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Chronic intestinal *Mycobacteria* infection: discrimination via VOC analysis in exhaled breath and headspace of feces using differential ion mobility spectrometry

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Abstract

Differential ion mobility spectrometry (DMS) is a method to detect volatile organic compounds (VOC) in the ppt range. This study assessed whether VOC analysis using DMS could discriminate subjects with an experimentally induced chronic intestinal infection caused by *Mycobacteria* from non-infected controls. The animal model consisted of two groups of goats orally infected with two different doses of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) and one group of non-infected healthy controls (each group: $n = 6$). Using DMS, exhaled breath and headspace of feces were analyzed on-line on an individual basis 9 months after inoculation of MAP. Data analysis included peak detection, cluster analysis, selection of discriminating VOC features (Mann–Whitney U test), and classification using a support-vector-machine. Taking the background of ambient air conditions into account, VOC analysis of exhaled breath as well as of feces revealed significant differences between chronically infected animals and non-infected controls. In both specimens, increasing as well as decreasing VOC features could be attributed to infection. Discrimination between infected and non-infected animals was sharper analyzing exhaled breath compared to headspace of feces. In exhaled breath, at least two VOC features were found to increase in a dose-dependent manner with increasing doses of MAP inoculated. Results of this study provide strong evidence that DMS analysis of exhaled breath has the potential to become a valuable tool for non-invasive assessment of VOC specifically related to certain diseases or infections.

(Some figures in this article are in colour only in the electronic version)

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1. Introduction

Exploring volatile organic compounds (VOCs) as non-invasive biological markers (biomarkers) of diseases or infections is a research area of growing interest in both human and veterinary medicine. Typical techniques applied to VOC analysis are gas chromatography with mass spectrometry detection (GC-MS), proton transfer reaction mass spectrometry (PTR-MS), selected ion flow tube mass spectrometry (SIFT-MS), laser spectrometry, ion mobility spectrometry (IMS) or differential ion mobility spectrometry (DMS), and sensors or sensor arrays such as electronic nose (e-nose) technologies. The latter techniques (particularly IMS, DMS and e-noses) offer the advantage of being portable and applicable directly at the patient’s side, but do not identify the biochemical origin of the compounds detected. Over the last few years, significant progress has been made in evaluating these different methods—based on the detection and analysis of volatiles present in clinical samples and coupled with multivariate data analysis—for rapid medical diagnoses.

Encouraging data has been reported for VOC analysis in exhaled breath with respect to respiratory and non-respiratory diseases in humans. For example, profiling of exhaled air could distinguish between patients with COPD or asthma, respectively, and healthy control subjects using GC-MS, DMS or e-noses (Dragonieri et al 2007, Fens et al 2009, Kikowatz et al 2009, Dallina et al 2010). Furthermore, several e-nose, IMS, GC-MS and PTR-MS technologies have been successfully exploited to identify patients with lung cancer (Phillips et al 1999, 2007a, 2008, Machado et al 2005, Bajtarevic et al 2009, Dragonieri et al 2009, Ligor et al 2009, Westhoff et al 2009). In addition to a breath test identifying women with breast cancer (Phillips et al 2010), a recent study showed that analyzing exhaled alveolar breath by nanosensors linked to GC-MS was successful in detecting and differentiating lung-, breast-, colorectal- and prostate cancer (Peng et al 2010).

Another area of interest is the detection of infectious agents or infectious diseases by VOC analysis. An e-nose differentiated between different Mycobacterium species—and between Mycobacteria and other pathogens of the respiratory tract—both in culture and in spiked sputum samples (Fend et al 2006). The detection of VOCs from microbes in vitro has been reported for E. coli using multi capillary column coupled IMS (Maddula et al 2010); and for cultures of different Mycobacteria spp. (Syhre and Chambers 2008) as well as Aspergillus and Fusarium spp. using GC-MS (Syhre et al 2008). In vivo, breath tests have been developed offering the possibility of identifying patients with tuberculosis (Phillips et al 2007b, Syhre et al 2009), and progress has been made recently toward a breath test for pulmonary aspergillosis (Chambers et al 2010).

