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SID 5 Research Project Final Report

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Project identification

1. Defra Project code: SE3221
2. Project title: Volatile organic compound analysis for the rapid diagnosis of disease: TB in badgers and cattle as proof of principle
3. Contractor organisation(s): Veterinary Laboratories Agency
   Woodham Lane
   New Haw
   Addlestone
   Surrey
   KT15 3NB
4. Total Defra project costs (agreed fixed price): £ 457,390
5. Project: start date .... 01 January 2006
   end date .... 30 June 2009

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Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

Background and approach

Culture of *Mycobacterium bovis* from clinical specimens obtained from the live animal is an insensitive and inappropriate method for the diagnosis of tuberculosis (TB) in cattle and badgers. We therefore undertook to explore the diagnosis of TB in animals, based on a holistic approach to the problem. Recently, significant progress has been made in developing tests for rapid diagnosis of disease based on the detection and analysis of volatile organic compounds (VOC) present in clinical samples, such as blood, urine and breath. The principle behind the approach is that clinical specimens from an infected individual produce a volatile chemical signature that is distinguishable from that obtained from uninfected individuals. The inherent advantages of this approach are that multiple markers of infection are examined simultaneously without needing prior knowledge of the underlying biology.

We wished to see if we could build on our earlier proof of principle that it was possible to differentiate badgers and cattle with TB from healthy controls by analysing the volatiles present in serum using an electronic nose (eNose). The advantages of the eNose are its small size, affordability, and ability to describe the sensed 'odour' as a multivariate dataset; thereby allowing powerful mathematical discriminatory methods to be used in the diagnosis of disease. One potential drawback of using an eNose for diagnosis is that the components of the odour signature are hard to dissect. Whilst this is of no consequence for diagnosis *per se*, identification of the individual components could lead to refined diagnostic tools and a greater understanding of host-mycobacterium interactions.

We also evaluated a new tool, SIFT-MS (selective ion flow tube mass spectrometry) that has been demonstrated to be able to monitor very low levels of trace gases in breath. By this approach, several molecules have been shown to be associated with disease states in humans. Compared to the eNose, SIFT-MS is currently a larger and more expensive device, but individual volatile compounds can be identified and quantified and the method is much more sensitive.

Gas chromatography-mass spectrometry (GC-MS) was also used in this project to analyse breath samples specifically. GC-MS is a very sensitive technique; that plus its large library of more than 100,000 compounds made it well suited to the analysis of volatile compounds in breath that might be associated with TB.

Samples for analysis from TB-infected badgers (serum) and cattle (serum, urine, breath) were provided from a variety of Defra-funded projects. Additional bovine serum samples from other disease settings were provided by collaborators at the Friedrich Loeffler Institute, Germany.

Objectives

01 Build a close-fitting moulded device for the collection of all exhalations by mouth and nostrils from cattle and determine if bag samples of breath can be correlated reliably to fresh samples.

02 Obtain samples from cattle and badgers for analysis and identify and validate a safe method with which to remove breath samples from TB infected cattle from the laboratory for analysis.

03 Determine the feasibility of using VOC analysis for the reliable detection of *M. bovis* infection and detail any compounds that appear to be consistently associated with TB.

Summary of findings and conclusions

1. Data generated by the eNose demonstrated that there were discernible changes to VOC liberated by serum and urine from cattle for a range of infections, including TB, Brucellosis, paraTB, and *Mannheimia haemolytica*. These differences only become apparent at the group level and not at the individual animal level. This was almost certainly a consequence of the considerable methodological variation associated with eNoses; a fact we discovered and published during the life of this project. This was true for two fundamentally different types of eNose. Considerable effort was invested in developing a methodological approach to minimise these differences. At present, current eNose technology holds little promise for diagnosis of TB (or other bovine diseases), although proof of principle was shown that there are different patterns of the VOC brought about by infection using eNose technology.
2. The SIFT-MS data confirmed this principle and allowed the identity of some disease related markers. The data this method generated were very complex. A mathematical modelling approach (PLSDA) proved the best method to analyse the data, although certain common compounds present in large amounts in all samples meant that the data had to be 'cleaned' extensively before analysis was meaningful. Analysis of serum from wild badgers revealed the potential of the approach. However, projections suggested that samples sizes in excess of 150 (preferably equally divided between TB positive and negative) would be needed to build a PLSDA model of approx. 80% accuracy. This number of samples will be hard to obtain and costly to analyse. On the other hand, the samples used in this study were not taken with VOC analysis in mind and contained sources of variation that could be minimised or eliminated with a defined methodology, hence this number could possibly be reduced. The data generated using the badger sera indicated a possible link between increased serum concentration of ammonia and TB. This is worth investigating further with specific sensors for ammonia.
3. A reliable and safe method was developed to obtain breath samples from experimentally infected cattle. The method could be applied to cattle elsewhere if the animal is adequately restrained (e.g. by using a crush). Due to the health risks posed by breath from infected cattle, the only safe method for handling breath samples was after immobilisation on trap-columns, followed by heat-treatment. Breath volatiles were therefore only amenable to analysis by GC-MS. Using this approach a few potential biomarkers associated with TB were identified. However, their concentration was extremely low and probably too low to make them useful as practical disease markers.
4. The sum of the data from all three methods suggests that disease is most likely associated with changes in concentrations of host volatiles and not the emergence of unique markers. If this is the case, a pattern recognition method using a more robust electronic nose, or SIFT-MS may be used but under these circumstances, the method may only be suitable as a screening tool.

Potential for future work

A few possible areas for continued work have been identified as a result of this project, as follows:

1. The hypothesis that serum concentrations of ammonia are increased in badger TB could be tested.
2. Similarly, there is potential in evaluating a sensitive gas monitor of ammonia to analyse the breath of anaesthetised badgers (and possibly cattle) with/without TB infection.
3. There is potential in continuing VOC analysis in the context of *Brucella* and paraTB infections infections and *Mannheimia heamolytica*.
4. There is application of methods deployed in this project to VOC analysis in the context of human TB.

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
   - the scientific objectives as set out in the contract;
   - the extent to which the objectives set out in the contract have been met;
   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and
   - any action resulting from the research (e.g. IP, Knowledge Transfer).

Objectives

01 Build a close-fitting moulded device for the collection of all exhalations by mouth and nostrils from cattle and determine if bag samples of breath can be correlated reliably to fresh samples (by 30/11/2006).

02 Obtain samples from cattle and badgers for analysis and identify and validate a safe method with which to remove breath samples from TB infected cattle from the CL3 laboratory for analysis (by 29/08/2008).

03 Determine the feasibility of using VOC analysis for the reliable detection of *M. bovis* infection and detail any compounds that appear to be consistently associated with TB (by 31/03/2009).

Background

Culture of *M. bovis* from clinical specimens obtained from the live animal is an insensitive and inappropriate method for the diagnosis of tuberculosis (TB) in cattle and badgers. For this reason, the most popular approach to the diagnosis of tuberculosis in the live animal relies on tests of the immune response of the host to infection. The principle behind these immunodiagnostic tests is that the host immune response is an amplified readout, signalling encounter with *M. bovis*. When expressed in these terms it is apparent why immunological assays sometimes lack both sensitivity and specificity. Furthermore, all such assays have to be underpinned by a fundamental understanding of the host immune response to infection, which in the case of tuberculosis, is incomplete.

These issues led us to explore the diagnosis of tuberculosis in animals, based on a holistic approach to the problem. Recently, significant progress has been made in developing tests for rapid diagnosis of disease based on the detection and analysis of volatiles present in clinical samples, such as blood, urine and breath [1]. The principle behind the approach is that clinical specimens from an infected individual produce a volatile chemical signature that is distinguishable from that obtained from uninfected individuals. The inherent advantages of this approach are that multiple markers of infection (not just immunological) are examined simultaneously without needing prior knowledge of the underlying biology.

We wished to see if we could build on our earlier proof of principle that it was possible to differentiate badgers and cattle with tuberculosis from healthy controls by analysing the volatiles present in serum using an electronic nose (eNose) [2]. The advantages of the eNose are its small size, affordability, and ability to describe the sensed 'odour' as a multivariate dataset; thereby allowing powerful mathematical discriminatory methods to be used in the diagnosis of disease. One potential drawback of using an eNose for diagnosis is that the components of the odour signature are hard to dissect. Whilst this is of no consequence for diagnosis *per se*, identification of the individual components could lead to refined diagnostic tools and a greater understanding of host-mycobacterium interactions.

We have also evaluated a new tool, SIFT-MS (selective ion flow tube mass spectrometry) that has been demonstrated to be able to monitor very low levels (a few ppb) of trace gases in breath. By this approach, several molecules have been shown to be associated with disease states in humans [3-5]. Compared to the eNose, SIFT-MS is currently a larger and more expensive device, but unlike the eNose, is able to generate a mass/charge spectrum of the sensed sample; thereby allowing individual volatile compounds to be identified and quantified. Both approaches are complementary, and each can use the same clinical sample to generate a result, as was this case in this project.

Gas chromatography-mass spectrometry (GC-MS) was also used in this project to analyse breath samples specifically. As described in detail later, GC-MS was the technique chosen because breath samples could be collected using thermal desorption tubes relatively easily within an ACDP Containment Level 3 (CL3) laboratory at the VLA, and the thermal desorption tubes decontaminated without affecting sample quality. This then enabled the tubes to be sent to Cranfield University for analysis. GC-MS is a very sensitive technique; that plus its large library of more than 100,000 compounds made it well suited to the analysis of volatile compounds in breath that might be associated with TB.

The bulk of the data presented in this report were generated by staff at Cranfield University, together with their analysis. Samples from TB-infected badgers and cattle were provided from a variety of Defra-funded projects by staff at the VLA. Additional bovine samples from other disease settings were provided by collaborators from the Institute of Molecular Pathogenesis at the 'Friedrich–Loeffler–Institut' (FLI; Federal Research Institute of Animal Health), Jena, Germany. The project was managed by Dr Mark A Chambers of the VLA. A project board comprising of the following people met three times a year to discuss results, strategy, and progress with the project.

SE3221 Project Board

Name	Affiliation	Role in project
Mark A Chambers	VLA	Project manager & provision of samples from badgers
Claire Turner	Cranfield University	Responsible for all work conducted at Cranfield University; generating data from SIFT-MS and GC-MS
Henri Knobloch, PhD student	Cranfield University	Generating eNose data, sample management and sample analysis using eNose and SIFT-MS, developing sampling methodology for eNose and parallel SIFT-MS analysis, developing alternative methods for eNose data analysis (combination of uni- and multivariate)
Andrew Spooner, PhD student	Cranfield University	Developing & conducting methods for the analysis of

		SIFT data
Martin Vordermeier & Adam Whelan	VLA	Providing samples from cattle and advice on biological interpretation of data
Heike Koehler	FLI, Germany	Providing samples from cattle and advice on biological interpretation of data
Petra Reinhold	FLI, Germany	Providing samples from cattle and advice on animal physiology and biological interpretation of data
John Richards, Private Contractor	Solutions for Research Ltd	Design, development, and build of a cattle nasal breath collection device
David Marlin, Private Contractor (formerly of the Animal Heath Trust)	David Marlin Consulting Ltd	Advice on animal physiology & practical assistance with evaluation of the nasal breath collection device
Toby Mottram, Private Contractor (formerly of the Silsoe Research Institute)	SilsoeResearch.org.uk	Initial design of a cattle nasal breath collection device and practical assistance with its evaluation
Ruth McNerney	London School of Hygiene & Tropical Medicine	Advice on use VOC methods for detection of TB (based on experiences from human TB)
Nicola Commander	VLA	Supply of samples from *Brucella* infected cattle and controls
Anthony Woodman	Formerly of Cranfield University	Initial project development, PhD staff recruitment and supervision

Methodology and Results

1. Optimisation of methods for generating volatiles from serum samples

Two methods were compared for the generation of volatiles from serum: a **static method**, whereby serum is placed in a Nalophan (low volatile, polyethylene-terephthalate material) bag, filled with bottled hydrocarbon free air, and then volatiles drawn off from the bag into the eNose followed by the SIFT-MS; and a **dynamic method** similar to the one published previously by VLA and Cranfield University [2], whereby volatiles are generated by drawing air from an external source through the serum sample and into the eNose and SIFT-MS. Data generated from a comparison of both methods supported the use of the static method over the dynamic. Signal intensity in the eNose diminished during sampling time using the dynamic method suggesting that volatiles were being depleted faster than they can be replaced from the sample. This was not observed with the static method, which by nature is closed to an external atmosphere. One of the variables identified that impacted results achieved with the eNose was the air flow rate across the sensor head, which could be affected significantly by the placement of filters or other inhibitors to the flow. Filters also led to retention of specific molecules and changed the VOC profile significantly which confirmed using SIFT-MS. These issues were more prevalent with the dynamic sampling method. Furthermore, the sensor response of the eNose was found to be influenced by temperature (both sample and ambient). The temperature dependence was pronounced, both in terms of the theoretical predictions of Henry's law (partition of a gas in liquid and vapour phase at different temperatures), and the actual response of the sensors. In general, an increase in sample temperature gave increased signal intensity.

1.1. Methodological variation using eNoses

Following the generation of preliminary data using a BH214 eNose, it became rapidly apparent that any biological differences between samples were being masked by intrinsic variations in the response of the eNose, which was shown to be critically sensitive to a number of variables such as temperature, sensor drift over time, and flow-rate. Early in the project, it became clear that many of these variables would need to be strictly optimised and controlled in order to realise any discriminatory power of the eNose. Considerable work over the first two years of the project focussed in this area.

1.1.1. Impact of flow-rate on BH214 eNose

Since the BH214 eNose doesn't have a flow adjusted pump, different flow rates may be applied, mainly according to the sampling method, resulting in different numbers of headspace molecules being monitored over time, thereby affecting reproducibility. Accurate control of the air flow (sample headspace) is crucial for reproducible and reliable headspace analysis. Flow rates were analysed, applying static sampling with two "identical" BH214 eNoses. In order to measure the flow, a mass flow meter sensor (Honeywell AWM 3300V) was used. The flow meter was connected to a 9V battery via a home made protection circuit. For calibration, the flow meter was connected to a zero grade air gas bottle with an adjustable valve and a mass flow regulator (ADM 1000; J&W Scientific). Adjusting different mass flows going through the Honeywell flow meter, the voltage was measured. In a second step the flow meter was attached to the eNose and headspace volatiles from serum in bags were analysed as usual using the calibration curve (static sampling - see SID4, 2006 for details). The number of replicates was 10; the analysis time was 30 seconds per replicate.

Results:

- eNose 1 had a significantly lower air flow in comparison to eNose 2 (14% higher).
- Nevertheless, both devices provided constant flows over six replicates before the flow started to drop which guarantees no disturbing influence in terms of flow changes across the analysed replicates.
- The first replicate showed some variance which may be due to inertia.
- Recalculating the volume which was analysed almost identical values were obtained (573mL and 574 mL).

