

Detection of Volatile Organic Compounds in *Brucella abortus*-Seropositive Bison

Alona Bayn,^{†,||} Pauline Nol,^{‡,||} Ulrike Tisch,[†] Jack Rhyan,[‡] Christine K. Ellis,[§] and Hossam Haick^{*,†}

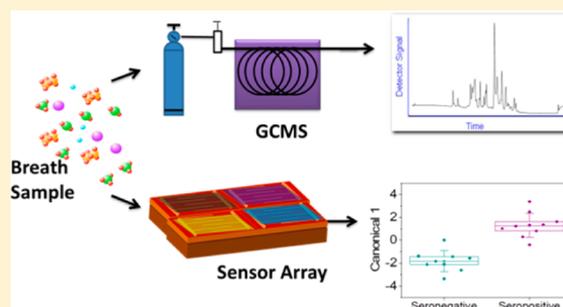
[†]The Department of Chemical Engineering and Russell Berrie Nanotechnology Institute, Technion—Israel Institute of Technology, Haifa 3200003, Israel

[‡]Wildlife Livestock Disease Investigations Team, U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services, National Wildlife Research Center, Fort Collins, Colorado 80521, United States

[§]U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services, National Wildlife Research Center, Fort Collins, Colorado 80521, United States

Supporting Information

ABSTRACT: Brucellosis is of great public health and economic importance worldwide. Detection of brucellosis currently relies on serologic testing of an antibody response to *Brucella* infection, which suffers from cross-sensitivities to other antibody responses. Here we present a new method for identifying *Brucella* exposure that is based on profiling volatile organic compounds (VOCs) in exhaled breath. Breath samples from *Brucella*-seropositive bison and controls were chemically analyzed and demonstrated statistically significant differences in the concentration profiles of five VOCs. A point-of-care device incorporating an array of nanomaterial-based sensors could identify VOC patterns indicative of *Brucella* exposure with excellent discriminative power, using a statistical algorithm. We show that the patterns were not affected by the animals' environment and that the discriminative power of the approach was stable over time. The *Brucella*-indicative VOCs and collective patterns that were identified in this pilot study could lead to the development of a novel diagnostic screening test for quickly detecting infected animals chute-side, pen-side, or even remotely in populations of free-ranging ungulates. The promising preliminary results presented encourage subsequent larger scale trials in order to further evaluate the proposed method.



Brucellosis, caused by bacteria of the genus *Brucella*, is a zoonotic disease of livestock that is of great public health and economic importance worldwide.¹ This disease results in annual losses of approximately 1 million dollars in the United States alone.¹ In domestic cattle (*Bos taurus*), *Brucella abortus* is the causative agent. However, other mammalian species are susceptible to *B. abortus* infection as well. In the United States, *B. abortus* was likely introduced to bison (*Bison bison*) and elk (*Cervus elaphus*) populations in the Greater Yellowstone Area (GYA) through cattle at the beginning of the 20th century. While national eradication campaigns have all but eliminated cattle brucellosis from the country, wild elk and bison herds of the GYA still maintain the disease and are potential transmission sources for domestic cattle sharing the range with wildlife.²

Ante-mortem detection of brucellosis currently relies on blood collection and serologic testing of animals and therefore depends on an antibody response to infection. Additionally, cross-reactivity of the serological tests to antibodies against other organisms, such as *Yersinia enterocolitica* O:9, can occur. Hence, there is a need for a less invasive, more accurate technique to detect *Brucella* infection. Ideally, this technique would allow remote detection of *Brucella* infection in wild animals.

The detection of disease-associated volatile organic compounds (VOCs), or patterns thereof, from exhaled breath^{3–8} may provide an efficient and accurate solution for identifying infectious diseases in both domestic and wild animal populations.⁹ Volatile organic compounds are currently being explored for detection of various disease processes.^{3–8} In the case of infectious diseases, unique VOC combinations and concentrations may be present in various host-derived biological products or tissues and may result from the interactions between host and agent.⁹ For example, VOC pattern differences could be detected in the headspace of serum samples collected from populations of brucellosis-infected and healthy cattle, using an array of conductive polymer sensors.¹⁰

