SYMPOSIUM II:
Respiratory Infections

NON-INVASIVE DETECTION OF PULMONARY INFECTIONS: THE CURRENT STATUS

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The concept that blood, urine, and other body fluids and tissues can be sampled and analyzed to yield clinical information for diagnosis of disease states or to monitor tissue injury or therapy is the foundation of pathology, clinical diagnosis and modern medical or veterinary practice. The use of breath as a collectable sample has not received similar clinical use mainly due to the low concentrations of potential marker molecules in breath.

The ability to exchange carbon dioxide with oxygen is essential for many life forms. In animals or humans, this gas exchange occurs at the alveolar-capillary membrane in the respiratory tract. Oxygen and carbon dioxide are passively transported from blood to breath or *vice versa* and the diffusion of these gases is governed by their concentration gradients across the alveolar-capillary junction. Any additional molecule present in the blood or in the inspiratory air will also pass into the breath or blood respectively. The only requirement for transport in the gas phase is that the molecule must exhibit a significant vapor pressure. The molecular profile of gaseous breath is the resultant of the composition of the inspiratory air and volatile molecules that are present in the blood. Additionally, cells or tissues in the mouth, nose, sinuses, airway and the gastrointestinal tract may also contribute molecules. The bulk matrix of gaseous breath (>99.999999) is a mixture of nitrogen, oxygen, carbon dioxide, water vapor and the inert gases and the remainder (<100 ppm) is a mixture of as many as 500 different compounds. These molecules will have both endogenous and exogenous origins, however, the concentrations of gaseous molecules will often be higher when their origins are exogenous. The concept that breath contains molecules originating from normal or abnormal physiology has its origins in the writings of Hippocrates, the father of medicine. For example, detection of the presence of water vapor in breath has been used as a noninvasive monitor of mortality for thousands of years. Additionally, distinctive breath odors have been used for centuries as indications of diseases now known as uncontrolled diabetes, liver disease, renal failure, infection or dental disease. However, the use of odors of breath for clinical diagnosis can be masked by the powerful odors that result from the ingestion of such materials as garlic, onions, spices, peppermint, and ethanol.

The identification and quantification of molecules present at trace concentrations in breath has a limited history even though breath can be sampled from any subject from the neonate to the elderly or from mouse to horse with relative ease, minimum invasion and multiple times.

Lavoisier reported the first quantitative analysis of carbon dioxide in 1784 and demonstrated conclusively that this compound was a product of normal respiration. In the interim there were a number of reports of breath analysis for molecules such as ethanol. The earliest publications of modern day breath analysis appeared in the late 1960s and early 1970s, which was the time of nascence for modern analytical chemistry. Researchers such as Pauling, Larsson, Chen, Cohen, and Phillips reported some of these pioneering studies. Many of these studies were only possible as a result of the improved separation of gas molecules by gas chromatography, increased selectivities of mass or optical spectrometers and improved limits of detection from high parts-per-million to low parts-per-billion. However, recent advances in analytical instrumentation have suggested that

the use of exhaled breath to study disease processes should now be re-examined. Currently, a number of marker molecules have been identified in breath that can be used to identify disease, disease progression, or to monitor therapeutic intervention. It is expected that this list will soon increase dramatically since the analysis of breath is ideally suited for multiple applications including veterinary medicine.

Endogenous Gas Phase Breath Molecules

compound	concentration	physiological basis
acetaldehyde	ppb	ethanol metabolism
acetone	ppm	decarboxylation of acetoacetate
ammonia	ppb	protein metabolism
carbon dioxide	%	product of respiration
carbon disulfide	ppb	gut bacteria
carbon monoxide	ppm	production catalyzed by *heme oxygenase*
carbonyl sulfide	ppb	gut bacteria
ethane	ppb	lipid peroxidation
ethanol	ppb	gut bacteria
ethylene	ppb	lipid peroxidation
hydrocarbons	ppb	lipid peroxidation/metabolism
hydrogen	ppm	gut bacteria
isoprene	ppb	cholesterol biosynthesis
methane	ppm	gut bacteria
methanethiol	ppb	methionine metabolism
methanol	ppb	metabolism of fruit
methylamine	ppb	protein metabolism
nitric oxide	ppb	production catalyzed by *nitric oxide synthase*
oxygen	%	required for normal respiration
pentane	ppb	lipid peroxidation
water	%	product of respiration

Collection of breath condensate is an exciting new direction for breath analysis because it allows for the collection of non-volatiles. Many of these larger molecules are of significant interest. Liquid droplets are produced during normal breathing when turbulent airflow across the airway surface, or over airway cilia, causes the airway-lining fluids to be nebulized. Airway-lining fluids can also be nebulized when closed respiratory bronchioles and alveoli pop-open during normal ventilation. The amount of airway fluids that are produced is dependent upon the dynamics of breathing and the composition of the liquid droplets will be similar to the composition of airway-lining fluid. Typically sufficient samples of breath condensate are obtained by chilling the breath that is exhaled during 5-15 minutes.