Besides human medicine, a number of veterinary studies—analyzing headspaces of blood samples—contributed significantly to current knowledge about VOC analysis in infectious diseases. E-nose technology discriminated between headspaces of serum samples obtained from cattle and badgers infected with Mycobacterium bovis and non-infected animals (Fend et al 2005). Field studies provided further evidence that cattle suffering from brucellosis (Brucella spp.), paratuberculosis (MAP, Mycobacterium avium subspecies paratuberculosis), and healthy ones could be distinguished based on a different pattern of VOCs present in serum headspaces (Knobloch et al 2009a). In a bovine model of acute pneumonia caused by gram-negative bacteria (Mannheimia spp.), our group was able to demonstrate that headspaces of sera provided significant changes in e-nose responses due to infection (Knobloch et al 2010).

MAP is known to be the causative agent of paratuberculosis (paraTB) or Johne’s disease; a contagious, chronic and sometimes fatal infection that affects primarily the small intestine of ruminants (for example, cattle, sheep, goats and wild ruminants). The etiologic role of this bacterium in Crohn’s disease—a chronic inflammatory disease affecting the intestinal tract of humans—has been controversial for many years and is yet to be defined (Grant 2005, Mendoza et al 2009). ParaTB in the early stages of disease is difficult to diagnose in vivo due to the low sensitivity of established direct (fetal culture, detection of the MAP genome) and indirect (antibody ELISA) diagnostic methods (Köhler et al 2008, McKenna et al 2005). Alternative methods improving the sensitivity of diagnosis of this disease are urgently required.

The rational behind analyzing VOCs in gaseous samples is to identify specific ‘smellprints’ representing the metabolism of the host and predictive for unique biochemical reactions related to certain diseases or infections. In this context, the particular study presented here aimed to evaluate the potential of VOC analysis by DMS to assess potential biomarkers of MAP infection in vivo. Thus, an already existing animal model was exploited supporting the hypothesis that volatile biomarkers measured by DMS allow discrimination between healthy animals and those infected with MAP. Exhaled breath and excrements (headspace of feces) were compared for their strength to express VOC clusters of diagnostic value under conditions of a chronic infectious intestinal disease. In addition, it was tested whether results of VOC analyses differed due to a different severity of infection (i.e. different dosages of MAP inoculated).

This ‘proof of concept study’ has direct implication for both human medicine and veterinary medicine. Independent of any particular mammalian species, this study provides strong evidence that VOC analysis by DMS will become a useful tool in diagnosing infectious diseases.

2. Material and methods

2.1. Animals

Goatlings aged 3–7 days were purchased from a local goatherd free of paraTB (local breed ‘Thüringer Waldziege’). After a quarantine period of 6 weeks to confirm clinically healthy status, animals were included in the study. In the animal facility of the institute, goats were kept under standardized and controlled conditions according to the guidelines for animal welfare. Animals were housed in three groups of six animals, each being separated from the other. They were fed twice
daily with milk replacer until 10 weeks of age and later on with a commercial grower diet without antibiotics. Water and hay were supplied ad libitum.

2.2. Study design

The study had a randomized, negatively controlled design and was performed at biosafety level 2. Ethical approval was obtained from the Commission for the Protection of Animals of the State of Thuringia, Germany (registration number 04-002/08). Two groups of six goats were orally exposed ten times, i.e. every 2–3 days, to 10 mg/day (group ‘paraTB I’) or 20 mg/day (group ‘paraTB II’) of wet weight of a low passage field strain of MAP (strain J1961). Six goats fed with pure milk replacer served as controls.