Further studies revealed that dynamic sampling caused large variations in flow (up to 50%) and that the maximum flow rate was only about 80 to 90% of the flow using static sampling.

Conclusion: using static sampling, constant flow rates could be maintained over six replicates before the flow started to drop. Therefore, static sampling was adopted as the method for all headspace analysis using eNose. Only the third to sixth replicates can be used for data analysis; the first two should be removed from analysis as they are not representative of the sample due to dead-space; the last of seven is removed due to declining flow rates.

1.1.2. Impact of sample bag size on data capture by BH214 eNose

Identical serum samples were placed in small (0.75mL) and large bags (3.5L). After incubation (25°C, 15 mins) both bags were analysed every two hours, four times beginning at 10:00. The serum volume in the small bag was 0.9mL; the volume in the large bag was 5mL. The sample volume to bag volume ratios were 1.2E-03 and 1.4E-03 respectively. The number of replicates per run was seven with a 20 second adsorption time per replicate. The first

two and the last replicate were omitted for the reasons shown at 1.1.1. Values were averaged concerning time and/or bags. The same data were generated for the second BH214 eNose.

Results:

- Data from both eNoses revealed a repetitive and systematic phenomena of a sine shaped development of sensor responses across the day. This is possibly due to semi- reversible adsorption which changes the values and the characteristics of the sensors across the day.
- The variation in sensor response (change in divergence) was comparably low. However, some sensors varied more than others across the day. The reason for this remains unclear.
- Small bags gave more negative values which equate to a bigger change in divergence on all sensors on both machines, although the differences between large and small bags were modest.
- Compared to the first eNose, the second machine gave lower variation across replicates but greater variation of some sensors across time points.
- Most significantly, the pattern in sensor response was significantly different between both machines although they were from the same company and manufactured in the same batch.

Conclusion: using small bags was preferable to using large, and required considerably less sample volume. Whilst the two eNoses performed similarly in some respects, the difference in sensor pattern response between two supposedly identical machines showed that the results obtained on one machine are not in any way comparable to the results obtained with another.

1.1.3. Use of alternative MOSFET/MOS eNose (NST)

The Bloodhound BH214 eNose operates on the principle of conducting polymer sensor technology. To see if the variations between machines was a feature of the sensor technology used, a different type of eNose was evaluated using metal oxide semiconductor/field-effect transistor (MOS/MOSFET) sensor technology - NST Lab Emission Analyser (Nordic Sensor Technologies, Sweden). In these eNoses, ten metal oxide semi-conducting field effect transistors (MOSFET), twelve MOS sensors and a humidity sensor are housed. Temperature sensors check the temperature in the sampling carousel which contains the samples in an isolated environment, and also the sensor temperature since MOSFET sensors require a high operating temperature (140°C - 170°C). Serum from one clinically healthy cow was used (FLI, Jena) to investigate a number of variables.

Influence of sample volume and repeatability

Different volumes of serum (50µL to 500µL) were dispensed twice into 30mL vials which were sealed with a septum and a lid.

- Differing sample volume had no influence on the results. The sample volume to vial volume ratio was comparable to static sampling using the BH214 eNose (1.67E-3 to 1.67E-2).
- Sampling the same vial again caused significantly lower responses compared to the original values.

Comparison of two NST machines

As for the BH214 eNoses, two similar NST eNoses were compared. Serum (200µL) was dispensed into three vials at four time points for both eNoses.

- The two MOSFET/MOS eNoses were also not comparable concerning the sensor patterns they generated. This was in relation to the signal intensity as well as the pattern of the signals.
- Variation across time differed for different sensors. While some sensors remained constant others varied considerably.
- Generally, the MOS sensors varied more than the MOSFET sensors.
- Sensors of the same class (MOS) revealed different sensor responses across time.

Conclusion: there was nothing to be gained in using eNoses based on alternative (MOSFET/MOS) sensing technology. A similar lack of comparability between machines was apparent. Use of the BH214 eNose was preferable to the NST. The former makes use of a static sampling method which allows simultaneous sampling with the SIFT-MS. This was not possible with the dynamic sampling method used for the NST eNose.

1.2. Methodological and biological variation in BH214 (CP-type) eNose data using serum from TB-free cattle

Since we found that significant variability existed in the data generated from serum samples using eNoses due to methodological variation largely beyond our control, we needed to establish the extent of the biological variation in samples from cattle. On the basis of such data it would be possible to understand the challenges of using eNose technology to discriminate TB infected from uninfected individuals against all background variation. With this objective in mind, we analysed ~900 serum samples obtained from TB-free cattle. These were supplied by the FLI, Germany and supplemented with sera from the *Brucella* national testing archive at VLA (Table 1).

The samples were split into two groups. The first were exclusively clinically healthy, and the second contained samples from animals with other non-tuberculous infections (see Table 1). Methodological variables studied were:

temperature; sample set analysed; sequence of analysis; eNose replicates. Biological variables studied were: health/disease status; animal ID, sample analysed.

The three cows (study 4, Table 1) were held under standardised conditions but fed a different diet depending on age: milk replacement, hay & oats (weeks 1-4); oats and hay (weeks 5-7); hay & groats (weeks 8-20). Serum was analysed three times (sets A to C) in a different randomised order using a BH214 eNose. Thirty samples were analysed each day. The time and temperature were recorded. The eNose data were analysed using linear regression and multifactor ANOVA. Multiple analyses were conducted but only the most pertinent results are reported here.

Results:

Methodological

- Significant day to day variation in results and also between the sequence of analysis.
- No variation across replicates of the same sample.
- Two strategies for data analysis found to minimise the methodological variation as far as possible.

Biological

- Variation across time for all animals (age dependent?). Greatest variation coincident with change in diet but could also be influenced by sexual maturation.
- Very similar patterns in sensor response between individuals.

Conclusions: the use of carefully standardised samples from cattle housed under identical conditions allowed the identification of a strategy for data analysis that minimised the methodological variation as far as possible. This was to randomise the order in which the samples were processed by the eNose, and use groups / subsets of the overall data rather than single values for testing statistically. Biological variation was observed as animals matured. This is unavoidable. Any changes caused by disease would be superimposed on this.

As a consequence of this work, it became clear that the methodological variation inherent with the eNose method masked any biological variation between individual samples. This essentially means that for the eNoses used in this project, diagnosis at the level of the individual animal would never be possible. A full presentation of the methodological variation found with the eNoses used in this project has been published in Sensors and Actuators B journal [6].

Table 1 List of samples supplied by the FLI, Germany (a.i. = ante infection, p.i = post infection)

Study	Total number	description	Aim
1	144	**6 healthy calves:** 8 samples per day and calf (two-hour intervals; 6:00 – 20:00) 3 repeated days per calf	Circadian variability & inter-subject variability
2	329	**20 calves:** experimental infection with *Mannheimia haemolytica* a.i.: 5 samples per calf (-28, -21, -14, -7, 0 days); p.i.: +3h, +6h, +12h, +24h, +48h, +3d, +4d, +5d (*not all samples in all animals because of spontaneous deaths at different time points*)	Specificity; respiratory infection caused by *Mannheimia* & with severe clinical symptoms
3	237	**30 calves:** experimental infection with *Mycoplasma bovis* a.i.: 2 samples per calf (-11, -5 days) p.i.: 3, 7, 10, 14, 17, 21, 35 days (*not all samples in all animals because of necropsies at different time points*)	Specificity; respiratory infection caused by *Mycoplasma* & clinically latent
4	60 (2 week interval)	**3 healthy cattle** Sampling started when animals were aged a few weeks and continued in 2-week-intervals	Influence of growth, changes in nutrition, and ambient conditions & comparison between species

2. eNose studies using serum from TB-infected badgers and cattle

2.1. Badgers

Approximately 250 serum samples from wild badgers and 14 serum samples from captive badgers (11 experimentally infected with *M. bovis* plus 3 uninfected controls) were analysed with eNose. The samples were obtained from the Randomised Badger Culling Trial (wild, naturally-infected badgers) and collaborative work performed in the Republic of Ireland by staff at University College Dublin (captive, experimentally-infected badgers). For each animal, corresponding data were available for interferon-gamma (IFNγ) ELISA, post mortem examination, and detailed culture.

Analysis of the badger eNose data proved complex and was undertaken by a bioinformatics PhD student under the supervision of Dr Conrad Bessant, Bioinformatics expert at Cranfield University. Different multivariate methods were evaluated to examine the data. Data from all other studies were analysed by the PhD student who executed the sample analysis. Divergence of the eNose data (i.e. maximum sensor response amplitude) was the only valid parameter identified of use for analysis. Due to the variability of the data obtained from experimentally infected badgers, there were too few such samples to analyse adequately. Considerably more samples were available from wild badgers but data from these showed even larger variations, most likely due to their uncontrolled diet and environment, which were thought to have a major impact on the volatiles present. Due to the variability of the data, it was not possible to find any "clustering" of samples, and it was not possible to tell the difference between TB positive and TB negative samples, despite previously promising results in this respect [2]. Analysis of the badger samples was made harder because of the ambiguous status of IFNγ positive, culture negative animals.

Due to the natural variation in wild badgers and the inherent uncertainty associated with their TB status, only those badgers culture positive (definitive TB-positive) and both culture negative, IFNγ negative (TB-negative) were included for analysis. As the day to day variation in data from the BH214 was considerable, only samples analysed on the same day were included. In this way it was hoped to maximise the discriminatory power of the analysis, as we were merely seeking proof of principle at this stage.

Initially, eNose data from seven culture positive, IFNγ positive badgers and seven culture negative, IFNγ negative badgers were used for analysis. The first observation was the drift in response seen over replicates. As the most significant difference between replicates was found between the first two and the last five, the first and second replicates were excluded from the classification. Even then, PCA was unable to classify the samples based on TB status. The positive and negative cases were mixed together. One possible reason was that any separation seen was caused simply by drift of the eNose instrument. To test for this, one of the samples was removed and then the replicates of this sample placed back into the PCA. The drift over the replicates was large for most of the sensors.

Conclusion: the lack of repeatability caused by sensor drift over time meant that PCA was unsuitable as a routine means to analysing data from the BH214 eNose. As sensor drift is an inherent property of the eNose, alternative methods of data analysis would be needed if the eNose was to be discriminatory.

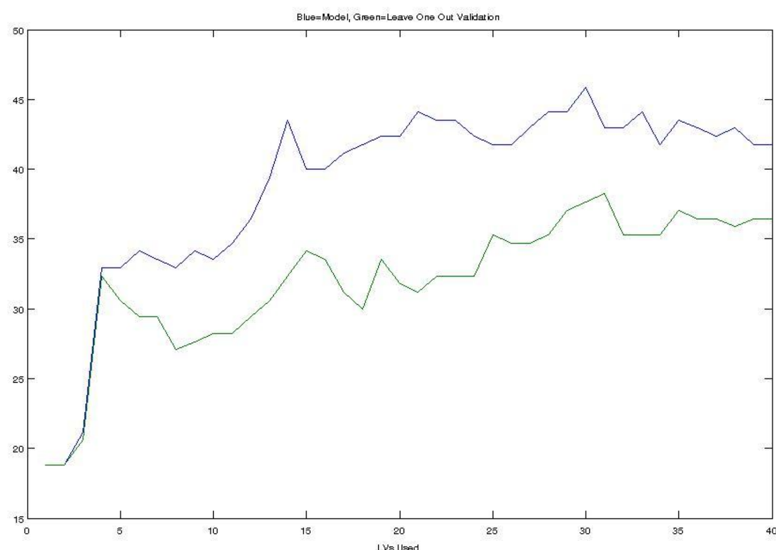
2.1.1. Application of Partial Least Squares Discriminate Analysis (PLSDA) to eNose data

The problem with PCA for eNose data is that a highly varying sensor response had a big impact on the first Principal Component even if the reason it was varying had nothing to do with the TB status of the sample. PLSDA provided a solution to this problem and was adopted as an improved method for analysing data from the eNose. PCA is an 'unsupervised' technique as no information about the dataset is given to the algorithm or is in fact taken into account. PLSDA is a 'supervised' technique. It requires the data to be split into classes e.g. TB positive and negative. The next steps follow a similar method to PCA and the data are rearranged into new dimensions that maximise the difference between the classes. In a simple two class problem this can be imagined as first finding the means of each response of class 1 and class 2, and then creating new a dimension that captures as much of this difference as possible. In reality the process is more complex, as it can cope with more than two classes and involves a calculation of statistical significance.

PLSDA of the available eNose data failed to find any significant discrimination between TB positive and negative badger samples. Figure 1 shows a 35% chance of correctly identifying the status of the badger from the eNose results. This is approximately chance (not 50% as there are uneven numbers of positive and negative samples). It also shows that the number of Latent Variables (LV's [a]) required to achieve this is 30. This means that the data are showing a large number of other factors that have greater impact on the results than the TB status of the sample. These factors could relate to the eNose (temperature, humidity, time of sample, etc.), the sample itself (age, sex, geographical location of badger, etc.), or both.

[[a] LV's are dimensions of variance and the PLSDA equivalent to Principle Components of PCA. When building a classification model, more LV's required indicate a greater percentage of the data needed to build the model, which in practice means delving deeper into the noise of the signal].

Figure 1 PLSDA of serum from wild badgers measured by the BH214 eNose



Conclusion: the inherent variability in the eNose methodology, combined with the biological variation seen in the badger samples used meant that the eNose could not be used in any meaningful way to diagnose TB in badgers. Although the conditions associated with experimental infection studies in badgers could be controlled better (e.g. diet, stage of infection), too few samples were available from such studies to evaluate the eNose further. Despite these disappointing results, the effort was not wasted as many of the methodological approaches used for the badger samples (e.g. generating volatiles and data analysis) were directly applicable to the work subsequently undertaken in cattle.

2.2. Cattle

2.2.1. TB Study 1 – discrimination of samples based on severity of disease
In this trial 13 serum samples were analysed using the two BH214 eNoses. The samples came from different cattle that were all experimentally infected with *M. bovis* as part of a vaccine efficacy study at the VLA. From that work, a pathology score was assigned to each animal at post-mortem. The objective of this study was to see whether the BH214 eNose could discriminate samples according to their associated pathology score.

Serum samples were defrosted and 1mL of serum was dispensed into small bags which had a volume of 0.75L (the sample volume to bag volume ratio was 1.33E-3). All samples were prepared approximately 30 minutes before analysis to allow equilibration with the head-space air. The number of replicates was seven and each replicate had an adsorption time of 20 seconds. All samples were analysed randomly but both eNoses were operated in parallel. Alternative univariate statistics were developed and applied by the PhD student who also performed the sample analysis.

