Final report

Breath sensing nanotechnology for bovine respiratory disease diagnosis

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Abstract

Technologies that differentiate between infection and disease (stage and severity of pathology) are required to ensure appropriate antimicrobial use. Research to calibrate such technologies against gold standards for infection and disease in live animals is required. This project has sought to develop and evaluate the use of breath sensing nanotechnology to determine stage and severity of bovine respiratory disease in live feedlot cattle. The results are very positive with a number of metabolites found to increase or decrease in the presence of bovine respiratory disease. The breath collection method of Agscent has developed significantly since the origins of this project which has, based on the results of this method being used for pregnancy detection, significantly improved the reliability of the findings. Unfortunately, due to the small number of cases and thereby samples, the e-nose device was not able to model the disease. However, again, based on the experience of Agscent with new sensors and larger sample numbers in pregnancy, this is likely to be effective in the future.

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Executive summary

This project has sought to develop and evaluate the use of breath sensing nanotechnology to determine stage and severity of bovine respiratory disease in live feedlot cattle. This pilot project has now demonstrated that it is possible to use the Agcent breath collection device and analytic methods to identify animals with BRD – albeit in a very small sample size and with the need to undertake further larger scale research.

A key factor in the ability to identify core breath biomarkers or compounds relevant to bovine respiratory disease was the use of the Agscent breath sampling device and our method of compound analysis. While the Cyranose e-nose device was not able to model disease due to its inadequate sensitivity but also the small number of samples, Agscent has made significant progress is identifying sensors for integration into their device which are likely to be more successful. This would result in a 'real time' assessment screening device for BRD and other lung diseases.

It is important to note that while the initial number of relevant compounds preliminary, there is a strong biological rationale each one and we expect additional compounds are likely to be found to be significant and potentially related to severity with further research.

1. Background

Bovine respiratory disease (BRD) is the most common and costly disease of feedlot cattle, and a major shortfall in mitigating negative disease impact is our inability to rapidly and accurately diagnose BRD. In addition to costs associated with performance loss and animal death, the cattle industry spends millions each year attempting to prevent and treat BRD. A major opportunity exists to improve BRD diagnostics thereby promoting efficacious therapy, animal well-being, and appropriate antimicrobial utilization. New nano technologies based on breath biopsy and metabolomics have the potential to identify biomarkers in breath of cattle which may indicate presence or absence of the disease. Agscent has developed a breath capturing device and breath sampling methodology which allows for breath to be captured in a time efficient and non-invasive way with the breath sample being able to be analysed using GC-MS methods and e-nose sensors. Their expertise in this has been demonstrated with pregnancy detection in cattle and this pilot project has applied their existing knowledge, expertise and invention to the potential detection of BRD in feedlot cattle.

2. Objectives

1. Develop and evaluate breath volatile signatures (algorithms) to predict stage and severity of bovine respiratory disease in live animals.

2. Determine feasibility of integrating volatile signatures (algorithms) into a practical handheld device.

3. Methodology

Project collaboration

This project will collaborate with a separate project (B.FLT.3010) to obtain breath samples from live animals prior to euthanasia/slaughter. Animal care and use committee approval was obtained for breath sampling and euthanasia/slaughter of animals by the B.FLT.3010 project leader (Quirindi Feedlot Services).

Training

An initial trip to Quirindi NSW by Agscent staff occurred to scope the feedlot research site and conduct training on breath sampling. On-site veterinarians (funded by B.FLT.3010) were trained to utilise the Agscent breath sampler and instructed on storage/handling/shipping of samples to Agscent for breath volatile analysis via gas chromatography–mass spectrometry (GC-MS) and Electronic Nose (Cyranose). Following the initial trip, Agscent staff were on hand as required and interacted regularly through the process.

Experiment Sampling

A single pen of mixed breed steers (*Bos taurus*) was sourced to a feedlot in NSW through normal procurement practices at high risk times of year over two successive years (Autumn 2020 & 2021). It was originally proposed that the sample population would ideally consist of 10 acute BRD cases, 10 moderate BRD cases and 20 control animals from each pen per year (i.e. 20 acute BRD cases, 20 moderate BRD cases, 40 control animals total over the project duration[1]). Case definition is based on "Visual Symptoms + Fever + Lung Pathology" is included in Table 1.

