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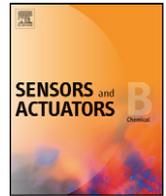
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Detection of volatile organic compounds in cattle naturally infected with *Mycobacterium bovis*

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ABSTRACT

We report here on a novel methodology in detecting *Mycobacterium bovis* (*M. bovis*) infection in cattle, based on identifying unique volatile organic compounds (VOCs) or a VOC profile in the breath of cattle. The study was conducted on an *M. bovis*-infected dairy located in southern Colorado, USA, and on two tuberculosis-free dairies from northern Colorado examined as negative controls. Gas-chromatography/mass-spectrometry analysis revealed the presence of 2 VOCs associated with *M. bovis* infection and 2 other VOCs associated with the healthy state in the exhaled breath of *M. bovis*-infected and not infected animals, yielding distinctly different VOC patterns for the two study groups. Based on these results, a nanotechnology-based array of sensors was then tailored for detection of *M. bovis*-infected cattle via breath. Our system successfully identified all *M. bovis*-infected animals, while 21% of the not infected animals were classified as *M. bovis*-infected. This technique could form the basis for a real-time cattle monitoring system that allows efficient and non-invasive screening for new *M. bovis* infections on dairy farms.

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1. Introduction

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis* (*M. bovis*), is a serious global disease with an impact on animal health, public health, and international trade [1–3]. The transmission of tuberculosis to humans via infected milk was considered a significant cause of morbidity and mortality from Victorian times until the Second World War [4,5]. Milk pasteurization and intensive eradication programs led to sharp declines of bTB in domestic livestock and humans, especially in developed countries [6]. However, the challenge of eradication remains, largely due to unauthorized

movement of infected animals and persistent wildlife reservoirs of the disease [7,8].

Accurate and efficient detection of bTB in animal populations remains of paramount importance to bTB control programs. Currently, tuberculosis testing in live cattle in the United States consists of a caudal fold test (CFT) as a screening test, with a comparative cervical test (CCT) or interferon gamma assay test as supplemental or confirmatory tests [3,9–11]. The CFT and CCT involve injecting tuberculin(s) intradermally and measuring any subsequent swelling at the site of injection 72 h later [12]. The interferon gamma assay is a confirmatory or supplemental blood test that relies on quantifying the amount of gamma interferon that is produced in animal blood samples cultured in the presence of tuberculin. The final diagnosis of bovine tuberculosis requires post mortem laboratory confirmation of disease via histopathology, polymerase chain reaction, and bacteriological culture [9,10].

Although these combined tests have good specificity and sensitivity depending on the stage of infection (over 80% sensitivity and over 90% specificity, respectively), conducting these tests at large dairies is expensive, time-consuming, logistically challenging, and must be performed by certified veterinarians [13]. Skin testing requires a second examination, and the interferon gamma assay is considerably more expensive in comparison with a skin test [14,15]. Both interferon gamma and skin testing results are delayed a minimum of 48–72 h [13,15]. The development of a

Abbreviations: bTB, bovine tuberculosis; *M. bovis*, *Mycobacterium bovis*; VOC, volatile organic compound; CFT, caudal fold test; CCT, comparative cervical test; GC–MS, gas chromatography–mass spectrometry; DFA, discriminant factor analysis; GNP, gold nanoparticles; TP, true positive; TN, true negative; FP, false positive; FN, false negative; CV, canonical variable.

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sensitive, specific, non-invasive, and efficient method for detecting *M. bovis* infection, which can be performed on the premises, would be highly beneficial to both regulators and managers in the livestock industry.

An emerging approach for diagnosing, an infectious disease at its earliest stages relies on volatile organic compounds (VOCs) that are emitted from the infectious agent and/or the host. The successful analysis of infectious disease-related VOCs is based on the following principles of cell biology. The bacterial cell membrane consists primarily of amphipathic phospholipids, carbohydrates and many integral membrane proteins that are distinct for different cell types. In disease formation, both host and invading cells can undergo structural changes, one example of which would be oxidative stress, i.e., a peroxidation of the cell membrane that causes VOCs to be emitted [16]. Some of these VOCs appear in distinctively different mixture compositions [17–22]. What is particularly significant about this approach is that each type of disease has its own unique pattern of VOCs; therefore, the presence of one disease would not mask other disease types [23]. These VOCs can be detected directly from: (i) cultured cells (i.e., the mixture of VOCs trapped above the cells in a sealed vessel) [20–22]; (ii) urine [24]; or (iii) exhaled breath [17–19].