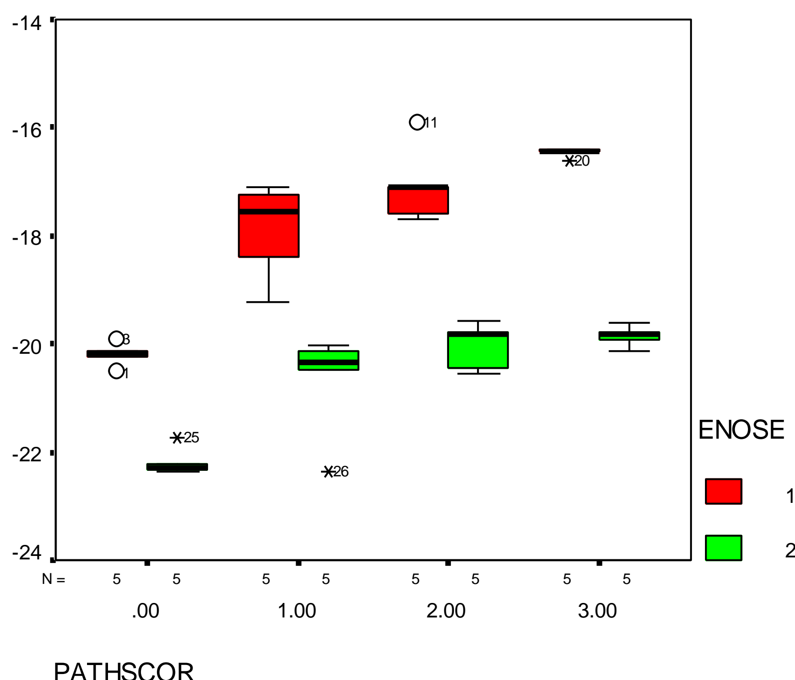
Results:
Analysing all data together, no discrimination was found on the basis of pathology score. Consequently, the dataset was separated according to both eNoses and the following factors investigated using linear regression and multifactor ANOVA ($P \leq 0.05$):
- replicate
- time
- temperature
- pathological score

Still no association was found with the pathology score using data from either eNose. The main problem seemed to be the fact that to complete the sample analysis took nearly six hours, and as reported previously, there is considerable variation in sensor response over time. Therefore, the data were grouped into four sets (*pathscore*: “0” = score 0; “1” = score 2-6; “2” = score 7-17; and “3” = score 18-28), averaging the two main methodological factors *temperature* and *time*. The average temperature was 23.75 (±0.89) °C, the average sampling time for each of the four groups was 116 (± 62) minutes.

Applying linear regression and multifactor ANOVA the averaged data from the first eNose identified responses at nine sensors that were significantly influenced by the *pathscore*. Encouragingly, three sensor responses were absolutely free from any other influences. These were sensors 9, 10 and 11. In comparison, data from the second eNose showed the response for eight sensors to be significantly influenced by the *pathscore*. As for the first eNose, three sensor responses were absolutely free from any other influences (sensors 4, 10 and 11), but only

two of these were common to the first eNose – sensors 10 and 11. Figure 2 shows the results for sensor 11 for both eNoses superimposed on the same divergence scale.

Figure 2 Variations in sensor (11) response for both BH214 eNoses as a function of *pathscore*



Conclusion: using either eNose, it was possible to differentiate cattle experimentally infected with *M. bovis* on the basis on their pathological score. A number of sensors were discriminatory and two were consistent between eNoses. Although all animals had TB, these data demonstrated proof of principle that volatiles in the serum of infected cattle change in proportion to the severity of infection.

2.2.2. TB Study 2 – change in eNose response over time infected

Having demonstrated proof of principle that volatiles in the serum of *M. bovis* infected cattle changed in proportion to the severity of infection, we next investigated whether the eNose would permit differentiation of serum samples pre and post –infection.

Sera from five different cows were supplied by the VLA. Each individual was sampled beginning 14 weeks before and ending 18 weeks after experimental infection with *M. bovis*. Due to limited sample volume (approximately 700µL per sample), samples were pooled according to Table 2. For this study, data were generated using a BH214 eNose. In order to analyse samples using all three methods (eNose, SIFT-MS and GC-MS), the volume of the bags used for static sampling was increased to 1L.

Table 2 Samples of five individuals were pooled according to the following scheme - three pooled samples were obtained; one *post-infection*, two *pre-infection*

	Post-infection		Pre-infection			
Weeks	+18	+8	-1	-4	-6	-14
Pool	1		2		3	

After defrosting, the samples were pooled inside the bag used for static sampling by dispensing 600µL per sample, filling the bag with zero grade air (BOC, England), smoothly inverting the bags and finally letting the bags incubate at a constant room temperature (25.9 C ± 0.5; mean ± SD). The pools were then analysed one after another, but following a random sequence. The number of replicates was seven; the adsorption time was 20 seconds. The sample volume per bag volume ratio was 1.2E-3 which was comparable to analyses carried out before.

Data were analysed using different statistical methods. First, SPSS 11.5 was used for linear regression and multifactor ANOVA. The level proving statistical significance was 95% ($P \le 0.05$). The influence of single factors (e.g. individual, infection status, etc.) on the overall result was expressed as a t value. Box plots were used to illustrate results. The line within each box equals the median. The upper and the lower end of the boxes describe the 75 and 25 percentile, respectively. The lowest and the highest values were displayed as whiskers outside the box. Second, principal component analysis (PCA) was was performed based on averaged data. By this approach, methodological variation could be eliminated and Components 1 and 2 could be plotted using a Matlab script. Discrimination was found.

Results:
Data were first analysed by considering the impact of factors: *individual*, *pooled sample*, *TB status*, and *replicate*. Variation between individuals and the pooled sample accounted for nearly all the variance. Only sensor 12 showed significant differences in the sensor response when *TB infection* status was considered. As the variance between different individuals was higher than between the infection status, each individual was considered separately.

Two analyses per individual were performed: *a)* considering the factors *TB status* and *replicate* only; and *b)* averaging the pooled samples 2 and 3 which were both pre-infection. For individual 1, no changes in sensor responses according to the TB status were found. However, for the remaining four individuals, there were changes in the sensor response according to TB status (e.g. Figures 3 & 4). PCA confirmed the SPSS results for these individuals as discrimination between samples pre and post -infection.

Combining data from all individuals, discrimination was clearly observed using PCA of data from sensors 4 and 5 only (Figure 5).

Conclusions: for the PCA shown in Figure 5 for all cattle, the data from pools 2 and 3 had to be averaged, suggesting that methodological and biological variation in cows before infection with *M. bovis* can mask differences in volatiles induced by infection. However, these data demonstrate proof of principle that as long as this variation can be reduced (e.g. through pooling samples), there is sufficient information in serum head-space volatiles to indicate the presence of *M. bovis* infection. Serum for this study was obtained from cows under controlled conditions and the post-infection samples were matched with samples from the same individuals pre-infection. Whether the discriminatory power will be lost when samples from field animals are used remains to be seen.

Figure 3 Response of sensors 1 (left) and 4 (right) illustrate the two significant kinds of response found using serum taken from a single cow before (pools 2 & 3) and after (pool 1) experimental infection with *M. bovis* - infection was either association with an *increase* in negative divergence (sensor 1) or a *decrease* in negative divergence (sensor 4, also 2, 3, 5 and 13 not shown)

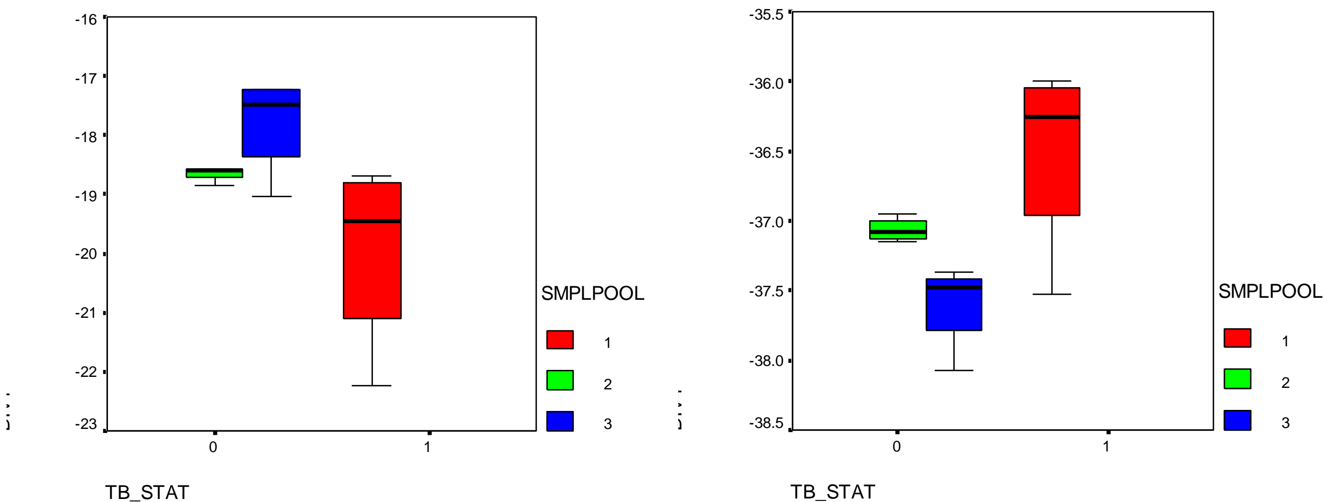


Figure 4 Response of sensors 1 (left) and 4 (right) with serum from a second cow pre- (pools 2 & 3) and post- (pool 1) experimental infection with *M. bovis*

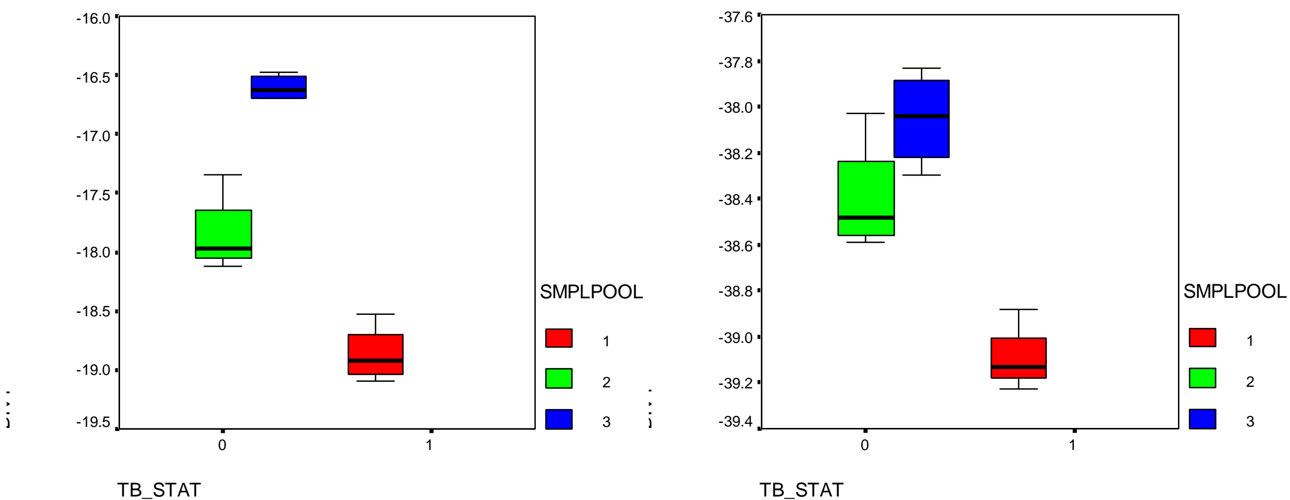
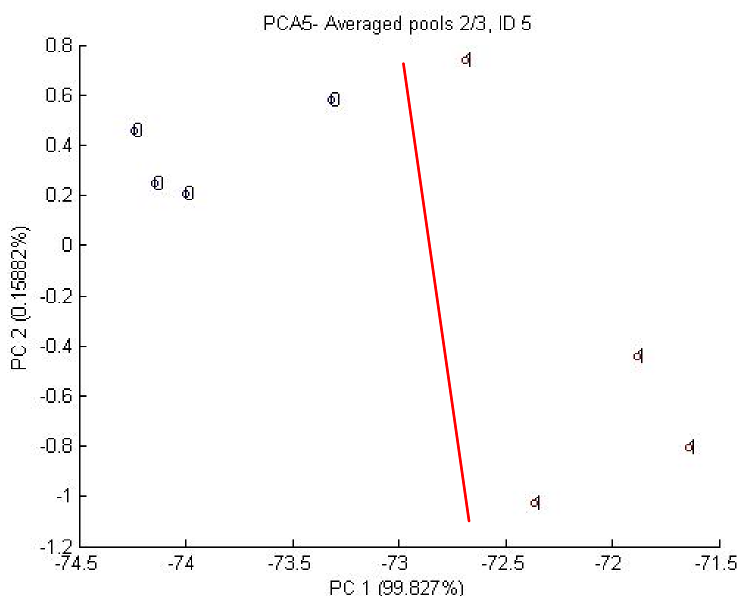


Figure 5 PCA plot of eNose data derived from five cattle pre- (“0”) and post- (“1”) experimental infection with *M. bovis* - the diagonal line was drawn to illustrate the clear discrimination between samples obtained pre and post –infection



2.2.3. TB Study 3

Serum was obtained from a further single experimental vaccination-challenge study at the VLA, with the following details. 10 cattle were vaccinated with BCG at week 0. Five of these were boosted subsequently with a recombinant adenovirus vaccine (Ad85) at week 8. All cattle (including five unvaccinated controls) were infected with *M. bovis* by endobronchial challenge at week 14. Post-mortems were conducted from week 28.

Treatment	Animal ID	Pre-infection samples	Post-infection samples		
Unvaccinated	4328-32	Week 14	Week 16	Week 17	Week 18
BCG vaccinated	4333-37	Week 14	Week 16	Week 17	Week 18
BCG vaccinated, Ad85A boosted	4338-42	Week 14	Week 16	NT	Week 18

NT = sample not taken

One unvaccinated, challenged animal (4330) was immunologically negative, had no lesions at post-mortem and was also negative for *M. bovis* culture. Therefore, this animal was subsequently removed from the analysis.

A pathology score was assigned to each of the remaining animals according to the findings at post-mortem, as follows:

Treatment	Animal ID	Pathology score	
Unvaccinated	4328	16	
	4329	14	
	4331	11	
	4332	8	Median = 12.5*
BCG vaccinated	4333	20	
	4334	5	
	4335	9	
	4336	2	
	4337	0	Median = 5*
BCG vaccinated, Ad85A boosted	4338	6	
	4339	0	
	4340	0	
	4341	8	
	4342	14	Median = 6*

*median pathology scores not significantly different from one another (P = 0.216, Kruskal-Wallis non-parametric ANOVA test).