Table 1: Case Definitions	
Healthy	CIS never > 1 at any time point; Temp less than 40 C at last evaluation, lung lesions less than 5% at autopsy
Case	at least one CIS equal or greater than 3; Temp greater or equal to 40 C at last evaluation, lung lesions greater or equal to 5% at autopsy

A total of 80 animals were proposed to be sampled and subsequently euthanised/slaughtered (within 24 hrs of breath sampling). Duplicate samples were to be collected from each animal with the original Agscent breath sampler expecting a total sample size of 160. It was expected that the total duration of sampling cattle will take up to 90 days (March to May, 2020). Delays and issues relating to case numbers meant that the first samples were taken late Autumn 2020 with the final samples in the March to May period of 2021.

While the initial contract proposed for breath samples to be taken using a 550ml canister which was to be sealed and secured. The purchase of bags and the additional canisters was completed,

[1] The original proposal was to utilise the CIS system which ranges from 0 (healthy) to 4 (extremely ill) with the specific criteria used for each level. The following criteria was used to determine each CIS level: CIS 1 = normal behaviour and appetite, CIS 2 = slight illness, mild depression, and/or a cough, CIS 3 = moderate illness, severe depression, laboured breathing, and/or cough, and CIS 4 = severe illness, where animals may be moribund or have little response to human approach. Clinical illness scores were to be conducted every 12 hours by a trained feedlot pen checker.

however, by the time sampling occurred, developments in our breath capturing method had led to the prototyping of the Agscent Breath Diverter device which was being used for pregnancy breath sample taking. This method did not use the original canister bags, but we designed and had custom made PTFE collection bags which attached to the Agscent Breath Diverter device. The breath collection using this device was simpler and far superior ensuring ambient air did not ingress into the sample. The custom bags were designed with a particular one-way valve and an exit mechanisms which could easily and securely be attached to a tube which allowed for safe and efficient decanting of the sample onto the sorbent tube for analysis at CSIRO. The sorbent tube used 1 litre of breath leaving the same sample from the same cow available also for the e-nose sensor device. This eliminated the need for duplicate samples. Sample bags were transported to CSIRO in Canberra for GC-MS analysis undertaken by Agscent and CSIRO staff. Support from CSIRO was part of a Kickstarter grant which also covered some activities relating to our (Agscent) ongoing pregnancy detection work. By having CSIRO engaged, we are very confident that the findings are robust and the GC-MS work is underpinned by significantly robust methodology.

Unfortunately, Agscent only received 23 samples in total being 8-Ambient air; 7-control; 6-case[2] and 2-non-formal cases. The non-formal cases were originally excluded from the model development. When we were clear on the model of metabolite separation, we identified the non-formal cases as 'control' as they did not present with the metabolites of a case. While we have not included them in the models here presented in images, we are able to demonstrate that by including them, they fit in the control and were not a case. Subsequently, we have been informed that these non-formal cases had CIS scores greater or equal to 3 and rectal temperatures of greater or equal to 40 C. However upon autopsy had no presence of lung lesions. This is supported by our assessment.

Finally, we had one specific challenge with transport rendering one day's samples (1 case, 1 control and one ambient) damaged. They were excluded from our findings due to deterioration in the sample as Australia Post held them inexplicably for a number of days prior to delivering them to us for processing.

Statistical methods

Data was analysed as per previous work on patterns of volatile organic compounds associated with pregnancy in cattle (MLA P.PSH.1166). Principal component analysis (PCA), PLS DA and Partial Least Squares Regression (PLSR) were used to identify volatile signatures and their relationship to BRD cases and controls. We only categorised the samples as case vs control vs ambient air.

Principal Component Analysis (PCA) is a method of analysis which provides a highly visual environment for detecting patterns in complex data, such as the total ion chromatographs (TICs) generated by GC-MS. It allows an analyst to see if there are any within group variations, for instance pregnant vs. non-pregnant animals in our previous research, and stages of BRD in this current research and any time dependent changes in the groups. The main advantage of the method of PCA is that it is highly interpretable and can be validated. Partial least squares DA is an alternative method to PCA that provides more direct modelling capabilities when the classes of data to discriminate between any changes between samples.

[2] One 'control sample was an outlier with significant ambient air ingress representative of a problem with sample taking and was excluded resulting in analysis being for 6 control and 6 case.

4. Results/key findings

The following charts outline the results; however it is important to note that with the small sample numbers, we would propose that further research be conducted to confirm the promising early results.

