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APPLICATION OF VOLATILOME ANALYSIS TO THE DIAGNOSIS OF MYCOBACTERIA INFECTION IN LIVESTOCK

APLICACIÓN DEL ANÁLISIS DEL VOLATILOMA AL DIAGNÓSTICO DE LA INFECCIÓN POR MICOBACTERIAS EN GANADERÍA

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1. Summary

Volatile organic compounds (VOCs) are small molecular mass metabolites which compose volatilome, whose analysis has been widely employed in different areas. This innovative approach has emerged in research as a diagnostic alternative to different diseases in human and veterinary medicine which still present constraints regarding analytical and diagnostic sensitivity. Such is the case of the infection by mycobacteria responsible for tuberculosis and paratuberculosis in livestock. The low sensitivity of the current diagnostic techniques against *Mycobacterium bovis* (or *Mycobaccterium tuberculosis* complex) and *Mycobacterium avium* subsp. *paratuberculosis*, as well as other hurdles such as low mycobacteria loads in samples, a tedious process of microbiological culture, inhibition of many variables or intermittent shedding of the mycobacteria highlight the importance of evaluating new techniques that open different options in the diagnostic paradigm. In this sense, volatilome analysis stands as a potential option because it fulfills mycobacterial diagnosis requirements. The aim of the present review is to compile the information related to the diagnosis of tuberculosis and paratuberculosis in livestock through the analysis of VOCs by using different biological matrixes. The analytical techniques used for the evaluation of VOCs are discussed focusing on the advantages and drawbacks offered compared to the routine diagnostic tools. In addition, the described differences in the bibliography among *in vivo* and *in vitro* assays, natural and experimental infections and the use of specific VOCs (target analysis) and complete VOCs pattern (untarget analysis) are highlighted. This review emphasizes how this methodology could be useful in the problematic diagnosis of tuberculosis and paratuberculosis in livestock and poses challenges to be addressed in future research.

2. Resumen

Los compuestos orgánicos volátiles (COVs) son metabolitos de bajo peso molecular que componen el volatiloma, cuyo análisis ha sido ampliamente utilizado en diferentes áreas. Este innovador enfoque ha surgido en el campo de la investigación como una alternativa diagnóstica a diferentes enfermedades en medicina humana y veterinaria con problemas de sensibilidad analítica y diagnóstica. Tal es el caso de la infección causada por las micobacterias responsables de la tuberculosis y la paratuberculosis en el ganado. La baja sensibilidad de las técnicas diagnósticas actuales (principalmente basadas en cultivo, PCR y ensayos inmunológicos) frente a *Mycobacterium bovis* (o complejo *Mycobacterium tuberculosis*) y *Mycobacterium avium* subsp. *paratuberculosis*, así como otros inconvenientes como la baja carga bacteriana en las muestras, el tedioso y laborioso proceso que el cultivo microbiológico supone, la inhibición asociada con diferentes variables o la eliminación intermitente de las micobacterias, ponen de manifiesto la importancia de evaluar nuevas técnicas que arrojen diferentes opciones al paradigma del diagnóstico de la infección por estas micobacterias. En este sentido, el análisis del volatiloma se presenta como una opción de potencial interés ya que cumple ampliamente los requerimientos exigidos para el diagnóstico de los procesos causados por micobacterias. El objetivo de la presente revisión es recopilar la información relativa al diagnóstico de la tuberculosis y la paratuberculosis en ganado mediante el análisis de COVs utilizando diferentes matrices biológicas. Así, en este trabajo se discuten las técnicas analíticas utilizadas para la evaluación de COVs poniendo el punto de mira en las ventajas e inconvenientes que ofrecen en comparación con las herramientas diagnósticas de rutina. Además, se destacan las diferencias descritas en la bibliografía entre los estudios *in vivo* e *in vitro*, las infecciones naturales y experimentales y entre el uso de volátiles concretos (análisis dirigido) y patrones de COVs completos (análisis no dirigido). Esta revisión resume como diferentes técnicas analíticas podrían ser útiles en el problemático diagnóstico de la tuberculosis y la paratuberculosis en ganado, y plantea desafíos para estudios futuros.

3. Introduction

Analysis of volatile organic compounds (VOCs) is an emerging research area in both human and veterinary medicine (Purkhart et al., 2011), which allows a non-invasive, fast and economic diagnosis as well as identification of new biomarkers as alternative to current diagnostic techniques (Maurer et al., 2019). VOCs are defined as a sub-category of small molecular mass substances within metabolites, which are characterised by its low boiling point and high-vapour pressure (Rioseras et al., 2017; Ebert et al., 2017). VOCs are produced into the environment, allowing a direct measuring in the gas phase and offering a minimum sample handling, a non-invasive monitoring and an easier sampling compared to other metabolites which have to be extracted from biological samples (Sinha et al., 2017; Singh et al., 2018). In this context, volatilome (or volatome) is the VOCs signature produced by an organism (Phillips et al., 2013; Amann et al., 2014a; Heddergott et al., 2014; Filipiak et al., 2016).

The volatilome has a wide variety of uses and applications, such as diagnosis of infectious diseases (Burciaga-Robles et al., 2009) and neoplasia (Sever et al., 2015), distinction between vaccinated and non-vaccinated animals (Stahl et al., 2015), monitoring of antibiotic treatment (Berendsen et al., 2015), differentiation of diet composition (Recharla et al., 2017; Perez-Calvo et al., 2019) and even evaluation of reproductive parameters (Karthikeyan et al., 2013). Since VOCs are constantly emitted during metabolic processes, the detection of VOC profiles might enable the development of novel non-invasive diagnostic tools (Amann et al., 2014a).

The identification of VOCs produced by pathogens, host-pathogen interactions and biochemical pathways, either associated with homeostasis or pathophysiological responses, has become the volatilome into an approach of growing interest for the diagnosis of infectious diseases (Ellis et al., 2014). Pathologic processes have the potential to influence VOCs either by producing new volatile substances or by the metabolic consumption of VOC substrates that are normally present (Probert et al., 2009). Consequently, the diagnostic potential of VOC analysis includes two perspectives, the search of new biomarkers and the identification of biomarkers lost along a pathological process (Purkhart et al., 2011).