Species found in Breath Condensate

Cytokines and Other Proteins
Eicosanoids
Isoprostanes
Leukotrienes
Nitrites and Nitrates
Prostanoids
Reactive Oxygen Species

This talk will discuss the collection and analysis of breath molecules in the gas and liquid phases that may be useful to diagnose and treat pulmonary infections in animals.

MYCOBACTERIAL PULMONARY INFECTIONS IN HUMANS

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Pulmonary infections due to mycobacteria are predominantly due to *Mycobacterium tuberculosis* (MTB). Approximately 1/3 of mankind is currently infected with MTB, causing significant morbidity and mortality with more than 2 million deaths per year worldwide. However, the vast majority of individuals who are infected with MTB will not develop active tuberculosis. Some may clear the initial infection in the course of the first line pulmonary defence, most others will remain latently infected (LTBI) for a lifetime.
While the incidence of tuberculosis is steadily declining in Western Europe, many countries of the world have experienced a steep rise in tuberculosis cases in the past decades as a consequence of the HIV-epidemic.
Immunization against tuberculosis with the Bacille Guérin Calmette (BCG) substrain of *Mycobacterium bovis* is still the most widely used vaccine overall, although its efficacy is only documented in the prevention of severe forms of the disease in children.
The tuberculin skin test (TST) introduced by Mantoux has been widely used as a screening test to identify individuals with LTBI. One problem in the clinical applicability of the Mantoux test is its cross-reactivity with antigens present in other mycobacteria, such as in the BCG-vaccine strain. Recently a new blood test has been developed that is both more sensitive and more specific for the diagnosis of MTB-infection than the TST and shows no cross-reactivity with prior BCG-immunization.
Mycobacteria other than tuberculosis (MOTT), also referred to as nontuberculous mycobacteria (NTM), are increasingly recognized as colonizers or pathogens in the human lungs. Nontuberculous mycobacteria are mainly found in the cause of chronic lung diseases like cystic fibrosis or other pulmonary diseases with bronchiectasis. The NTM that most commonly cause pulmonary disease are *M. abscessus, M. avium-intracellulare*, *M. cheloneae M. kansasii* and *M. malmoense*. While on standard sputum examination NTM cannot be differentiated from MTB, all NTM are resistant to standard first-line antituberculosis combination therapy. The clinical presentation, cultural identification and knowledge of the antimicrobial resistant patterns of the different mycobacteria are therefore necessary for identification of the associated diseases and successful treatment of the patients in the case of infections.

TUBERCULOSIS IN VETERINARY MEDICINE – IN AN OFFICIALLY TUBERCULOSIS-FREE MEMBER STATE OF THE EUROPEAN UNION

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Tuberculosis in humans and animals is caused by members of the *Mycobacterium* (*M.*) *tuberculosis* complex (MTC). Tuberculosis in veterinary medicine normally refers to tuberculosis in cattle caused by *Mycobacterium* (*M.*) *bovis*/*caprae*. It is a notifiable animal disease according to the OIE and it is causing disease also in humans. The taxonomic classification of *M. caprae* as separate species or subspecies of *M. bo*vis is still a matter of debate. Differences in some molecular characteristics and geographical distribution rather than in host specificity and virulence seem to distinguish these pathogens. In Germany, both *M. bovis* and *M. caprae* are present with *M. caprae* as more frequently identified species. Until the end of the second world war, approximately 60% of the cattle farms with approximately 45% of slaughtered cows were positive for tuberculosis. Therefore, cattle tuberculosis was in Germany economically and regarding public health the most important animal disease. Ten years of eradication campaign 50 years ago and milk pasteurisation since then have totally revised the situation. Germany is recognized as being free from bovine tuberculosis since 17.12.1996 according to EU decision (87/76/EG). This does not mean that cattle tuberculosis never occurs in German cattle farms. However, nowadays normally less than 10 outbreaks occur every year including only single cases, small herds or even farms of more than 1000 heads. Nevertheless, this is far below the level tolerated to maintain the status "free from tuberculosis". As zoonotic agent infecting animals involved in food production *M. bovis* can principally be transmitted to humans via direct contact or ingestion of food. The eradication campaign and milk pasteurisation, however, have caused a reduction of human cases of bovine tuberculosis from more than 10% before 1950 to approximately 1.3% today in Germany. *M. bovis* in humans can evoke a severe illness, mostly with extra-pulmonary manifestation, but in contrast to *M. tuberculosis* is normally not transmitted to other persons.

Other veterinary relevant members of the MTC, *M. microti* and *M. pinnipedii*, are found in mice as well as small predators and aquatic mammals, respectively. They are rarely isolated from human patients but still have zoonotic potential. All these members of the MTC may infected domestic animals and domestic wildlife as well as exotic animals living in zoos and game parks. In the latter case tuberculosis may threaten endangered species Due to the poor prognosis treatment is normally not recommended.

Another cycle of tuberculosis infection threatening humans and animals may be the transmission of *M. tuberculosis* from infected humans to animals and *vice versa*.