The process of analysis is to confirm that breath samples (case and control) based on GC-MS analysis are significantly distinct from ambient air. The Agscent breath diverter device opens and closes as a cow breath using a number of sensors to ensure that ambient air is excluded and lung or end tidal breath is collected. The following figure (Figure 1) shows that there is significant separation between cow breath and ambient air.

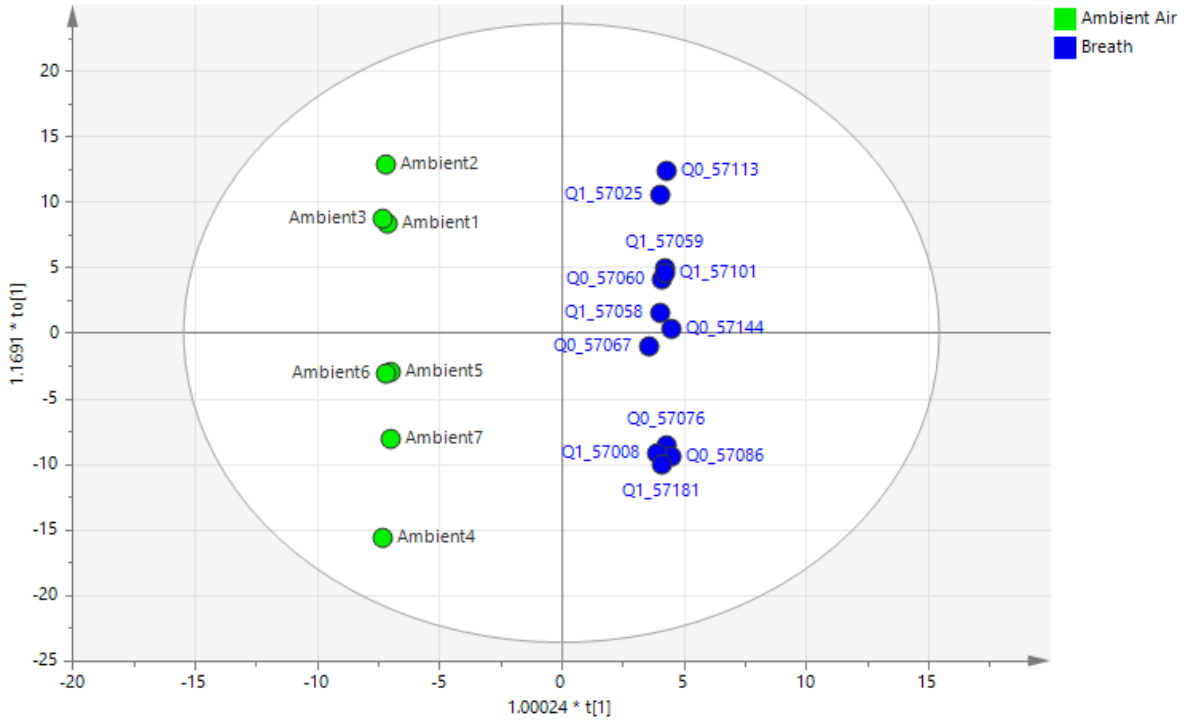

Figure 1: Score plot of ambient air and breath sample (by OPLS model). OPLS explained most of the variance in the data (R2: 0.999; Q2:0.457) which shows that breath sample could be discriminated easily (qualitatively Y variable).

Using a heat map visualisation, figure 2 shows significant metabolites related to cow breath, threshold *P*-value of 0.05 with Bonferroni correction. Blue cell: decrease; Red cell: increase. Sample size: 7-ambient air and 12-breath sample (6-control and 6-case).