In regard to exhaled breath, the principle is that disease-related changes in blood chemistry are reflected in measurable changes to the breath through exchange via the lungs. In certain instances, breath testing offers several potential advantages, such as (a) breath samples are non-invasive and relatively easy to obtain, and (b) breath testing has the potential for direct, inexpensive and eventually real-time monitoring.

In this paper, we explore the utility of breath testing for the detection of *M. bovis* infection in cattle. We analyze breath samples collected from cattle using gas-chromatograph/mass-spectrometry (GC-MS) to identify the VOC patterns linked with the disease conditions. Based on the detected VOC patterns, a nanotechnology-based array of sensors, termed Nano Artificial NOSE (NA-NOSE) [25–30], was tailored for the detection of bTB disease from exhaled breath. NA-NOSE is an artificial olfactory system based on an array of cross-reactive, nanomaterials-based, chemical gas sensors, which can identify and separate different gaseous mixtures, even if their constituent analytes are present at very low concentrations and their differences are very subtle. The results obtained indicate that the NA-NOSE could efficiently detect *M. bovis* infection from breath samples of cattle.

2. Materials and methods

2.1. Breath collection from cattle

Breath samples were collected and tested from 14 cattle from an *M. bovis*-infected dairy in the southern part of the state of Colorado, USA. Ten of these animals were identified as bTB-positive based on conventional tests. Nine of 10 cattle were culture positive for *M. bovis* at necropsy. One animal was culture negative but had gross and microscopic lesions compatible with bTB which were polymerase chain reaction positive. Nine of 9 animals tested were positive on CFT, 9 of 10 animals tested were positive on CCT, 8 of 10 animals tested on interferon gamma assay were positive and 2 were suspect; all 10 animals had gross lesions and 8 animals had microscopic lesions compatible with bTB. The remaining four animals from the same dairy were deemed bTB-negative based on the following: none of the animals had gross lesions, three of three animals tested were negative on CFT, one of one animal tested was negative on CCT, and one of one was negative on gamma interferon. Only one animal was cultured and it was negative, whereas the others were not cultured because they were negative on skin tests and

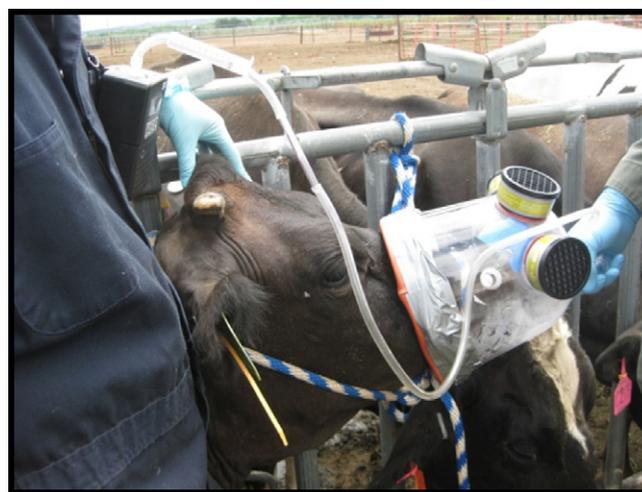


Fig. 1. Photo illustrating the system employed for breath sample collection in the cattle. Inspired air first passes into the mask through three charcoal filters and one-way valves to remove environmental VOCs. Expired air passes out of the mask through two one-way valves and through the tubing inserted into a hole in the front of the mask. Air in the tubing passes through a glass cartridge containing sorbent material (Tenax™) and is exhausted through the hand-held suction pump.

gamma interferon tests and they had no evidence of disease on post mortem examination. Additionally, breath samples from 13 cattle from two bTB-negative dairies located in northern Colorado were also tested. These animals served as negative controls, as well as to exclude confounders caused by farm and feed differences. These animals were not skin tested.