Infection by slowly growing mycobacteria, such as *Mycobacterium bovis* or *M. avium* subsp. *paratuberculosis* (MAP), is one of the diseases of livestock which might take advantage of the development of faster and sensitive diagnostic techniques. Taking into account the growth requirements of these mycobacteria as well as other factors associated with the host immune response after infection, diagnosis of mycobacterial infection becomes a challenge, especially in livestock sector. The diagnosis of the infection by mycobacteria is currently based on different tedious, expensive, laborious and time-consuming methodologies (Biet et al., 2005; Nienhaus et al., 2011; Ratiu et al., 2017). Thus, the analysis of VOCs has been proposed as an alternative for the diagnosis of these infections (Table 1) supported by the fact that, historically, people suffering tuberculosis had a characteristic breath smell (Spooner et al., 2009).The research carried out in this context has used different biological matrices, such as serum (Fend et al., 2006; Weiner et al., 2012), breath (Phillips et al., 2010; Weiner et al., 2012), faeces (Stahl et al., 2015; Ellis et al., 2017) and microbiological culture (Pavlou et al., 2004; McNerney et al., 2012; Purkhart et al., 2017; Küntzel et al., 2018) in order to identify biomarkers related to diseases produced by mycobacteria.

Despite the use of VOCs obtained from different biological samples to diagnose diseases is considered as a big hope with a promising future, at the moment it remains at a developing step (Ratiu et al., 2019a). One of the main hurdles against the development of this new strategy is the lack of standardisation between studies which often leads to non-comparable results (Franchina et al., 2018; Ratiu et al., 2019a). Few detailed *in vivo* studies are available on the analysis of VOCs as a diagnostic tool for mycobacterial infection in animals. In light of these premises, the present review collects the available literature to evaluate methodologies and procedures used for the diagnosis of infection by mycobacteria in livestock, focusing in the infection by *Mycobacterium bovis* and MAP, to point out future research lines of interest to be implemented.

4. *Mycobacterium bovis* and *Mycobacterium avium* subsp. *paratuberculosis* as mycobacteria target of study

Mycobacteria belong to the genus *Mycobacterium* which includes the *M. tuberculosis* complex (MTBC), with all the causative species of human and mammal's tuberculosis; the *M. avium* complex (MAC), which also comprises species of relevance in human and veterinary medicine, such as MAP; as well as environmental rapid and slow-growing non-

tuberculous mycobacteria. These all are aerobic and immobile bacilli with specific growing conditions which include pathogenic, opportunistic and saprophytic species (Pontiroli et al., 2013; Rahman et al., 2014). While there are many species, such as *M. tuberculosis* and *M. bovis*, known for being the etiological agents of important human and animal diseases; rapid- and slow-growing non-tuberculous mycobacteria use to be minority species, which should be considered because of their interference with the currently established diagnostic systems (Biet and Boschiroli, 2014).

4.1 Mycobacterium bovis

M. bovis is the aetiological agent responsible for bovine tuberculosis (bTB), also considered as the main cause of animal tuberculosis due to the multi-host character of this bacterium (Michelet et al., 2018). Animal tuberculosis is a zoonotic disease with great impact on public health, agriculture, wildlife and trade areas (Biet et al., 2005; Schiller et al., 2010a). In this sense, although the majority of cases reported as human tuberculosis are caused by *M. tuberculosis*, approximately 30 % of these cases are related to *M. bovis* infection (zoonotic tuberculosis) (Ellis et al., 2017), especially in developing countries (Müller et al., 2013) where prevalence of livestock bTB becomes substantial (Cosivi et al., 1998; Grange, 2001; Cleaveland et al., 2007). Despite huge efforts are currently focused on the eradication of bTB, there are many difficulties, mainly associated with the performance of the different diagnostic techniques, which make it very difficult in endemic countries (Skuce et al., 2012). Therefore, zoonotic tuberculosis is often under-reported, emphasising the importance of providing appropriate diagnostic tools in livestock to reach the eradication of *M. bovis* and reduce zoonotic tuberculosis cases.

4.2 Mycobacterium avium subsp. *paratuberculosis*

Paratuberculosis (PTB) or Johne's disease is a chronic infection that affects the small intestine of ruminants resulting in a marked reduction of animal productivity (Bergmann et al., 2015) and sometimes in death (Purkhart et al., 2011). Its causative agent is MAP and it is also believed to be related to Crohn's disease, a chronic bowel disease in humans, although this fact is yet to be defined (Mendoza et al., 2009; Chiodini et al., 2012; Roda et al., 2020).

The main importance of PTB comes from the great economic losses in animals due to reduced milk and meat yields as well as slaughter value (Kasbohm et al., 2017). MAP

diagnosis becomes a challenge because of its pathogenesis: while the main clinical signs are only present in the late progression of the disease, when the body condition is severely affected, the animals intermittently spread bacteria during a previous subclinical phase. These features result in a low sensitivity of the current direct (faecal culture and genome detection) and indirect (specific antibodies detection) diagnostic methods (McKenna et al 2005, Köhler et al 2008). Hence, reliable and alternative diagnostic methodologies are of key importance to identify infected animals and improve the sensitivity of the diagnosis.

5. Routine diagnostic techniques against *M. bovis* and *M. avium* subsp. *paratuberculosis*

The diagnosis of mycobacterial infection is currently at the centre of attention because, although well-established and reliable, it has its own limitations. Apart from being a tedious process, special consideration must be given to the lack of an optimal diagnostic sensitivity and the different variables which may interfere with the methods and techniques in use (Nienhaus et al., 2011; Maurer et al., 2019). Therefore, an accurate and reliable diagnostic methodology of the infection by mycobacteria is the cornerstone of their control (Fend et al., 2005).

