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ORIGINAL ARTICLE

Exploring MALDI-TOF MS approach for a rapid identification of *Mycobacterium avium* ssp. *paratuberculosis* field isolates

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diagnosis, identification, MALDI-TOF MS, *Mycobacteria*, proteomics, rapid methods.

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Abstract

Aims: The aim of the study was to explore the suitability of matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) for a rapid and correct identification of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) field isolates.

Methods and Results: MALDI-TOF MS approach is becoming one of the most popular tests for the identification of intact bacterial cells which has been shown to be fast and reliable. For this purpose, 36 MAP field isolates were analysed through MALDI-TOF MS and the spectra compared with two different databases: one provided by the vendor of the system employed (Biotyper ver. 3·0; Bruker Daltonics) and a homemade database containing spectra from both tuberculous and nontuberculous *Mycobacteria*. Moreover, principal component analysis procedure was employed to confirm the ability of MALDI-TOF MS to discriminate between very closely related subspecies. Our results suggest MAP can be differentiated from other *Mycobacterium* species, both when the species are very close (*M. intracellulare*) and when belonging to different subspecies (*M. avium* ssp. *avium* and *M. avium* ssp. *silvaticum*).

Conclusions: The procedure applied is fast, easy to perform, and achieves an earlier accurate species identification of MAP and nontuberculous *Mycobacteria* in comparison to other procedures.

Significance and Impact of the Study: The gold standard test for the diagnosis of paratuberculosis is still isolation of MAP by cultural methods, but additional assays, such as qPCR and subculturing for determination of mycobactin dependency are required to confirm its identification. We have provided here evidence pertaining to the usefulness of MALDI-TOF MS approach for a rapid identification of this mycobacterium among other members of *M. avium* complex.

Introduction

Mycobacterium avium ssp. *paratuberculosis* (MAP) is the aetiological agent of paratuberculosis (Johne's disease) in cattle (Li *et al.* 2016). This disease can affect virtually all domestic ruminant species. Moreover, MAP can also infect other wildlife animals, both ruminants or nonruminants. A good example of the wide host susceptibility to MAP is represented by rabbits, which are considered the reservoir in some districts (Stevenson *et al.* 2009).

Clinical signs of the disease in cows are severe gastroenteritis, diarrhoea, weight loss and reduced milk production, leading to premature culling and economic losses (Motiwala *et al.* 2006). A potential role for MAP has been also suggested for the aetiology of Crohn's and other human diseases. However, so far no definitive evidence has proved its implication in the developing mechanism of these diseases (Chiodini *et al.* 2012).

MAP is an aerobic Gram-positive and acid-fast staining bacillus belonging to the *Mycobacterium* genus, which

includes many human and animal pathogens, such as *M. tuberculosis*, *M. leprae*, *M. bovis*, *M. intracellulare* and *M. avium* (Li *et al.* 2016). In particular, *M. avium* and *M. intracellulare* are included in the *Mycobacterium avium* complex (MAC) (Mackenzie *et al.* 2009), a group encompassing also the species *M. chimaera*, *M. colombiense*, *M. arosiense*, *M. boucheduhonense*, *M. maeseillense*, *M. timonense* and *M. vulneris* sp. nov. (van Ingen *et al.* 2009; Cayrou *et al.* 2010). Although these last seven human pathogens have only been investigated quite recently (Cayrou *et al.* 2010), *M. avium* and *M. intracellulare* are instead well characterized and are considered pathogens for both humans and animals.

According to Thorel *et al.* (1990), *M. avium* further encompasses three different subspecies: MAP, *Mycobacterium avium* ssp. *avium* (MAA) and *Mycobacterium avium* ssp. *silvaticum* (MAS). A fourth subspecies, designated *Mycobacterium avium* ssp. *hominissuis* (MAH) has been proposed for human/porcine-type *M. avium* isolates (Mijs *et al.* 2002; Ronai *et al.* 2015).

Based on to their genetic differences, MAP strains have been classified into two major groups (Stevenson 2015): type S (sheep) or I and type C (cattle) or II. In addition, a subtype of type S, named type III (intermediate) and a sublineage of type C, designated type B (bison), have been described. However, this classification only partially reflects their host-specificity, being the types named after the host from which they were originally isolated (Stevenson 2015).

Among the *Mycobacteria* previously cited, MAP is the slowest growing, requiring for the first isolation, at least 4 weeks of incubation for type C field isolates and even more for types S. Moreover, it requires the presence of mycobactin, a specific siderophore, in order to grow in solid or liquid media (Anonymous 2014). The OIE manual states that, for a correct identification of MAP, the subculturing of suspect colonies into media containing mycobactin or not is required to demonstrate mycobactin dependency of suspected colonies. Because of the aforementioned slow growth rate of MAP, this step can require even more than 1 month. In addition, confirmatory PCR targeting specific sequence (IS900 or f57) is also required to reach a final identification (Anonymous 2014).