A nanomaterial-based sensor array developed by Haick and co-workers has been proven useful for identifying diseases in humans and livestock based on analysis of VOC profiles in exhaled breath samples, including infectious diseases,^{9,11} renal disease,^{12–14} neurodegenerative diseases,^{15,16} and different types of cancer.^{17–20} In particular, Peled et al.⁹ found that cattle with bovine tuberculosis could be identified using a

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nanomaterial-based breath test. In this paper we explored VOC concentration profiles in exhaled breath samples collected from bison naturally infected or exposed to *B. abortus*, using methods of quantitative chemical gas analysis. We then tested the discriminative power of a point-of-care diagnostic method that utilized an array of nanomaterial-based sensors combined with a statistical data-analysis algorithm and its insensitivity against changes in the local environment of the tested animals. The stability of the process over time was verified in a follow-up sampling event of one of the previously tested cohorts.

■ EXPERIMENTAL SECTION

Animals. A total of 38 female bison were used for this study. Seventeen bison were housed in the United States Department of Agriculture (USDA)/Animal Plant Health Inspection Service (APHIS)/Animal Population Health Institute/Wildlife Research Facility (WRF) in Fort Collins, Colorado. Nine of these animals originated from a *Brucella*-infected herd in Wyoming and were identified as seropositive for *B. abortus* on standard tests. These animals had been housed at the WRF for 17 months. The remaining eight bison served as negative controls, were members of a commercial herd that was acquired for another research project, and had been on the premises for 7 months. Twenty-one bison were housed at the USDA/APHIS Bison Quarantine Facility (BQF) in Corwin Springs, Montana and were also part of an immunocontraception vaccine study. These bison had been captured and transported from Yellowstone National Park in April, 2012. In total, 11 bison in this cohort were seropositive for *B. abortus*; 10 bison were seronegative and served as negative controls. In July 2012, they were divided and transported to Site 1 and Site 2, which were approximately 11 km to the north of the BQF site, and approximately 1 mile apart. Five seropositive and five seronegative bison were held at Site 1. Six seropositive and five seronegative bison were held at Site 2. The six seropositive animals at Site 2 had been vaccinated with a GnRH immunocontraceptive vaccine after the first sampling in May 2012. All animal work, including the peripheral studies, was approved by the Institutional Animal Care and Use Committees of the Bison Quarantine Feasibility Study and Colorado State University.

Brucella Serology. Blood was collected from the jugular vein of each animal at the time of breath collection to perform standard serologic testing for brucellosis and blood culture. Blood samples were placed in two 10 mL clot tubes and one 7 mL heparinized tube. Serum was collected after centrifugation. Both whole blood and serum were stored at $-70\text{ }^{\circ}\text{C}$ until shipment for testing. Testing was done at the Montana State Veterinary Diagnostic Laboratory, Bozeman, MT and/or at the USDA-APHIS National Veterinary Services Laboratory in Ames, Iowa. Serologic tests included fluorescence polarization assay, complement fixation test, rivanol test, card test, buffered acidified plate antigen test, and standard plate agglutination test. Procedures for the isolation of *Brucella* bacteria from whole blood, as well as subsequent biochemical identification, has been described in Alton et al.²¹

Breath Sample Collection. Breath was collected from the 17 bison at WRF in April 2012 and the 21 bison at BQF in May 2012. Bison were restrained in a squeeze chute and breath samples were collected by use of a mask and pump as described in Peled et al.,⁹ with a few modifications. Briefly, a modified equine nebulization mask (Aeromask, Trudell Medical International, London, ON, Canada) was used for breath sample

collection. Breath samples were collected using a vacuum pump (AirChek XRS000, SKC Inc., Eighty Four, PA). Breath was drawn through a 5 cm section of Tygon tubing ($3/8$ in. o.d., $1/4$ in. i.d.) (Saint-Gobain Performance Plastics, Akron, OH) emerging from the mask, followed by a three-piece bioaerosol cassette (SKC Inc., Eighty Four, PA) containing a 37 mm, $0.22\text{ }\mu\text{m}$ PTFE filter (Tisch Scientific, North Bend, OH) and a 37 mm cellulose pad (SKC Inc. Eighty Four, PA), followed by a 20 cm section of Tygon tubing connecting a glass cartridge containing inert sorbent material (Tenax; SKC Inc. Eighty Four, PA), followed by a 20 cm section of Tygon tubing leading to the pump. The mask was held over the animal's muzzle and breath samples were collected at a rate of 1 L/min for 2 min. A total of three Tenax cartridges were collected from each animal. Three ambient air samples were collected at 1 L/min for 2 min at the beginning of collection, after approximately half of the animals were sampled, and at the end of sampling.