5.1 Current ante-mortem and postmortem diagnostic techniques against M. bovis

Field and ante-mortem surveillance tests against *M. bovis* infection are mainly based on the detection of a delayed-type hypersensitivity response to the intradermal skin test (IST) through the inoculation of purified protein derivative from *M. bovis* (bPPD; tuberculin protein); and on quantifying the concentration of gamma interferon (IFN-γ) after culturing blood samples in the presence of tuberculin, in the case of IFN-γ assay test, a supplemental or confirmatory test (Peled et al., 2012). IST is currently considered as the official diagnostic screening technique in many countries worldwide, being compulsory the slaughtering of those animals with a positive result (Bezos et al., 2014). Although IST and IFN-γ assay have reasonable sensitivity (66,06-69,41% and 74,00%, respectively) and good specificity (≥ 99% for both tests) (Alvarez et al., 2012; Nuñez-Garcia et al., 2018), both techniques requires a minimum of 48–72 hours to obtain a result (Schiller et al., 2010b; Nienhaus et al., 2011) besides presenting other disadvantages and limitations. On the one hand, IST requires visiting the farm and restraint of the animals twice and a delicate procedure and interpretation of skin results (Peled et al., 2012; Cho et al., 2015); on the other hand, IFN-γ assay implies a complex laboratory methodology (Ellis et al.,

2014), a considerably more expensive price than a skin test (Schiller et al., 2010b; Katsenos et al., 2011) and suffer from cross-reactivity with other related mycobacteria resulting in false positive test results (Maurer et al., 2019). In addition, performance of these tests can be compromised by factors associated with the immune response and health status of the animal leading to a misinterpretation of the results (Kaneen and Pfeiffer, 2006). Development and use of a pre-screening test before field tests would be useful to reduce work efforts and diagnostic time (Cho et al., 2015).

Although microbiological culture is considered the gold standard approach for the diagnosis of mycobacterial infection, it is characterised by a long incubation time to confirm the presence of mycobacteria (around 8-12 weeks) (Maurer et al., 2019). Furthermore, the isolation of mycobacteria needs specific compounds such as mycobactin, which determine the viability and growth of the mycobacteria, and sometimes, additional steps such as decontamination. For all these reasons culture becomes a tedious and laborious, although necessary, option in *M. bovis* diagnosis.

Other *in vitro* assays, such as serologic assays (ELISA) or polymerase chain reaction (PCR), have limitations associated with accuracy and execution that restrict their use (De la Rua-Domenech et al., 2006). While ELISA sensitivity is affected by the delayed and irregular antibodies response in bTB (Hanna et al., 1992), PCR is considered a post-mortem diagnostic option with promising findings but still under development, focused on the search of markers that ensure diagnostic sensitivity (Lorente-Leal et al., 2019). Therefore, the reliability of these tests depends on the stage of infection and, in addition, these require transporting of animal samples to the laboratory, which finally increases diagnostic time too (Ratiu et al., 2019a), highlighting the interest on the availability of portable equipment.

5.2 Current ante-mortem and postmortem diagnostic techniques against M. avium subsp. *paratuberculosis*

The intermittent and sometimes low shedding of the mycobacteria as well as the irregular sero-conversion in the subclinical phase of PTB (Kruger et al., 2014; Miekisch et al., 2014) gives a limited sensitivity to the *in vivo* diagnosis (Kasbohm et al., 2017), currently based on serological assays (ELISA) and PCR from faeces. Although ELISA has a limited sensitivity, the irregular spread of bacteria via faeces has raised serology as the most

common technique used for the monitoring of PTB (Ezanno et al., 2005). In addition, faecal shedding and immune response vary individually to a large extent (Köhler et al., 2015). For example, the sensitivity of PCR methods can be affected by the variable bacterial load in samples and the co-purification of PCR inhibitors during DNA extraction (Sevilla et al., 2014).Therefore, there is a need for diagnostic tests with higher sensitivity and decreased processing time to reduce false negative results and enable effective disease control strategies.

In short, against the present situation it would be helpful to have an ante-mortem diagnostic methodology capable of detecting mycobacterial infection with repeatability, a good quality/price ratio, high sensitivity and specificity, and rapid detection and obtaining of results. VOCs strategy could be an option because it mostly fulfils these objectives and it has been successfully used for mycobacterial diagnosis in many animal species (Table 1). Moreover, volatilome evaluation has been capable of discriminating mycobacterial infection before clinical illness occurs, offering an early diagnosis and time advantage (Purkhart et al., 2011).

6. Impact of the experimental setting on the VOCs profile

6.1 In vitro vs in vivo studies

Analysis of VOCs as a diagnostic alternative for mycobacterial disease has been evaluated both *in vivo* and *in vitro*. Compared to *in vivo* assays, the large number of existing *in vitro* studies, which basically consist in mycobacteria culturing, reveals the early stage of development where this research area stands (Pavlou et al., 2004; McNerney et al., 2012; Chingin et al., 2016; Küntzel et al., 2016; Küntzel et al., 2018). The reviewed literature in the present study suggests some drawbacks related to those *in vitro* studies.