A fast approach to bacteria identification is represented by whole-cell matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). This approach has been reported to be able to yield effective species-level identification (Karlsson *et al.* 2015; Patel 2015). Particularly, this method has been shown to be suitable for a rapid identification of different species belonging to the genus *Mycobacteria*, but some complications for the differentiation between *M. tuberculosis* and *M. bovis* were reported (Shitikov *et al.* 2012). Moreover, this method has been successfully applied also for the identification of nontuberculous *Mycobacteria*, such as *M. avium, M. abscessus* and others (Fangous *et al.* 2014; Rodriguez-Sanchez *et al.* 2015, 2016).

So far, this promising approach has not been applied yet to the rapid identification of MAP field isolates. The aim of the work herein presented was to explore the suitability of MALDI-TOF MS for the identification of this pathogen and, more generally, for the identification of different species and subspecies belonging to MAC.

Materials and methods

Culture of strains of *Mycobacteria*

One of the critical aspects of this technique is the significant effect that variations in cultural media, incubation conditions and length of incubation had on the spectra produced (Balazova *et al.* 2014; Karlsson *et al.* 2015). Therefore, in order to achieve reliable and reproducible species-level identification of micro-organisms from MALDI-TOF MS identification, standardized culture procedures have been adopted. The selected strains were subcultured (passage between three to six) in tubes containing 7H9 Middlebrook broth (Difco Middlebrook BD, Milan, Italy), enriched with acid-albumin-dextrose-citric acid (ADC; Difco Middlebrook BD) and mycobactin J (2 mg l^{-1}) (Institut Pourquier, Montpellier, France). The tubes were incubated at 37°C in the presence of 5% CO_2 and checked daily for growth. The cultures used for the study were all in logarithmic phase to reach a high concentration (approximately 10^5–10^8 CFU per ml). All procedures described above were performed under biosafety level-3 (BSL-3) conditions.

Although we did not analyse any type S field isolates in this study, liquid medium 7H9 was chosen because this medium supports the growth of both type S and C isolates well, while other solid media, such as Herrold's Egg Yolk Medium supplemented with mycobactin, support the growth of type S isolates poorly (Stevenson 2015).

All strains had been grown on 7H9 culture medium, and processed when the *Mycobacteria* were in the logarithmic phase of growth (Balazova *et al.* 2014).

Selectivity of the assay

In accordance with the OIE Terrestrial manual (Anonymous 2012), selectivity of the assay was tested analysing both inclusivity and exclusivity.

A total of 35 field isolates of MAP collected and identified at the Italian Reference Centre for Paratuberculosis (hosted at the Istituto Zooprofilattico of Lombardia and

Emilia Romagna) and one reference strain (ATCC 19698) were used for testing the inclusivity. Field isolates, all type C (see Table 1), were recovered from different hosts and sources. All isolates were tested for their dependency on mycobactin for growth, Ziehl-Neelsen staining and specific PCR (targeting IS900 and f57 sequences). For testing the exclusivity of the method, 152 different strains of *Mycobacteria* were tested, encompassing both reference strains and field isolates (Table 2).

Inactivation and preparation of the strains for MALDI-TOF MS identification

The method for inactivation and preparation of the strains for MALDI-TOF MS analysis was slightly modified from that previously described by Shitikov *et al.* (2012). Briefly, 3 ml of liquid culture were centrifuged at 3500 ***g*** and the pellets treated with 900 µl of ethanol, mixed thoroughly, washed and heated for 30 min at 95°C in a Thermomixer (Eppendorf, Milan, Italy). The samples were then washed again and 1200 µl of pure pre-cooled ethanol were added. After centrifugation for 2 min at maximum speed (16 000 ***g***) and careful removal of any residual ethanol, the pellets were allowed to dry at room temperature. Fifty microlitres of bead suspension (Zirconia/Silica; BioSpec, Bartlesville, OK, USA) were added to the pellets and the tubes were first treated with 25–50 µl of acetonitrile and centrifuged for 2 min 16 000 ***g***; then 25–50 µl of 70% formic acid were added, depending on the volume of the pellets. After a final

Table 1 List of *Mycobacterium avium* ssp. *paratuberculosis* field isolates tested to determine the inclusivity. Asterisks indicate strains with misleading identification