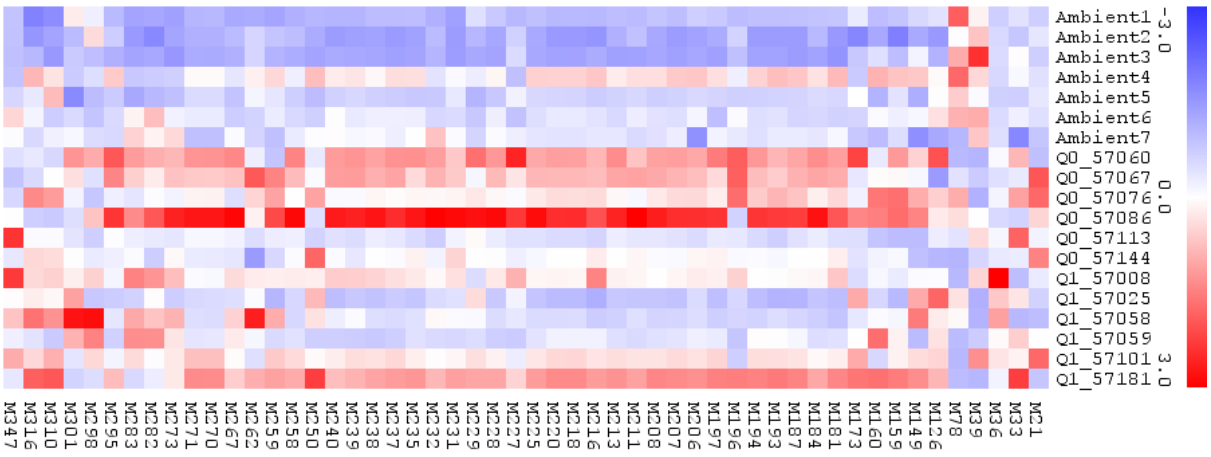

Figure 2: Heat map of cow breath to ambient air metabolite up and down regulation

Step two in our analysis was to model potential significant differences between case and control biomarkers using GC-MS. Again, it is important to recognise that the small numbers can only be interpreted as 'indicative' of potential significance and further research is required to confirm these initial findings. However, the results are very positive. Figure 3 represents the score plot of Control and Case (by OPLS model). OPLS explained most of the variance in the data (R2: 0.9; Q2:0.693) which show that disease sample could be discriminated easily (qualitatively Y variable). In each case, n=6.

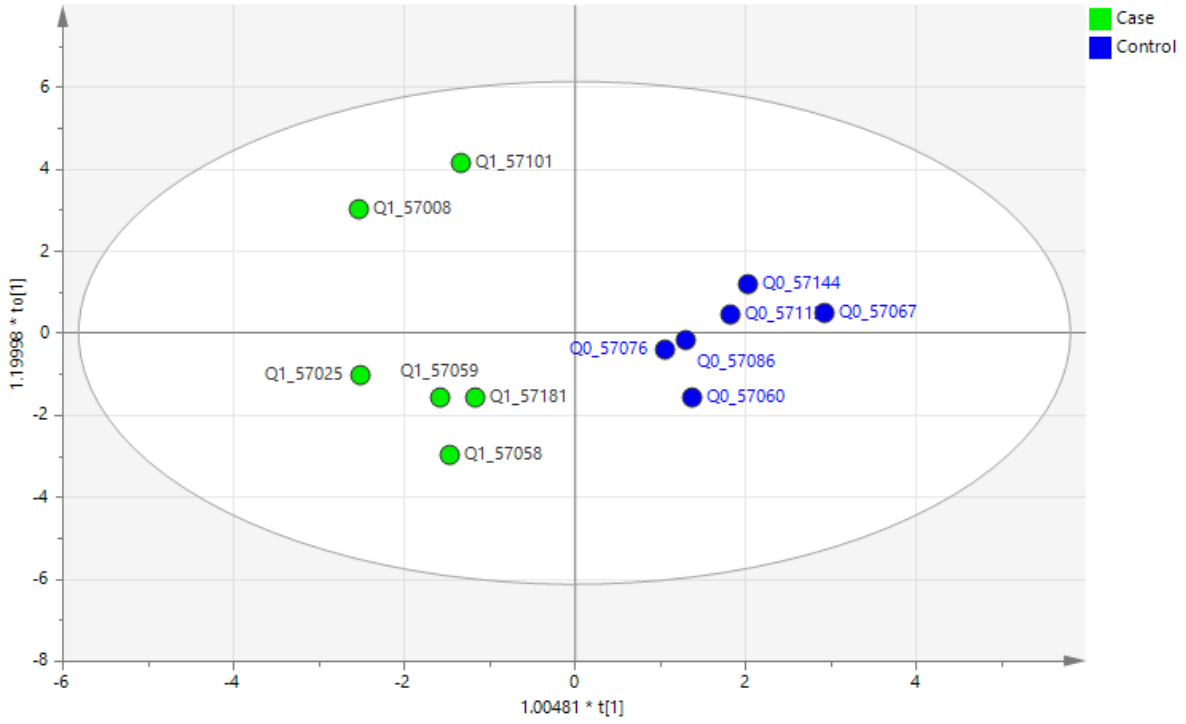

Figure 3: Case versus control

When considering the differences between the metabolites which are statistically significantly different between case and control, we observed that some increase in representation and others decrease. Figure 4 uses a heatmap to demonstrate the key metabolites of significance related to disease, threshold *P*-value of 0.05 with Bonferroni correction. Blue cell: decrease; Red cell: increase. In each case, n=6.