Firstly, mycobacteria growth, which as above mentioned requires several weeks or even months, is required to identify changes in the analysis of VOCs from microbiological culture to allow the distinction between negative and positive samples. In other words, although *in vitro* experiments are able to detect VOCs changes related to different stages of the mycobacteria growth (Trefz et al., 2013; Küntzel et al., 2016), it still takes long time to identify these, which is one of the main disadvantages linked to the current diagnostic methodology. Accordingly, researchers point to reduce the diagnostic time by

Table 1. *In vivo* studies evaluating VOCs analysis as a diagnostic tool for mycobacterial infection in animals

Animal species	Matrix	Analytical technique	Kind of infection	Sensitivity	Specificity	References
Mycobacterium bovis						
Cattle	Exhaled breath	GC-MS	Experimental	83.8-96.4%	97.4-99.2%	Ellis et al., 2014
White-tailed-deer	Faeces	GC-MS	Experimental	78.6%	91.4%	Stahl et al., 2015
Cattle	Faeces	GC-MS	Experimental	83-100%	100%	Ellis et al., 2017
Cattle	Serum	EN	Natural	-	-	Cho et al., 2015
Badger	Serum	SIFT-MS	Natural	88%	62%	Spooner et al., 2009
Cattle	Exhaled breath	GC-MS EN	Natural	- 100%	- 79%	Peled et al., 2012
Mouse (C57BL/6J)	Exhaled breath	GC-GC-MS	Experimental	-	-	Franchina et al., 2018
Cattle	Serum	EN	Experimental	-	-	Fend et al., 2005
Badger	Serum	EN	Natural and experimental	-	-	Fend et al., 2005
Cattle	Exhaled breath	ATD-GC-MS	Experimental	-	-	Turner et al., 2012

Animal species	Matrix	Analytical technique	Kind of infection	Sensitivity	Specificity	References
Mycobacterium avium subsp. Paratuberculosis						
Goat	Exhaled breath and faeces	GC-MS	Experimental	-	-	Bergmann et al., 2015
Goat	Exhaled breath and faeces	DMS	Experimental	-	-	Purkhart et al., 2011
Cattle	Serum	EN	Natural	-	-	Knobloch et al., 2009
Goat	Exhaled breath and faeces	GC-MS	Experimental	Exhaled breath: 90.3% Faeces: 86.6%	Exhaled breath: 81.8% Faeces: 85.0%	Kasbohm et al., 2017
Mycobacterium tuberculosis						
Cynomolgus macaque	Exhaled breath	GC-GC-MS	Experimental	97%	97%	Mellors et al., 2018
Cynomolgus macaque and rhesus macaque	Exhaled breath	GC-GC-TOFMS	Experimental	-	-	Mellors et al., 2017

Abbreviations: *ATD-GC-MS* Thermal desorption-gas chromatography-mass spectrometry, *DMS* Differential ion mobility spectrometry, *EN* Electronic nose, *GC-MS* Gas chromatography-mass spectrometry, *GC-GC-MS* Two-dimensional gas chromatography-mass spectrometry, *GC-GC-TOFMS* Two-dimensional gas chromatography time-of-flight mass spectrometry, *SIFT-MS* Selected ion flow tube mass spectrometry

avoiding the limiting step of culturing and suggesting other innovative techniques such as VOCs measurement directly *in vivo* (Kasbohm et al., 2017).

It is also important to highlight the low correlation existing between results obtained from cultured bacteria compared with those VOCs produced from other biological samples studied in *in vivo* experiments (Ratiu et al., 2019a). For example, Kasbohm et al. (2017) detected two compounds only present above MAP cultures which were ranked among the top discriminating VOCs in their statistical analysis. However, in the comparison with their *in vivo* results, these two compounds tended to be in lower concentration in MAP-inoculated animals compared with non-inoculated animals. This situation enhances the caution required when adopting *in vitro* findings to *in vivo* conditions since the influence from the host, its microbiome and host–microbiome interactions (Zhu et al., 2013), as well as the influence from environmental factors, such as diet, age or drugs use (Ratiu et al., 2019a) needs to be considered. In addition, other *in vitro* hurdle is related to the different VOCs profiles obtained depending on the substrate where the mycobacteria grow resulting in incoherent findings (Dang et al., 2013).

The effectiveness of *in vivo* approach is supported by the results of many studies where VOCs from biological samples have been used to distinguish between infected animals with different mycobacteria species and non-infected animals (Purkhart et al., 2011; Ellis et al., 2014; Bergmann et al., 2015; Mellors et al., 2018). Many different biological matrices such as serum, breath or faeces have been studied as a source of information for VOCs analysis in this field, existing great differences between their nature and characteristics. This constitutes another problem in the comparison between *in vitro* vs *in vivo* experiments, giving inconsistent results. When Bergmann et al. (2015) compared *in vivo* results obtained from faeces and breath samples with the *in vitro* VOCs profiles obtained from different MAP strains' culture by Trefz et al. (2013), their conclusions were not very clarifying: from more than 100 substances detected in faeces and breath, only 15 and 5 of them, respectively, were found in the bacterial *in vitro* pattern.

6.2 Experimental vs natural infection

Another variable to take into account for the evaluation of VOCs as an alternative for mycobacterial diagnosis in animals is the type of infection: natural or experimental. Although experimental infections are logically the most common and easy option for this

kind of approximation, experiments in naturally infected animals are of paramount importance. Experimental infections allow controlling different environmental conditions that may impact on the results, being the most studied option for mycobacterial diagnosis through the analysis of VOCs (Table 1). However, assays with natural infections are needed to validate the results obtained from any new diagnostic tools, such as volatilome analysis, in experimental settings. Along this review only a single article has been found to include the analysis of VOCs from both experimentally and naturally infected animals (Fend et al., 2005). These researchers found that differences between negative and positive animals were more pronounced in the natural infection group than in the experimentally infected one. This fact highlights the importance of performing studies in field conditions in the future to compare with those with experimentally infected animals and to validate the results from the latter ones.

7. Species under study

The analysis of VOCs has been used in many species for the diagnosis of mycobacterial infection. Livestock species are the most frequent ones, probably because of the importance and repercussion of bTB and PTB for farm animals. As expected, bovine is the most studied animal model with this innovative approach, followed by goats (Table 1). Our findings are consistent with the wide variety of diseases that have been tested through this methodology in cattle, such as bovine respiratory disease (Burciaga-Robles et al., 2009), mastitis (Dervishi et al., 2017), brucellosis (Knobloch et al., 2009), ketosis (Zhang et al., 2013) or ketoacidosis (Elliot-Martin et al., 1997; Mottram et al., 1999).