Clinical observations were recorded twice daily, and included general behavior, feed intake, appetite, rectal temperature, respiratory rate and the presence or absence of diarrhea. Results of clinical examinations were averaged per animal per week. Infection of the MAP-inoculated goats was confirmed by monthly fecal cultures and by necropsy of all animals in 51–52 weeks post-inoculation. Exhaled breath and headspaces of feces were analyzed 41 weeks post-inoculation using DMS.

2.3. DMS methodology

In comparison with other types of ion mobility spectrometry, DMS uses the dependence of ion mobility on electric field strength which enables detection of both positive and negative ions simultaneously. Methodological principles of DMS have been described recently (Kikowatz et al 2009). In this study, the DMS micro-analyzer (SIONEX Corp., Boston, USA, Microanalyzer, Serial No 0149) was used.

A gaseous sample was aspirated into a trap by a pump with a flow of 1.5 cc per second (s). After an aspiration time of 20 s, the inlet closed and the sample was pumped through a multi-capillary column into an ionization chamber using air as carrier gas. Trap and column (both given by the producer) were heated in a special time course to 300 °C (trap) and accordingly 120 °C (column). In the ionization chamber, the sample was ionized by a radioactive 63Ni-source (93 MBq). To identify different ions, an oscillating asymmetric radio frequency (RF) electric field (1000 V) and a direct current (dc) compensation electric field were applied across two parallel plates. The tunable dc-field was superimposed on the oscillating asymmetrical field and kept the ions of interest centered between the plates and thus detectable by electrometers (other ions which got in contact with the plates were neutralized). The dc-field changed stepwise between −20 and +5 V one time per second (100 steps). Consequently, one positive and one negative spectrum between −20 and +5 V were stored per second. Measurement time was settled to 270 s resulting in 540 (270 positive + 270 negative) spectra stored per measurement.

2.4. Analysis of exhaled breath

The individual animal undergoing the test wore a tightly fitting face mask of an appropriate size in relation to the animal’s head (figure 1(a)). Valves for inspiration and expiration ensured that (i) only expired breath was directed into a spacer adapted to the exhalation part, and (ii) approximately 500 mL of expired breath (i.e. volume reservoir) was kept in the spacer during the subsequent inspiration (figure 1(b)). The breathing time for each test was 30 s while the first 10 s of exhaled breath were rejected.

A tube (30 cm length, ID: 1.5 mm, wall thickness 0.25 mm, Teflon) was connected to the inlet of the micro-analyzer. The internal suction pump took a 20 s sample with an aspiration volume of 30 mL (1.5 mL s−1).

The procedure was well tolerated by all animals and the total time taken for exhaled breath analysis did not exceed 5 min per animal. Ambient conditions in the animal house during collection were similar to normal room conditions (ambient temperature: 18–22 °C; ambient relative humidity: 60–65%).

2.5. Analysis of feces

Fecal samples were collected on an individual basis and were stored in screw capped polystyrene vials overnight at 4 °C. Before DMS analysis, fecal samples were warmed in an incubator for 2 h up to 37 °C. DMS analysis was performed in a safety cabinet at room temperature. The vials containing the pre-warmed samples were opened; headspace above the feces was collected for 20 s in the same manner as breath analyses and was analyzed immediately.
2.6. Data analysis

The entire data set consisted of 18 samples for breath analysis (6 for each group) and 16 samples for analysis of feces (4 controls, 6 for each paraTB group). Using DMS, ionization of molecules by a radioactive $^{63}$Ni-source mainly resulted in positive ions (proton transfer). Consequently, the positive spectra of each measurement contained most of the information. Thus, only the positive spectra were used for further analysis.

2.6.1. Pre-processing and peak detection. The most important information of DMS-spectra is contained in signals forming different peaks of different intensity, where each peak represents a specific VOC. Prior to peak detection, all data had been pre-processed. The spectra had been background-adjusted so that baseline value was equalized across all samples. In addition, the spectra had been filtered using a moving average filter to reduce noise of data.