In January 2013, breath samples were collected from 19 of the 21 bison previously sampled at BQF in 2012. The second collection occurred after they had been transported to Sites 1 and 2 using a moderately different system than that described above. Bison were restrained in a squeeze chute and allowed to breathe freely into a plastic reservoir with an approximate volume of 40 L. After four exhalations, the reservoir was sealed. Three separate reservoirs were filled per animal. The reservoirs were then transported to a vehicle whose interior temperature remained between 8 and $13\text{ }^{\circ}\text{C}$. The reservoir contents were then collected via the same tubing/filter/Tenax/pump apparatus described above. One Tenax was used per reservoir, and reservoir contents were collected for 2 min at 2.4 L/min. At each site, three air samples were collected from a clean 40 L reservoir for 2 min at 2.4 L/min at the beginning of collection, after approximately half of the animals were sampled, and at the end of sampling. Following each collection event, the Tenax cartridges were sealed and stored at $-70\text{ }^{\circ}\text{C}$ until shipment for GC/MS and NA-NOSE analyses.

Gas Chromatography/Mass-Spectrometry (GC/MS). Chemical analysis of exhaled breath and air samples was performed using gas chromatography/mass-spectrometry (GC/MS; GCMS-QP2010, Shimadzu Corporations, Japan), combined with a thermal desorption (TD) system (TD20; Shimadzu Corporation). A SLB-5 ms capillary column was used (5% phenyl methyl siloxane; 30 m length; 0.25 mm internal diameter; $0.5\text{ }\mu\text{m}$ thicknesses; from Sigma-Aldrich Ltd., Rehovot, Israel). Prior to the analysis, the sorbent material from each separate Tenax sorbent cartridges was transferred to a precleaned glass TD tube (Sigma-Aldrich; compatible with the TD system). The TD tubes were injected into the GC-system in splitless mode at 30 cm/s constant linear speed and under 0.70 mL/min column flow; oven temperature profile: (a) 10 min at $35\text{ }^{\circ}\text{C}$, (b) $4\text{ }^{\circ}\text{C}/\text{min}$ ramp until $150\text{ }^{\circ}\text{C}$, (c) $10\text{ }^{\circ}\text{C}/\text{min}$ ramp until $300\text{ }^{\circ}\text{C}$, and (d) 15 min at $300\text{ }^{\circ}\text{C}$. The TD temperature was set to $250\text{ }^{\circ}\text{C}$. The measured GC/MS chromatograms were analyzed using the postrun analysis program for GC/MS (GCMS solutions version 2.53SU1, Shimadzu Corporation); the compounds were tentatively identified via spectral library match, using the compounds library of the National Institute of Standards and Technology (NIST; Gaithersburg, MD). Confirmation of compound identity and compound quantification were performed through measurements of external standards (heptanal, 2-ethyl hexanol, acetophenone, benzaldehyde, octanal; all from Sigma-Aldrich, Israel). The gaseous standards were produced using a

commercial permeation/diffusion tube dilution (PDTD) system (Umwelttechnik MCZ, Germany); purified dry N₂ (99.9999%) from a commercial N₂ generator (N-30, On Site Gas Systems; equipped with an N₂ purifier) was used as carrier gas. A temperature controlled oven (Dynacal, VICI Metronics, Poughkeepsie, NY) was used to mix a constant mass flow of the vaporized VOC of interest with a constant flow (100 ± 1 cm³/min) of purified N₂. The VOC/N₂ mixture exiting the PDTD system was diluted again with purified N₂ to achieve the desired concentrations ranging from single ppb_v to several ppm_v, depending on the VOC. The VOC concentration was set by (i) controlling the diffusion tubes' temperature; (ii) controlling the mass flow rate of the vaporized VOC; and (iii) controlling the total N₂ volume flow rate. The calibration gas mixtures were pumped through Tenax sorbent cartridges of the same type as were used for the exhaled breath and ambient air sampling at a rate of 1 L/min for 2 min. The calibration samples were analyzed under the same experimental conditions as the breath samples.