			MALDI-TOF MS log(scores)			
	Strains	ID code strain	Biotyper 3·0	Home made	Origin/Source	Origin
1	*M. avium* ssp. *paratuberculosis*	ATCC 19698	1·96*	>2·5	–	Italy
2	*M. avium* ssp. *paratuberculosis*	*75/15*	2·2	>2·5	Faeces/bovine	Italy
3	*M. avium* ssp. *paratuberculosis*	*616/13*	1·91	>2·5	Faeces/bovine	Italy
4	*M. avium* ssp. *paratuberculosis*	*Carne 51/13*	2·1	>2·5	Meat/bovine	Italy
5	*M. avium* ssp. *paratuberculosis*	*83/15*	1·86	>2·5	Bowel/goat	Italy
6	*M. avium* ssp. *paratuberculosis*	*22/14*	1·94	>2·5	Faeces/bovine	Italy
7	*M. avium* ssp. *paratuberculosis*	*Buffalo B/15*	2·05*	>2·5	Bowel/buffalo	Italy
8	*M. avium* ssp. *paratuberculosis*	*85/15*	2·1*	>2·5	Bowel/goat	Italy
9	*M. avium* ssp. *paratuberculosis*	*284/14*	1·85*	>2·5	Faeces/bovine	Italy
10	*M. avium* ssp. *paratuberculosis*	*23/15*	1·95	>2·5	Faeces/bovine	Italy
11	*M. avium* ssp. *paratuberculosis*	*171/14*	2·18	>2·5	Lyophilized faeces/bovine	UK
12	*M. avium* ssp. *paratuberculosis*	*192/14*	2·04	>2·5	Faeces/bovine	Italy
13	*M. avium* ssp. *paratuberculosis*	*31/15*	1·82	>2·5	Faeces/bovine	Italy
14	*M. avium* ssp. *paratuberculosis*	*82/15*	1·86	>2·5	Faeces/bovine	Italy
15	*M. avium* ssp. *paratuberculosis*	*Carne 27/13*	1·89	>2·5	Meat/bovine	Italy
16	*M. avium* ssp. *paratuberculosis*	*22013/21*	1·83	>2·5	Faeces/goat	Italy
17	*M. avium* ssp. *paratuberculosis*	*25/15*	2·05	>2·5	Faeces/bovine	Italy
18	*M. avium* ssp. *paratuberculosis*	*285/14*	2·0*	>2·5	Faeces/bovine	Italy
19	*M. avium* ssp. *paratuberculosis*	*35/15*	1·87	>2·5	Bowel/goat	Italy
20	*M. avium* ssp. *paratuberculosis*	*160/15*	1·96	>2·5	Faeces/bovine	Italy
21	*M. avium* ssp. *paratuberculosis*	*22013/13*	1·86*	>2·5	Faeces/goat	Italy
22	*M. avium* ssp. *paratuberculosis*	*76/13*	1·98*	>2·5	Environmental/bovine manure	Italy
23	*M. avium* ssp. *paratuberculosis*	*159/15*	2·14*	>2·5	Faeces/bovine	Italy
24	*M. avium* ssp. *paratuberculosis*	*327/14*	1·97	>2·5	Faeces/bovine	Italy
25	*M. avium* ssp. *paratuberculosis*	*83/14*	2·1*	>2·5	Faeces/bovine	Italy
26	*M. avium* ssp. *paratuberculosis*	*917/11*	2·1	>2·5	Environmental/bovine manure	Italy
27	*M. avium* ssp. *paratuberculosis*	*415/14*	1·91*	>2·5	Faeces/bovine	Italy
28	*M. avium* ssp. *paratuberculosis*	*534/13*	1·8	>2·5	Faeces/bovine	Italy
39	*M. avium* ssp. *paratuberculosis*	*172/14*	2·07	>2·5	Lyophilized faeces/bovine	UK
30	*M. avium* ssp. *paratuberculosis*	*630/13*	1·81	>2·5	Faeces/bovine	Italy
31	*M. avium* ssp. *paratuberculosis*	*607/13*	2·2	>2·5	Faeces/bovine	Italy
32	*M. avium* ssp. *paratuberculosis*	*482/14*	1·85*	>2·5	Bowel/deer	Italy
33	*M. avium* ssp. *paratuberculosis*	*83/14*	2·1*	>2·5	Faeces/bovine	Italy
34	*M. avium* ssp. *paratuberculosis*	*187/11*	1·92*	>2·5	Bowels/buffalo	Italy
35	*M. avium* ssp. *paratuberculosis*	*86/13*	2·08	>2·5	Faeces/bovine	Italy
36	*M. avium* ssp. *paratuberculosis*	*195/14*	2·2	>2·5	Bowels/goat	Italy

Table 2 List of *Mycobacteria* sp. used in the study for testing exclusivity

	Mycobacterium sp. analysed	Field isolates	Reference strains
1	*M. tuberculosis*	81	1 (ATCC 27294)
2	*M. bovis*	1	1 (ATCC 35734)
3	*M. avium* ssp. *avium*	13	1 (ATCC 25291)
4	*M. avium* ssp. *intracellulare*	19	1 (ATCC 13950)
5	*M. avium* ssp. *silvaticum*	–	1 (ATCC 49884)
6	*M. abscessus* ssp. *abscessus*	3	1 (ATCC 19977)
7	*M. xenopi*	3	1 (ATCC 19250)
8	*M. gordonae*	2	1 (ATCC 14470)
9	*M. terrae*	–	1 (ATCC 15755)
10	*M. smegmatis*	1	1 (ATCC 19420)
11	*M. fortuitum*	1	1 (ATCC 6841)
12	*M. chelonae*	1	1 (ATCC 35752)
13	*M. peregrinum*	3	1 (ATCC 14467)
14	*M. vaccae*	–	1 (ATCC 15483)
15	*M. flavescens*	–	1 (ATCC 14474)
16	*M. hiberniae*	–	1 (ATCC 49874)
17	*M. malmoense*	–	1 (ATCC 29571)
18	*M. marinum*	–	1 (ATCC 927)
19	*M. scrofulaceum*	–	1 (ATCC 19981)
20	*M. szulgai*	–	1 (ATCC 35799)
21	*M. nonchromogenicum*	–	1 (ATCC 19530)
22	*M. haemophylum*	–	1 (ATCC 29548)
23	*M. simiae*	–	1 (ATCC 25275)
24	*M. gastri*	–	1 (ATCC 15754)
	Total strains analysed	128	24

