of non-volatile concentrations in EBC. Third, the dilution of aerosols depends on the amount of water vapour exhaled per unit time (Effros et al. 2005), but may also vary with the efficiency of the condenser. In accordance with McCafferty et al. (2004), the latter has also been found to be significantly influenced by breathing pattern. Further sources of variability that include capture of droplets and assay variability (McCafferty et al. 2004) were not assessed in this study.

To reduce the confounding effects of these various methodological and biological factors on the interpretation and comparison of condensate data, it is recommended that one express the concentration of total protein in the EBC in relation not only to the condensate volume, but also to 100 litres exhaled breath. The concentration of total protein/100 litres exhaled breath, however, can be regarded as a baseline to express the composition of EBC with respect to other non-volatile components (e.g. interleukins).

pH of exhaled breath condensate

In human medicine, the pH in EBC has been proposed as a marker of airway inflammation, lung injury or pneumonic infection under clinical conditions. Compared with healthy subjects, a significant acidification of EBC has been reported in patients suffering from pneumonia, in ventilated patients with mechanical stress and lung injury, in patients with chronic obstructive pulmonary disease, chronic cough, bronchiectasis and asthma, or in patients with cystic fibrosis, especially when affected from an infective exacerbation (Hunt et al. 2000, Kostikas et al. 2002, Tate et al. 2002, Gessner et al. 2003, Anaev & Chuchalin 2004, Carpagnano et al. 2004, Niimi et al. 2004, Borrill et al. 2005, Carraro et al. 2005, Ojoo et al. 2005). In those studies, some authors examined de-aerated EBC samples while others used non-degassed ones.

Since pH has been thought to be significantly influenced by the concentration of exhaled CO_2 in EBC, most authors in the literature have suggested to de-aerate EBC with argon or with nitrogen before pH measurement in order to remove at least the soluble CO_2 fraction. Because physical solubility of gases is higher at lower temperatures, a high percentage of CO_2 is thought to be removed from the EBC collected near the freezing point. In general, EBC pH becomes significantly higher after argon de-aeration (Borrill et al. 2005) leading to pHs between 7.3 and 7.8 in average in healthy humans, while those in patients with disease were still acidic (Lehmann & Rothe 2003, Vaughan et al. 2003, Carpagnano et al. 2004). The EBC pH in clinically healthy dogs after argon degassing has been reported to be around 7.6 −7.8 (Hirt et al. 2003).

Data reported in the present study are from clinically healthy animals free of respiratory symptoms and are used to evaluate the range of physiological data and to compare baseline values between different species. Samples were not de-aerated and

pCO_2 was measured in parallel in order to clarify the influence of pCO_2 on pH in native EBC samples. Immediately after finishing the sampling process, pHs ranging from 5.6 to 6.2 were measured in native EBC of both calves and pigs without any significant difference between species. This finding is in general agreement with the results reported in humans showing that fluid condensed from the breath of healthy volunteers is originally acidic with means $<6.0–6.5$ (Tate et al. 2002, Lehmann & Rothe 2003, Anaev & Chuchalin 2004, MacGregor et al. 2005, Ojoo et al. 2005).

pCO_2 but not pH was significantly negatively dependent on the duration of collection period (doubled in pigs compared with calves due to differences in body weight and ventilation per time). Despite significant differences in pCO_2, nearly identical pHs were found in fresh EBC samples of both species. In accordance, Tate et al. (2002) could not show any significant correlation between pH in EBC and the peak alveolar CO_2 concentration. These findings suggest that pH in fresh native EBC must be influenced by multiple factors, of which pCO_2 plays a minor role. It has been demonstrated in isolated distal bronchi from pigs that intact distal airways secrete both, acid and base equivalents (Inglis et al. 2003). However, the acid–base balance of ASL is not completely understood nor is its influence on pH in EBC. Various factors are under controversial discussion that may potentially contribute to understanding the determinants of EBC pH. Despite the volatile cation NH_4^+ having been regarded as the principal buffer in EBC (Effros et al. 2003, 2005), 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 (Wells et al. 2005). Some authors found significant correlations between pH and ammonia (Gessner et al. 2003, Carraro et al. 2005) while others did not (MacGregor et al. 2005). The presence of further cations such as sodium and potassium (Svensson et al. 2005), a reduced secretion of buffering anions such as bicarbonate (Inglis et al. 2003) or chloride (Niimi et al. 2004), lactate (Gessner et al. 2003), glutaminase (Hunt et al. 2002), and transport mechanism for protons (e.g. Na^+/H^+-antiporter, H^+-ATPase or 'voltage gated 'H^+ channels) are under consideration. Due to this complexibility, de-aeration may remove volatile components that make an important biological contribution and non-degassed samples may more closely reflect the pH on the airway surface. Factors responsible for the 'physiological' acidity of EBC, however, need to be clarified in future.