Contaminants of the Tenax sorbent material were identified through GC/MS analysis of pristine Tenax material from unused Tenax sorbent cartridges. Five VOCs (tentatively identified by spectral library match as methylene chloride, acetaldehyde, L-cysteine sulfonic acid, malonic acid, and naphthalene) were identified and are most likely contaminants of the Tenax sorbent material. These compounds were disregarded in the subsequent comparative analysis.²² Volatile organic compounds with a high tendency to break-through (e.g., ethanol, pentene) were also excluded during the data analysis.

Nanomaterial-Based Sensor Array. An array of nanomaterial-based sensors was used to derive patterns indicative of *Brucella*. The sensor array was comprised of cross-reactive, but chemically diverse chemiresistors that were based on two types of nanomaterials: (i) organically functionalized spherical gold nanoparticles (GNPs, core diameter, 3–4 nm), and (ii) single-walled carbon nanotubes (SWCNTs). The chemical diversity of the sensors was achieved through 21 different organic functionalities, 10 for the GNP sensors and 11 for the SWCNT sensors (see Supporting Table S1 in the Supporting Information).

The organically functionalized GNPs were synthesized as described in refs 18, 23, and 24 and dispersed in chloroform, toluene, or nonane. Chemiresistive layers were formed by drop-casting the solution onto 10 pairs of semicircular interdigitated (ID) gold electrodes (outer diameter of electrode area, 3 mm; electrode width, 20 μm; electrode-separation, 20 μm) on device grade silicon wafers (Silicon Quest International, Nevada) with 1 μm thermal oxide. The device was dried for 2 h at room temperature in ambient atmosphere and then baked overnight in a vacuum oven at 50 °C. In these layers, the GNPs' gold cores enabled electric transduction and the organic ligands provided broadly cross-selective absorption sites for the breath-VOCs. The SWCNTs were purchased from ARRY International LTD, Germany (~30% metallic, ~70% semiconducting, average diameter = 1.5 nm, length = 7 μm) and were dispersed in dimethylformamide (DMF, from Sigma Aldrich Ltd., >98% purity). Electrically continuous random networks of SWCNTs (RN-SWCNTs) were formed, as described in refs 25 and 26 by drop-casting the solution onto the preprepared electrical transducers (10 pairs of 4.5 mm wide, interdigitated Ti/Pd electrodes on device grade silicon wafers (Silicon Quest International, Nevada) with 1 μm thermal oxide. The devices

were dried under ambient conditions overnight to evaporate the solvent. The following day the RN-SWCNTs were organically functionalized with different cap-layers organic monomer, sulfated β-cyclodextrin, polycyclic aromatic hydrocarbon (PAH) derivatives, and hexabenzocoronene (HBC) derivatives (see Supporting Table S1 in the Supporting Information) and were again dried under ambient conditions.^{25,26} The fabricated GNP and SWCNT sensors responded rapidly and reversibly to typical breath-VOCs and had low sensitivity to water.^{20,25,26}

The TD tubes containing Tenax were inserted into a stainless steel chamber (volume, 400 mL; preheated to 270 °C); the samples were thermally desorbed at 270 °C for 10 min inside the chamber; thereafter the gas was inserted into the vacuumed exposure chamber (containing the array of sensors). The electrical resistance of the sensors was recorded as a function of time by an Agilent 34980A multifunction switch device. The AC voltage to the sensors (0.2 V at a frequency of 1 kHz) was supplied by a SR830 DSP lock-in amplifier controlled by an IEEE 488 board. Before each measurement, the chamber was cleaned with a 5 min vacuum procedure (>50 mtorr). In a typical exposure cycle, the sensors' baseline responses (R_0) were recorded for 5 min in vacuum, followed by 5 min under exposure to the breath sample, followed by another 5 min in vacuum. The resistance response of the sensors to the breath samples was rapid and reversible. Four normalized sensing features were extracted from the time-dependent resistance response, as illustrated in Supporting Figure S1 in the Supporting Information: (i) normalized resistance response at the beginning of the signal, $(\Delta R/R_0)^{\text{BEG}}$; (ii) normalized resistance response in the middle of the signal, $(\Delta R/R_0)^{\text{MID}}$; (iii) normalized resistance response at the end of the signal, $(\Delta R/R_0)^{\text{END}}$; and (iv) area under the curve, AUC. We have verified that the sensors' baseline resistances did not fluctuate or shift during the measurement period, indicating stable sensing performance (see Supporting Figure S2 in the Supporting Information).