To aid analysis, the animals/samples were grouped not according to treatment (vaccinated or non-vaccinated), but according to post-mortem pathology score, so there was approximately the same number of animals in each grouping as follows.

Group 0 = 0 path score (animals = 4337, 4339, 4340)
Group 1 = 1-6 path score (animals = 4334, 4336, 4338)
Group 2 = 7-11 path score (animals = 4331, 4332, 4335, 4341)
Group 3 = 12-20 path score (animals = 4328, 4329, 4333, 4342)

First, week 18 only data were analysed as a function of the above grouping. Second, the data from all post-infection samples (weeks 16, 17, 18) were pooled for each animal.

By either approach, however, no association between divergence of any sensor and pathology grouping was found, in contrast to the results obtained in TB Study 1 (Figure 2).

Despite the fact that there was no significant difference in the median pathology scores between vaccinated and unvaccinated groups at the week 28 post-mortem time point, we nonetheless examined if there was any association between the eNose data and treatment group. For this analysis, data from all pre-challenge samples were pooled (Table 3, group 1) and compared with the week 18 samples from each of the treatment groups (Table 3, groups 2-4). We first tested whether it was legitimate to pool all pre-challenge samples since some animals had been vaccinated and others had not. By mANOVA and multiple-range testing, there was no significant difference between the three treatment groups at the week 14 pre-challenge time point. We therefore concluded it was legitimate to pool all pre-challenge samples.

Multiple range testing was used to compare the responses for each sensor, with the following outcome.

Table 3 Overview of multiple range test results of all 4 groups over all sensors

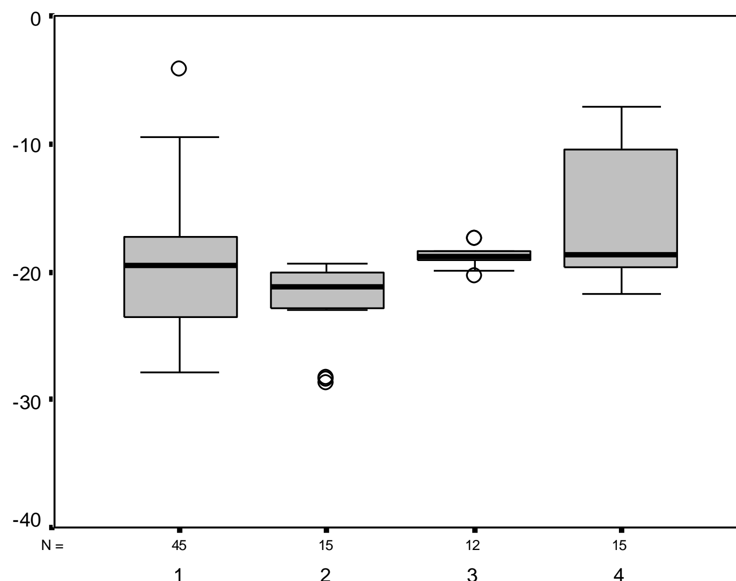
	Sensors												
Group	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
1: all pre-challenge samples pooled	a	a	a	a	b	a	a	ab	b	b	b	ab	b
2: unvaccinated, week 18	a	a	a	a	a	a	a	a	a	a	a	a	a
3: BCG vaccinated, week 18	a	a	a	ab	b	a	a	ab	b	b	b	ab	b
4: BCG vaccinated, Ad85A boost, week 18	b	b	b	b	b	b	b	b	b	b	b	b	b

Different letters indicate significant differences (LDS, P ≤ 0.05)

For the sensors highlighted in the table, multiple range testing suggested that the eNose response for the vaccinated groups at week 18 (post-challenge) was indistinguishable from the pre-challenge samples, whereas the response at week 18 for the unvaccinated animals was significantly different from either of the vaccinated groups at the same time point, as well as from the pre-challenge time point (see Figure 6, by way of example). This result provides evidence that VOC analysis using eNose might have the potential to detect the positive effect of vaccination, at least at the group level. This effect was apparent at week 18 (four weeks after infection). Although the individual pathology scores at the time of post mortem at week 28 suggested that vaccination had been effective in many cases, the variance of the scores in each group meant that any differences were not statistically significant. It is tempting to speculate that the eNose data four weeks after infection were a genuine indicator of vaccine efficacy at that time point. Unfortunately, serum was unavailable for testing at the time of post mortem (week 28).

Data were also grouped and analysed according to the week of the study. These data were compared with the corresponding BOVIGAM IFNγ results. No correlation could be found between the two (data not shown).

Figure 6 Response of sensor 9 illustrating difference between unvaccinated (control) samples from week 18 and pre-challenge samples, which were indistinguishable from week 18 samples from either of the two vaccinated groups (see also Table 3)



1= pre-challenge, 2= controls, 3= BCG vacc., 4= AD85a vacc.

3. eNose studies using urine from TB-infected cattle

Urine was obtained from a similar but different experimental vaccination-challenge study at the VLA to the one described in the preceding section, with the following details. Cattle were vaccinated with BCG at week 0 and all cattle infected with *M. bovis* by endobronchial challenge at week 14. Post-mortems were conducted from week 28.

Treatment	Animal ID	Pre-infection samples		Post-infection samples	
Unvaccinated	4141-45	Week 12	Week 13	Week 25	Week 28
BCG vaccinated	4146-50	Week 12	Week 13	Week 25	Week 28

A pathology score was assigned to each animal according to the findings at post-mortem, as follows:

Treatment	Animal ID	Pathology score	Culture Result
Unvaccinated	4141	4	Positive
	4142	12	Positive
	4143	2	Positive
	4144	11	Positive
	4145	28 Median = 11*	Positive
BCG vaccinated	4146	0	Positive
	4147	0	Positive
	4148	0	Negative
	4149	7	Positive
	4150	0 Median = 0*	Negative

*median pathology scores significantly different from one another (P = 0.0317, Mann-Whitney Test).

As performed for the cattle serum studies (1 & 3), the animals/samples were grouped not according to treatment (vaccinated or non-vaccinated), but according to post-mortem pathology score, so there was approximately the same number of animals in each grouping as follows.

Group 0 = 0 path score (animals = 4146, 4147, 4148, 4150)
Group 1 = 1-7 path score (animals = 4141, 4143, 4149)
Group 2 = 8-28 path score (animals = 4142, 4144, 4145)

First, week 28 only data were analysed as a function of the above grouping. No association between divergence of any sensor and pathology grouping was found. Second, the data from the two post-infection samples (weeks 25, 28) were pooled for each animal. Here, there was some suggestion by multiple range testing (MRT) that sensor divergence decreased from groups 0 to 1 to 2. However, this finding was not substantiated by SPSS or heat-mapping.

The data from this study were also analysed to see if there was any association between sensor response and treatment group, as for TB Study 3 (Table 3). The data from all pre-infection samples (weeks 12, 13) were pooled, but since half of these animals were vaccinated and half were not, we first tested whether vaccination influenced the eNose data. By all means of analysis, data from vaccinated and non-vaccinated animals were indistinguishable. On that basis, we compared the pre-challenge pooled data with the data from week 28 for each treatment group (Table 4).

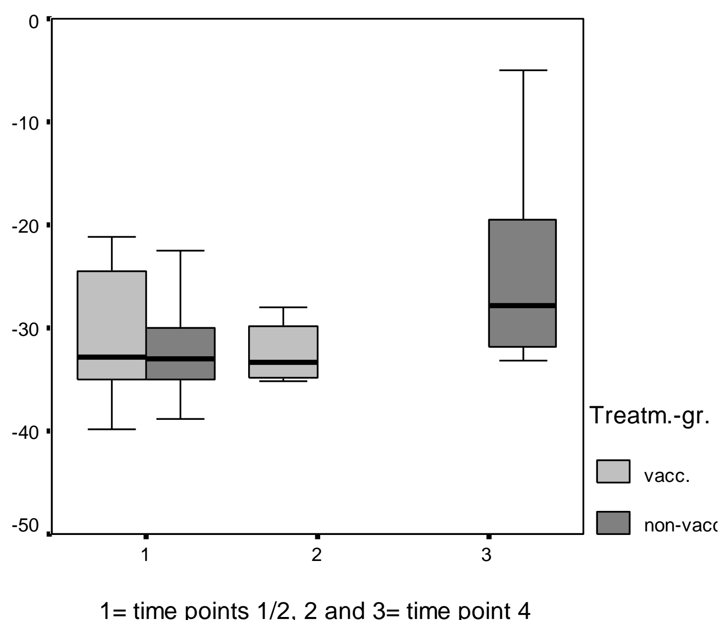
Table 4 Overview of multiple range test results of all 3 groups over all sensors

	Sensors												
Group	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
1: all pre-challenge samples pooled	a	a	a	a	a	a	a	a	a	a	a	a	a
2: unvaccinated, week 28	a	a	a	a	a	a	a	a	ab	a	a	a	a
3: BCG vaccinated, week 28	b	b	b	b	b	a	a	b	b	b	b	a	a
mANOVA result (F, p values)	NS	NS	NS	NS	2.178, 0.033	NS	NS	2.515, 0.014	2.480, 0.015	2.169, 0.033	2.419, 0.018	NS	NS

Different letters indicate significant differences (LDS, P ≤ 0.05)

For the sensors highlighted in the table, MRT suggested that the eNose response for the vaccinated groups at week 28 (post-challenge) was indistinguishable from the pre-challenge samples, whereas the response at week 28 for the unvaccinated animals was significantly different from either of the vaccinated groups at the same time point, as well as from the pre-challenge time point (see Figure 7, by way of example). This result adds further evidence to the results found with serum in TB Study 3 (Table 3) that VOC analysis using eNose might have the potential to detect the positive effect of vaccination, at least at the group level using either serum or, as in this case, urine.

Figure 7 Response of sensor 5 illustrating difference between unvaccinated (control) samples from week 28 (x axis, group 3) and pre-challenge samples (x axis, group 1), which were indistinguishable from week 28 samples from the BCG vaccinated group (x axis, group 2) (see also Table 3). The pooled pre-challenge samples from weeks 12 & 13 are shown (x axis, group 1) separated into unvaccinated and vaccinated groups, showing their similarity and hence the legitimacy of pooling these data

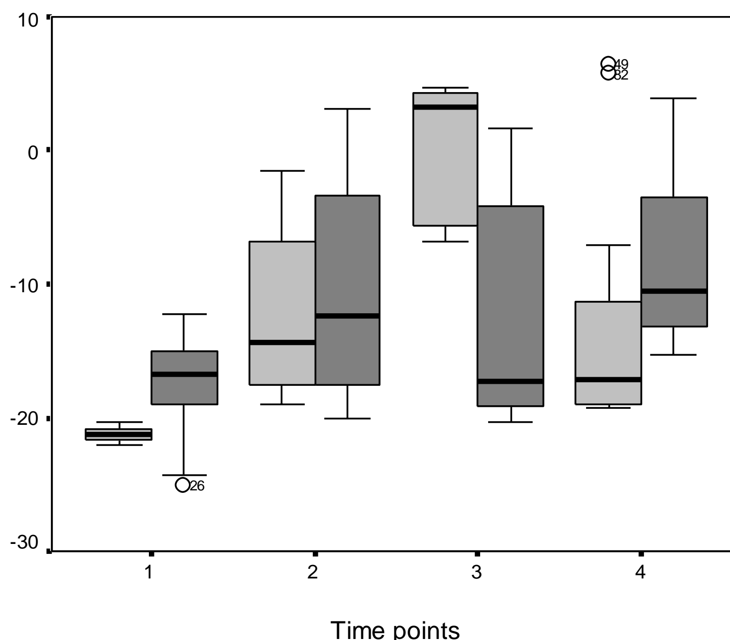


1= time points 1/2, 2 and 3= time point 4

Data were also grouped and analysed according to the week of the study. There was a marked and significant difference in the response of sensor 1 and of sensors 8-13 at time point 3, corresponding to 11 weeks after infection, for the unvaccinated group compared with the vaccinated. The result for sensor 9 is shown in Figure 8

by way of illustration. These data were compared with the corresponding BOVIGAM IFNγ results but no obvious correlation was apparent (data not shown).

Figure 8 Divergences of sensor 9 – light grey bars = unvaccinated group; dark grey bars = BCG vaccinated group



4. SIFT-MS studies using serum from TB-infected badgers and cattle

4.1. Badgers

SIFT-MS analysis was employed for the same badger serum samples analysed by eNose (section 2.1.). Numerous volatile compounds were found to be present in the serum at high concentrations (see Figure 9 for example of SIFT-MS data) but with the same high levels of inter-animal variation. All data (both raw and normalised) were entered into a database. No unique markers associated with TB were identifiable by eye. Nonetheless, initial evaluation using principle component analysis (PCA) was encouraging. Ions present ubiquitously in living things and which were responsible for most of the variance were removed from the spectra followed by PCA. Although there was no clustering, the positive samples and negative samples did trend towards opposite directions (Figure 10).