Remarkably, wildlife has been also used to perform diagnosis through volatilome, more specifically with deer and badgers (Fend et al., 2005; Spooner et al., 2009; Stahl et al., 2015), with much effort put into the development of a better disease surveillance methodology on these species. Among lab animals, non-human primates have been used to study the mycobacteria species which usually affect humans, *M. tuberculosis*; identifying 19 breath molecules that discriminate pre- and post-infection status, detecting two new potential breath biomarkers, as well as heptanal, a previously confirmed biomarker in human medicine (Mellors et al., 2017, 2018). The murine model has also been used to assess the use of breath for mycobacterial infection (Franchina et al., 2018), with 23 VOCs being identified to discriminate between infected and non-infected mice.

The encouraging results obtained in these studies with different animal species highlight the great potential of this methodology in MTBC diagnosis. However, there is a lack of studies in other species of interest, such as the pig, an animal model with an increasing interest in biomedical research (Käser et al., 2018); or the wild boar, noted for its interference with bTB control programs in many European countries (Diez-Delgado et al., 2018). Furthermore, the marked differences that exist among different animal species make feasible that different approaches may be necessary for each species. This review highlights the starting point where this new diagnostic approach stands and the necessity of further studies and research.

8. Biological matrices

VOCs can be detected directly from different biological samples such as blood, serum, breath, faeces, sweat, skin, urine or vaginal fluids (Klemm et al., 1987; Ma et al., 1995; Shirasu et al., 2011; Cho et al., 2015; Stahl et al., 2015), opening up huge opportunities for this new diagnostic methodology. Although samples should be initially selected according to the disease and the pathogenesis of the agent, there are multiple options that allow collection of alternative samples. For example, the predominantly respiratory character of bTB would place exhaled breath as the most appropriate sample to study this disease. However, there are studies that show interesting results for the analysis of VOCs from *M. bovis* infected animals using different biological matrices such as faeces (Stahl et al., 2015; Ellis et al., 2017) or serum (Spooner et al., 2009; Cho et al., 2015). A similar situation occurs with PTB. MAP is a mycobacteria characterised by causing digestive disorders, making feasible to find these alterations directly reflected in the faecal volatilome. Despite of this, exhaled breath (Purkhart et al., 2011; Bergmann et al., 2015; Kasbohm et al., 2017) and serum (Knobloch et al., 2009) have given promising findings in different animal species.

The rationale for analysing exhaled breath in a model of chronic intestinal infection or faeces in a primarily respiratory disease is based on the hypothesis that they do not only contain substances originated from the airways or from the digestive system. These also contain metabolites released via the lung or the intestine but originated and related to the whole metabolic or health state of the subject (Purkhart et al., 2011).

The three most used biological samples for VOCs analysis of mycobacterial diseases in animals are exhaled breath, serum and faeces (Table 1).

8.1 Exhaled breath

The principle of using exhaled breath lies in its capability for discerning disease-related changes and biomarkers in the organism that are reflected into the breath through exchange via the lungs (Peled et al., 2012), because of its ability to cross the alveolar membranes prior to being exhaled (Turner et al., 2012). The use of exhaled breath offers several advantages because it is a non-invasive sample produced in ample supply, having the potential for direct, inexpensive and eventually real-time monitoring (Peled et al., 2012; Amann et al., 2014b). Although in the literature it is considered as a relatively easy to obtain sample, its sampling methodology in animals is diverse, revealing a lack of standardisation: from modified equine nebulization masks or nostril samplers for cattle, specific ventilators for mice or intubation for macaques, to automated alveolar sampling devices for goats. VOCs from breath are normally concentrated to sorbent materials, such as Tenax or Carbopack Y, Carbopack X and Carboxen 1000 (Ellis et al., 2014; Mellors et al., 2018), which simplify its transport and storage, and later these are used to quantify and evaluate the volatile substances with different analytical techniques.

Healthy and diseased animals have been successfully distinguished in mycobacterial infections by identifying volatile molecules in exhaled breath (Table 2). Ellis et al. (2014) performed breath collection and analysis in *M. bovis*-inoculated cattle with two strains obtaining good sensitivity and specificity: 83.8% to 96.4% and 97.4% to 99.2%, respectively, using the microbiological culture as reference technique. In addition, Peled et al. (2012) reported the measurement of two VOCs from breath linked with *M. bovis* infection and other two VOCs associated with samples from negative individuals, obtaining sensitivity and specificity values of 100% and 79%, respectively.

The studies included in this revision evaluating exhaled breath in the context of mycobacteria infection highlight some important variables to take into account (Franchina et al., 2018). The use of different animal species models, the *Mycobacterium* species and strain used, the infection phase, the breath volume collected and the sorbent phases used to concentrate VOCs are factors that often differ between the existing assays. Considering all the above information, a comparison between the existing results is a challenge.

8.2 Faeces

Faeces are regarded as the most accessible sample for research (Deda et al., 2015). Taking into account that faeces constitute the main media for eliminating metabolic products, these are an important source of information about the internal homeostasis (Karthikeyan et al., 2013). The reason for testing changes in VOCs in faeces is based on the common assumption that any abnormality in the activity or composition of the intestinal microbiota and in the whole organism may alter the odour of this matrix (Purkhart et al., 2011); which is supported by studies from both human (Garner et al., 2007; Tait et al., 2014; Aggio et al., 2017; Ubeda et al., 2019) and animal medicine (Garner et al., 2008; Kizil et al., 2015; Blake et al., 2019; Summers et al., 2020). Consequently, examination of volatile faecal emission could be a very useful non-invasive diagnostic approach (Purkhart et al., 2011).