Breath specimens were collected by use of a mask designed to deliver nebulized medication to horses (Aeromask®, Trudell Medical International, London, Ontario, Canada) modified so that inspired air passed through charcoal filter cartridges (North Safety Products by Honeywell, Cranston, RI, USA) and air in the mask was pumped via Tygon® tubing (Saint-Gobain Performance Plastics, Akron, OH, USA) through a glass cartridge containing inert sorbent material (Tenax™ Catalog No. 226-35-03, SKC Inc. Eighty Four, PA, USA) by means of a handheld pump (Air Check XR5000, SKC) (Fig. 1). This approach is necessary to reduce as much as possible any confounders or contaminants that occur external to the animals we are targeting. Air was sampled from the mask at a rate of 1 L/min for 2 min. The sorbent material concentrated the VOCs in the 2 L gas sample that passed through the tube. Following exposure, the sorbent tubes were sealed and stored at -70°C until shipment to Israel for GC-MS and NA-NOSE analyses. The experiment was performed in compliance with the U.S. laws for the humane treatment of animals and was done in conjunction with disease management procedures of the Colorado Department of Agriculture and the U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services.

2.2. Breath analysis using the GC-MS

The chemical composition of the breath samples collected from all 27 cattle was analyzed employing a Gas Chromatography–Mass Spectrometry equipment (GC-MS-QP2010; Shimadzu Corporation, Japan), combined with a thermal desorption system (TD20; Shimadzu Corporation, Japan). The GC oven temperature profile used was (i) 35°C , hold for 10 min; (ii) ramp of $4^{\circ}\text{C}/\text{min}$ until 150°C ; (iii) ramp of $10^{\circ}\text{C}/\text{min}$ until 300°C ; and (iv) hold for 15 min at 300°C . VOCs were chromatographically separated using an SLB-5ms, $30\text{m} \times 0.25\text{mm}$, $0.5\ \mu\text{m}$ film thickness, with 5% phenyl methyl siloxane, capillary column (Sigma Aldrich Ltd., Rehovot, Israel). The injection port was configured in a splitless injection mode

at 23.4 kPa for 2 min, resulting in airflow with a constant linear velocity of 30.0 cm/s and a column flow of 0.70 ml/min. Samples in the sorbent tubes were desorbed at 250 °C. The molecular structures of the VOCs were determined by spectral library matching, using the Automated Mass Spectral Deconvolution and Identification System software. Ion profiles (m/z) were processed using the open source XCMS package version 1.22.1 for R environment (<http://metlin.scripps.edu/xcms/>).

2.3. Breath analysis using the NA-NOSE

Twenty-two breath samples collected from the cattle (8 bTB-positive and 4 bTB-negative from the infected dairy, and 10 animals from the non-infected dairies) were analyzed with our specifically designed NA-NOSE system (Fig. 2a). Two sorbent tubes from animals confirmed as bTB-positive and three tubes from the tuberculosis-free dairies were damaged during shipment and could not be analyzed with the NA-NOSE. For the NA-NOSE, an array of six chemiresistive films of gold nanoparticle (GNP) sensors (GNP with either octadecanethiol (2 items), decanethiol, 2-naphthalenethiol, 2-mercaptobenzoazole or 2-nitro-4-trifluoromethylbenzenethiol) was selected from an initial pool of 18 sensors (Fig. 2b) based on the results of the GC-MS analysis, whereby the organic functionalities provided broadly cross-selective absorption sites for the breath VOCs [25–29,31,32]. The sensors were produced by successively drop casting the molecularly modified GNP solutions onto pre-prepared circular Ti/Au interdigitated electrodes (24 pairs of Au electrodes; 5 μm width and 25 μm spacing between the adjacent electrodes) on a silicon wafer with 1000 nm SiO_2 film and by wire-bonding the electrodes to TO5 package holder (National Semiconductor, US) – see Fig. 2c. In these sensing films (Fig. 2d), the gold particles provide the electric conductivity and the organic film component provides sites for the sorption of analyte (guest) molecules. Details of the sensing materials synthesis have been described elsewhere [25–29,31,32].