Statistical Analysis: GC/MS. Binary comparisons between all 20 *Brucella*-seropositive bison and all 18 negative controls (from WRF and BQF) were performed for each tentatively identified compound by means of nonparametric Wilcoxon/Kruskal–Wallis rank sum tests at a significance level of $p < 0.05$.²⁷ The statistical tests were performed using SAS JMP, version 8.0 (SAS Institute Inc., Cary, NC, 1989–2005).

Statistical Analysis: Sensor Array. Each of the 20 sensors in the array responded to all or to a subset of the breath-VOCs. Models were created according to the different groups present (i.e., *Brucella*-seropositive vs -seronegative samples) and were derived from the collective resistance responses, using discriminant factor analysis (DFA). Discriminant factor analysis is a linear supervised pattern recognition method that effectively reduces multidimensional experimental data, in which the classes that should be discriminated are defined before the analysis is performed. Additionally, DFA served as a heuristic for selecting the most relevant sensing features and filtering out noncontributing ones. Selection of a certain set of sensing features was directly derived from their ability to identify the suspected brucellosis-patterns. The DFA input variables were the four sensing features that were obtained from the time-dependent response of each sensor, as described above. From these linear combinations were determined such that the variance within each class was minimized and the variance between classes was maximized. The DFA output

variables (i.e., canonical variables) were obtained in mutually orthogonal dimensions; the first canonical variable (CV1) had the most powerful discriminating power. The classification success was estimated through leave-one-out cross-validation in terms of the number of true positive (TP), true negative (TN), false positive (FP), and false negative (FN) predictions. Given n measurements, the model was computed using $n - 1$ training vectors. The validation vector that was left out during the training phase was then projected onto the model, producing a classification result. All possibilities of leaving-one-sample-out were considered, and the classification accuracy was estimated as the averaged performance over the n tests. DFA and data classification were conducted using MATLAB (The Math-Works).

RESULTS AND DISCUSSION

Chemical Analysis of Exhaled Breath. During the first phase of the experiment, 38 breath samples from 20 seropositive and 18 seronegative bison (collected during April/May 2012; 17 bison from the WRF; 21 from the BQF) were chemically analyzed as described in GC/MS to explore potential relationships between the VOCs and *Brucella* seropositivity/seronegativity.

Five VOCs were excluded *ab initio* because they were known contaminants of the Tenax material in the collection tubes: methylene chloride, L-cysteine sulfonic acid, malonic acid, acetaldehyde, and naphthalene. These VOCs had previously been tentatively identified in the GC/MS chromatogram of pristine Tenax.²⁸ Signatures of 158 VOCs were observed by GC/MS in at least one of the individual breath samples; 150 of these were present in >85% of the 38 breath samples. While attempting to identify these 150 VOCs by spectral library match (NIST), we applied a similarity criterion of greater than 90%. This resulted in exclusion of 74 VOCs due to their low similarity to the tabulated information, emphasizing the limitations in using existing databases. Shapiro–Wilcoxon tests demonstrated that the null hypothesis for normal distribution of the GC/MS data was not fulfilled for the 76 remaining compounds. The binary comparative analysis between the seropositive and seronegative states was performed using nonparametric Wilcoxon/Kruskal–Wallis tests. Statistically significant differences in the concentration profiles of six VOCs were observed when comparing their abundance (normalized peak areas), using a cutoff value of $p = 0.05$. One of these compounds (tentatively, styrene) was excluded after comparison with the ambient air samples because it was found in very high concentration (2 orders of magnitude higher than in the breath samples) in the ambient air at both sampling locations. The identity of the five remaining statistically significant VOCs (2-ethyl-1-hexanol, acetophenone, octanal, heptanal, and benzaldehyde; Table 1) were tentatively confirmed and their absolute concentrations determined by measuring external high-purity standards. We observed that the average concentrations of all significant VOCs present in the exhaled breath of the seropositive bison was generally lower than in the breath of the seronegative controls. However, the VOC concentrations in the ambient air samples were of the same order of magnitude as the concentration of all compounds found in the breath samples. All statistically significant VOCs (2-ethyl-1-hexanol, acetophenone, octanal, heptanal, and benzaldehyde) shared the same concentration profile: seronegative controls > ambient air > seropositive states. Further systematic studies are required to confirm the trends that were