However, as a remarkable fraction of VOCs found in faeces is generated by gut commensal microbiota (Guarner and Malagelada, 2003), a well matched control group and knowledge on these bacteria are necessary to identify VOC patterns of pathogenic conditions (Bergmann et al., 2015). Despite this shortcoming, using faeces as matrix has many advantages; besides an easier sampling, it is not necessary to restrain the animals, eliminating the stressful situation that it implies. Moreover, and in contrast with human medicine, faeces offer many different possibilities in terms of sampling protocol: per rectum, after sacrifice, using laboratory animal cages or just after defecation are some options in veterinary research. The studies using faeces reviewed in the present work highlight the existing heterogeneity between the published results (Table 2). However, the obtained results have placed faecal volatilome analysis as an innovative diagnostic approach in the current research context for mycobacterial infections. In this sense, attention has been focused not only on the discrimination between infected and healthy individuals (Purkhart et al., 2011; Bergmann et al., 2015; Stahl et al., 2015; Kasbohm et al., 2017), but also in the use of faecal VOCs profile for other purposes, such as identification of vaccinated animals in white-tailed deers (Stahl et al., 2015) and cattle (Ellis et al., 2017).

8.3 Serum

Serum is the sample of choice in many studies because of its relatively ease to obtain, store and safely distribution (Spooner et al., 2009). Blood or serum is the means of

transport of many different substances, compounds and markers through the organism, existing a complex exchange with the lung or the intestine, among other systems (Harper et al., 2004). Alterations in VOCs from serum can be detected when a disease, an infection or a pathologic condition occurs (Kurada et al., 2019).

Serum has been used to distinguish the infection by *M. bovis* or MAP in different animal species through volatilome evaluation (Table 2) obtaining very interesting results. For example, Knobloch et al. (2009) were able to discriminate MAP and brucellosis infection in cattle through VOCs analysis; and Cho et al. (2015) reported an analysing time of only 20 minutes to differentiate between bTB infected and bTB-free bovine sera. However, and although blood and serum could be the most routine samples used in diagnostic field, its collection supposes a stressful situation as it is an invasive method that requires individual immobilisation.

In conclusion, three different biological samples have been discussed as source of information in mycobacterial diagnosis in animals through volatilome analysis. Although interesting and useful findings have been shown, there is still a lack of homogeneity among many different study conditions. This often leads to incomparable and inconsistent results. For example, despite of studying the same pathogen (MAP), and using the same biological samples (exhaled breath and faeces) and animal species (goats), contradictory conclusions can be found in the literature: while ones showed that differences in VOC profiles were less pronounced from breath than those obtained in faeces (Bergmann et al., 2015; Kasbohm et al., 2017); however, others suggest that volatilome evaluation from exhaled breath might be superior compared with the one from faeces (Purkhart et al., 2011). In fact, the researchers usually acknowledge that their hypotheses should be verified by future studies, considering their findings as starting points (Purkhart et al., 2011; Kasbohm et al., 2017). Hence no reliable comparisons or conclusions can be made with the available information, being advisable to carry out studies where the biological matrices are used simultaneously with the same methodological conditions.

Table 2. VOCs related to mycobacterial infection in different animal species

Mycobacteria species	Potential discriminatory VOCs	Animal species	Matrix	Analytical technique	References
Mycobacterium bovis	Thioether, Thiophene, Aldehyde, Organosulfur (sulfone), Imine, Pyridine derivative, Amino acid, Ketone, Alcohol, Indole, Diterpenoid alkane, Fatty acyl (amino acid derivative), Diterpene alcohol, Dicarboxylic acid and derivative	Cattle	Faeces	GC-MS	Ellis et al., 2017
Mycobacterium bovis	4-hydroxy-4-methyl-2-pentanone, Benzaldehyde, 1-ethyl-2-pyrrolidinone, α, α -dimethyl-benzenemethanol, Nonanal	Cattle	Exhaled breath	GC-MS	Ellis et al., 2014
Mycobacterium bovis	>100 compounds (acetone, dimethyl sulphide and 2-butanone as the most abundant)	Cattle	Exhaled breath	ATD-GC-MS	Turner et al., 2012
Mycobacterium bovis	Decyl-cyclohexane, methanethiol, 3,4-diethyl-2-hexene, dimethyl sulphite, 2,2,4,4-tetramethyl-3-pentanone, Acetaldehyde, Acetone, 2-butene, 3-oxo cyclopenten-1-yl-(2E)-penta-2,4-dienoate, 4-methyl-decane, Ethylbenzene, 2,4,4-trimethyl-1-pentene, 4-methylene-1-(1-methylethyl)-bicyclo[3.1.0]hexane, 2-methyl-(E)-2-butenal, 2-ethyl-3-methylbutanal, 2-methylheptane, Butanal, 3-methylheptane, (2-aziridinylethyl)amine, 3-methyl-(Z)-4-nonene, 2-decen-1-ol, 4-[2-(methylamino)ethyl]-phenol, 3,4-dimethylpentanol	Mouse (C57BL/6J)	Exhaled breath	GC-GC-MS	Franchina et al., 2018

Mycobacteria species	Potential discriminatory VOCs	Animal species	Matrix	Analytical technique	References
Mycobacterium bovis	2,3-Dimethyl, 1,3- Pentadiene, 1,3-Dimethylbutyl Cyclohexane	Cattle	Exhaled breath	GC-MS	Peled et al., 2012
Mycobacterium bovis	Methylbenzene, Hexanal, 2-Methyl pyridine, 2,4-Dimethyl pyridine, 2-(1,1-Dimethoxy)-ethanol *, 2-Ethyl-1-hexanol, Benzene acetaldehyde, 3,7-Dimethyl-6-octenyl-(2E)-2-butanoate, Acetophenone *, 4-Methyl-phenol, 2-Decanone *, (-)-Beta-Fenchol, 1-Decanol, Indole, 3-(1,1-dimethylethyl)-4-methoxy-phenol, 1-Octadecanol, 2-Dodecanone	White-tailed-deer	Faeces	GC-MS	Stahl et al., 2015
Mycobacterium avium subsp. *paratuberculosis*	45 compounds. Top-3 (random-forest): 3-methylfuran, 2,3-butanedione, Methyl acetate	Goat	Faeces	GC-MS	Kasbohm et al., 2017
Mycobacterium avium subsp. *paratuberculosis*	51 compounds. Top-3 (random-forest): 3-methylpentane, 2-ethyl-1-hexanol, 2-methylpentane	Goat	Exhaled breath	GC-MS	Kasbohm et al., 2017
Mycobacterium avium subsp. *paratuberculosis*	1-Propanol, 2-Butanone, Acetone, Benzene, 2-methyl-butanal, Ethylbenzene, Hexanal, Nonanal, Styrene	Goat	Exhaled breath	GC-MS	Bergmann et al., 2015
Mycobacterium avium subsp. *paratuberculosis*	Pentane, Hexane, Heptane, Acetone, 2-Butanone, 2-pentanone, 2-hexanone, 2-heptanone, 3-Octanone, 3-methyl-2-Butanone, 3-methyl-2-Pentatone, Methyl Isobutyl Ketone, Isoprene, Methyl acetate, Dimethyl sulfide, Dimethyl disulfide, Furan, 2-ethylfuran, 2-methylfuran, 3-methylfuran, 2-pentylfuran	Goat	Faeces	GC-MS	Bergmann et al., 2015