For analyzing the breath samples with the NA-NOSE, the breath samples collected were introduced into a 400 mL sealed test chamber, housing the sensors (see Fig. 2a). Samples were thermally desorbed at 270 °C from the TenaxTM cartridge using a 750 mL gas stream into the sample chamber. Sensors output was monitored for a change in resistance using a custom program (LabView, National Instruments). All sensors were monitored simultaneously through an Agilent 34980A multifunction switch. A Stanford Research System SR830 DSP lock-in amplifier controlled by an IEEE 488 bus was used to supply the AC voltage signal to the sensors (0.2 V at 1 kHz), and to measure the corresponding current (<10 μA in the studied devices). This setup allows for measuring normalized changes in conductance as small as 0.01%. The sensors system was degassed under vacuum for 5 min at a pressure of <50 mtorr, prior to analyzing another sample, in order to purge the test chamber.

2.4. Data analysis

The GC-MS results for a given identified compound were compared across three treatment groups using the Wilcoxon rank sum test at a significance level of $p\text{-value} < 0.05$ [33]: bTB positive animals from the infected dairy, bTB negative animals from the infected dairy, and animals from the tuberculosis-free dairies. NA-NOSE sensors responses, defined as the relative resistance change experienced by the sensors immediately after exposure to the breath sample, were used as inputs for Discriminant Factor Analysis (DFA) pattern recognition algorithm [34,35]. Data were selected to be used as training and validation data sets. The classification prediction was calculated employing the leave-one-out cross-validation method, as described elsewhere [36]. For this purpose, DFA was computed using a training data set that excluded one