The aim of the present study was to evaluate methodological influences on pH in EBC. No effect of the collection process itself or the pattern of breathing (McCafferty et al. 2004) could be found on pH measured immediately after finishing EBC collection. However, storage of EBC for only 1 h led to significant decreases in EBC pCO_2 that were significantly correlated with increases in pH. To avoid any falsification of pH (either by an escape of volatile components or by ongoing biochemical processes), pH measurements should be carefully undertaken immediately after collection of EBC.

Conclusions

Collection of EBC does not affect lung function in spontaneously breathing subjects. Despite the minute volume of ventilation being the most important factor affecting the collection of EBC, expiratory airflow rates, and the ratio between dead space volume

and tidal volume may also influence EBC collection and its composition. Consequently, care should be taken to standardize both EBC collection and the analysis of samples of EBC as far as possible. To clarify the physiological background of the pH of EBC, further studies are necessary. The data obtained in this study may help to avoid possible methodological pitfalls of EBC collection in different species.

Acknowledgements

The authors thank Dr rer. nat. Michael Rothe and Dr med. Gunther Becher (Research Company of Lung and Chest Diseases Ltd., Berlin, Germany) for protein analysis, David Marlin, BSc PhD (Animal Health Trust, Newmarket, UK), for a critical reading, and Annelie Langenberg, Gerhard Foedisch, Sylke Stahlberg, Ines Lemser and Heike Friedrich (FLI, Jena) for skilful assistance.

References

Amann A, Smith D, editors. 2005. Breath analysis for clinical diagnosis and therapeutic monitoring. Singapore: World Scientific.

Anaev E, Chuchalin A. 2004. Die Azidose des Atemkondensates als Marker bei Pneumonien. Pneumologie 58(Suppl. S1):S46 (V54). [abstract]

Borrill Z, Starkey C, Vestbo J, Singh D. 2005. Reproducibility of exhaled breath condensate pH in chronic obstructive pulmonary disease. European Respiratory Journal 25:269–274.

Carpagnano GE, Barnes PJ, Francis J, Wilson N, Bush A, Kharitonov SA. 2004. Breath condensate pH in children with cystic fibrosis and asthma: a new noninvasive marker of airway inflammation? Chest 125:2005–2010.

Carraro S, Folesani G, Corradi M, Zanconato S, Gaston B, Baraldi E. 2005. Acid–base equilibrium in exhaled breath condensate of allergic asthmatic children. Allergy 60:476–481.

Deaton CM, Marlin DJ, Smith NC, Smith KC, Newton RJ, Gower SM, Cade SM, Roberts CA, Harris PA, Schroter RC, Kelly FJ. 2004. Breath condensate hydrogen peroxide correlates with both airway cytology and epithelial lining fluid ascorbic acid concentration in the horse. Free Radical Research 38:201–208.

Dwyer TM. 2004. Sampling airway surface liquid: non-volatiles in the exhaled breath condensate. Lung 182:241–250.

Effros RM, Biller J, Foss B, Hoagland K, Dunning MB, Castillo D, Bosbous M, Sun F, Shaker R. 2003. A simple method for estimating respiratory solute dilution in exhaled breath condensates. American Journal of Respiratory and Critical Care Medicine 168:1500–1505.

Effros RM, Dunning MB, Biller J, Shaker R. 2004. The promise and perils of exhaled breath condensates. American Journal of Physiology/Lung Cellular and Molecular Physiology 287:L1073–L1080.

Effros RM, Su J, Casaburi R, Shaker R, Biller J, Dunning M. 2005. Utility of exhaled breath condensates in chronic obstructive pulmonary disease: a critical review. Current Opinion in Pulmonary Medicine 11:135–139.

Gessner C, Hammerschmidt S, Kuhn H, Seyfarth HA, Sack U, Engelmann L, Schauer J, Wirtz H. 2003. Exhaled breath condensate acidification in acute lung injury. Respiratory Medicine 97:1188–1194.

Gessner C, Kuhn H, Seyfarth H-J, Pankau H, Winkler J, Schauer J, Wirtz H. 2001. Factors influencing breath condensate volume. Pneumologie 55:414–419.

Harrison CM, Andersen CC. 2005. Exhaled breath measures of inflammation: are they useful in neonatal chronic lung disease? Archives of Disease in Childhood. Fetal and Neonatal 90:F6–F10.

Hirt RA, Gütl A, Delvaux F, Gustin P, Marlin D, Kirschvink N. 2003. Hydrogen peroxide and pH analysis in exhaled breath condensate of healthy dogs. Journal of Veterinary Internal Medicine 17: 746. [abstract]

Horváth I, Hunt J, Barnes PJ. 2005. Exhaled breath condensate: methodological recommendations and unresolved questions. European Respiratory Journal 26:523–548.