Mycobacteria species	Potential discriminatory VOCs	Animal species	Matrix	Analytical technique	References
Mycobacterium tuberculosis	49 compounds with 19 putative identifications: (Z)-3-Tetradecene, 1,1'-Bicyclohexyl, 2,2-Dimethylheptane, 2,6,11-trimethyldodecane, 2-Ethylhexyl isohexyl ester sulfurous acid, 3-Methyl-dodecane, Dodecane, Hexadecane, Hexylcyclohexane, Octylcyclohexane, Tridecane, 2-Heptanone, Acetic acid, phenyl ester, Allyl heptanoate, 4-Methylene-1-(1-methylethyl)-bicyclo[3.1.0]hexane, 2-Methylbutyl ester butanoic acid, n-Amyl isovalerate, o-Cymene, Trans-á-Ocimene	Cynomolgus macaque and rhesus macaque	Exhaled breath	GC-GC-TOFMS	Mellors et al., 2017
Mycobacterium tuberculosis	38 compounds with 11 putative identifications: 2,3,6-trimethylnapthalene, 4-methyl-1-decene, 4-ethyl-2,2,6,6-tetramethylheptane, 2,2,3-trimethylhexane, 6-phenyl-4-(1-phenylethoxy)-1-Hexene, Butyl acetate, 1,2,3-trimethylbenzene, Indane, 2,2,4,6,6-pentamethylheptane, 4-tert-Amyl phenol, Ethyl butyrate	Cynomolgus macaque	Exhaled breath	GC-GC-MS	Mellors et al., 2018

Abbreviations: *ATD-GC-MS* Thermal desorption-gas chromatography-mass spectrometry, *GC-MS* Gas chromatography-mass spectrometry, *GC-GC-MS* Two-dimensional gas chromatography-mass spectrometry, *GC-GC-TOFMS* Two-dimensional gas chromatography time-of-flight mass spectrometry

* = Statistically significant trends identified for vaccinated and infected animals but not in non-vaccinated and infected animals

9. Instrumental techniques

A large spectrum of available analytical instrumentation techniques allows VOCs identification. Although gas chromatography with mass spectrometry (GC-MS) is referred very often as the "gold standard" for VOCs analysis (Phillips et al., 2012; Zhang et al., 2011), selected ion flow tube-mass spectrometry (SIFT-MS), proton transfer reaction mass spectrometry (PTR-MS) and secondary electrospray ionization mass spectrometry (SESI-MS) are other mass spectrometry based options available for bacterial VOCs analysis (Ratiu et al., 2019a). Moreover, various types of ion mobility spectrometers (IMS), such as classical time of flight IMS (ToF-IMS), aspiration IMS (a-IMS), differential mobility spectrometers (DMS), field-asymmetric wave IMS (FAIMS) or multi capillary column ToF IMS (MCC-IMS) have been successfully used in identification of bacterial VOCs as well (Ratiu et al., 2017).

In the present review, different analytical techniques have been evaluated to assess VOCs as a diagnostic alternative for mycobacterial infection in animals (Table 2): different GC-MS modalities, various electronic noses (EN) models and DMS, being the first two options by far the most frequent approaches. In this sense, as other researchers have previously indicated, the diverse methods of VOC collection and analytical systems that have been used are likely to have contributed to the results' variability (Ellis et al., 2014). Supporting this context, each analytical method offers both advantages and limitations.

9.1 Gas chromatography with mass spectrometry (GC-MS)

GC–MS has become one of the most preferred methods for identification of bacterial markers with a very good sensitivity (Ratiu et al., 2019a). It has a huge potential for both identification and quantification of unknown VOCs from complex matrices (Buszewski et al., 2018; Ratiu et al., 2019b). Ellis et al. (2014) found that 4-hydroxy-4-methyl-2-pentanone, benzaldehyde, 1-ethyl-2-pyrrolidinone, α, α - dimethyl-benzenemethanol and nonanal were present in significantly greater concentration in *M. bovis* infected animals than in control ones. Moreover, Bergmann et al. (2015) found 16 and 3 VOCs in faeces and breath, respectively, with detectable differences at any infection time between MAP-inoculated and non-inoculated animals. GC-MS has the capacity of detecting VOCs within a range of parts per billion range, or lower, good reproducibility and linearity (Peled et al., 2012; Ratiu et al., 2017). In other words, GC-MS not only seems to be the most suitable for bacterial biomarkers search; in fact, it is the most utilised technique to

diagnose these infections (Ratiu et al., 2019a). The present review highlights GC-MS usefulness as an analytic tool to evaluate VOCs changes due to mycobacteria infection employing different biological samples such as exhaled breath or faeces (Table 2).

In spite of these many advantages, GC-MS has also several drawbacks: most GC-MS equipments are still not implemented as a portable tool; it requires high levels of expertise, qualified personnel and pre-concentration techniques; and it is currently an expensive instrumentation (Ratiu et al., 2019). Therefore, given the above-mentioned cons and the significant sampling and analysis time that it implies, GC-MS is not suitable for being used in end-user or point-of-care sites (Peled et al., 2012; Ratiu et al., 2019a).