centrifugation (2 min 16 000 ***g***), 1 μl of the sample were spotted on the MALDI-TOF target plates (MSP 96 target ground steel; Bruker Daltonics, Billerica, MA, USA) and 1 μl of matrix added (HCCA, α-cyano-4-hydroxycinnamic acid). The spectrum was manually acquired following manufacturer's instructions. Samples were processed immediately after the extraction procedure. Sterility control tests were performed to assess the safety of the extraction procedure, and allow the analysis of the samples outside of the BSL-3 environment.

Parameters for MALDI-TOF MS analysis

Mass spectra of all clinical isolates were obtained by Microflex LT benchtop (Bruker Daltonics) via the Autoflex III smart beam of the MALDI-TOF MS system (Bruker Daltonics), using a laser frequency of maximum 60 Hz, and manually acquired in a linear positive mode. Mass signals were detected in the mass-to-charge (m/z) range of 2000–20 000. Ion sources one (IS1) and two (IS2), as well as the lens were adjusted to 20·08, 18·57 and 6·02 kV respectively. Ten technical replicates were performed per sample (the system performs between 100 and 1000 laser shots per sample). Results were then analysed using the MALDI Biotyper 3·0 software (Bruker Daltonics) and compared with the internal database spectra profiles and with a 'homemade' database generated including only the spectra of *Mycobacteria* species analysed in the study. The 'MBT FC.-par' standard flexControl method was chosen for both analyses, while the instrument calibration was performed with Bruker bacterial test standard (Myende 2013). The maximum deviation was always observed to be below ±300 ppm as recommended by the manufacturer (Myende 2013). The quality of spectra was evaluated through elimination of the m/z signals with a mass deviation above 3 Da, particularly for peaks that occurred at mass range between 6 and 7 kDa. The result of these procedures was the generation of specific mass spectra profiles (MSP), libraries and dendrograms. The dendrogram was built by MALDI-TOF Biotyper 3·0 software analysing the main spectra profiles through the statistical toolbox Matlab 7·1 (The MathWorks Inc., Natick, MA, USA), which is integrated in the MALDI Biotyper software. The distance measure was set at 'Euclidian' and linkage set at 'complete'. Each main spectrum of the data set was compared with each other main spectrum resulting in a matrix of crosswise distance scores. This matrix was used to calculate the distance values among the main spectra. Moreover, we also conducted the principal component analysis (PCA), a type of multivariate analysis integrated into Biotyper 3·0 software, which is able to reduce factors when a redundancy in the data is present. This method is based on calculation and comparison of the parameters showing high variances and has already been used for clustering correlated species and subspecies (Zautner *et al.* 2013; Vidigal *et al.* 2014). The software generates new co-ordinates (m/z-values) representing the principal components or PCs. In more detail, the co-ordinates are ordered according to the percentage of variance in the original data (Fig. S3) and the first three components are used to visualize the data as dots in a 3-dimension graph. PCA was conducted using at least two technical replicates of spectra acquired for each strain of MAA (14), MAP (36) and *M. intracellulare* (19), while for the only available MAS, we used all the technical replicates.

Creation and validation of the *Mycobacteria* homemade database

Before introducing each MSP into our homemade database, a total of 10 technical replicates were incorporated for each strain. Resulting spectra were analysed by FlexAnalysis software (ver. 3·3) and also estimated using the composite correlation index analysis, according to Bruker manufacturer's instructions for postprocessing. Only good quality spectra, showing correlation index over than 95% among the replicates of the same strains were accepted and chosen to create an MSP. In order to build our database, the MSPs belonging to each MAP

strains (with the exception of ATCC 19698) were matched against each other, avoiding self-matching. After this analysis, the scores obtained for all strains were over 2·5 (see Table 1). To further verify our approach, the spectra of the ATCC 19698 strain were matched using our database and the average score was still over 2·5 (see Table 1).

The reproducibility of MALDI-TOF MS approach was tested analysing two different batches containing the same strain (MAP IZSLER 171/14) growth at different times (Fig. S2).

Results

For testing inclusivity, we analysed 35 strains of MAP, isolated from different hosts and sources, and one laboratory strain (ATCC 19698) (Table 1). For each strain included in this group, the correctness of species identification was verified as previously reported in Materials and Methods section.