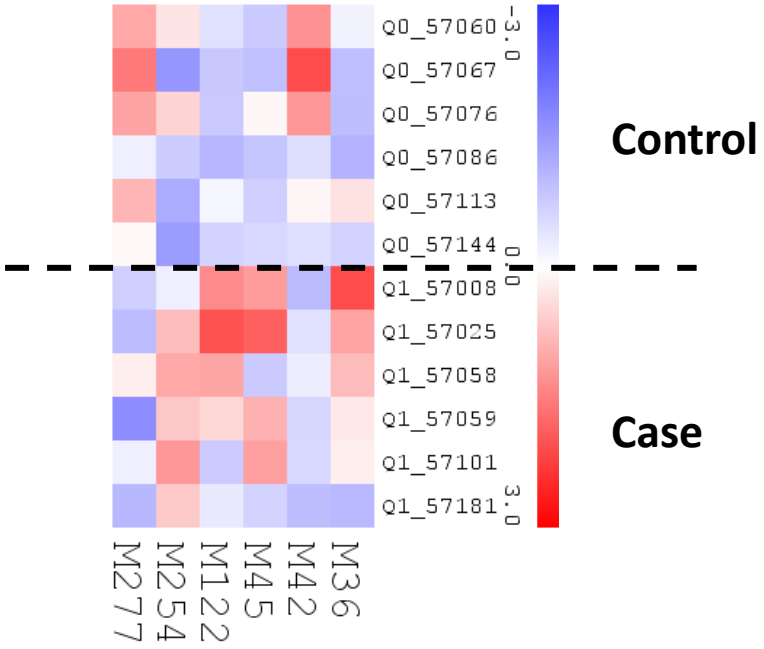

Figure 4: Heat map of metabolites of significance for disease

Our findings are that M36, M45, M122 and M254 are up-regulated in diseased sample, while M42 and M 277 are down-regulated in diseased sample.

Identifying volatile organic compounds from metabolites of significance from GC-MS.

The process of identifying the compound which is represented by retention times (GC) and M/z (mass) at this early stage is done by reference to the National Institute of Standards and Technology (NIST)'s electron ionization library. This database is the product of an ongoing comprehensive evaluation and expansion of the world's most widely used mass spectral reference library.

Identification using the NIST library provides a score as to the likelihood of the categorisation of the compound being accurately identified with over 700 demonstrating high confidence. Further confirmation of identification is generally conducted using chemical standards, however at this early stage (pilot project with few cases), reference to the NIST library is appropriate and is the basis of our findings.

The full table of potential metabolites is attached as Appendix 1, however a reduced list of most likely compound identifications and their biological rationale based on existing literature is as follows. These equate to metabolites 36, 45, 122 and 254:

2-nitropropane

2-Nitropropane is a colourless liquid that is primarily used as a solvent. 2-Nitropropane serves as an intermediate in the synthesis of some pharmaceuticals, dyes, insecticides, and textile chemicals. Chronic inhalation exposure to 2-nitropropane can cause nausea, vomiting, diarrhoea,

severe headaches, and pulmonary irritation. Princivalle et al. (2018) found 2-nitropropane in volatile organic compounds in alveolar air of patients with pancreatic cancer. Patients with pancreatic cancer have signs of respiratory diseases, pulmonary metastases, and tumour in the lung (Deeb et al., 2015).

Furan

Furan is a volatile, colourless, and flammable liquid consisting of a five-membered aromatic ring with four carbon atoms and one oxygen, mostly found in mint plants and naturally occurring food ingredients.

Furan has been identified as a major VOC of primary lung cancer in alveolar breath of patients diagnosed with lung cancer (Filipiak et al., 2008). Hashoul and Haick (2019) emphasised a relationship of furan with many respiratory diseases. 2-pentylfuran a derivative of furan has been found in VOCs of patients with respiratory disease (Chambers et al., 2009).

Furan has been identified as a potent lung toxicant (Boyd, 1980). In rats and mice, exposure to furan resulted in fluid accumulation and inflammatory reactions in lung (Egle and Gochberg, 1979). An association between furan and lung microsomal proteins has been observed (Ravindranath et al. 1984). Furan in the form of substituted furan is a highly specific pulmonary toxin in rats, guinea pigs, and rabbits (Dutcher and Boyd, 1979). It can produce edema, congestion, and haemorrhage with specific damage to bronchiolar epithelial cells (Boyd, 1977) by activating bovine lung enzyme CYP4B to form a reactive agent.