It is also worth mentioning that comprehensive two-dimensional GC-MS (GCxGC-MS), stands out for the possibility of analysing VOCs coming from complex matrices (Ratiu et al., 2019) and for providing a more complex and unparalleled separation as well as three dimensional chromatograms' visualisation (Ibrahim et al., 2019).

9.2 Electronic nose (EN)

The EN is an artificial instrument based on chemical sensors combined with a pattern recognition system (Gardner and Bartlett, 1994), able to detect different VOCs, such as odours, flavours and vapours (Rock et al., 2008; Macias et al., 2013). The main advantages of this methodology are the ease of use, its low price and the rapid analysis time (Cho et al., 2015). Furthermore, EN methodology avoids sample transport to laboratory, positioning itself as one of the optimal techniques for pen-side use (Cho et al., 2015). However, it has problems with background separation, it does not identify substances detected and sometimes its detection limit is high, giving insufficient sensitivity (Bergmann et al., 2015; Majchrzak et al., 2018).

The huge variety of applications where EN has shown effectiveness could be also considered as another of its strengths: versatility. In this sense, the reviewed information reveals the applicability of many types of EN sensors for different species of mycobacteria diagnosis (Table 1). Despite of the good and interesting results obtained, the ease of transport of this device has not been exploited in depth due to the fact that the majority of studies using EN has analysed VOCs from serum (Table 1) and not from other type of samples such as faeces or exhaled breath. The above-mentioned information

enhances the importance of carrying out future studies using EN focused on non-invasive biological matrices which would permit to develop a portable tool. In this sense, Peled et al. (2012) used their GC-MS results to tailor an artificial olfactory system to detect bTB in cattle exhaled breath. Although their new system successfully identified all infected animals (100% sensitivity), it wrongly classified 21% of the non-infected individuals (79% specificity).

9.3 Other minor techniques

DMS is an IMS modality that has been occasionally used for volatilome assessment in mycobacterial infections (Table 1). This instrumentation has a lower cost and it can be used alone or coupled with a GC column which acts as a pre-separation stage (Ratiu et al., 2019). Its relatively low price, robustness, reliability and miniaturisation turn IMS technology into one of the potential alternatives for portable VOCs analysis in disease diagnostic (Ratiu et al., 2019). As with EN, one of its main drawbacks is its lack of capacity to identify specific VOCs (Purkhart et a., 2011). This analytical device used by Purkhart et al. (2011) permitted to discriminate healthy from MAP-infected goats, noting a direct correlation among postmortem findings and *in vivo* measurements.

SIFT-MS is a quantitative technique for trace gas analysis based on the ionization of these volatile compounds by positive precursor ions along a flow tube. Although its main advantages are a rapid analysis time and a lower mass range, biological samples usually provide complex data which need computational assistance to be analysed (Spooner et al., 2009). Spooner et al. (2009) applied multivariate analysis for the first time to SIFT-MS data to evaluate serum headspace analysis as a faster screening tool for *M. bovis* infection in badgers, obtaining a much faster diagnosis. However, the insufficient accuracy achieved (88% of true positive and 38% of false positive) makes this approach unsuitable as an alternative for conventional diagnostic techniques.

10. Targeted analysis versus non-targeted analysis

The diagnosis of an infection using VOCs analysis can be reached by identifying specific substances related to the pathologic process or by detecting significant alterations in the whole VOCs profile. Most of the research has attempted to isolate unique VOC biomarkers (targeted analysis**)** that would indicate the presence of mycobacterial

infection, with little work done investigating potential changes within the whole VOC profiles (non-targeted analysis**)**.

There are VOCs that can be present in many different situations, hampering to find a specific substance for a particular infection or process. This is the case of methyl-nicotinate, a compound proposed as *M. tuberculosis* biomarker (Ellis et al., 2017). In this sense, although tentative biomarkers have been associated with mycobacterial infection in both human (Phillips et al., 2007; Phillips et al., 2010; McNerney et al., 2012; Nawrath et al., 2012) and veterinary medicine (Table 2), the influence of different factors makes the identification of indicative VOCs difficult (Ellis et al., 2017). According to the literature, these factors may be related to host biological variables, environmental conditions, symbiotic and infectious microbe-host interactions, pathophysiological responses, the method of sample collection and differences in analytical methods used for sample analysis (Knobloch et al., 2009; Shirasu et al., 2011; Mellors et al., 2017). The bias induced by these factors is exemplified by the comparison of two studies which aimed to use exhaled breath VOCs as a source of information to diagnose *M. bovis* infection in cattle (Peled et al., 2012; Ellis et al., 2014): using the same animal species, pathogen and biological sample, only two VOCs were consistent between both studies, highlighting the challenge that this approach suppose.

On the other hand, there are already studies in the literature which have used the entire VOCs profile (non-targeted analysis**)** to successfully discriminate between disease and non-infected animals (Ellis et al., 2017). In this way, many research groups have highlighted the importance of considering the entire profile of VOC released by specific pathogens and how these profiles can help discriminating between infecting pathogens, rather than relying on a limited number of biomarkers (targeted analysis) (Graham, 2013).

11. Conclusion

In conclusion, the number of *in vivo* assays which study the implementation of the analysis of VOCs for mycobacterial diagnosis in animal research is considered scarce. Although there is currently an important research trend that evidences the potential of VOCs emitted in mycobacterial infections in animals as a diagnostic tool, it is still in an initial phase and presents some difficulties. The lack of standardisation and the differences in the current methodology and the use of biological matrices are the main

hurdles, usually resulting in inconsistent and incomparable results. The high number of research groups that have studied this new approach worldwide contribute to the lack of standardisation because they usually use different protocols, reason that makes more difficult to reproduce their results. Further and thorough studies using several biological matrices with constant conditions are required to overcome these drawbacks in the near future. This will open new possibilities in the questioned diagnosis of mycobacterial infection.

12. References

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