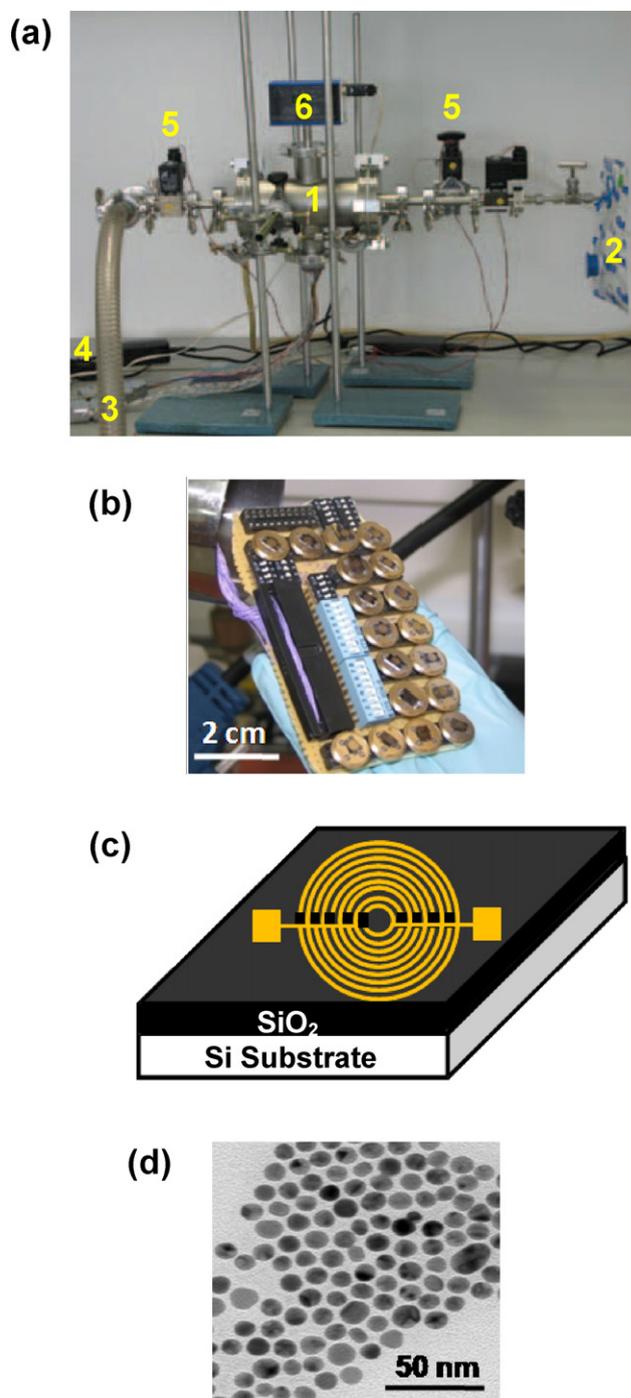


Fig. 2. (a) Photo of the NA-NOSE measurement system: 1 – sensors test chamber, 2 – breath sample bag, 3 – tubing to the vacuum pump, 4 – data acquisition board, 5 – automatically controlled valves, 6 – vacuum meter; (b) photo of the array of chemisensors; (c) schematic representation of sensors substrate (not drawn to scale); (d) tunneling electron micrograph image of the GNP sensing film.

test sample. After the DFA computation, the test sample was projected onto the DFA model that was calculated using the training set. In this way, the test sample was blind for the DFA model, so that its class affiliation was unknown. In a two-group classification case, the discrimination is obtained through the first canonical variable (CV1). The classification of the unknown sample was determined using standard cluster analysis based on the distance to groups centers on CV1-axis. All possibilities of leaving out one sample were tested, and the left-out sample was classified as true positive (TP), true negative (TN), false positive (FP) and false negative (FN). bTB

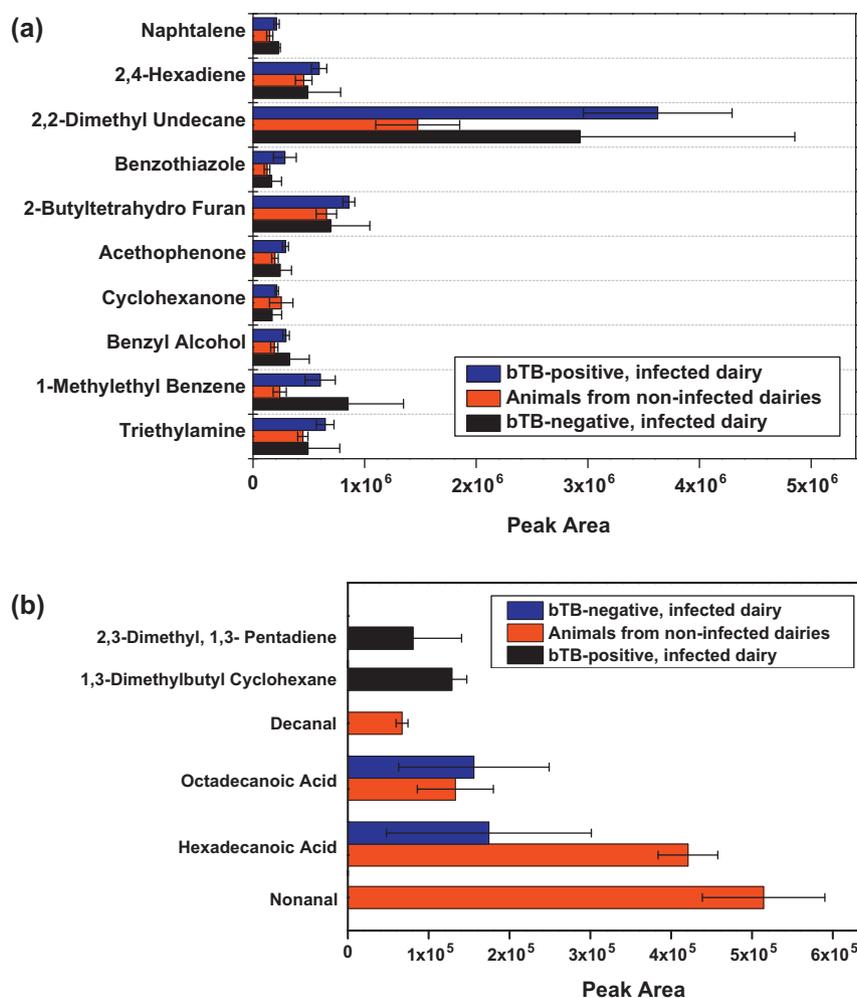


Fig. 3. The chemical composition of the breath samples identified by GC–MS: (a) predominant VOCs in animals' breath samples and (b) exclusive VOCs for bTB positive and bTB negative cattle.