Acetic acid, ethoxy-, 1-methylethyl ester

Acetic acid, ethoxy-, 1-methylethyl ester is also known as isopropyl acetate. Isopropyl acetate is an ester, an organic compound which is the product of esterification of acetic acid and isopropanol. Lim et al. (2016) found that isopropyl acetate is in high amount in people infected with tuberculosis induced respiratory disease. Banday et al. (2011) also found a link between pulmonary tuberculosis and isopropyl acetate.

Propanoic acid ester

Propanoic acid ester is a colourless volatile liquid with a pineapple-like odour. Presence of propanoic acid ester has been observed in patients with lung cancer and respiratory disease (Filipiak et al., 2008). Chuang et al. (2020) also found propanoic acid ester as a potential volatile biomarker for lung injury.

Further research outside this project is required to confirm additional compounds which are significant to BRD and additional work to confirm these against standards will be required.

E-Nose analysis

While the e-nose device was able to distinguish between ambient air and breath samples, there were insufficient numbers of samples (case vs control) for the algorithm to make an effective model disease predictive model. In addition, the sensitivity of the Cyranose is inadequate as we have found with pregnancy to be unaffected by some volatile organic compounds which are present in air and also present in breath. It lacks differential selectivity. We are working with NASA from the US and Scentroid from Canada on different e-nose sensors to integrate into our breath collection device which can be ‘tuned’ to identified VOC’s such as this in the future.

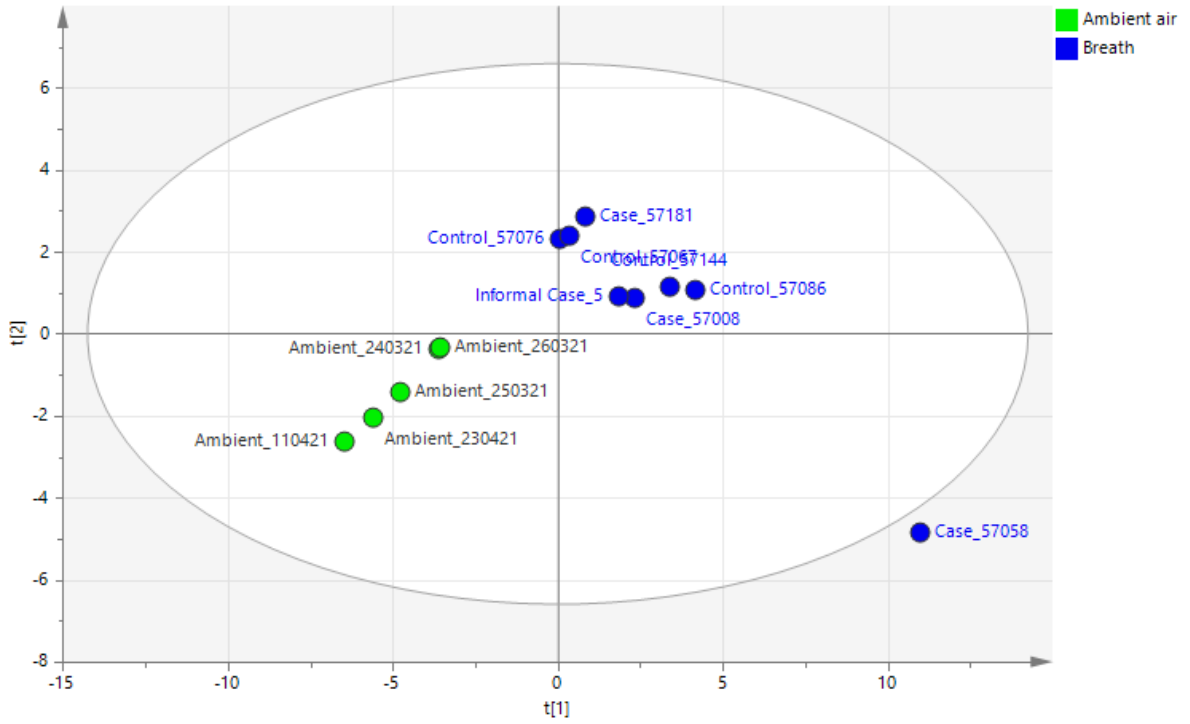

Figure 5: Cyranose data separating ambient air and cow breath

5. Benefits to industry

Our research has identified potential volatile organic compounds which are present in diseased cattle and not in healthy ones. While we were not provided severity information which may add to the selectivity of VOC's according to severity, the small sample numbers would make this assignment challenging from a statistical significance point of view. The model would not hold enough data to be able to actually work.