Hunt J, Erwin E, Palmer L, Vaughan J, Malhotra N, Platts-Mills TAE, Gaston B. 2002. Expression and activity of pH-regulatory glutaminase in the human airway epithelium. American Journal of Respiratory and Critical Care Medicine 165:101–107.

Hunt JF, Fang K, Malik R, Snyder A, Malhotra N, Platts-Mills TAE, Gaston B. 2000. Endogenous airway acidification: implications for asthma pathophysiology. American Journal of Respiratory and Critical Care Medicine 161:694–699.

Inglis SK, Wilson SM, Olver RE. 2003. Secretion of acid and base equivalents by intact distal airways. American Journal of Physiology/Lung Cellular and Molecular Physiology 284:L855–L862.

Kharitonov SA, Barnes PJ. 2001. Exhaled markers of pulmonary disease. American Journal of Respiratory and Critical Care Medicine 163:1693–1722.

Kharitonov SA, Barnes PJ. 2002. Biomarkers of some pulmonary diseases in exhaled breath. Biomarkers 7:1–32.

Kirschvink N, Delvaux F, Leemans J, Clercx C, Marlin D, Sparkes A, Gustin P. 2005. Collection of exhaled breath condensate and analysis of hydrogen peroxide as potential marker of lower airway inflammation in cats. Veterinary Journal 169:385–396.

Klein C, Reinhold P. 2001. Analysis of respiratory mechanics by impulse oscillometry in non-sedated and diazepam-sedated swine. Research in Veterinary Science 70:181–189.

Klein C, Smith H-J, Reinhold P. 2003. Respiratory mechanics in conscious swine: effects of face mask, head position and bronchoconstriction evaluated by impulse oscillometry. Research in Veterinary Science 75:71–81.

Kostikas K, Papatheodorou G, Ganas K, Psathakis K, Panagou P, Loukides S. 2002. pH in Expired Breath Condensate of Patients with Inflammatory Airway Diseases. American Journal of Respiratory and Critical Care Medicine 165:1364–1370.

Laakso O, Haapala M, Kuitunen T, Himberg JJ. 2004. Screening of exhaled breath by low-resolution multicomponent FT-IR spectrometry in patients attending emergency departments. Journal of Analytical Toxicology 28:111–117.

Latzin P, Beck J, Bartenstein A, Griese M. 2003. Comparison of exhaled breath condensate from nasal and oral collection. European Journal of Medical Research 8:505–510.

Lehmann C, Rothe M. 2003. Der pH-Wert im Atemkondensat-Einflussgrößen und mögliche Bedeutung. Pneumologie 57: 115. [abstract]

MacGregor G, Ellis S, Andrews J, Imrie M, Innes A, Greening AP, Cunningham S. 2005. Breath condensate ammonium is lower in children with chronic asthma. European Respiratory Journal 26:271–276.

Marczin N, Kharitonov SA, Yacoub MH, Barnes PJ, editors. 2003. Disease markers in exhaled breath. New York, NY: Marcel Dekker.

McCafferty JB, Bradshaw TA, Tate S, Greening AP, Innes JA. 2004. Effects of breathing pattern and inspired air conditions on breath condensate volume, pH, nitrite, and protein concentrations. Thorax 59:694–698.

Montuschi P. 1999. Isoprostanes and other exhaled markers in respiratory diseases. European Respiratory Review 9:249–253.

Montuschi P. 2005. Exhaled breath condensate analysis in patients with COPD. Clinica et Chimica Acta 356:22–34.

Moser B, Bodrogi F, Eibl G, Lechner M, Rieder J, Lirk P. 2005. Mass spectrometric profile of exhaled breath — field study by PTR-MS. Respiratory Physiology and Neurobiology 145:295–300.

Mutlu GM, Garey KW, Robbins RA, Danziger LH, Rubenstein I. 2001. Pulmonary perspective: collection and analysis of exhaled breath condensates in humans. American Journal of Respiratory and Critical Care Medicine 164:731–737.

Niimi A, Nguyen LT, Usmani O, Mann B, Chung KF. 2004. Reduced pH and chloride levels in exhaled breath condensate of patients with chronic cough. Thorax 59:608–612.

Ojoo JC, Mulrennan SA, Kastelik JA, Morice AH, Redington AE. 2005. Exhaled breath condensate pH and exhaled nitric oxide in allergic asthma and in cystic fibrosis. Thorax 60:22–26.

Papineni RS, Rosenthal FS. 1997. The size distribution of droplets in the exhaled breath of healthy human subjects. Journal of Aerosol Medicine 10:105–116.

Phillips M, Gleeson K, Hughes JM, Greenberg J, Cataneo RN, Baker L, McVay WP. 1999b. Volatile organic compounds in breath as markers of lung cancer: a cross-sectional study. Lancet 353:1930–1933.