For testing exclusivity, several *Mycobacteria* species were analysed (Table 2). The species analysed included laboratory reference strains and several field isolates clinically recovered from 2014 to 2015 at the Microbiology lab of 'Istituto Nazionale per le Malattie Infettive 'L. Spallanzani'. The correctness of species identification was performed by 16s rDNA sequencing and/or by *hsp65* gene (Telenti *et al.* 1993).

Optimization of extraction protocol for *Mycobacteria*

Working with *Mycobacteria* in the open work area of the laboratory poses significant risks to laboratory personnel. To address this point, we used 'heat-killing protocols' as previously reported for inactivation and protein extraction (Shitikov *et al.* 2012). This protocol proved to be rapid and obtained good spectra profiles for each *Mycobacteria* tested (see Fig. S1). Moreover, none of the species tested in the study grew after 45 days of incubation at 37°C. The heat-killing step was tested by inoculating 20 μl of inactivated *Mycobacteria* suspensions in both Löwenstein–Jensen medium tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and 7H9 liquid medium. The absence of any visible growth after this period was taken as evidence of effective inactivation, indicating that the extraction protocol could be performed safely in the open laboratory after this killing step (data not shown).

Fifty spectra (using 10 plate positions and five times shooting) were obtained for each sample. The number of strains tested is reported in Table 1. Overall, the comparison of MALDI-TOF MS collected for different *Mycobacteria* species revealed several significant differences in peak positions (Fig. S1).

In particular, it is possible to manually analyse, using Bruker Daltonics flexAnalysis ver. 3·3, species-specific peaks and shared peaks into MAC to discriminate species. Figure 1 shows four species of MAC which were representative of all the strains analysed; peaks at 3765, at 5640 and at 11 310 *m*/*z* appeared in all strains analysed and we can assume that they must be characteristic of the complex. Moreover, the uniqueness of the shapes of these peaks could be also useful for the discrimination among the difference species. Furthermore, spectra analysis allowed the assessment of the presence/absence of dimensionally defined peaks, which can be allocated in species-specific manner. More specifically, we observed that peak at 7489 *m*/*z* was exclusively for MAA strains but absent in other MAC; in the same way we found a peak at 5389 *m*/*z* into MAP spectra, a peak at 4963 *m*/*z* MAS and a peak at 8088 *m*/*z* in *M. intracellulare*.

Biological and technical MAP replicates confirmed our results and ruled out the possibility that the presence/absence of a peak is due to the case (Fig. S2).

Comparison between Biotyper ver. 3·0 and homemade *Mycobacteria* database

The spectra obtained were then compared with those present in two different databases: one provided by Bruker Daltonics (MALDI Biotyper ver. 3·0; Bruker Daltonics), where 6000 bacteria strains are included, and another 'homemade *Mycobacteria* database' where reference strains and clinical isolates of *Mycobacteria* have previously been collected. The number of reference, clinical and field isolates was 188 (see Tables 1 and 2). According to the Bruker manufacturing manual, ranges in log(score) values between 2·0 and 3·0 are considered suitable for an identification up to species level, values from 1·6 to 1·999 for genus identification and <1·6 are considered unacceptable for a correct identification.

For 16 out of 36 strains herein analysed, the matching of MAPs spectra profiles with the reference spectra profile already present in the Biotyper database showed a mean log(score) values ≥2·0, while for the remaining strains (20), these values fell between 1·80 and 1·999 (see Table 1). Furthermore, 13 strains (36% of the total) were identified by Biotyper database as mix *M. avium* ssp. *avium/paratuberculosis* (both identifications were alternated), suggesting a misleading identification (Table 1, scores marked with asterisks).

To improve the ability of identification (log(score) values) and solve mixed identification, the respective *Mycobacteria* and MAP main spectra were collected into a new library containing single mass spectra for every *Mycobacteria* strain and the original spectra were then matched against this new library. In this way, the