Figure 9 Example SIFT-MS trace using serum from a wild badger (precursor ion: H3O+)

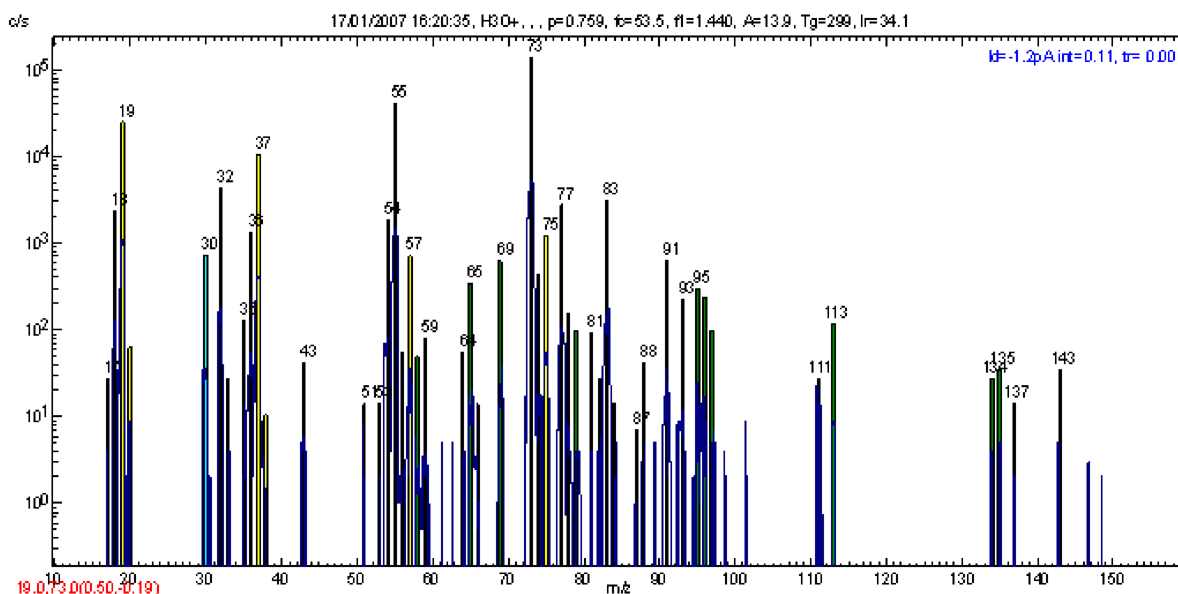
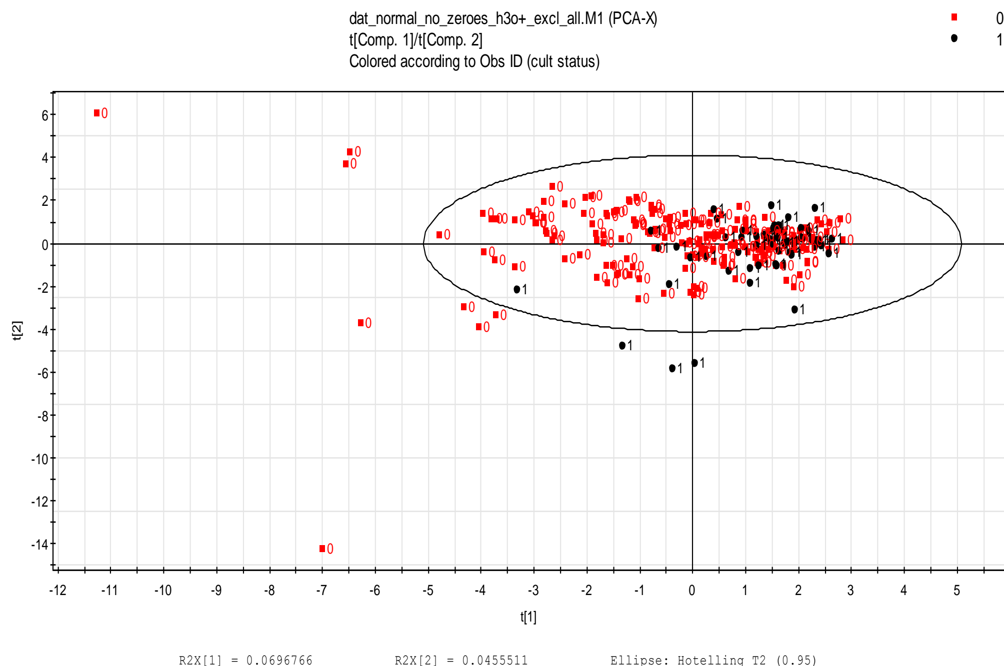
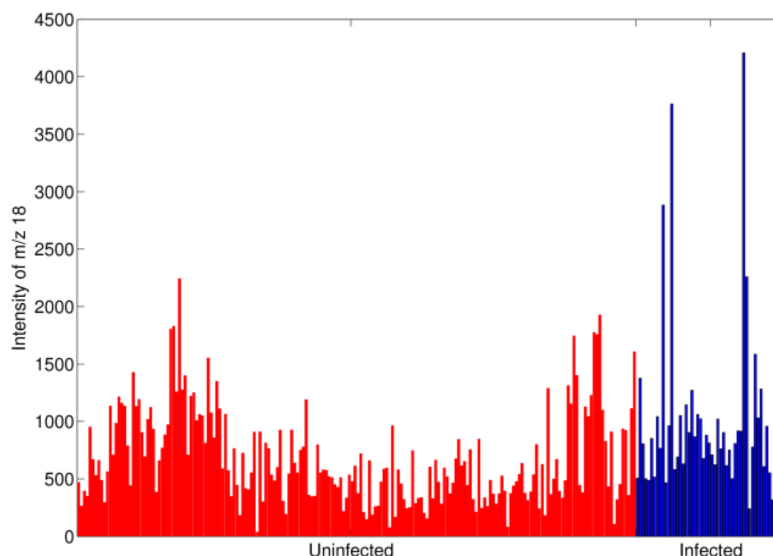


Figure 10 PCA plot of 250 badger sera analysed by SIFT-MS (ubiquitous ions responsible for most of the variance were removed before analysis)
0 = TB negative by culture; 1 = positive by culture



The mass-charge (m/z) spectra contributing to each principle component were then examined individually. Ammonia was apparent at m/z 18 and m/z 54 from the H3O+ spectra and m/z 17 from the O2+ spectra. Toluene was also present in all three (m/z 93 on H3O+ and 92 for NO+ and O2+). Acetone also appeared on m/z 77 on H3O+ and m/z 88 on NO+, and ethanol at m/z 83 on H3O+. Concentrating on the H3O+ spectra, the levels of acetone, ethanol, and toluene did not appear to vary in relation to TB status. It was possible that both acetone and ethanol are influenced by the diet of the badgers. Levels of toluene may also vary due to the use of an injected anaesthetic cocktail, of which toluene is a major breakdown product. However, the levels of ammonia (m/z 18) did appear to be higher on average in TB positive badgers (Figure 11) and was the most efficient component associated with the discrimination emerging in Figure 10. The difference suggested by Figure 11 in the levels of ammonia between TB positive and negative badgers was tested statistically. In the first instance, a chi2gof test (goodness of fit) was used to see if the data were significantly different from normally distributed. Analysis revealed that the data appeared to log normal. On this basis a t-test was performed to see if the difference in the means could be caused by chance resulting in a p value of 0.0044. Even when the data were analysed on the basis of being non-normal (wilcoxon test), the median concentration of ammonia in the serum head-space of TB positive badgers was significantly higher than in TB negative badgers (p = 0.0134).

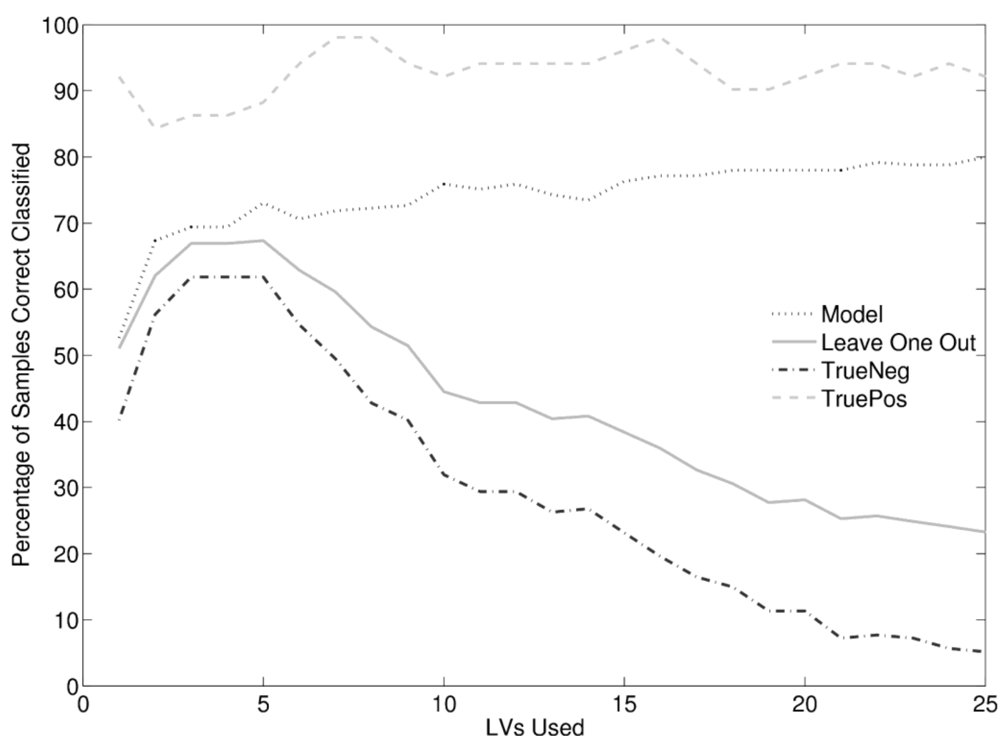
Figure 11. Levels of ammonia (m/z 18) in the headspace of serum from badgers of different TB status as determined by SIFT-MS



4.1.1. Application of PLSDA to SIFT-MS data

As demonstrated for the eNose, the problem with PCA for SIFT-MS data is that a highly varying m/z peak value has a big impact on the first Principal Component even if the reason it is varying has nothing to do with the TB status of the sample. Therefore, PLDSA was applied to analysis of the SIFT-MS data as well. In comparison to the eNose data, PLSDA analysis of the SIFT-MS data from badger serum showed much more promise. PLSDA has the potential to distinguish between samples that could not be separated using PCA because it maximises the co-variance between the acquired data and the sample classifications (control *versus* TB). This gives the possibility of distinguishing between samples. In Figure 12, an estimate of this ability has been produced (Model). More important is the result produced using the Leave One Out (LOO) algorithm (solid line) in which models are built using all but one sample and then the “unknown” sample is classified by the model. The number of correctly classified positives (TruePos) and correctly classified negatives (TrueNeg) is also shown as a percentage. The LOO validation on the complete dataset shows that an overall accuracy of 67% can be achieved with 88% correctly classified positives and 62% correctly classified negatives using the currently available dataset. All percentages are estimates.

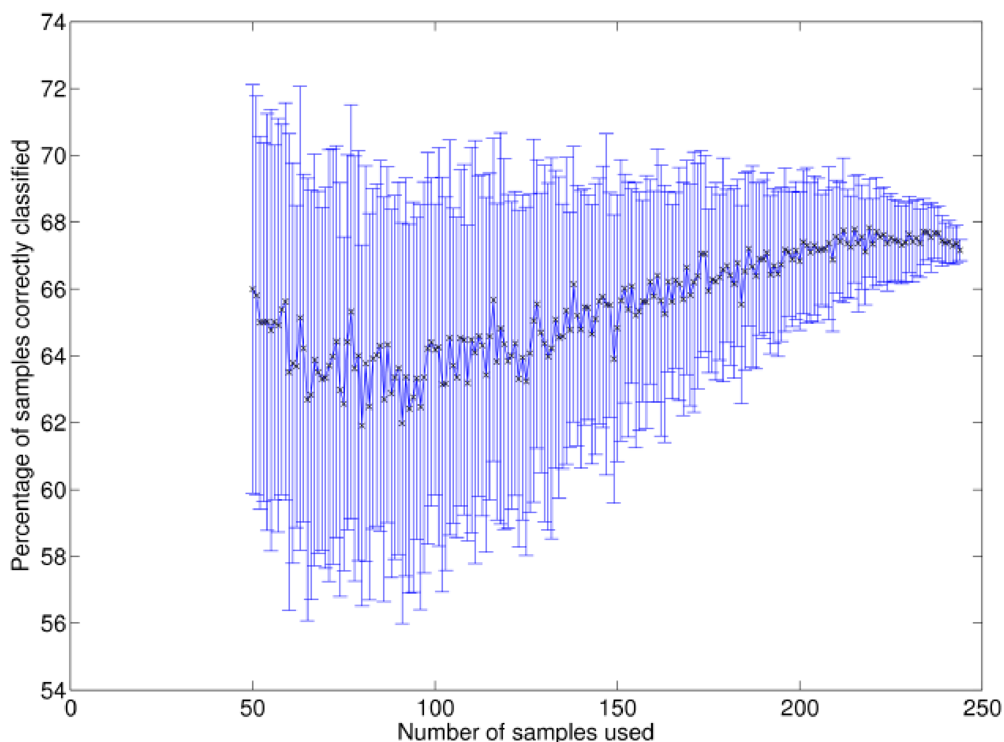
Figure 12 PLDSA of SIFT-MS data generated from wild badger serum samples



The relationship between the accuracy of the PLSDA LOO performance and the size of the training dataset is shown in Figure 13. It can be see that the maximum accuracy of the PLSDA has yet to be reached, although the average improvement in accuracy reduces as the number of samples increases, suggesting that provision of additional samples might not substantially improve diagnostic performance. It can also be seen that in this specific example a minimum number of samples required to produce a repeatable model is 150. Below this, the accuracy of the model (65%) is stable but the errors associated are very large and increase with fewer samples. It appears that using more than 250 samples is unlikely to substantially increase the accuracy of diagnosis.

Conclusion: the application of SIFT-MS to badger serum followed by PCA and PLSDA analysis show that TB discrimination is clearly possible using SIFT-MS but that to build a truly accurate model would require analysis of >150 samples and even then there is no guarantee that an accuracy >68% could be obtained. Spectral analysis reveals an interesting association between elevated serum ammonia and TB that deserves further attention. The analytical methods developed in this project for the analysis of the SIFT-MS data have been accepted for publication in the Analyst journal [7].

Figure 13 The mean and standard deviation of PLSDA LOO corrected classified results over a range of dataset sample sizes using 64 bootstraps. Error bars indicate one standard deviation, capturing 66% of the models produced



4.2. Cattle

4.2.1. TB Study 1

After eNose analysis of the cattle serum from TB Study 1, the bags were connected to the SIFT-MS. For each analysis 10 replicates (duration 5 seconds per replicate) were carried out using the three precursor ions H_3O^+, NO^+ and O_2^+ at a m/z ratio ranging from 10 to 160. SIFT-MS traces from the cattle samples showed no discernible differences when scanned by eye. By PCA, the main variance in the data was not the result of the severity of TB infection (pathology score used as variable).

There were too few samples generated from the experimental infection studies in cattle to analyse the resulting SIFT-MS data by PLSDA.

5. Obtaining breath samples from TB-infected cattle in a safe and effective manner

Sampling breath from cattle has rarely been attempted; although some earlier work had been carried out by Toby Mottram, an initial partner in this project. As the sampling and analysis of cattle breath was identified as a potential mechanism for detecting the presence of TB in this project, a breath sampler was developed to enable the sampling of bovine breath into individual sampling bags.

5.1. Design, production and evaluation of a bovine breath sampler (BBS) for the collection of exhaled breath from cattle

An initial design of breath sampler was tested on cattle and found to be successful in obtaining a sample directly into a sample bag relatively easily without causing the cattle distress. This was carried out twice; once at the Cambridge University Veterinary School Farm, and then at Cranfield University in September 2006, during which the bags were analysed for water and a number of volatile compounds using both SIFT-MS and eNose. It was found that the first design of sampler in use during the Cranfield University cattle breath tests did not enable the collection of representative concentrations of volatiles. The concentrations of key marker volatiles such as water and acetone were a fraction of what was expected and it was believed that these were either being “diluted” some way during the sampling process, or were condensing and being lost in the large dead space of the sampler.