To detect peaks in spectra, local maxima were calculated using 8-neighborhood connectivity. For further analysis, only peaks higher than a threshold of 0.01 for exhaled breath or 0.007 for headspace of feces, respectively, were used in order (i) to reduce dimensionality and (ii) to make sure that no noise-originated peaks were included in further analysis. Group-specific peaks occurred at compensation voltages ($V_c$) of $-7$ and higher. Therefore, the dimensionality of data could be additionally reduced by just including these peaks in further analysis. For breath analysis a total of 408 peaks were detected (figure 2(a)) while in headspace of feces, 763 peaks were found (data not shown).

2.6.2. Feature construction and selection. A main problem in the comparison of different measurements is that the peak position of the same VOC may vary between measurements. To overcome this problem, a cluster analysis over all peak positions was performed to find groups (clusters) of corresponding peaks, assuming that these peaks are representing the same VOC.

Using a hierarchical clustering algorithm, 47 clusters were found for exhaled breath (figure 2(b)) and 68 for headspaces of feces (data not shown). These clusters were used to construct new peak variables (i.e. features), calculated for each measurement as the height of the highest peak in the determined clusters (zero if no peak belonged to the cluster). Because several features did not show normal distribution, the Mann–Whitney U test was used to test significant differences between different groups of animals for each of the features. Based on these results, most important features were selected for exhaled breath as shown in figure 2(c).

2.6.3. Classification. A support-vector-machine was exploited to classify new data sets. Classifying an unknown sample, accuracy of a prediction was estimated using a leave-one-out cross validation. In each round, a single observation from the original data set was used as validation data, and the remaining observations were used to train the support-vector-machine (with linear kernel) using features that showed significant differences between the investigated groups (without the left-out sample). An example of exhaled breath is given in figure 2(c). Accuracy was calculated as the mean correct rate over all rounds (correct rate for one round is either 1 or 0).

3. Results

3.1. Health status of the animals

VOC analyses of exhaled breath and feces took part about 10 months (41 weeks) after inoculation. At this time point,
As shown in table 1, no significant differences were seen between controls and paraTB groups or between the two groups inoculated with different doses of MAP, respectively, with respect to clinical data (body temperature, heart rate, respiratory rate), white blood cells, or body weight (data not shown). Appetite and food intake were normal in groups inoculated with different doses of MAP, respectively, between controls and paraTB groups or between the two groups (table 1; P = 0.06). Due to intermittent mild diarrhea that occurred occasionally, consistency of feces in group paraTB II reached maxima of 0.8 scores (which corresponds to mild abnormalities) compared to scores of 0.1 on the two other groups (table 1).

### 3.2. Lesions characteristic of paratuberculosis postmortem

Animals were euthanized and necropsied 10 weeks after DMS analysis (51–52 weeks after inoculation). All goats of group paraTB I and five out of six goats of group paraTB II had granulomatous lesions characteristic of paratuberculosis.

In group paraTB I, lesions were seen in both intestine and intestinal lymph nodes of five animals and only in intestinal lymph nodes of one goat. Intestinal lesions were mild and mainly restricted to Peyer’s patches in jejunum. *Mycobacteria* were cultured, but not detected by immunohistochemistry in tissues.

In group paraTB II, lesions were present in both intestine and intestinal lymph nodes (three goats) and either in intestine or intestinal lymph nodes (one goat each). Intestinal lesions were more extensive throughout the jejunum than in group paraTB I. *Mycobacteria* were detected by immunohistochemistry in tissues of two goats.

### 3.3. Exhaled breath

The 47 clusters detected in exhaled breath resulted in 47 features that were included in statistical analyses. Taking a probability level of $P \leq 0.05$ into account, 9 features were found to be statistically different between infected and non-infected animals. The 3 features presented in figure 3 (i.e. features 26, 38 and 47) revealed the highest statistically secured discrimination ($P \leq 0.01$). While 2 features were increased in infected goats (figures 3(a) and (b)), 1 feature was found to be decreased in comparison to non-infected controls (figure 3(c)).