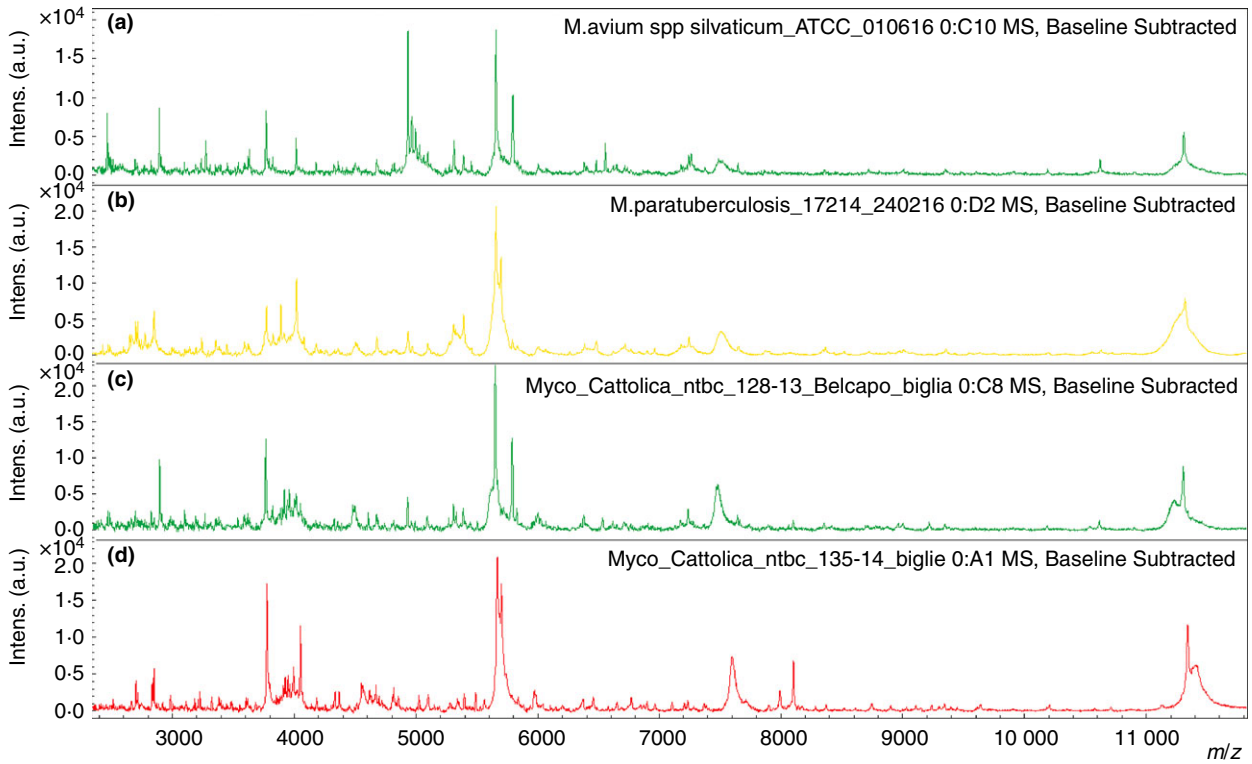

Figure 1 Mass spectra profiles of some member of *Mycobacterium avium* complex (MAC): *M. avium* ssp. *silvaticum* (a), *M. avium* ssp. *paratuberculosis* (b), *M. avium* ssp. *avium* (c) and *M. intracellulare* (d). The representative peaks patterns can be used discriminate among the different MAC species and subspecies. The relatives intensities of the ions are shown on the *y* axis, and mass to charge ratios are shown on the *x* axis. [Colour figure can be viewed at wileyonlinelibrary.com]

identification performed with this 'homemade *Mycobacteria* database', showed very good log(score) values (over 2·5) for all MAP strains tested, which, according to the above statements, are considered valid at species-level identification.

Dendrograms and PCA analysis

To further verify the closeness among the members of MAC and the different subspecies inside *M. avium* species, the main spectra of the member of the MAC were used to build one dendrogram (Fig. 2). The figure shows how all MAP strains were clustered into a major group, while the rest of MAC were separated from this cluster. However, the MAP group also contains the MAS reference strain.

To further investigate the closeness and the distances obtained by MALDI-TOF MS spectra among the organisms of the MAC, the PC analysis was performed. Figure 3 shows a three-dimensional graph in which the four different 'clouds of dots', belonging to the four members of MAC, at species or subspecies level, are differentially distributed in the dimensional spaces, allowing a clear discrimination between the different species and subspecies. Notably, this last analysis revealed how the dots from MAS were very close to those belonging to MAA strains.

Discussion

Paratuberculosis is a widespread disease, affecting domestic animals and wildlife in many countries. Despite the disease being well known for more than a century (Li *et al.* 2016), diagnosis still remains difficult. At the moment, the most popular approaches for the diagnosis of paratuberculosis are: indirect enzyme-linked immunosorbent assays in serum and milk, MAP DNA direct PCR from different matrices (milk, faeces, organs) and culture assays. Each of these approaches has its own advantages and disadvantages, but, according to the 'OIE Terrestrial Manual for the detection of paratuberculosis' (Anonymous 2014), the gold standard for the diagnosis of paratuberculosis in live animals is still the is still the isolation of MAP from culture. These methods, due to the slow growth rate of MAP and the complexity of the matrices, are technically demanding and require long periods of incubation (Anonymous 2014). Moreover, additional confirmatory assays, such