Table 1. Statistically Significant VOCs Extracted from the GC/MS Analysis, When Comparing Breath Samples of *Brucella*-Seropositive and Seronegative Bison That Were Collected at the Wildlife Research Facility (WRF) and the Bison Quarantine Facility (BQF) and Ambient Air^a

compound	chemical group	retention time (min)	m/z	CAS no.	LOD (ppb)	LOQ (ppb)	VOC concentrations [ppb]												p -values		
							seropositive (WRF and BQF, April/May 2012)						seronegative (WRF and BQF, April/May 2012)							air sample	
							mean \pm SD	median	interquartile (Q3–Q1)	mean \pm SD	median	interquartile (Q3–Q1)	mean \pm SD	median	interquartile (Q3–Q1)	mean \pm SD	median	interquartile (Q3–Q1)			
heptanal	alkyl aldehyde	20.58	44	111-71-7	0.36	1.21	4.66 \pm 1.55	4.15	1.67	1.27	5.33 \pm 1.44	5.34	1.27	4.94 \pm 1.00	4.79	1.59	0.053				
2-ethyl-1-hexanol	fatty alcohol	26.88	57	104-76-7	0.05	0.16	169.48 \pm 62.97	144.15	65.67	154.34	234.35 \pm 91.02	239.30	172.01 \pm 64.07	140.30	91.40	0.0214					
acetophenone	aromatic ketone	28.70	105	98-86-2	5.19	17.13	39.68 \pm 11.42	38.69	17.34	10.42	49.20 \pm 11.42	46.89	45.62 \pm 15.37	46.81	16.59	0.01					
benzaldehyde	benzene ring with formyl derivatives	23.83	77	100-52-7	4.00	13.2	31.14 \pm 11.89	29.95	9.48	32.84	44.54 \pm 19.76	41.59	38.55 \pm 15.04	42.81	26.38	0.0232					
octanal	aldehyde	25.71	43	124-13-0	1.27	4.20	10.00 \pm 2.44	9.78	2.69	6.78	13.86 \pm 4.62	14.79	12.13 \pm 4.29	12.36	6.12	0.013					

^appb: parts per billion; m/z : ion profile; CAS no: Chemical Abstracts Service Registry; LoD: limit of detection; LoQ: limit of quantification.