Both features that increased in infected animals did so in a dose-dependent manner, resulting in a difference between both infected groups (paraTB I versus paraTB II) that was statistically most impressive for feature 26 ($P < 0.01$, figure 3(a)).

Interestingly, the most outstanding results in exhaled breath analysis of infected animals (i.e. outlier value in group paraTB I+II; figures 3(a) and (c)) belonged to one goat (no 89; group paraTB II). At necropsy, this particular animal was the only one in its group which did not present typical lesions and growth of MAP could not be detected in the gut or in intestinal lymph nodes. Furthermore, this goat had stopped excreting the pathogen 24 weeks before VOC analysis (i.e. 17 weeks after challenge).

### 3.4. Headspace of feces

In headspace of feces, 10 of the 68 features statistically discriminated infected animals from non-infected controls ($P \leq 0.05$), and 5 of them reached a probability level of $P \leq 0.01$. Examples of 3 relevant features (17, 33 and 64) are given in figure 4. While 2 features were significantly reduced in each of the infected groups compared to non-infected controls (figures 4(a) and (b)), 1 feature increased due to infection (figure 4(c)). In feces, no significant difference was observed between both paraTB-groups challenged with different doses of MAP.

Again, outlier values of features 17 and 64 (figures 4(a) and (c)) represent data provided by the only goat that did not develop typical signs of infection (no 89; group paraTB II). The outlier for feature 33 (figure 4(b)), however, represents
controls paraTB I paraTB II paraTB I+II

0.03

0.06

0.09

values of feature 26

P < 0.01

P < 0.01

P < 0.01

P < 0.01

(a)

values of feature 47

P < 0.01

P < 0.05

P < 0.01

P < 0.01

(b)

values of feature 38

P < 0.01

P < 0.01

P < 0.01

P < 0.01

(c)

Figure 3. Most important features detected in exhaled breath of healthy non-infected goats (controls: n = 6) and 12 goats experimentally infected with different doses of MAP (paraTB I: n = 6; paraTB II: n = 6). Box-and-Whisker Plots represent lower quartile, median and upper quartile values (box). Whiskers extend from each end of the box to the adjacent values in the data—by default, the most extreme values within 1.5 times the interquartile range from the ends of the box. Outliers are data with values beyond the ends of the whiskers (+). (a) Feature 26. The corresponding cluster was located at 253 retention time and −1.67 V compensation voltage. (b) Feature 47. The corresponding cluster was located at 148 retention time and −2.66 V compensation voltage. (c) Feature 38. The corresponding cluster was located at 77 retention time and −6.88 V compensation voltage.

Figure 4. Most important features detected in headspace of feces of healthy non-infected goats (controls: n = 4) and 12 goats experimentally infected with different doses of MAP (paraTB I: n = 6; paraTB II: n = 6). Box-and-Whisker Plots represent lower quartile, median and upper quartile values (box). Whiskers extend from each end of the box to the adjacent values in the data—by default, the most extreme values within 1.5 times the interquartile range from the ends of the box. Outliers are data with values beyond the ends of the whiskers (+). (a) Feature 17. The corresponding cluster was located at 88 retention time and −1.90 V compensation voltage. (b) Feature 33. The corresponding cluster was located at 129 retention time and −3.09 V compensation voltage. (c) Feature 64. The corresponding cluster was located at 154 retention time and −1.79 V compensation voltage.

3.5. Prediction model

The results of the prediction for different groups, calculated via the support-vector-machine and leave-one-out cross validation are shown in table 2. The best accuracy of 100% was achieved for exhaled breath testing non-infected controls versus all paraTB-infected animals. Considering the same groups of a different animal (goat no 85). This particular goat was the only one in its group that never showed any abnormalities in feces consistency (despite continuous excretion of the pathogen).
test subjects, headspaces of feces yielded a lesser accuracy of 81%.