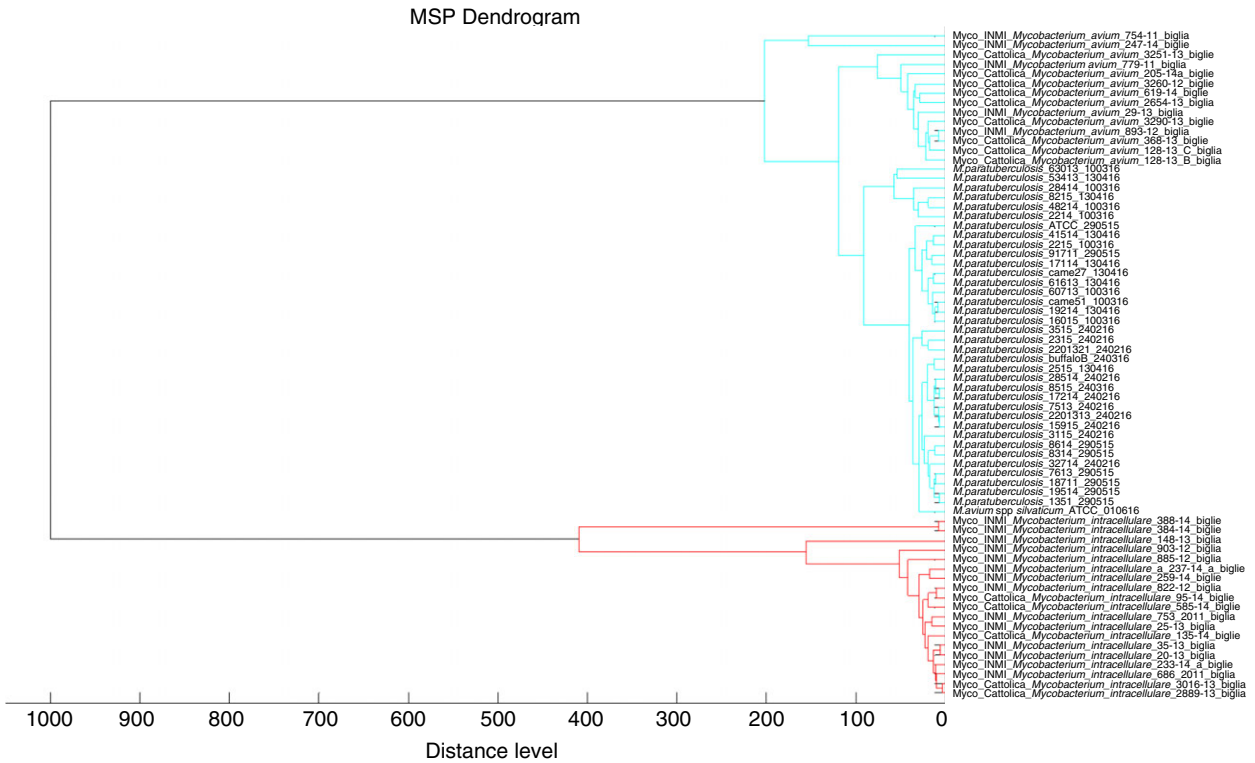

Figure 2 The dendrogram of *Mycobacterium avium* complex shows a clusterization at species and subspecies level, which essentially confirms the log(score) identification values, but underlined that, with this Euclidean analysis, *Mycobacterium avium* ssp. *silvaticum* and *M. avium* ssp. *paratuberculosis* clustered together. [Colour figure can be viewed at wileyonlinelibrary.com]

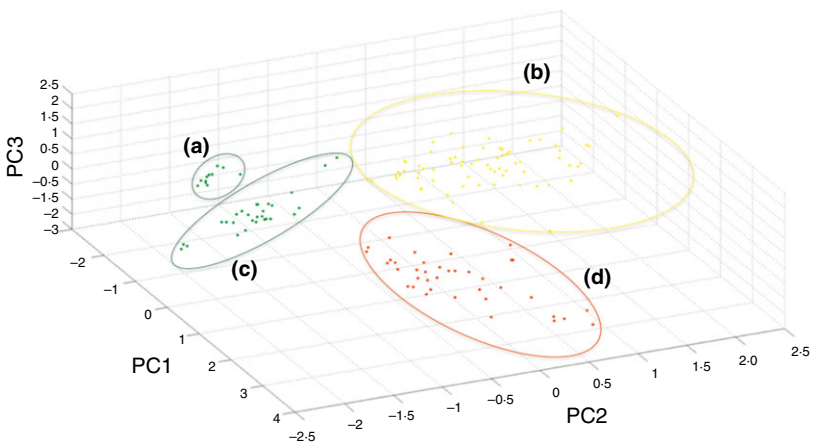

Figure 3 Principal component analysis of *Mycobacterium avium* ssp. *silvaticum* (MAS) (a, green), *M. avium* ssp. *paratuberculosis* (b, yellow), *M. avium* ssp. *avium* (c, green) and MAS (green) and *M. intracellulare* (d, red) based on MALDI-TOF MS spectra profiles. [Colour figure can be viewed at wileyonlinelibrary.com]

as PCR, Ziehl-Neelsen staining and subculturing, are needed before achieving a final identification, leading to a further increase in the time required for identification and diagnosis.

For these reasons, a tool allowing a rapid and prompt identification could be very useful for the identification of MAP. Our results suggest as MALDI-TOF MS could be a good choice to overcome many of the aforementioned steps. In fact, it requires only the field isolate, while the preparation of the samples for this analysis needs only a few steps, which take approximately 2 h to perform.