This research does a very positive picture of a potential non-invasive and cost-effective screening option for all stages of BRD using breath analysis. Being able to screen all animals quickly and potentially in an automated fashion (which is a goal of Agscent), would provide a significant positive benefit in the management of BRD. By being able to breath screen for BRD markers at induction of all cattle, separation of potentially infected animals could occur prior to integration with other non-infected animals.

6. Future research and recommendations

While there are limitations as to the level to which findings from such a small sample size can be generalised to a full cohort of infected animals, these early results regarding VOC's relevant to BRD as evidenced in breath are very positive. The Agscent method of breath collection and analysis has demonstrated that it is possible to identify potential breath biomarkers which relate to BRD however the sample size was too small to train the e-nose device.

We believe that this proof of concept trial demonstrates that a further trial on larger numbers of animals is warranted to both confirm biomarkers and VOC's that are evidenced in BRD and to identify which are prevalent in early to late stage of the disease.

Further, by using the Agscent breath sensing device with new sensors we are currently trialing under license from NASA and other places, we believe we may be able to train our algorithm to potentially screen all animals prior to admission into the hospital and/or induction with those indicating potential biomarkers of BRD being either excluded or segregated and monitored. This would reduce the contagious effect of the disease upon induction and further reduce the prevalence of BRD in feedlots in Australia. We would like to take this research further and take breath samples from a much larger number of animals in a feedlot and undertake GC-MS analysis to target the presence of currently identified biomarkers and train our algorithm using the new sensors to potentially identify any cases.

We believe this would allow us to customise our breath sampling device and associated algorithms to function as a non-invasive, simple and easy to use BRD screening device in feedlots, thereby improving animal welfare and productivity.

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Appendix 1: Likely Metabolites relating to BRD

RT (mins)	Metabolite	Metabolites related to Case	Metabolite_1	NIST score	Formula	Metabolite_2	NIST score	Formula	Metabolite_3	NIST score	Formula	Metabolite_4	NIST score	Formula	Metabolite_5	NIST score	Formula	Metabolite_6	NIST score	Formula	Metabolite_7	NIST score	Formula	Metabolite_8	NIST score	Formula
3.542	M36	increased (in Case)	Propane, 1-nitro-	757	C3H7NO2	Propane, 2-nitro-	762	C3H7NO2	Propanesulfonylacetonitrile	680	C5H9NO2S	Oxalic acid, allyl pentyl ester	747	C10H16O4	Butane, 1-isocyano-	736	C5H9N	Propanoic acid, 2-methyl-, 2-propenyl ester	620	C7H12O2	Oxetane, 2,2-dimethyl-	625	C5H10O	Methyl isobutyrate	649	C5H10O2
4.002	M42	decreased (in Case)	Formamide	981	CH3NO	Ethylamine	974	C2H7N	Dimethylamine	971	C2H7N	Isopropyl Alcohol	935	C3H8O	4-Penten-2-ol	916	C5H10O	Acetaldehyde, methoxy-	925	C3H6O2	Methyltartronic acid	924	C4H6O5	Acetoin	870	C4H8O2
4.618	M45	increased (in Case)	2,5-Furandione, dihydro-3-methylene-	750	C5H4O3	Furan	875	C4H4O	1H-Imidazole	701	C3H4N2	7-Oxabicyclo[2.2.1]hept-5-en-2-one	697	C6H6O2	3-Butyn-2-amine, 2-methyl-	618	C5H9N	2H-Pyran-2-one, 5,6-dihydro-	667	C5H6O2	1H-Pyrazole	619	C3H4N2	2-Cyclohexen-1-one	613	C6H8O
13.873	M122	increased (in Case)	Ethanol, 1-methoxy-, acetate	767	C5H10O3	2-Propanesulfonic acid, methyl ester	516	C4H10O3S	Acetic acid, ethoxy-, 1-methylethyl ester	755	C7H14O3	Ethanimidic acid, ethyl ester	743	C4H9NO	Guanidine	835	CH5N3	Diacetamide	704	C4H7NO2	Propanoic acid, 2-hydroxy-2-methyl-, methyl ester	748	C5H10O3	2-Hydroxy-2,4-dimethyl-3-pentanone	489	C7H14O2
27.025	M254	increased (in Case)	2-Butanone, 3-[(methylsulfonyl)oxy]-	683	C5H10O4S	2-Methanesulfonylethanamine	510	C3H9NO2S	Methanesulfonylacetone	481	C4H8O3S	Propanedinitrile, (acetyloxy)methyl-	472	C6H6N2O2	1,7-Octadien-3-ol, acetate	447	C10H16O2	trans,trans-Hexa-2,4-dienyl acetate	431	C8H12O2	Cyclohexanamine, 5-methyl-2-(1-methylethyl)-, (1α,2α,5β)-	423	C10H21N	2-Propanone, 1-cyclohexylidene-	423	C9H14O
29.767	M277	decreased (in Case)	Cathinone	611	C9H11NO	Benzenemethanol, α-(1-aminoethyl)-, [R-(R*,R*)]-	609	C9H13NO	Cathine	593	C9H13NO	1-Methyl-2-phenoxyethylamine	588	C9H13NO	Norephedrine, (±)-	583	C9H13NO	Paradrine	573	C9H13NO	Phenylephrine	550	C9H13NO2	Metaraminol	526	C9H13NO2