Separating the two groups infected with MAP, the accuracy of prediction in comparison to non-infected controls was higher for group paraTB I than for group paraTB II; and this was true for exhaled breath as well as for feces. Testing the two infected groups for the hypothesis that different infectious dosages resulted in differences with respect to VOC analysis, a higher accuracy was obtained for exhaled breath (83%) compared to headspace of feces (67%).

### 4. Discussion

The purpose of this study was (1) to assess DMS technology for its potential to discriminate healthy animals from those experimentally infected with *Mycobacteria*, (2) to compare VOC signals in exhaled breath and feces for their ability to reflect a chronic intestinal inflammatory disease, and (3) to evaluate the sensitivity of VOC analysis due to the severity of disease or the infectious load. Results of this animal study clearly showed that VOC analysis of both exhaled breath as well as feces revealed significant differences between healthy individuals and those chronically infected. Furthermore, DMS results of exhaled breath revealed dose-dependent courses of different VOC features due to increasing infection dosage.

Although information is based on a single domestic animal model, it contributes to the general understanding in biomarker research based on VOC analysis. Domestic animal models like the one exploited in this study offer a unique opportunity to evaluate diagnostic methods and techniques (usually produced for and applied to humans) under controlled and standardized *in vivo* conditions that can never be reached in patients. Besides the fact that experimentally induced chronic mycobacterial infection will never become possible in human subjects for ethical reasons, controlled conditions as achieved in this study can hardly be guaranteed in clinical trials. For example, cohorts of animals included in this study represented homogeneous groups with respect to sex, age, nutrition and ambient conditions. Furthermore, no confounding influences deriving from smoking, alcohol, drugs, co-infections or additional diseases requiring therapeutic substances or influencing metabolism were present.

Summarizing the level of standardization guaranteed in this study, the variability of results was most likely much lower compared to the results gained from studies in human medicine or from field studies in veterinary medicine. In so far, the relatively small number of animals included (*n* = 6 per group) has not been considered as a limitation factor. Further unique advantages of this study were that (1) direct correlation of postmortem findings with *in vivo* measurements performed previously was possible, and (2) presence of the pathogen (MAP) in feces and in organs after necropsy was evaluated in parallel.

#### 4.1. DMS analyses and Mycobacteria infection

MAP was used as a prototype of bacteria causing chronic-persistent infection in its mammalian host with an unusual long incubation period of months or even years without the presence of obvious clinical signs. In this incubation period, host–pathogen interactions already occur that may result in the production of substances that can be analyzed from the host and used as indicators of a certain biologic state (biomarkers). In the study presented here, we were able to show that VOC analysis had the power to discriminate MAP-infected individuals from healthy ones already in the 9th month of incubation period. At this time point, marked clinical illness was still lacking in most of the animals. This result is encouraging because it offers time advantage in exploring VOC analysis for the detection of infectious diseases prior to clinical signs (early diagnosis).

Among the huge amount of available data and clusters provided by DMS, several VOC features allowed discrimination between infected and non-infected animals. However, similar to e-nose technology (Knobloch *et al* 2009b), DMS does not allow identification of the biochemical background. In order to identify discriminating compounds biochemically, additional analytical techniques need to be implemented in further studies.

Whether one single biochemical component will emerge as a discriminating biomarker is yet to be defined. Due to experience in other medical fields—specifically breast cancer (Phillips *et al* 2010)—it is likely that a combination of volatile substances need to be employed in a multivariate algorithm to develop diagnostic test systems with acceptable sensitivity and specificity.