identification sensitivity and specificity were calculated from Eq. (1):

$$\text{Sensitivity} = \frac{TP}{TP + FN}; \quad \text{Specificity} = \frac{TN}{TP + FP} \quad (1)$$

Features extraction and data classification were conducted under the MATLAB® (The MathWorks) environment. Statistical analysis was carried out using SAS JMP, Version 8.0 (SAS Institute Inc., Cary, NC, USA, 1989–2005).

3. Results

3.1. Chemical analysis of the breath samples

The chemical composition of the breath samples analyzed by GC–MS included the following types of compounds: two ketones, two aromatic compounds, one methylated alkane, one cycloalkane, one ether compound, one alcohol, one benzene derivative, one amine, two dienes, two aldehydes, and two acids. All but six compounds were found in the exhaled breath of the majority of cattle, being present in at least 80% of bTB positive cattle's breath samples and in at least 75% of bTB negative cattle's breath samples (Fig. 3a). These compounds cannot be associated with bTB infection since the relative concentrations of the 10 analytes common to both groups, based on differences in peak areas for an identified compound, were not statistically similar for bTB negative animals independent of their dairy of origin, whereas

statistically different from the bTB infected animals, as resulting from the Wilcoxon tests performed. The remaining six compounds were further investigated due to their presence in the breath of more than 75% of the specimens from one group and in less than 25% of the specimens from the other group. These compounds were: one cycloalkane and one diene for bTB, and two aldehydes and two acids for not infected animals (Fig. 3b). As the two aldehydes were found only in the breath of the animals from the tuberculosis-free dairies, and not in the breath of the bTB negative animals from the infected dairy, they were excluded from the development of the GNP sensors array.

3.2. Analysis of breath samples with NA-NOSE

The NA-NOSE ultimately comprised an array of six cross-reactive GNP sensors which were selected based on their ability to discriminate the VOC patterns identified by the GC–MS. The six chemiresistive films of GNP were coated with either octadecanethiol (2 sensors), decanethiol, 2-naphthalenethiol, 2-mercaptobenzoazole or 2-nitro-4-trifluoro-methylbenzenethiol. Although employing the same active material, the two sensors coated with octadecanethiol were not identical because they presented different baseline resistance values. Each of the six GNP sensors of the reservoir responded either to all or to a certain subset of the VOCs found in the samples, because the organic ligands of the GNPs provided only a moderate chemical selectivity. Each

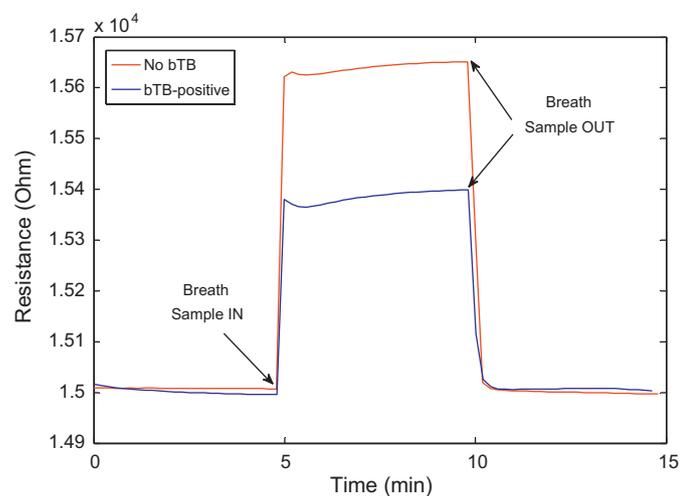


Fig. 4. Response of the GNP coated with octadecanethiol sensor to the breath samples of one animal from a tuberculosis-free dairy (red curve) and one bTB-positive animal from the *M. bovis*-infected dairy (blue curve). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