Approximately 7 000 newly infected persons are registered every year in Germany. The infection of cattle by humans excreting *M. tuberculosis* is possible, but confirmed only in very few cases. However, pet animals, dogs, cats, even horses, elephants or parrots can acquire a *M. tuberculosis* infection transmitted from their infected owners or care taking peoples and are presented to veterinary practices and hospitals. Using modern molecular tools the identity or at least the close epidemiological relatedness of the isolates from humans and their pet animals can be confirmed. Due to the poor prognosis and the zoonotic risk treatment is normally not recommended.

In conclusion, Germany is officially free from bovine tuberculosis, but animal tuberculosis has not totally disappeared from Germany.

THE POTENTIAL OF VOLATILE ORGANIC COMPOUND ANALYSIS TO DIAGNOSE *MYCOBACTERIUM BOVIS* INFECTION IN BADGERS AND CATTLE

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It is estimated that more than 50 million cattle are infected with *Mycobacterium bovis* worldwide, resulting in severe economic losses. Current diagnosis of tuberculosis (TB) in cattle relies on tuberculin skin testing, and when combined with the slaughter of test-positive animals, it has significantly reduced the incidence of bovine TB. The failure to eradicate bovine TB in Great Britain has been attributed in part to a reservoir of the infection in badgers (*Meles meles*). Accurate and reliable diagnosis of infection is the cornerstone of TB control. Bacteriological diagnosis has these characteristics, but only with samples collected *post mortem*. Unlike significant wild animal reservoirs of *M. bovis* that are considered pests in other countries, such as the brushtail possum (*Trichosurus vulpecula*) in New Zealand, the badger and its sett are protected under law in the United Kingdom. Therefore, an accurate *in vitro* test for badgers is needed urgently to determine the extent of the reservoir of infection cheaply and without destroying badgers. Current antibody tests for badgers lack sufficient sensitivity. A gamma interferon assay developed recently by the Veterinary Laboratories Agency for badgers represents a significant improvement on the existing antibody tests, but the fact remains that the gamma interferon assay depends on skilled laboratory staff and takes a minimum of two days from receipt of the blood sample. For cattle, a rapid on-farm test to complement the existing tests (the skin test and bovine gamma interferon assay) would be highly desirable.

The principle behind conventional immuno-diagnosis of TB is that the host immune response is an amplified readout, signalling encounter with *M. bovis*. When expressed in these terms it is apparent why immunological assays sometimes lack both sensitivity and specificity. Furthermore, all such assays have to be underpinned by a fundamental understanding of the host immune response to infection, which in the case of TB, is incomplete. These issues have led us to explore the diagnosis of TB in animals based on an holistic approach to the problem. Recently, significant progress has been made in developing tests for rapid diagnosis of disease based on the detection and analysis of volatiles present in clinical samples, such as blood, urine and breath. The principle behind the approach is that clinical specimens from an infected individual produce a volatile chemical signature that is distinguishable from that obtained from uninfected individuals. The inherent advantages of this approach are that multiple markers of infection (not just immunological) are examined simultaneously without needing prior knowledge of the underlying biology.

We are evaluating two complementary methods of this so-called Volatile Organic Compound (VOC) analysis: an electronic nose (EN) and a Selective Ion Flow Tube Mass Spectrometer (SIFT-MS). ENs uses a series of chemical sensor arrays that produce semi-quantitative outputs when exposed to VOC down to ppm concentrations. The advantages of the EN are generally its small size, affordability, and ability to describe the sensed

‘odour’ as a multivariate dataset; thereby allowing powerful mathematical discriminatory methods to be used in the diagnosis of disease. One potential drawback of using an EN for diagnosis is that the components of the odour signature are hard to dissect. Whilst this is of no consequence for diagnosis *per se*, identification of the individual components could lead to refined diagnostic tools and a greater understanding of host-mycobacterium interactions. By comparison, the SIFT-MS has been demonstrated to be able to monitor even lower levels (a few ppb) of trace gases in breath, and several molecules have been shown to be associated with disease states in humans. Compared to an EN, the SIFT-MS is currently a larger and more expensive device, but unlike an EN, is able to generate a mass/charge spectrum of the sensed sample; thereby allowing individual volatile compounds to be identified and quantified. Both approaches are complementary, and each can use the same clinical sample to generate a result within minutes.

Proof of principle was demonstrated initially by us for the EN using serum samples obtained from both experimentally infected badgers and cattle, as well as naturally infected badgers. Without exception, the EN was able to discriminate infected animals from controls as early as three weeks after infection with *M. bovis*; the earliest time point examined. These results were published in the Journal of Clinical Microbiology in April 2005 and will be presented in full. More recent studies have focussed on comparison of the SIFT-MS with the EN, as well as methods for collecting and analysing cattle breath in real-time. Progress with these studies will be presented.

If VOC analysis fulfils its potential, the technology underpinning the EN and SIFT-MS could be used to develop rapid diagnostic tests which could be performed on farms or in the field. Such a test for TB would complement current immuno-diagnostic assays such as tuberculin skin testing, blood-based gamma interferon assays and serology. In the longer term such tests could be developed for use by farmers (or local vets) to monitor their own livestock for a range of infectious diseases.