With respect to the biological background, differentiating features may result from the metabolism of the pathogen, the metabolism of host, or a combination of both. *In vitro* studies

<table>
<thead>
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<th>Table 2. Accuracy of prediction when classifying an unknown sample.</th>
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<tr>
<td><strong>Prediction based on exhaled breath spectra</strong></td>
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<td>(using the three features with the most significant differences)</td>
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<td><strong>Test</strong></td>
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<tr>
<td><strong>Accuracy</strong></td>
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<tr>
<td><strong>Prediction based on spectra of the headspace of feces</strong></td>
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<tr>
<td>(using the three features with the most significant differences)</td>
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<td><strong>Test</strong></td>
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<td><strong>Accuracy</strong></td>
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have shown that different bacteria provide different gaseous spectra based on combinations of numerous volatile substances (Pavlou et al 2004, Schulz and Dickschat 2007). Recently, first proof of concept has been provided for Mycobacterium tuberculosis—the causative agent of human tuberculosis (TB)—indicating that volatile metabolites produced by the bacteria in vitro (specifically methyl nicotinate) can be detected subsequently in exhaled breath of TB infected patients (Syhre et al 2009).

Based on the results presented here, the question whether VOC features that discriminate infected from non-infected animals indicate the presence of the pathogen and/or result from host–pathogen interactions cannot be answered. In a previous study performed by our group, VOC patterns detected by e-nose responses in experimentally infected calves correlated significantly with markers of acute phase reaction of the host (Knobloch et al 2010).

Pathologic processes have the potential to influence VOCs either by producing new volatile substances or by the metabolic consumption of VOC substrates that are normally present (Probert et al 2009). Consequently, the diagnostic potential of VOC analysis includes at least two aspects: the search for biomarkers produced and the search for biomarkers lost. In this study, we could not identify any VOC feature present in MAP-infected groups which otherwise is completely absent in healthy controls. Vice versa, some VOCs present in healthy non-infected animals were significantly reduced or even absent in infected groups. A marked reduction in detectable VOCs was true for exhaled breath as well as for headspace of feces, and in strong agreement with findings published recently for headspace of blood serum samples from infected calves (Knobloch et al 2010) and feces from preterm children suffering from necrotizing enterocolitis (Garner et al 2009).

In summary, results of this study indicate that VOC analysis offers potential to develop rapid and non-invasive tests assessing biomarkers of MAP infection in vivo.

4.2. DMS analyses of different biological matrices (exhaled breath versus feces)

In the literature, VOC analyses have been described for different biological specimens (for example, exhaled breath, headspaces of blood, urine, stool or feces), but data comparing different samples of the same subject is lacking. This study compared VOC results obtained from exhaled breath and feces with respect to their potential to reflect an intestinal infection.

The rationale for testing headspaces of feces was based on the common assumption that abnormality in the activity and/or composition of intestinal microbiota may alter the odor of feces. Consequently, examination of volatile fecal emission could be a very useful non-invasive way of diagnosing diseases of the gastro-intestinal tract. In support of this hypothesis, studies from human medicine already provided evidence that volatile patterns from feces differed significantly between healthy donors and patients with gastrointestinal diseases (ulcerative colitis) or infections (Clostridium difficile, Campylobacter jejuni) (Garner et al 2007). Furthermore, feces of chicken with and without Campylobacter jejuni differed significantly including six of the extracted VOCs from their feces in further mathematical analysis (Garner et al 2008).

The rationale for analyzing exhaled breath in a model of chronic intestinal infection was based on the hypothesis that exhaled breath—primarily the product of alveolar gas exchange and airway water loss—does not only contain substances originating directly from the airways and the lung, but also contains metabolites released via the lung. Almost 40 years ago, the first modern breath analysis using gas liquid partition chromatography quantitatively determined about 250 different volatile substances in a sample of exhaled breath (Pauling et al 1971). Based on the hypothesis that blood born substances in exhaled breath might provide comprehensive information about the metabolic state of the subject (as known for fetor hepaticus in liver failure, uremic smell in kidney failure, or diabetic ketoacidosis), potential biomarkers of various systemic or local diseases are exhaled and possibly may provide so-called breathprints typical for a certain disease.