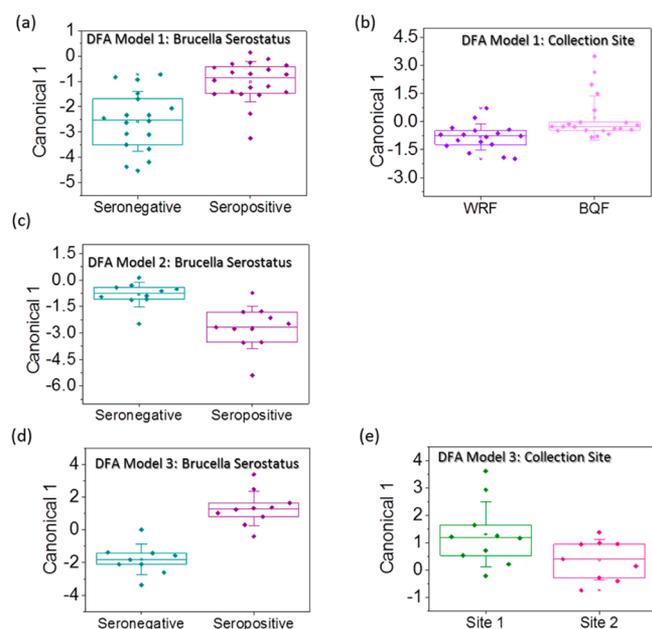


Figure 1. Distribution of the first canonical variable obtained by discriminant factor analysis (DFA). (a) DFA model 1 discriminating between seropositive ($n = 20$) and seronegative ($n = 18$) bison. Samples were collected April/May 2012 at the Wildlife Research Facility (WRF) and the Bison Quarantine Facility (BQF); (b) DFA model 1 not distinguishing between the collection sites (WRF and BQF); (c) DFA model 2 discriminating between seropositive ($n = 11$) and seronegative ($n = 10$) bison from the BQF only (May 2012); (d) DFA model 3 discriminating between the follow-up breath samples (collected January 2013 from 19 of the original 21 BQF bison that were transferred to Sites 1 and 2) of seropositive ($n = 10$) and seronegative ($n = 9$) bison; (e) DFA model 2 not distinguishing between Site 1 and Site 2.

observed in this pilot study and to clarify their biochemical origin. Heptanal could be of endogenous origin, since it has been found in the headspace of bison muscle,¹ bovine milk,²⁹ and in raw and extruded bovine rumen.³⁰ Octanal and acetophenone have been previously observed in bovine breath samples by others,³¹ 2-ethyl-1-hexanol have been detected in cattle rumen headspace,³² and benzaldehyde have been known to be present in dairy cows and/or their waste,³³ consequently any of those materials could have metabolic relevance.

Although these compounds cannot be attributed to a specific host/agent activity or response, the observed differences in the concentrations of the five statistically significant VOCs may reflect changes in VOC production related to the infection process. In addition, we cannot with absolute certainty exclude the possibility that the identified compounds are of exogenous

origin and that the observed concentration differences are a coincidental outcome of the applied statistical analysis.

In the second phase of the experiment, we derived collective patterns from the same 38 breath samples (20 seropositive and 18 seronegative bison) that were chemically analyzed, using an array of 21 broadly cross-reactive nanomaterial-based sensors (see Supporting Table S1 in the Supporting Information) and the statistical algorithm DFA, as described in the sections Nanomaterial-Based Sensor Array and GC/MS. Several potential sensing feature combinations were identified that appeared to distinguish between seropositive and seronegative states. The observation of multiple possibilities for DFA models with similar discriminative power increases the reliability of the separation between the two states. Figure 1a shows a representative example for a DFA model (viz. DFA model 1, based on 4 sensing features from three GNP sensors, see Supporting Table S1 in the Supporting Information) with good discriminative power. The CV 1 output parameters of DFA model 1 formed well-defined and well-separated clusters for the seropositive and the seronegative states (see Figure 1a). The statistical classification success of model 1 was derived by leave-one-out cross validation, as described in the section GC/MS and is listed in Table 2. Classification results achieved sensitivity, specificity, and accuracy levels, 72%, 90%, and 82%, respectively.

In order to study the possible confounding effect of the bison's environment, DFA model 1 was then applied to separating the breath samples collected at the WRF from the ones collected at the BQF. Figure 1b shows that the CV1 values for the two locations overlapped strongly and leave-one-out cross validation yielded arbitrary classification results. This indicates that the discriminative power of the derived DFA model for *Brucella* seropositivity in bison was not affected by the animals' environment. This feature is highly relevant for developing a future universally applicable breath test.

Figure 1c provides a second example for a DFA model with good discriminative power for 11 seropositive and 10 seronegative bison from a uniform environment (BQF sample collection, May 2012), based on three sensing features from two different types of sensors (one GNP sensor and one SWCNT sensor; see Supporting Table S1 in the Supporting Information). Well-defined and well-separated CV 1 clusters were again observed for the two states (see Figure 1c). However, interestingly, the maximal overall classification success that could be achieved was not higher than for the mixed cohort from two different environments (accuracy, 81%). This further validates the universal applicability of the developed method.