Phillips M, Greenberg J, Cataneo RN. 2000. Effect of age on the profile of alkanes in normal human breath. Free Radical Research 33:57–63.

Phillips M, Herrera J, Krishnan S, Zain M, Greenberg J, Cataneo RN. 1999a. Variation in volatile organic compounds in the breath of normal humans. Journal of Chromatography B 729:75–88.

Rahman I, Kelly F. 2003. Biomarkers in breath condensate: a promising new non-invasive technique in free radical research. Free Radical Research 37:1253–1266.

Reinhold P, Becher G, Rothe M. 2000. Evaluation of the measurement of leukotriene B4 concentrations in exhaled condensate as a noninvasive method for assessing mediators of inflammation in the lungs of calves. American Journal of Veterinary Research 61:742–749.

Reinhold P, Deaton C, Marlin D. 2005. Potential for and limitations of exhaled breath analysis in animal models. In: Amann A, Smith D, editors. Breath analysis for clinical diagnosis and therapeutic monitoring. Singapore: World Scientific. p. 503–514.

Reinhold P, MacLeod D, Lekeux P. 1996. Comparative evaluation of impulse oscillometry and a monofrequency forced oscillation technique in clinically healthy calves undergoing bronchochallenges. Research in Veterinary Science 61:206–213.

Reinhold P, Smith H-J, Close R, Genicot B, Lekeux P. 1998a. Validation of Impulse Oscillometry in Friesian and Blue Belgian Calves with respect to changes in extrathoracic upper airway resistance. Research in Veterinary Science 65:93–102.

Reinhold P, Smith H-J, Langenberg A, Lekeux P. 1998b. Measurement of respiratory impedance in healthy calves using the impulse oscillation technique — physiological and methodological aspects. Veterinary Journal 155:27–38.

Risby TH, Jiang L, Stoll S, Ingram D, Spangler E, Heim J, Cutler R, Roth GS, Rifkind JM. 1999. Breath ethane as a marker of reactive oxygen species during manipulation of diet and oxygen tension in rats. Journal of Applied Physiology 86:617–622.

Risby TH, Sehnert SS. 1999. Clinical application of breath biomarkers of oxidative stress status. Free Radical Biology and Medicine 27:1182–1192.

Rosias PP, Dompeling E, Hendriks HJ, Heijnens JW, Donckerwolcke RA, Jöbsis Q. 2004. Exhaled breath condensate in children: pearls and pitfalls. Pediatric Allergy and Immunology 15:4–19.

Scheideler L, Manke H-G, Schwulera U, Inacker O, Hammerle H. 1993. Detection of nonvolatile macromolecules in breath. A possible diagnostical tool? American Review of Respiratory Diseases 148:778–784.

Sparkes AH, Mardell EJ, Deaton C, Kirschvink N, Marlin D. 2004. Exhaled breath condensate (EBC) collection in cats — description of a non-invasive technique to investigate airway disease. Journal of Feline Medicine and Surgery 6:335–338.

Svensson S, Isacsson AC, Ljungkvist G, Torén K, Olin AC. 2005. Optimization and validation of an ion chromatographic method for the simultaneous determination of sodium, ammonium and potassium in exhaled breath condensate. Journal of Chromatography B 814:173–177.

Tate S, MacGregor G, Davis M, Innes JA, Greening AP. 2002. Airways in cystic fibrosis are acidified: detection by exhaled breath. Thorax 57:926–929.

Vaughan J, Ngamtrakulpanit L, Pajewski TN, Turner R, Nguyen T, Smith A, Urban P, Hom S, Gaston B, Hunt J. 2003. Exhaled breath condensate pH is a robust and reproducible assay of airway acidity. European Respiratory Journal 22:889–894.

Wells K, Vaughan J, Pajewski TN, Hom S, Ngamtrakulpanit L, Smith A, Nguyen A, Turner R, Hunt J. 2005. Exhaled breath condensate pH assays are not influenced by oral ammonia. Thorax 60:27–31.

Wood LG, Gibson PG, Garg ML. 2003. Biomarkers of lipid peroxidation, airway inflammation and asthma. European Respiratory Journal 21:177–186.

Wyse CA, Hammond J, Arteaga A, Cumming D, Cooper JM, McEwan JD, Yam PS. 2004a. Collection and analysis of exhaled breath condensate hydrogen peroxide in conscious healthy dogs. Veterinary Record 155:744–746.

Wyse CA, Preston T, Yam PS, Sutton DG, Christley RM, Hotchkiss JW, Mills CA, Glidle A, Cumming DR, Cooper JM, Love S. 2004b. Current and future uses of breath analysis as a diagnostic tool. Veterinary Record 154:353–360.