Under conditions of this study focusing on an intestinal infection, both kinds of specimens (exhaled breath and feces) were suitable biological matrices presenting increasing as well as decreasing VOC features related to MAP infection. With respect to sensitivity, exhaled breath allowed assessment of disease activity by separating mild infected animals (lower dosage of MAP inoculated; paraTB I) from heavily infected ones (higher dosage of MAP inoculated; paraTB II) statistically while in headspaces of feces no significant differences were found between both paraTB groups. To the best of our knowledge, this is the first report about dose-dependent effects of VOC analysis in an infectious disease.

Results of this study further imply that VOC analysis in exhaled breath might be superior compared to VOC analysis in headspace of feces even for intestinal diseases. This hypothesis has to be verified by future studies including larger group sizes and various conditions related to different diseases or infections.

4.3. Accuracy of DMS analyses

Discriminating healthy goats from paraTB goats using the combination of the three features proving the strongest significant differences, a 100% correct classification was obtained for exhaled breath (while accuracy of prediction was only 81% for headspace of feces). Using a total of eight components detected in exhaled air by GC-MS, discrimination between asthmatic and healthy children was possible with an accuracy of 92% (Dallinga et al 2010). Using e-nose, breathprints from patients with asthma were separated from patients with COPD (accuracy 96%), from nonsmoking control subjects (accuracy 95%), and from smoking control subjects (accuracy 92.5%) (Fens et al 2009). Consequently, the level of accuracy obtained from this animal model is superior compared to data published from clinical studies in humans which confirms the need of most standardized conditions as long as a new technique is undergoing validation.

By exhaled breath analysis, goats with low-dose infection (paraTB I) could be distinguished from non-infected ones to 100% indicating that this group of infected animals was more...
homogeneous compared to group paraTB II in which outlier data reduced accuracy to 92%. After looking into the details, outlier data in DMS analysis of exhaled breath were attributed to one single animal (no 89) that did not acquire infection after inoculation of MAP. Following this, outliers closed to the range of healthy controls (feature 38; figure 3(c)), are logically interpretable. In contrast, outliers in feature 26 (figure 3(a)) are far away from control data and even exceeded DMS data of infected animals. Whether this indicates a pattern that might have been protective can only be speculated. However, there is already evidence in the literature that VOC analysis might have the potential to identify individuals at risk of developing a certain disease (Garner et al. 2009).

In feces, outliers of feature 17 (figure 4(a)) and feature 64 (figure 4(c)) again reflected goat no 89—the only animal that did not get infected. A second animal (no 85) of group paraTB II presented one remarkable outlier in VOC analysis of feces (feature 33; figure 4(b)) but not in exhaled breath analysis. Interestingly, this animal was clearly infected from the microbiological point of view (excreting MAP and developing antibodies) as well as from the pathological point of view (pathologic lesions at necropsy), but never showed any abnormalities in feces consistency. Being aware that individual observations do not allow the drawing of final conclusions, they provide hypotheses of what kind of detailed information might be obtained from VOC analyses.

4.4. Conclusions and future directions

DMS technology was found to be suitable for breath analysis in large animals as well as for analyzing the smell of feces. The characteristic VOC pattern in both biological matrices discriminated healthy from infected goats. It is yet to be defined whether the features presented by VOC analysis in this study are specific for this particular infectious disease (paratuberculosis), the infectious agent (Mycobacteria spp. or particularly MAP), or whether they are linked to the host response. Thus, further in vitro and in vivo studies including additional analytical techniques are necessary to identify and to compare VOCs released by MAP and other Mycobacteria in bacterial cultures as well as VOCs or the VOC pattern presented by infected hosts.

The vision of future is an immediate or in-time detection of volatile biomarkers to assess the presence and/or the severity of different infectious diseases as early as possible. Specific and sensitive ‘breathprints’ would be ideal and are of particular interest because collection of exhaled breath is completely non-invasive, repeatable and does not necessarily require patient cooperation—only spontaneous breathing. Since exhaled breath collection is easily applicable to all mammalian species (humans, large and small animals), the development of breath tests for disease diagnosis is an exciting goal and promising target for both human and veterinary medicine.

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