FINAL PROJECT REPORT

Date: 27/11/2015

Name:

Amy Scott-Thomas

Project Title:

Development of a Non-Invasive Breath Test for Legionnaires' Disease

Please copy the "Specific Objective(s)" statement, entered on your application form, in the space below.

Objective 1: Detection and identification of "specific" microbial volatile organic compounds (VOCs) from in vitro cultures of *Legionella pneumophila*, *Legionella longbeachae*, *Legionella micdadei* and other *Legionella* species; alongside *Streptococcus pneumoniae* and *Staphylococcus aureus*.

Objective 2: Detection and identification of VOCs released by *Legionella* strains incubated with macrophages and neutrophils in order to determine if there is a change in the production of volatiles compared to the pure in vitro cultures investigated in Objective 1.

Objective 3: Determine the best sampling parameters for the sensitive detection of each volatile determine in Objective 1 & 2.

Objective 4: Investigation into the prevalence of the identified volatiles in food products, beverages, medications and the environment, which may cause confounding results.

Briefly describe how successful you were in achieving the stated objective(s). If the objective(s) was not achieved, explain why that is the case and describe what you did manage to achieve.

Objective 1: This objective has been completed in its entirety. This step took longer than first anticipated as Legionella species are fastidious and require specific media for growth (buffered charcoal yeast extract – BCYE). Unfortunately this media contained charcoal which posed a number of issues. Charcoal is efficient at scrubbing volatiles and as such much of the volatiles produced by the growing bacteria were sequested by the charcoal. I therefore looked at reducing the charcoal concentration in the media in order to allow the release of bacterial volatiles. While there was an increase in the level of bacterial volatiles I detected many charcoal related compounds in the headspace. These were in large quantities measuring in mega-counts. A non-charcoal containing media was therefore essential in allowing for the analysis of Legionella headspace. PhD student Ali Mohammadi was looking into media as part of his work and after consulting with Legionella experts and an exhaustive literature search, a liquid media free of charcoal was determined (buffered yeast extract – BYE). The Legionella species all grew relatively slowly on this media hitting mid exponential phase after 1.5 days.

The following species were all grown in BYE in triplicate with each vial re-tested three times:

Legionella longbeachae – 1 x ATCC, 2 x clinical isolates
Legionella pneumophila – 3 x clinical isolates
Legionella micdadei – 1 x clinical isolate
Pseudomonas aeruginosa – 1 x clinical isolate
Staphylococcus aureus – 1 x clinical isolate
Streptococcus pneumoniae – 1 x clinical isolate
Moraxella catarrhalis – 1 x clinical isolate

BYE did not support the growth of *M. catarrhalis*, *S. pneumoniae* or *H. influenza*. To overcome this a small supplement of Luria Burtani media was added to each culture including a negative media control (monitor background media volatiles). Both *M. catarrhalis* and *S. pneumoniae* grew however *H. influenza* did not. Because it was important to keep the media background as similar as possible for all cultures no further work was performed with *H. influenza*.

Although in the past we have looked for specific volatiles released by the different species, a new approach was determined for this objective. Alongside looking for specific volatiles of interest, detecting the complete volatile fingerprint was performed and then principal component analysis (PCA) was applied to this data. The data is still to be analysed by our statistician but it is hoped that it will have the ability to separate each species from each other. A similar approach will then be used for the analysis of patient breath.

Objective 2: Unfortunately due to time constraints this objective has not been completed. The assay has been set up for the incubation of Legionella species with neutrophils and/or macrophages; however no results were obtained. Ali Mohammadi (PhD candidate) will complete this section of work under my guidance early 2016.

Objective 3: When analysing a complex sample for unknown volatiles of interest determining the best sampling protocol is crucial. For this section of work it was determined that for the analysis of whole breath samples a 50/30um DVB/CAR/PDMS solid phase micro-extraction fibre (SPME) was the best. This fibre is a useful for extracting both low molecular weight volatiles and polar analytes. This SPME fibre was coupled to a ZBWax column which is good for the separation of complex polar mixtures and also is widely used for "fingerprint" profiling. For the analysis of the *in vitro* cultures the SPME exposure time within the headspace had to be determined. After trialling a number of exposure times 1 minute was established as the optimal exposure time period. Anymore and the machine struggled to cope with the high level of compounds delivered onto the column which increased the possibility of contamination between runs. For breath analysis (which was completed during this contract) the SPME fibre was inserted into the sample and left for 24 hours at room temperature prior to analysis.

Objective 4: This objective was not completed during this contract and the main reason was because we focused on the whole volatile "fingerprint" rather than specific volatiles. However in saying this I am currently re-analysing all *in vitro* samples for the detection of volatiles of interest. This will take some time but at the end a list will be produced at which stage analytical standards will need to be purchased to confirm identification. Once confirmed a range of products, beverage and foods will be analysed for the detection of these compounds. This is important work as understanding the prevalence of the volatile of interest assists with reducing the number of false positive results.

Other work completed as part of this grant.
During this contract we took breath samples from patients suffering from pneumonia. We recruited 60 breath samples which were analysed in full scan analysis; using the DVB/CAR/PDMS SPME and ZBWAX column. This data set is currently waiting to be analysed by the statistician using PCA. These samples will also be further analysed for the presence of any specific markers of interest that may have been detected in the *in vitro* cultures.

Briefly describe any interesting outcomes which might not have been considered in your original objectives (if any).

Nil

Conference poster:
Scott-Thomas, A., Mohammadi, A., Murdoch, D. & Chambers, S. (2015). **The effect of media on the production of volatiles from *Legionella* species**. International Association of Breath Research Conference, Vienna, 14-16 September 2015.