In the third phase of this study we investigated the stability of the method's discriminative power and its insensitivity to the local environment over time. In addition, we studied the tolerance of the approach against variation of the breath sample

Table 2. Success of Statistical Classification of *Brucella*-Seropositive and Seronegative Bison Using Discriminant Factor Analysis Models Represented in Figure 1, Derived by Leave-One-Out Cross Validation^a

DFA model	target group	control group	no. of animals	TN	FP	FN	TP	sensitivity (%)	specificity (%)	accuracy (%)
1	seropositive bison (WRF and BQF, April/May 2012)	seronegative bison (WRF and BQF, April/May 2012)	38	18	2	5	13	72	90	82
2	seropositive bison (BQF, May 2012)	seronegative bison (BQF, May 2012)	21	8	3	1	9	90	73	81
3	seropositive bison (sites 1 and 2, January 2013)	seronegative bison (sites 1 and 2, January 2013)	20	9	1	1	8	89	90	89

^aFN, false negative; FP, false positive; TN, true negatives; TP, true positives; sensitivity = TP / (TP + FN); specificity = TN / (TN + FP); accuracy = (TP + TN) / (TP + TN + FN + FP).

volume. Figure 1d presents the separation between the seropositive and -negative states, using breath samples that were collected 8 months later, in January 2013, from 19 of the 21 previously tested bison at the BQF. The herd had been separated in July 2012, after the first breath collection, into two groups of mixed seropositive and seronegative animals, and had been transferred, with the exception of two animals (1 positive and 1 negative) to two sites north of the BQF. The DFA statistical treatment yielded again multiple possibilities of sensing feature combinations with good discriminative power. However, we could not achieve the maximal discriminative power by applying the DFA models 1 or 2 from the previous analysis. This could be due to the differences in the sampling procedure, especially the increased breath sample volume (2 L in May 2013; 4.8 L in January 2013). Figure 1d depicts the well-separated CV 1 clusters of the two states that were obtained using a representative DFA model providing maximal discrimination (based on three sensing features from one GNP sensor and two SWCNT sensors). Good classification success after cross-validation was achieved in this case as well (sensitivity, specificity, and accuracy, 89%, 90%, and 89%, respectively; see Table 2). The slightly better overall accuracy of DFA model 3 could be due to physiological factors, such as extent of infection and gestation stage, causing more pronounced VOC profiles. Chemical analysis was not performed for the follow-up breath samples because of the relatively small sample size.

Attempting to separate with DFA model 3 the bison according to their location (sites 1 and 2) did not succeed: The CV 1 values of the two sites overlapped (see Figure 1e) and the classification success after cross-validation was arbitrary. These results indicate that the discriminative power of the tested nanomaterial-based sensor and the insensitivity of the method to the animals' local environment were stable over time. Variation of the breath testing protocol might require adjusting the sensing features but does not seem to impede discrimination of seropositive and seronegative animals.

SUMMARY AND CONCLUSIONS

Brucellosis causes the livestock industries great losses in time, resources, and money.¹ We have presented a feasibility test of a new method for identifying *Brucella*-seropositive bison that is based on profiling of VOCs in the animals' exhaled breath. A point-of-care diagnostic method that utilized an array of nanomaterial-based sensors combined with a statistical algorithm could identify well-separated patterns with good discriminative power and predictive capability for *Brucella* exposure. We demonstrated that the VOC patterns were not affected by the animals' environment, by including bison herds from different locations. We further demonstrated that the discriminative power of the approach and the insensitivity to the environment were stable over a time span of at least 8 months. Complementary quantitative chemical analysis identified five VOCs that were statistically significantly different in the concentration profiles of *Brucella*-seropositive bison versus controls. However, attributing these five VOCs to specific host responses to infection or agent–host interactions is beyond the scope of this pilot study. More systemic work is needed to understand the metabolic processes in individual host and bacterium species and the alterations that result when exposure or infection occurs.

The VOC concentration profiles and collective VOC patterns that were identified in this pilot study could form

the basis for the development of a novel diagnostic test to quickly detect *Brucella*-seropositive animals chute-side, pen-side, or even remotely in populations of free-ranging ungulates. However, the limited sample size of this study impedes far reaching conclusions at the preset stage. The promising preliminary results presented here support future work in the form of a large scale trial in order to validate the proposed method.

ASSOCIATED CONTENT

Supporting Information

Table of the nanomaterial-based sensors in the array and the sensing features used for the three DFA models, a schematic illustration of the feature extraction method from the time-dependent resistance response, and baseline resistance as a function of time. This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Author

*E-mail: hhossam@technion.ac.il. Phone: +972(4)8293087. Fax: +972-77-8871880.

Author Contributions

[†]These authors have contributed equally to the manuscript.

Notes

The authors declare no competing financial interest.

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