As a result, alternative sampler configurations were built and tested at the VLA in November 2006 during a training day for animal handling staff who would later be tasked with obtaining breath samples from cattle. Because the SIFT-MS equipment was not transportable, immediate analysis of the samples to assess volatile and

water levels in the sample bags was not possible. Instead, CO_2 and eNose analyses were performed at the VLA, and further samples taken for later analysis at Cranfield University by SIFT-MS, eNose, and CO_2 measurement. Five types of BBS were tested. Five samples were taken with each of the five samplers in random order. Three samples from each sampler were used for the analysis of CO_2 immediately after being taken, and one sample was used for the immediate analysis by eNose. A further sample was taken for return to Cranfield University for subsequent analysis by SIFT-MS and eNose, and transported in black plastic bags to reduce UV-induced changes in volatiles. The three samples for CO_2 analysis were also taken off site for subsequent CO_2 analysis to determine CO_2 / sample stability.

Observations were also recorded regarding the degree of difficulty each BBS provided for the operator to use. In general, the animals were relaxed while providing breath samples into the BBS until the bag was full; the subsequent back pressure made it more difficult to breathe and the animal then pulled away. Details of the results obtained at this stage in the development of the BBS were previously reported in the first annual project report (06-07), so are not reproduced again here. The combined results all indicated that the most representative samples were obtained by directly sampling from the animal's nostril. This was the simplest device, consisting of a sampling bag fitted with a one-way valve to enable the animal to inhale into the bag after which the operator needed to move the bag to allow the animal to inhale. This method, therefore, requires more operator skill than the other methods but comments from the animal handling staff at VLA at the time indicated that it was not particularly difficult to use.

The principle of collection behind this method was used subsequently in the production of four different configurations of the nostril sampler (Table 5) and assessed by measuring CO_2 and volatile compound concentration (using SIFT-MS) and analysing eNose profiles (using the two BH214 eNoses) of samples taken using each sampler. Six samples were taken from a single cow with each of the four samplers. Two samples from each sampler were used for the analysis of CO_2, (and some samplers had additional samples taken for CO_2 analysis, where results of duplicate samples were markedly different) and two samples were taken for immediate analysis by eNose and SIFT-MS. A further two samples were taken for subsequent analysis after five hours by SIFT-MS and eNose. These samples were kept at room temperature (26°C) in black plastic bags to reduce UV-induced changes in volatiles until analysed. After five hours, the CO_2 samples were re-analysed, and these five hour data were used to assess bag stability.

Table 5 Configuration of the nostril samplers

Nostril Sampler	Valve/opening diameter	Body length	
"Long-narrow"	3 cm	8 cm	
"Long-thick"	4.5 cm	8.5 cm	
"Short-narrow"	3.5 cm	4 cm	
"Short-thick"	4.5 cm	4 cm	

Results:

CO_2

- The values obtained with all four BBS were similar to those recorded previously at VLA for direct nostril sampling.
- There was no apparent effect of sample order.
- The coefficient of variation between the samplers was small (8.9%) and less than that for replicates taken with the same sampler (15.8%).
- Between sampler differences were not statistically significant.
- In addition, the size and dead-space for each sampler were similar and quite small (~200ml) in relation to the volume of exhaled gas collected (~5 litres), i.e. the dead-space volume of the sampler to gas ratio was about 1:25 or around 4% of the volume of exhaled gas collected.

Two additional samples were taken using each nostril sampler for CO_2 analysis; one using a larger bag and one using a smaller bag. Results showed that the normal sized bag used (volume approximately four litres) gave higher values (~40% higher) than a smaller (approximately two litres) bag. This indicated that it is important to examine the effect of bag size or carefully standardise the bag size.

SIFT- MS

Four samples were taken from each nostril sampler; two of these were analysed shortly after being taken, and two were left in the laboratory at 26^oC for five hours inside black plastic bags to eliminate exposure to UV light. Quantitative analyses were performed on each sample to determine concentrations of the following compounds: methanol, ethanol, (iso)propanol, water, acetaldehyde, ammonia, acetic acid, isoprene and acetone. In addition to quantitative analysis of selected compounds, full spectral scans were carried out on each sample using all three SIFT-MS precursor ions (H_3O^+, NO^+ and O_2^+). The SIFT-MS spectra were taken between 10 and 160 m/z for seven seconds with 10 replicates for each sample. The data were then averaged.

The results of the quantitative analysis demonstrated that almost all compounds increased in concentration after five hours. The exceptions to this were water, which was reduced from a mean value of 3.43% to a mean value of 1.58%; acetone, which decreased slightly (although not statistically significantly) and isoprene, which is highly volatile and was present at very low concentrations to begin with. This was a counterintuitive result and we speculate that this arises in the following way: water is lost from the bags through pores, and this rate of loss is increased at higher temperatures. This is supported by other studies. The volatiles which remain in the bag are not sticking to the bag surface after being dissolved in small water droplets from aerosols and hence increase in concentration. Previous studies have shown that acetone is stable in sample bags over longer periods and this was supported by these data.

It was assumed that the percentage water and acetone concentration in the bag gave the best indication of which sampler produced the best results (i.e. most representative samples). Water concentration in the breath of healthy humans at 37^oC is about 6%, which implies that the breath is fully saturated with water (6% is the amount of water in air at 37^oC if fully saturated). Bovines have greater “dead volume” in their breath and as a result it is possible that their breath is less humid than human breath and accordingly, was approx. 3.5 to 4% in this experiment.

Results from the full mass spectra provided data on other compounds present in cow breath. It has previously been reported that dimethyl sulphide (DMS) is present in bovine breath, and some DMS was detected in these samples, at a concentration of up to 100ppb. Apart from the compounds used for the quantitative analysis, another compound was present at relatively high concentration of a few hundred ppb: methyl ethyl ketone or 2-butanone. This was an interesting finding that distinguishes bovine breath from human breath in which 2-butanone is undetectable (at the detection limit of a few ppb using SIFT-MS).

eNose

The objective here was to determine the sampler that gave the best results with respect to signal intensity. The intensity represented the concentration of water or VOCs in the breath sample. A high concentration of VOCs was assumed to indicate good sampling quality due to a minimised dead space in the sampling system. Based on previous experience, the signal intensity expressed as change in divergence (%) across all sensors (1-13) was the most appropriate means of evaluating the eNose data generated by each sampler.

In this trial, both BH214 eNoses were used. The same samples used for SIFT-MS analysis were first analysed with both eNoses and then analysed after five hours storage in a black plastic bags at room temperature (26°C). For data analysis, a data matrix consisting of all datasets was generated. The first two readings of each analysis were omitted as they are not representative (dead space of sample line and sensor head). After creating modified matrices according to the factors for analysis, MS Excel and SPSS (version 11.5) were used for data analysis.

The factors influencing the responses of the eNose sensors were the eNose device used, the sampler, and the time points (morning vs afternoon readings). The specific time of each analysis was recorded but not considered as a major factor (all analyses were conducted within 65 minutes each other); neither was temperature (average temperature 25.5±0.5°C in the morning and 26.5±0.5°C in the afternoon).

The averaged sensor responses representing the four different samplers were analysed for each eNose across time points. While the values for eNose 1 in the morning showed approximately 10% change in divergence, the values in the afternoon were very close to zero which represents a loss in signal intensity of approx. 90% between the morning and afternoon. Similar effects were observed for eNose 2. The values in the afternoon were extremely low and could simply be due to random noise. It is interesting that the major difference between the morning and afternoon SIFT-MS analyses was the water concentration, hence it seems likely that many of the eNose sensors are responding to water, which is present at much higher concentrations than any of the other volatile compounds.

Conclusions: The results from the CO_2, SIFT-MS and eNose analyses all supported the conclusion that nostril sampler 1 (long and narrow sampler) was the best. Samples taken with this sampler gave the highest CO_2 concentrations, the highest water vapour concentration (by SIFT-MS) and the greatest divergences using both eNoses. However, the difference between the samplers was not large and any of these nostril samplers was

considered adequate for taking cattle breath samples. The differences between samplers were much smaller than those encountered with earlier designs of BBS and nostril sampling was very clearly better than the whole nose sampling evaluated previously. Taking other factors into consideration it was decided that sampler 2 (long and thick) would be used for all future sampling of cattle breath. This was on the basis that the thicker sampler has a larger valve area and thus less resistance, which would be preferable for use with larger cows, or for those that are particularly sensitive to this resistance. Cattle breath samples stored for five hours (even in the dark) were no longer suitable for eNose analysis. Therefore, analysis of cattle breath by eNose should be conducted as soon as possible after the sample has been taken.

5.2. Development of methods for the safe handling of breath from TB-infected cattle

In order to properly assess the risk of infection presented by breath obtained from TB infected cows it would have been necessary to attempt to isolate *M. bovis* from at least 50 animals, since shedding is likely to be a rare event (as demonstrated by the screening large numbers of nasal mucous samples by VLA). As this was impractical, we evaluated the use of UV light as a means to sterilise bagged cattle breath. The CL3 TB laboratory suite at VLA has a through-the-wall pass box with high intensity UV lamps positioned on all six inside faces of the box. The box was designed specifically for the UV sterilisation of small pieces of laboratory equipment and consumables when moving them from the CL3 suite to outside. For the purposes of this evaluation, we substituted BCG for *M. bovis* for time and safety reasons, as the two should be equally susceptible to UV killing.

Nalophan breath bags were prepared and supplied by Cranfield University and inflated using a laboratory pump with in-line 0.2μm filter. BCG ($2x10^7$ CFU) was injected in 5mL volumes into each of nine pre-inflated bags. The bags were then exposed to UV in the pass box for varying periods of time. Two bags were untreated as controls. After treatment, each bag was rinsed with 10mL sterile PBS and then 500μl samples plated on Middlebrook 7H11 agar from neat over a tenfold dilution series. Bacterial colonies on the agar were enumerated after four weeks of incubation (Table 6). It was noted that during activation of the UV lamps that the interior of the pass box was heated. The temperature at the end of the treatment was recorded using a thermometer.

Table 6 Ability of UV irradiation to kill BCG contained in Tedlar breath bags

Bag	UV exposure time (minutes)	CFU of each dilution			Max. temp. of pass box (°C)
		Neat	10^{-1}	10^{-2}	
1	10, intermittent	TMD	142	19	ND
2	1, constant	TMD	TMD	>600	34
3	1, constant	TMD	TMD	>600	34
4	5, constant	TMD	TMD	232	55
5	5, constant	TMD	TMD	82	55
6	10, constant	TMD	12	5	73
7	10, constant	0	0	0	73
8	Not exposed	TMD	TMD	>600	ND
9	Not exposes	TMD	TMD	>600	ND

Key: TMD = Too many to determine
ND = Not determined

Results:

- UV exposure for treatment periods of up to 10 minutes had negligible capacity to inactivate BCG.
- Where killing of BCG was observed, this was most likely the result of heat-inactivation caused by the elevated temperatures inside the pass box.
- The elevated temperatures resulting from constant use of the pass box caused the air inside the bags to expand to the point where fluid from within the bag was observed to escape from the ends of the inflated bag. Even though it should be possible to obtain an efficient seal of breath bags, this clearly represented an unacceptable safety risk when using bags potentially containing *M. bovis*.

Samples of the Tedlar bags as well as Nalophan as an alternative bag material were sent to Jenton International Ltd (JIL) as experts in UV science and applications, for UV permeability testing. Both bag materials blocked >99% of UVC light, which would account for the results obtained in the BCG trial. JIL found that FEP (fluoropolymer) gave the best UV transmissibility, blocking only 33% of the UVC light source.

Conclusions: The elevated temperatures resulting from constant use of the pass box may cause insurmountable problems, not least if bags are prone to leak when they expand. Therefore, although UV treatment of bagged breath may provide a safe means to handle infected breath samples outside of the CL3 laboratory as long as bags can be made from FEP with low residual volatile production it was decided that the risks associated with this procedure meant it could not be an acceptable means to removing potentially infected breath from the CL3 laboratory.

Since it was not possible to bring the eNose or SIFT-MS into the CL3 laboratory as subsequent decontamination of the equipment by fumigation with formalin vapour would wreck the equipment, an alternative means to the safe removal of potentially infected cattle breath was devised, as follows.

Bagged breath samples were placed within a constant temperature incubator within a class I microbiological safety cabinet within the CL3 laboratory. The incubator was based on equipment used to warm rodents prior to tail-bleeding and was modified by the manufacturer according to Dr Chambers' specification to be suitable for these studies. Bags containing breath were given five minutes to reach 40°C before 500ml of sample was withdrawn across an in-line conditioned thermal desorption tube (TD tube) (Carbotrap/Tenax TA) using a constant-flow pump for five minutes. The tube was capped and tightly sealed then heat treated at 80°C for one hour to kill any *M. bovis* bacteria that may be present before being removed from the CL3 facility. The tubes were then posted to Cranfield University where they could be handled in their routine laboratory. As sample capture on thermal desorption tubes was the only safe way we could devise for the removal of breath samples for analysis, it did mean that cattle breath was only available for analysis by GC-MS.

6. Searching for biomarkers for TB in cattle serum and breath using GC-MS

6.1. Serum

Where sample sizes of serum in this project were sufficiently large (>1.5ml), GC-MS was used in addition to SIFT-MS to analyse headspace and provide additional information. After eNose analysis, the bags were connected to the SIFT-MS, then finally attached to ATD tubes (Carbotrap/Tenax TA). The tubes were connected to the incoming side of a flow adjustable pump (set to 125mL per minute) for two minutes. The air stream of the pump led to a loading of the tubes which were then put into the GC-MS sample carousel for analysis.

The headspace of serum samples contained hundreds of volatile compounds and the approach was to find and identify those compounds that were present in TB positive samples but absent in TB negative samples; as these are potential TB markers. The same serum pools described in section 2.2.2. from TB Study 2 (Table 2) were subjected to GC-MS. Around 300 components were detected, of which 160 were identifiable from a library of known compounds. Nevertheless, the identity of each target had to be determined manually, which was extremely time consuming and required experience. Compounds were first identified in pool 1 (post-infection) that were not in pools 2 and 3 (pre-infection). Then for each compound, their presence in each pool 1 sample, and their absence from each pool 2/3 sample was sought. However, no compound was found exclusively in all TB positive animals. This does not necessarily mean they are not TB-associated markers. Some of the more interesting compounds present post-infection but frequently not pre-infection were, isopropyl butane, 3,4 dimethyl decane, and tetra methyl hexane. None of these compounds are obviously associated with mycobacteria (Dr Paul Wheeler, personal communication).