The purposes of having a homemade database are to increase the ability to identify species and to use MALDI-TOF MS as a powerful tool to discriminate down to subspecies level. These may be obtained by increasing the

number of acquired strains, corresponding to an enlargement of phenotypic heterogeneity (protein expression) in closely related micro-organisms.

In fact, the optimal log(score) values should be over 2·0 in order to state, with a certain degree of confidence, the true identification of the unknown bacterium at species level (Manual of Microflex LT; Bruker Daltonics). However, mycobacterial identification by MALDI-TOF MS for very close species was considered difficult (Fangous *et al.* 2014) and, for this reason, log(score) values 1·6 and 1·8 were proposed as 'new mycobacteria thresholds for low- and high-confidence identification levels, respectively' (Rodriguez-Sanchez *et al.* 2016). On the other hand, recent papers have shown how the use of specific databases containing several references from specific species of *Mycobacteria* can considerably improve the log(score) values obtained (Fangous *et al.* 2014). Moreover, just increasing the number of references belonging to different mycobacterial species can lead to the same result in term of log(score) (Rodriguez-Sanchez *et al.* 2016). These considerations can be extended to other bacterial species (Barberis *et al.* 2014).

In our study the log(score) values for MAP were over 2·5 when the spectra were matched against the 'home-made *Mycobacteria* database', while the match against the database provided by the vendor (MALDI Biotyper ver. 3·0; Bruker Daltonics) showed lower log(score) values and mixed identification. These results confirm that more specifically developed databases are needed when high-confidence level of identification between close species or subspecies is requested.

A recent review emphasized how the level of resolution to be expected from MALDI-TOF MS analyses applied to whole micro-organism can be limited, in some cases, to species-level differentiation and identification (Karlsson *et al.* 2015). In this regard, differentiation among MAP, MAA, *M. intracellulare* and MAS is achieved by specific PCR-based identification using MAC-specific gene targets (Ronai *et al.* 2015). However, in our study we demonstrated the possibility to identify the species belonging to MAC complex with MALDI-TOF MS analysis. In fact, a representative spectrum for each of the species belonging to MAC (Fig. 1) shows that each spectrum has specific peaks of peptides, which are variable in intensity and shape. According to these findings, the MALDI-TOF MS allows us to identify and distinguish each single species and subspecies.

The dendrogram built considering only MAC members showed only minor differences among the *M. avium* subspecies (Fig. 2), eventually confirming the closeness among the subspecies analysed. In particular, MAS, which shows some similarities to MAP for growth (both subspecies are mycobactin-dependent and slow growing) (Ronai *et al.* 2015), but is almost identical to MAA in its genome (Ronai *et al.* 2016), clustered within the MAP group. On the other hand, PCA approach permitted a clear discrimination and clusterization of all members of MAC. Moreover, as reported in Fig. 3, dots belonging to MAS spectra profile are closer to dots of MAA than to those of MAP, confirming the genomic closeness between MAS and MAA strains. The differences between the two cluster analytical methods (i.e. dendrogram building and PCA) are mainly due to the fact that dendrogram generates clusters with Euclidian distance calculation using all parameters of the spectra profiles (this is done by simply pairing all *m*/*z*-value coordinates, also those expressing low variances). PCA instead, is a multivariate analysis which takes into account only the three parameters (PC1, PC2, PC3) expressing the highest levels of variance. Very similar results were obtained for *Campylobacter jejuni* subgroups and *Stenotrophomonas maltophilia* (Zautner *et al.* 2013; Vidigal *et al.* 2014), in which the authors underlined how Euclidean distance analysis are suitable for calculating the degree of similarity, while PCA is more appropriate for catching the differences among the subjects analysed.

In conclusion, our data highlight the potential ability of MALDI-TOF MS to correctly identify type C MAP field isolates. Our results need to be validated on a larger set of samples, encompassing MAP type S strains, MAH and more MAS strains, but this approach is undoubtedly very attractive because the procedure employed is fast, easy to perform, and achieved an accurate identification of type C MAPs and other *Mycobacteria* earlier than other methods. If the quality and breadth of databases, as well as the identification performance of MALDI-TOF MS coupled with the use of multivariate approaches like PCA will improve, the laboratory diagnosis of *Mycobacteria* could become faster, more accurate and more cost-effective, with great benefits to the clinical management of mycobacterial infections, such as paratuberculosis. In particular, once the entire approach is validated, MALDI-TOF MS could be a valid alternative to PCRs and mycobactin dependency to confirm MAP identity.

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Conflict of Interest

The authors have no conflicting financial interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The figure shows spectra relative to each *Mycobacteria* species analysed in the study.

Figure S2. A strain of *Mycobacterium avium* ssp. *paratuberculosis* was tested with MALDI-TOF MS two times in same growth condition as described in the text (Materials & Methods).

Figure S3. The principal components (PCs), ordered according to the level of variance expressed for all the strains analysed are displayed in the figure.