sensor from the array underwent a rapid and fully reversible change (increase) in electrical resistance upon exposure to the samples. Fig. 4 shows a typical response of one of the sensors, whose resistance increased as a consequence of a swelling effect produced when its active (sensing) material trapped the VOCs from the breath samples [37,38]. This figure suggests a higher affinity of the sensor to the VOC pattern correlated to an uninfected animal.

DFA analysis was then performed with the aim of identifying the bTB-infected cattle from the other animals based on breath analysis. The DFA plot obtained employing the features extracted from sensors responses is shown in Fig. 5. Using a blind leave-one-out cross-validation procedure, the NA-NOSE system correctly identified all bTB positive animals, while three out of the 14 bTB negative cows were misclassified; one from the infected dairy and two from the non-infected dairies. The contingency table of samples classification is shown in Table 1. Overall, based on our small study of 22 animals, the sensitivity and specificity were 100% and 79%, respectively.

4. Discussion

So far, GC-MS is beneficial for detection of VOCs that are above the instrument's limit of detection. For cases where the GC-MS can detect and identify bTB breath VOCs, several factors impede its practical implementation in point-of-care or end-user sites. These limitations include the need for expensive equipment, the high levels of expertise required to operate such instruments, the speed required for sampling and analysis, and the need for preconcentration techniques. For bTB breath analysis to become a reality, we have utilized the GC-MS results obtained in the current study and designed a tailor-made NA-NOSE that is small, easy-to-use, inexpensive, and can detect VOCs in the presence of water vapor without the need for preconcentration and/or dehumidification techniques.

Table 1
Contingency table of bTB identification obtained by the NA-NOSE system.

		Actual value	
		Positive	Negative
Prediction outcome	Positive	TP = 8	FP = 3
	Negative	FN = 0	TN = 11

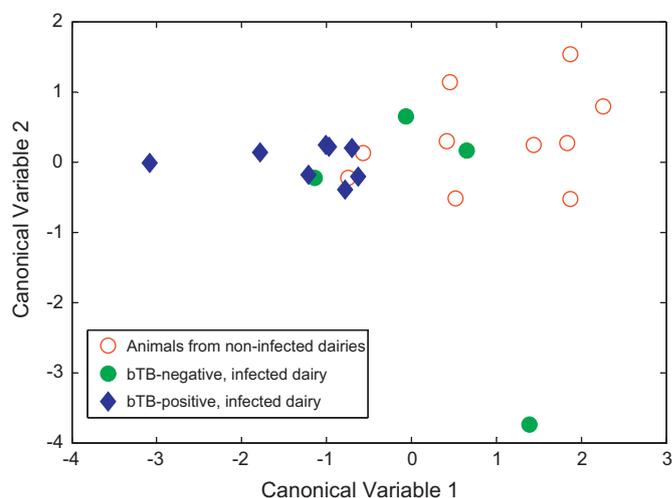


Fig. 5. DFA plot showing the discrimination between bTB-negative (circles; $n = 14$) and bTB-positive (diamonds; $n = 8$) cattle. An insight of the bTB-negative animals from the different dairies shows the complete mixing of the animals from *M. bovis* infected (green filled circles; $n = 4$) and tuberculosis-free (red open circles; $n = 10$) dairies. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

With our NA-NOSE system, we were able to correctly identify cattle naturally infected with *M. bovis* and 79% of bTB negative animals in this study. This is the first known report of application of VOC analysis to detect *M. bovis* in cattle breath. Previous studies in cattle have demonstrated different profiles of VOCs in the air over serum samples (headspace) from *Brucella*-infected, MAP-infected, *M. bovis*-infected, and normal cattle using an e-nose [39,40]. Studies in humans to detect tuberculosis in breath VOCs have been performed with some success [41–44].