6.2. Breath

Pulmonary TB will produce lesions in the lungs of infected animals and it is anticipated that these lesions may give rise to VOCs. These may be due to the host response to TB, or to the bacterial metabolism. The aim of this part of the project was to determine whether individual VOCs could be detected in breath. It was the original intention to be able to collect breath from infected cattle and to subsequently analyse by eNose, SIFT-MS, and GC-MS. However, as described in section 5.2., the only method for the safe removal of breath from a CL3 laboratory meant that samples could only be analysed using GC-MS.

A subset of the same cattle as described in TB Study 3 was used (see section 2.2.3.). Five animals were unvaccinated (labelled 4328C – 4332C) and five were vaccinated with BCG (labelled 4333B to 4337B), 14 weeks prior to infection with *M. bovis*. All animals were sedated with rompun (23.32 mg xylazine hydrochloride and 1mg methyl 4 - hydroxybenzoate as a preservative per ml) prior to challenge with *M. bovis*. There is a possibility that this could have introduced volatile aromatic derivatives into the animal, however it is unlikely to persist several weeks post infection. A sample of breath was taken from each animal prior to experimental infection with *M. bovis*, and then at two, three, and five weeks post-infection. Breath samples were taken from animals housed in CL3 facilities at the VLA into Nalophan bags via the BBS described in section 5.1. The standard stainless-steel ATD sorbent cartridges, containing dual packing comprising 50% Tenax TA and 50% Carbotrap (Markes International Limited, Llantrisant, UK) were used following conditioning. Conditioning was carried out by purging with helium carrier gas for 2 min at 25°C followed by 30 min at 335°C. Conditioned cartridges were sealed with locking caps and stored at 4°C until required for use.

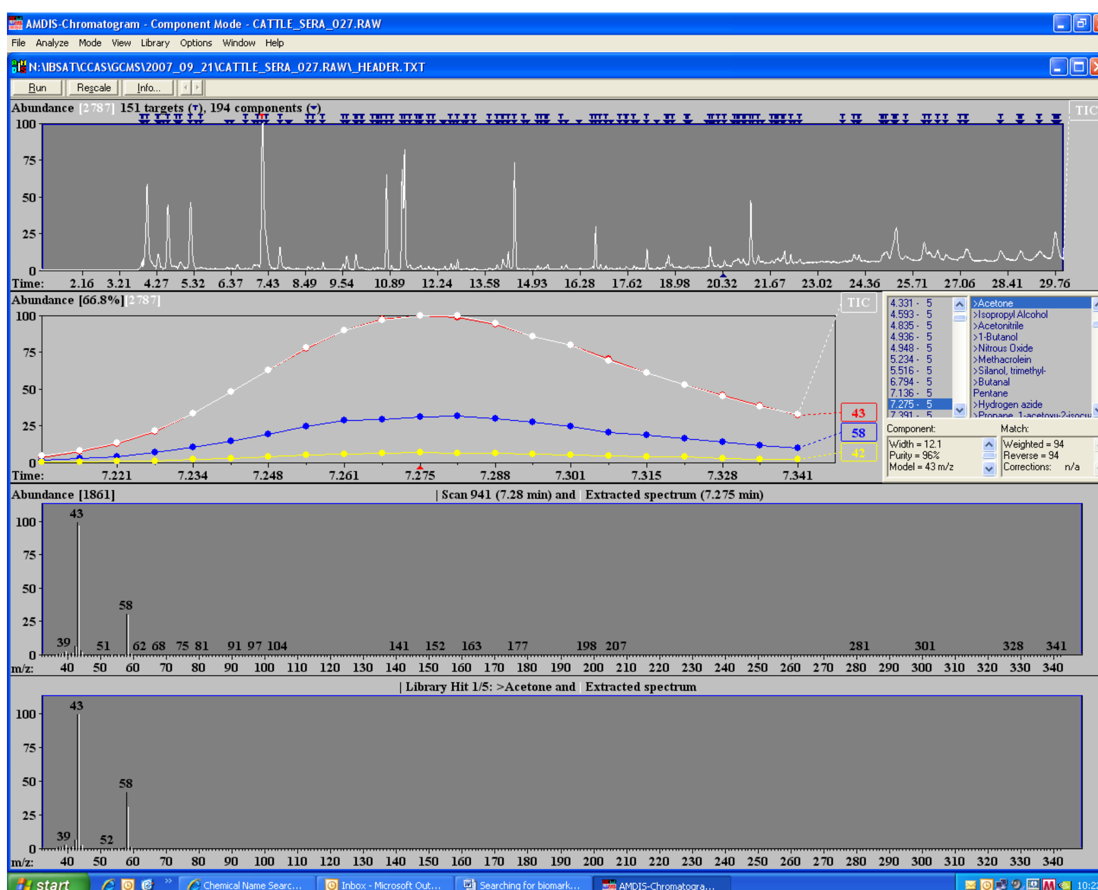
The captured volatiles were then analysed using an AutoSystem XL gas chromatograph equipped with an ATD 400 thermal desorption system and TurboMass mass spectrometer (Perkin Elmer, Wellesley, MA). The carrier gas used throughout was CP grade helium (BOC gases, Guildford, UK). Cartridges were desorbed by purging for 2 min at ambient temperature then for 5 min at 300ºC. Volatiles purged from the cartridge were captured on a cold trap which was initially maintained at 30ºC. Once desorption of the cartridge was complete, the trap was

heated to 320ºC using the fastest available heating rate and maintained at that temperature for 5 min whilst the effluent was transferred to the gas chromatograph via a heated (180ºC) transfer line coupled directly to the chromatographic column.

A Zebron ZB624 chromatographic column was used (Phenomenex, Torrance, CA). This is a wall-coated open tubular column (dimensions 30m×0.4mm×0.25mm ID), the liquid phase comprising a 0.25 µm layer of 6% cyanopropylphenyl and 94% methylpolysiloxane. The gas chromatograph oven was maintained at 50ºC for 4 min following injection and was then raised at 10ºC min^{-1} to 220ºC for 9 min. Separated products were transferred by heated line to the mass spectrometer and ionised by electron bombardment. The spectrometer was set to carry out a full scan from mass/charge ratios (m/z) 33 to 350 using a scan time of 0.3s with a 0.1s scan delay. The resulting mass spectra were combined to form a total ion chromatogram (TIC) by the GC-MS integral software (TurboMass 4.1). These were eventually analysed using the AMDIS (Automated Mass Spectral Deconvolution and Identification System) software where compounds were identified by automatically searching against the library of compounds in the NIST05 mass spectral database based on ionisation pattern. Figure 14 shows a typical result from an AMDIS analysis. The top graph shows the chromatogram of total ion counts as measured by the mass detector against time after injection into the GC. Thus the time refers to retention time of the compound. The third graph down shows the ion spectrum of what comes out of the GC column at that particular retention time, and the bottom graph shows a comparison of that spectrum against the best library match. The compound highlighted in figure 14 is acetone, having the retention time and spectrum characteristic of acetone.

Automated analysis of the compounds present using AMDIS correctly identifies about 80% of compounds present. However, a number are incorrectly identified. This may be due to a number of factors including incomplete separation of compounds, differences in instruments and methods etc. For these reason, it was necessary to go through each compound in each sample and manually check the spectrum obtained against library spectra. This was done for four samples from each of 10 cattle (one pre infection and three post-infection from weeks 2, 3 and 5). Samples of air from the cattle facility were also taken so that background air compounds could be excluded as potential markers. Compounds in each of the samples were identified and then all compounds present post-infection were compared with pre-infection sample data and background air data. Those that were present in at least two post infection samples but absent from all pre-infection samples and background air samples were considered to be *possible* markers. It is to be emphasised that these tentative markers would need to be confirmed in further experiments by looking for them in breath samples of known infected animals while at the same time, also testing uninfected controls to ensure they are absent. But in this experiment, they fulfilled these criteria.

Figure 14 Example of AMDIS analysis of GC-MS data - the spectrum shown in the third panel is for acetone



Results:
On post mortem examination, it was noted that one animal (4330) had no visible lesions at post-mortem and was both immunologically and culture negative. Therefore it was eliminated from the analysis.

The possible *M. bovis* breath markers were limited and are shown in Table 7.

Table 7 Possible biomarkers found in the breath of cattle experimentally infected with *M. bovis*

Possible marker	No. of occurrences & weeks post infection	Animal identities[1]
3-Hexanone, 2,2-dimethyl-	2; 1 week 2, 1week 3	4331C; 4238C
2-pentanamine	5; 1 week 2, 4 week 3	4333B; 4335B; 4331C

[1]C refers to unvaccinated animal; B refers to animal vaccinated with BCG

2-pentanamine was present in two different weeks' samples and in five different samples so seems the more likely potential biomarker. Syhre and Chambers [8] found four potential biomarkers in the headspace of *in vitro* cultures of *M. bovis* and *M. tuberculosis* (methyl phenylacetate, methyl *p*-anisate, methyl nicotinate and *o*-phenylanisole) but it is not clear whether these same compounds would be markers *in vivo*, given that there may be a host response to TB infection or whether their production *in vivo* may cause the markers to be modified in some way.

Semi-quantitative analysis of the compounds present is possible. GC-MS with thermal desorption cannot be accurately quantified, however because of the number of steps and the indirect nature of the techniques. For instance, the different binding coefficients of the compounds on the thermal desorption material, the varying quantities of material adsorbed and the nature of those materials is always highly variable so may not behave exactly as a standard. Taking this into account, the concentrations of the candidate biomarkers is likely to be <10 ng/L. By contrast, acetone is typically present at a concentration of 400 ng/L. This shows that these potential markers are present at very low concentrations which would be difficult to detect using field-deployable equipment.

7. eNose analysis of infections other than TB in cattle

In an effort to establish the extent of the biological variation in samples from cattle using eNose, we obtained ~900 serum samples from cattle either experimentally infected or naturally infected with a variety of common bovine bacterial pathogens (see section 1.2, Table 1). Through the course of their analysis, it was also possible to evaluate the eNose for its ability to discriminate infection with these pathogens compared with uninfected control animals. The results of these analyses are summarised here.

7.1. *Brucella abortus* and *Mycobacterium avium subspecies paratuberculosis* (MAP)

We investigated the possibility of detecting differences in serum headspace of non-standardised field samples from cattle infected with *Brucella sp*. or *Mycobacterium avium* subsp. *paratuberculosis*. Biological and methodological variation affecting the responses was assessed.

7.1.1. Experimental Design and Methods

Four groups of samples from two different regions and with different disease status were obtained. Sera from 43 dairy cattle naturally infected with paraTB and from 24 control cattle from paraTB non-suspect herds were collected under field conditions on different farms in Germany. 26 sera from cattle naturally infected with brucellosis and 24 from non-infected control cattle were collected and provided by VLA, UK. Samples were kept at -80°C during storage and shipment. For analysis, 1mL of serum was thawed and dispensed into Nalophan bags. After 15 minutes of incubation at 25°C the headspace of the blinded samples was statically analysed in a random order using the conducting polymer-based ST214 eNose. Replicates 3 to 5 were used for data analysis [6]. Univariate statistics such as multifactor ANOVA, linear regression and Spearman's rank correlation were applied to assess statistically significant differences associated with methodological and biological variation. Multiple range testing was used to investigate differences between diseased and healthy populations rather than a trend over all groups. Significant differences are indicated using different letters starting with "a" for the group with the lowest responses. The level of the least significant difference for all techniques applied was 5% (LSD; P≤0.05). SPSS (version 11.5) Statgraphics (version 4.0) and Matlab (version 2006b) including the PLS toolbox were used. Mean-centring is a way of normalising data. Here, the mean over all groups is calculated for a certain sensor and the mean or median of an individual group is then referred to the all-group-average. The sensor related means per group are then displayed as a colour from a spectrum; the red of which is above the sensor average while the blue end is below (Heat-maps).

7.1.2. Results

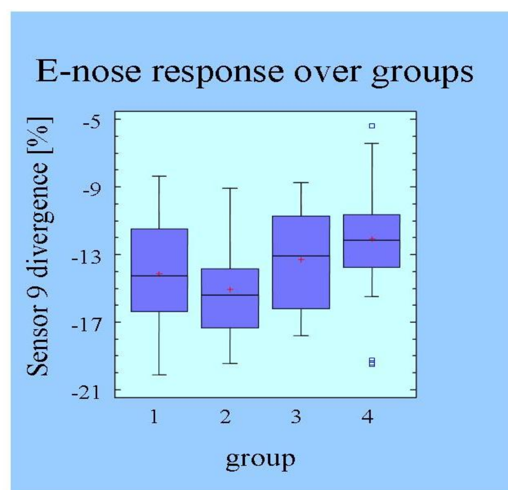
The samples were grouped into four subsets according to the disease status: 1= paraTB negative; 2= paraTB positive; 3= Brucella negative; 4 = Brucella positive. According to multifactor ANOVA, almost all sensors significantly changed due to the infection status. Applying multiple range testing, significant differences between groups were found. For all sensors, group 2 (paraTB positives) showed the lowest responses ("a") while group 4 (Brucella positives) showed the highest responses ("c" or "d"). Groups 1 (paraTB negative) and 3 (Brucella negative) were found to be identical (sensors 2, 10, 11) or close to each other (most remaining sensors, table 8).

Table 8 Results of multiple range testing for sensors

Sensor	Group 1	Group 2	Group 3	Group 4
1	a	a	b	c
2	b	a	b	c
3	b	a	c	d
4	b	a	c	d
5	b	a	c	d
6	a	a	b	c
7	b	a	c	d
8	b	a	c	d
9	b	a	c	d
10	b	a	b	c
11	b	a	b	c
12	b	a	c	d
13	b	a	c	d

Data can also be displayed as Box-and-Whisker plots. By way of example, Figure 15 shows responses of sensor 9 over all groups. Group 2 (paraTB positive) was below the control groups 1 and 3, while group 4 was above.

Figure 15 The response of sensor 9 is shown for all groups

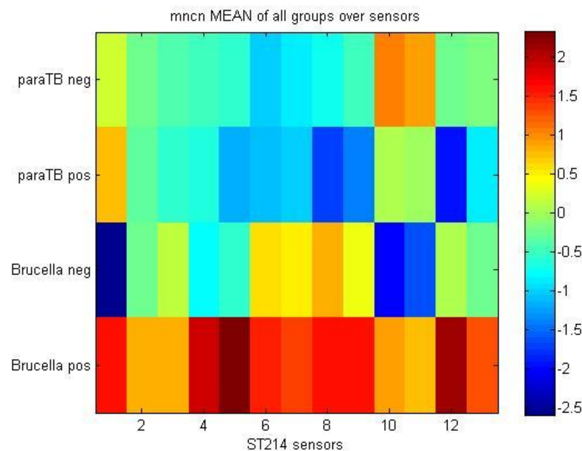


Key: group 1= paraTB neg., group 2=paraTB pos, group 3= Brucella neg., group 4 = Brucella pos

Box-and-Whisker plots indicated a broad overlapping in sensor responses mainly due to methodological variation. This was also confirmed using multifactor ANOVA. To aid the summary of results, sensor responses were mean-centred and displayed as a heatmap (Figure 16). ParaTB positives showed "colder areas" (towards blue) especially for sensors 8, 9 and 12 while brucella positives had higher responses expressed as "warm areas" (towards red) (see sensors 4 to 9, 12, 13).