Appendix 2: Additional Information on cases:

animal ID	Feedlot ID	CLASSIFICATION	Agscent Classification	REPLICATE	lesions Y/N	OBS SCALE	Breed	Sex	Induction Weight	U/S at entry	CIS -48	CIS -36	CIS -24	CIS -12	CIS -euth	R temp at euth
2	57002	CASE	NA	2	Y	5	British-Euro	C male	300	nil lesions	1	1	2	2	3	40.5
8	57008	CASE	Case	2	Y	5	British-Euro	C male	364	nil lesions	1	1	2	2	2	40.2
25	57025	CASE	Case	2	Y	5	British-Euro	C male	361	nil lesions	1	1	1	2	3	40.6
58	57058	CASE	Case	2	Y	5	British-Euro	C male	329	nil lesions	1	1	2	2	3	40.1
59	57059	CASE	Case	2	Y	4	British-Euro	C male	321	nil lesions	1	1	2	2	2	40.5
101	57101	CASE	Case	2	Y	5	British-Euro	C male	468	nil lesions	1	1	2	2	3	40.3
181	57181	CASE	Case	2	Y	3	British-Euro	C male	459	nil lesions	1	1	2	2	2	40.6
89	34217	CASE	na	1	Y	3	British-Euro	C male	315	nil lesions	1	1	2	2	2	40.9
110	34239	CASE	na	1	N	0	British-Euro	C male	267	nil lesions	1	1	2	2	2	40.8
130	34260	CASE	na	1	Y	5	British-Euro	C male	290	nil lesions	1	1	2	2	2	40.8
168	34299	FALSE NEGATIVE	na	1	Y	3	British-Euro	C male	264	nil lesions						39.8
60	57060	CONTROL	Control	2	N	0	British-Euro	C male	331	nil lesions						39.2
67	57067	CONTROL	Control	2	N	0	British-Euro	C male	295	nil lesions						39.1
76	57076	CONTROL	Control	2	N	0	British-Euro	C male	347	nil lesions						39.0
86	57086	CONTROL	Control	2	N	0	British-Euro	C male	424	nil lesions						39.0
113	57113	CONTROL	Control	2	N	0	British-Euro	C male	362	nil lesions						38.7
134	57134	CONTROL	na	2	N	0	British-Euro	C male	-	nil lesions						39.8
144	57144	CONTROL	Control	2	N	0	British-Euro	C male	453	nil lesions						39.2
25	34151	CONTROL	na	1	N	0	British-Euro	C male	320	nil lesions						39.4
60	34187	CONTROL	na	1	N	0	British-Euro	C male	301	nil lesions						39.6
92	34219	CONTROL	na	1	N	0	British-Euro	C male	312	nil lesions						39.1
109	34238	FALSE POSITIVE	na	1	N	0	British-Euro	C male	261	nil lesions	1	1	2	2	2	40.2
113	34242	FALSE POSITIVE	na	1	N	0	British-Euro	C male	251	nil lesions	1	1	2	2	2	40.0
159	34290	FALSE POSITIVE	na	1	N	0	British-Euro	C male	300	nil lesions	2	3	2	2	2	40.2

na = sample lost in transit or CSIRO data loss