It remains unknown why the three negative animals were misclassified as positive. Since the negative animals selected from the southern Colorado dairy did not undergo complete workups such as the positive animals did, it is possible that these cattle were exposed to *M. bovis* and were in very early stages on infection. There is also the possibility that these cattle, especially the animals from the bTB-free dairies, are undergoing an infection process that produces volatiles that “cross-react” in the NA-NOSE system used in this study. It is important to note that the bTB negative animals were independent of their dairy of origin, this affirmation being supported by the complete mixing of the bTB negative animals (Fig. 5), which indicates that the NA-NOSE was not affected by confounding factors determined by the dairy of origin. Our results suggest that the trained NA-NOSE system is responsive to a volatile biomarkers pattern related to *M. bovis* infection.

Importantly, the cyclohexane and the pentadiene identified as exclusive VOCs for *M. bovis* infection could not be found as prevalent in the breath of the bTB negative animals from the infected dairy, therefore they are not dairy dependent and could indeed represent tentative biomarkers for *M. bovis* identification. The two saturated fatty acids not found in the breath of the bTB infected animals were found in the breath of all bTB negative animals, also independent of their dairy location, which indicates that they were reduced during disease growth, which is in agreement with previous findings that fatty acids can be taken up by *M. tuberculosis* from the triton-fatty acid complex and utilized as a source of carbon for growth [45]. The two aldehydes that were excluded from the NA-NOSE sensor array are probably associated with feed differences among the different dairies. Most of the VOCs found in this study are similar in structure to the compounds found in previous studies done by Phillips et al. both in humans and *in vitro* [41,42]. However, there were some compounds found by Phillips et al. which were not

found in this study (i.e., more numerous alkane derivatives, alkene and ester), while no alcohols, amine and aromatic compounds were found in their studies. This difference may be due to the fact that previous studies were conducted on humans and on *in vitro* cultures and this study was in cattle.

Identification of the sensors that contributed most to differentiating bTB positives from negatives was crucial for identifying the bTB patterns. GNP sensors developed by Haick and coworkers have been shown to be sensitive to typical breath VOCs such as aldehydes, alkanes, ketones, alcohols, and benzene derivatives, with typical detection limits for the separate VOCs of 1–5 parts per billion (ppb), and showed a very low response to water [27], an important feature because the high background humidity in breath samples could easily mask the signal to the much lower concentrations of the VOCs that indicate bTB state. Breath specimens from the bTB positive and bTB negative animals were characterized by subtle differences in the concentration of a multitude of metabolites. On the other hand, the concentrations of many other metabolites remained unaffected. Some of the GNP sensors were especially sensitive to the classes of bTB-infected specific VOCs. Nevertheless, the majority of the sensors were more sensitive to the VOCs that were unaffected by the bTB state, and mainly added noise, therefore they were discarded.

5. Conclusions

We report on a new methodology in detecting *M. bovis* infection in cattle, based on identifying unique VOCs or a VOC profile in the breath of cattle. GC–MS analysis revealed the presence of two VOCs associated with *M. bovis* infection and two other VOCs associated with the healthy state in the exhaled breath of *M. bovis*-infected and not infected animals, yielding distinctly different VOC patterns for the two study groups. Based on these results, a custom-made nanotechnology-based array of sensors (NA-NOSE) was then tailored for detection of *M. bovis*-infected cattle via breath. Our system successfully identified all *M. bovis*-infected animals, while 21% of the not infected animals were classified as *M. bovis*-infected.

The NA-NOSE system we present here shows great promise as a screening technique for bovine tuberculosis in animal populations. The NA-NOSE has advantages over the GC–MS technique because it is faster, cheaper, and portable. It could be placed beside the milking line or at the barn entrance as a screening tool for bTB in cattle. This method might also hold potential as a screening test for other diseases, such as bovine brucellosis and paratuberculosis. Further studies on experimentally and naturally infected populations, spread over a larger geographical area, are necessary to fully verify and/or refine the observed breath biomarker patterns for bTB diagnosis.

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