However, results were less clear compared to Box-and-Whisker plots or multiple range tests due to the fact that heatmapping does not discriminate the different types of biological and methodological variation present in the data.

Figure 16 Differences in pattern between the four groups of samples. Lower responses were found for sensors 8, 9 and 12 (cold areas) for paraTB positives while for Brucella positives higher responses were found



7.1.3. Discussion & Conclusions

The eNose can differentiate sera from brucellosis and paratuberculosis infected animals and healthy animals at the population level. Statistically significant population level differences were shown in the responses of various sensors between paraTB positives, paraTB negatives, Brucella negatives and Brucella positives. In this order, responses ranged from low to high. Samples from control animals lay between samples from the two infected groups indicating that differences in sensor responses due to the sample origin was less than between the two infected groups.

Biological variation may be caused by inherent biological variability (age, sex, breed, and general physiological status) or environmental influences (diet, climate, season, husbandry practices, intercurrent infections). Eliminating these influences does not seem to be possible for a natural population. Therefore, they cannot readily be predicted, quantified or normalised to a sufficient extent to reduce their influence on the eNose sensor response. Furthermore, methodological variation during sample analysis (time and order of sample analysis, ambient temperature, sample storage conditions etc.) can also significantly affect eNose sensor responses, and it is recognised that reproducibility of data between eNose devices can be a significant problem.

Due to methodological variation the eNose technology in its present form using this particular device would not be suitable for direct diagnosis of the disease status of an individual animal because of the broad overlap of sensor responses. However, despite the limitations mentioned, the results of the study show the potential of VOC analysis for differentiation of infectious diseases in animals.

7.2. *Mannheimia haemolytica*

Mannheimia haemolytica is a gram-negative bacterium causing respiratory infections in cattle that may also lead to systemic disorders or even septicaemia. As a consequence, the regulation of electrolyte composition and acid-base balance might be altered, and the body odour may be significantly influenced by the changed pattern of VOCs present in the body. Therefore, the potential of rapid VOC analysis using eNose technology for detecting this gram-negative bacterial infection *in vivo* was assessed.

Blood samples were collected before and after an induced infection with *Mannheimia haemolytica* and were analysed for both (i) changes in sensor responses and (ii) the rectal temperature as a surrogate marker for non-specific host response and inflammation. Univariate data analysis was applied to investigate changes in the sensor response due to the infection status, and to eliminate confounding methodological variation overlapping with biological variation and masking changes in sensor responses due to the infection status.

7.2.1. Animals, materials and methods

Animals

Twenty conventionally cross-bred calves aged 2 to 3 months were housed in four groups (each group consisted of five calves) under controlled conditions according to the guidelines for animal welfare in the European Union. Daily clinical observation confirmed their healthy status before being included in the experiment. All phases of the study were performed in a specialised veterinary institute (Federal Research Institute for Animal Health, Germany) under supervision of a veterinarian, and had ethical approval.

Study design

This study was designed as an intra-individually controlled study in order to decrease the number of animals used for experimental purposes for ethical reasons. Thus, each animal was carefully and consecutively characterised before challenge (non-infected status or baseline data) as well as after challenge (infected status) by identical examinations and techniques. Daily clinical examination was performed in each animal throughout the full study (including monitoring of respiratory rate, nasal secretions, ocular secretions, rectal temperature, appetite, and body weight).

Experimental challenge

All calves were inoculated with *Mannheimia haemolytica* biotype A serotype 1 (*M. haem.* A1) intratracheally as described by Schimmel [9]. Ten ml of the culture containing 1.5 - 2.0 x 10^9 cfu per ml were administered per calf and day on two consecutive days (the interval between the two inoculations was 30 hours). Surviving calves (n=14) were euthanized for necropsy at 5 d after first inoculation.

Collection of blood and serum preparation

From each animal, two blood samples were collected before experimental infection (7 days and one hour pre infection), and 8 blood samples were obtained post infection: 3h, 6h, 12h, 24h, 48h, 3d, 4d, and 5d after the first bacterial inoculation. Jugular venous blood was collected using plastic syringes for serum production (S-Monovette®-Serum, Sarstedt AG & Co, Nuembrecht, Germany). Serum was harvested by centrifugation (20 minutes at 1500*g*) and stored at -80°C until analysis. Acute phase proteins (APPs) were determined, as well as headspace VOC profiles using eNose.

eNose headspace analyses

For eNose analysis, the conducting polymer based ST214 eNose was used. After thawing, the serum samples were dispensed into Nalophan bags which were sealed and incubated at 25°C for 15 minutes. The headspace generated was analysed by attaching the bag to the eNose and responses of replicates 3 to 5 were used for data analysis since they provide a maximum of stability during static sampling process as described [6].

Statistical analyses

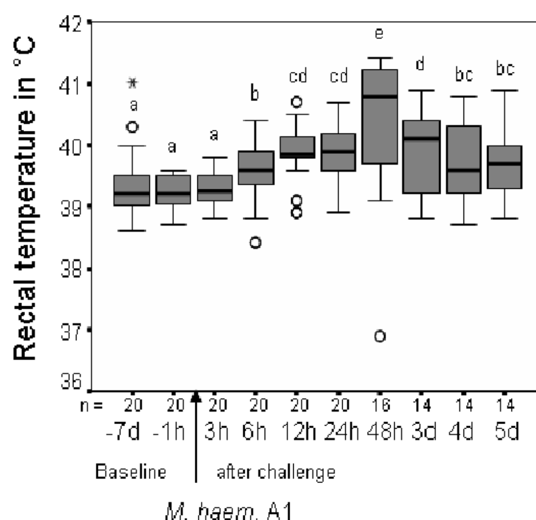
Linear regression (Pearson) and multifactor analysis of variance (mANOVA) was used to identify linear correlations between variables (sensor responses and methodological or biological variables) and to separate biological from methodological variation. For eNose data analyses, divergence, i.e. maximum amplitude of signal in sample analysis, was used. Multiple range testing was used to investigate differences between groups (e.g. over time). The level of significance for all statistical methods applied was $P \leq 0.05$.

7.2.2. Results

Clinical course of the study

After inoculation of *M. haem*. A1, four calves died 24-48 hours, and two 48-72 hours, after infection. The remaining calves (n=14) showed clinical symptoms such as loss of appetite or changes in behavior (moribund and unresponsive). The respiratory rate and rectal temperature started increasing six hours after infection. The maximum temperature was measured 48 hours after infection. Both rectal temperature (Figure 17) and breathing frequency (data not shown) remained significantly increased until the end of the study compared to pre-infection data. Surviving calves (n=14) were euthanized for necropsy (i.e. 5 d after first inoculation).

Figure 17 Rectal temperature significantly increased after *M. haem. A1* infection and reached a maximum 48h after challenge

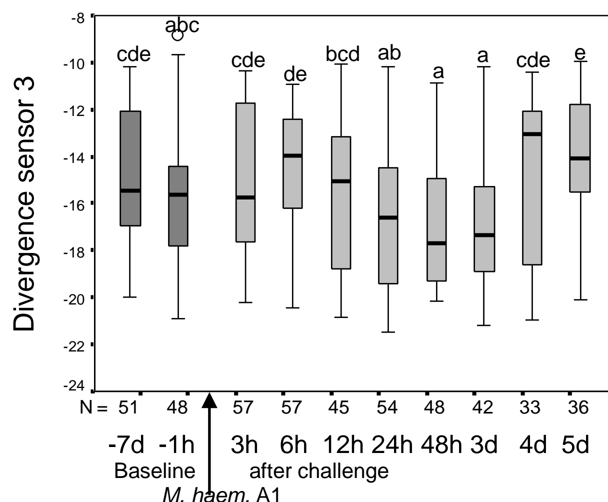


Different letters indicate significant differences between time points (mANOVA).

eNose analyses: changes over time

The sensor responses were analysed for significant changes over time. Sensors 3, 6 and 7 showed overall differences between pre- and post challange samples. Divergences of sensors 2 to 5, 10 and 11 decreased after challenge showing a temporal profile. Multiple range testing confirmed the findings and found significantly decreased divergences at days 2 and 3 post-infection. Figure 18 illustrates a typical temporal profile of a sensor response during course of the experiment.

Figure 18 Sensor responses changed significantly over time. Minimum Divergences were obtained at days 2 and 3 after challenge



Most sensors were significantly affected by methodological influences such as the time and day of analyses and temperature of the room in which the eNose was housed (data not shown). The subject (animal) did not influence the profile (data not shown). Comparing surviving individuals with the ones that died, no differences between both groups was found.

Correlation between eNose results and rectal temperature

Comparing eNose responses and rectal temperature (the latter as an indicator for the severity of the disease), significant correlations were found between two sensor responses (sensors 8 and 9) and rectal temperature. However, eNose responses followed the the trend of rectal temperature over time for almost all sensors (see profiles above). An increase in rectal temperature was associated with a decrease in sensor response. Strong correlations were also found between eNose responses and the concentration of APPs (data not shown).

7.2.3. Discussion

The main objectives of this study were to assess the possibility of using eNose technology for detecting changes in VOC composition and to underline the findings by comparing them to another independently obtained surrogate marker of disease (rectal temperature), using experimentally induced *M. haem.* A1 infection as the model. A temporal profile was observed for various eNose sensor responses and the rectal temperature. This indicates significant changes (i) in the headspace composition of the blood sampled at different time points during the study and (ii) between pre- and post- infection time points. It generally indicated that VOC analysis has real potential for disease diagnostics. However, the eNose technique is not able to elucidate the nature of the molecules leading to the change in sensor responses and furthermore this particular device did not allow discrimination between individual animals since methodological variation led to unpredictable changes in sensor responses. The changes in sensor responses in this trial may be due to the presence of compounds liberated by the bacteria themselves, and/or the host inflammatory response to infection, as indicated by increased rectal temperature. Temperature and sensor responses showed the same temporal profile with a temporal shift of approximately 24h, and two sensors were found to correlate directly.

7.2.4. Conclusions

As for the TB studies, eNose technology was found to have potential for analysing serum headspace VOCs and non-specifically identifying infections, e.g. *M. haem.* A1. Temporal changes in eNose sensor response associated with infection with *M. haem.* A1, and during the course of the infection, could be found at the group level and were found to correspond to changes in body temperature, demonstrating the eNose was tracking real physiological responses to infection. Further methodological improvements may enable discrimination between individuals.

Overall conclusions from project SE3221

1. eNose data demonstrate that there are discernable changes to VOC in the headspace of serum and urine from cattle for a range of infections, including TB, Brucellosis, paraTB, and *Mannheimia haemolytica*. These differences only become apparent at the group level and not at the individual animal level. This is almost certainly a consequence of the considerable methodological variation associated with eNoses [6]. This is true for two fundamentally different types of equipment. Considerable effort was invested in developing a methodological approach to minimise these differences. At present therefore, current eNose technology holds little promise for diagnosis of TB (or other bovine diseases), although proof of principle has been shown that there are different patterns of the VOC present in headspace samples brought about by infection.
2. SIFT-MS data has confirmed this principle and allowed the identity of some disease related markers. The data this method generates are very complex. A PLSDA modelling approach has proved the best method to analyse the data, although certain common compounds present in large amounts in all samples means that the data must be 'cleaned' extensively before analysis is meaningful. Analysis of serum from RBCT badgers has revealed the potential of the approach. However, projections suggest that samples sizes in excess of 150 (preferably equally divided between TB pos and neg) are needed to build a PLSDA model of approx. 80% accuracy [7]. This number of samples will be hard to obtain and costly to analyse. The data generated using the RBCT sera have indicated a possible link between increased serum concentration of ammonia and TB. This is worth investigating further with specific sensors for ammonia.
3. A reliable and safe method was developed to obtain breath samples from experimentally infected cattle. The method could be applied to cattle elsewhere if the animal is adequately restrained (e.g. by using a crush). Due to the health risks posed by breath from infected cattle, the only safe method for handling breath samples is after immobilisation on trap-columns, followed by heat-treatment. Breath volatiles are therefore only amenable to analysis by GC-MS. Using this approach a few potential biomarkers associated with TB were identified. However, their concentration was extremely low and probably too low to make them useful as practical disease markers.
4. The sum of the data from all three methods suggests that disease is most likely associated with changes in concentrations of host volatiles and not the emergence of unique markers. If this is the case, a pattern recognition method using a more robust electronic nose, or SIFT-MS may be used but under these circumstances, the method may only be suitable as a screening tool.

Potential for future work

A few possible areas for continued work have been identified as a result of this project, as follows:

1. The hypothesis that serum concentrations of ammonia are increased in badger TB could be tested by SIFT-MS analysis of serum from experimental infection studies in badgers, derived from Defra projects CB0116 and SE3246. Or through biochemical methods to quantify the amount of ammonia in serum samples.
2. In relation to point 1, there is potential in evaluating a sensitive gas monitor of ammonia to analyse the breath of anaesthetised badgers (and possibly cattle) with/without TB infection.
3. There is potential in continuing VOC analysis in the context of *Brucella* and paraTB infections.
4. Ruth McNerney is interested in VOC analysis in the context of human TB and is exploring this already with Claire Turner. There is the potential through read-through into animals if successful.

5. Heike Koehler and Petra Reinhold remain interested in the prospects for VOC analysis related to infection with other mycobacterial species (i.e. paraTB) as well as bovine infectious diseases that might be of differential diagnostic value.

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

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- Knobloch, H., C. Turner, A. Spooner and M. Chambers (2009). “Methodological variation in headspace analysis of liquid samples using electronic nose.” Sensors and Actuators B: Chemical 139:353-60.
- Spooner, A. D., C. Bessant, C. Turner, H. Knobloch and M. Chambers (2009). “Evaluation of a combination of SIFT-MS and multivariate data analysis for the diagnosis of *Mycobacterium bovis* in wild badgers.” Analyst, 17th July 2009 (doi: 10.1039/b905627k).

Presentations arising from this project

- M. Chambers. 2006. “The potential of volatile organic compound analysis to diagnose *Mycobacterium bovis* infection in badgers and cattle”. Invited speaker and chair at the 24th Syposium of the Veterinary Comparative Respiratory Society meeting – Respiratory Infections and Zoonoses, Jena, Germany.
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- H. Knobloch. 2009. ”Development of a cattle breath sampler and its use in analysing animals infected with *M. bovis*”. Poster at Breath 2009: International Conference on Breath and Odour Research, Dortmund, Germany.
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