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Instrumental analytical tools for mycobacteria characterisation

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Abstract: Mycobacteria in drinking water and in the water of swimming pools, whirlpools, hydrotherapy facilities and aquaria contribute significantly to human exposure to triggers of immune regulated chronic inflammatory and autoimmune diseases. Technological elements of water distribution systems, especially their inner surface, taps, shower heads and blind spots where sediments settle, affect the number of mycobacteria in the water. The review presents the possibilities of using analytical instruments for rapid determination of mycobacteria and for their typing as an alternative to classical culture and a method of monitoring specific nucleic acid sequences by polymerase chain reaction (PCR). Information about the use of flow cytometry (FCM), matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) spectrometry, Raman and infrared (IR) spectroscopy and biosensors are presented.

Keywords: flow cytometry; MALDI-TOF spectrometry; Raman spectroscopy; infrared spectroscopy; biosensors

The genus *Mycobacterium* contains about 190 species and subspecies, including several major human pathogens, known to cause tuberculosis and leprosy, as well as other environmental species (Gupta et al. 2018). Environmental opportunistic mycobacteria are distinguished from the members of the *Mycobacterium tuberculosis* complex (and *M. leprae*) since they are not obligate pathogens but are normal inhabitants of the environment (Primm et al. 2004). These nontuberculous mycobacteria (NTM) represent the majority of species in the genus *Mycobacterium* and are important opportunistic pathogens of humans, animals, poultry, and fish. NTM can be found in habitats such as natural waters, drinking water distribution systems, soils, dust, or aerosols (Tortoli 2006; Falkinham 2009). There are more than 100 documented species of slowly or rapidly growing pathogenic or non-pathogenic NTM, many of which are environmental saprophytes, but also commensals and symbionts (Primm et al. 2004; Falkinham 2009; Monteiro et al. 2018).

Although NTM, in contrast with *M. tuberculosis*, possess low virulence and lack human-to-human transmission, the pathogenicity of the NTM should not be underestimated, particularly in the immunocompromised patients (Tortoli 2006) and as a source of nosocomial infections (Perkins et al. 2009; Kuehl et al. 2018; Kaelin et al. 2020). The NTM most commonly infecting humans are *M. avium*, *M. intracellulare*, and *M. avium* complex (Falkinham 2011). NTM lung disease most commonly affects subjects with an underlying lung disease such as chronic obstructive pulmonary disease, bronchiectasis, or cystic fibrosis; however, healthy in-

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dividuals can also be infected. Less frequently, NTM can cause skin or soft tissue infections and lymphadenitis (Honda et al. 2015).

Cell wall-derived compounds from NTM have immunomodulatory activity and therefore the ability to trigger immunologically mediated chronic inflammatory and autoimmune diseases, such as type 1 diabetes mellitus, Crohn's disease, multiple sclerosis, psoriasis, asthma, allergies, degenerative inflammation of large joints, or sarcoidosis (Primm et al. 2004; Hruska and Cepica 2019). Especially newborns and infants are vulnerable to exposure to mycobacterial triggers due to their immature immune system. The consequences manifested as serious chronic diseases can arise with the delay of several years. In addition, not only mycobacteria act as immunomodulators. Other bacterial pathogens, which may release triggers of chronic inflammatory diseases as well, are only relevant in cases when they multiply in the host organism (Hruska and Cepica 2019).

NTM such as *M. avium*, *M. kansasii*, and *M. xenopi* have been frequently isolated from public and hospital drinking water distribution systems. Important factors contributing to their colonisation and persistence are mainly biofilm formation, amoeba-associated lifestyle, and resistance to chlorine due to their waxy outer membrane layer (Vaerewijck et al. 2005; Perkins et al. 2009). These aquatic mycobacteria tend to colonise biofilms at air-water and solid-water interfaces, and the latter seem to be an important proliferation site in oligotrophic habitats such as tap water (Schulze-Röbbecke and Fischeder 1989; Schulze-Röbbecke 1993).

Besides *M. avium* inhabiting hospital water supplies, the presence of *M. mucogenicum* and *M. gordonae* has also been detectable in the health care setting, notably in showerheads, water tanks, and faucets (Perkins et al. 2009). Findings also suggest the association of inflammatory lung diseases with attending spas and therapy pools contaminated with NTM that could have become aerosolised during normal use and operation (Glazer et al. 2007). Mycobacteria are known to selectively transfer from standing source water (e.g. pool water) into the aerosol by the 'bubble burst' mechanism from the water film due to their hydrophobic cell membranes (Perkins et al. 2009). NTM, particularly *M. chimaera*, can also contaminate water reservoirs of heater-cooler units used during open-heart surgery and disseminate into the air leading to devastating surgical site infections (Kuehl et al. 2018; Kaelin et al. 2020). Mycobacteria present in the air are also associated with dust or particles originating from soil (Hruska and Kaevska 2012). It was proved that house dust may contain a high diversity of mycobacterial species, especially *M. avium* complex and *M. terrae* (Torvinen et al. 2010; Nishiuchi et al. 2017).

Owing to the increased availability of modern genetic techniques and instrumentation, it is nowadays possible to detect and accurately identify a large number of species of the *Mycobacterium* genus (Tortoli 2006). Molecular techniques used for mycobacteria detection typically test for deoxyribonucleic acid (DNA) sequence on a chromosome and predominantly include polymerase chain reaction (PCR), fluorescence *in situ* hybridisation (FISH) or rolling circle amplification (Brehm-Stecher 2008; Schopf et al. 2011; Sevilla et al. 2015; Baliga et al. 2018).

Mostly PCR-based techniques are used for mycobacterial genotyping. PCR-restriction fragment length polymorphism (RFLP) analysis has been widely used, utilising restriction enzymes to generate fragments from genomic DNA. The most often analysed sequence in mycobacterial genotyping is the insertion element IS6110 as well as 16S rDNA, and genes rpoB, recombination protein A (recA), and heat shock protein 65 (hsp65) (Castro-Escarpulli et al. 2015). Another commonly used method is spacer oligonucleotide typing, or spoligotyping, which is based on PCR amplification of a highly polymorphic direct repeat locus in the *M. tuberculosis* genome (Gori et al. 2005; Castro-Escarpulli et al. 2015). Similar to spoligotyping is the analysis based on the variable-number tandem repeats of mycobacterial interspersed repetitive units (MIRU-VNTR) (Cowan et al. 2002; Castro-Escarpulli et al. 2015).

Methods for mycobacteria detection also involve immunoassays based on the production of specific mycobacterial antigens and their reaction with antibodies (Notermans and Wernars 1991). As for *M. tuberculosis* identification, usually, these *M. tuberculosis*-secreted antigens such as mycobacterial protein from species tuberculosis-64 (MPT64), early secreted antigenic target 6 kDa protein (ESAT-6), 10 kDa culture filtrate protein (CFP-10), and antigen 85B (Ag85B) serve as markers for the diagnosis (Mustafa et al. 2006; Zhang et al. 2015). Enzyme-linked immunosorbent assay (ELISA) is the most common immunoassay for mycobacteria identification from clinical samples (Verstijnen et al. 1991; Ahmad et al. 1995; Attallah et al. 2003; Wayengera et al. 2020). During ELISA, an inoculum of a sample is introduced to a well coated with primary antibodies, which correspond to the antigen of interest. After adding labelled secondary antibodies, the change in colour or fluorescence indicates the presence (or absence) of the antigen (Law et al.

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2015). Another technique represents immunohistochemical detection of mycobacterial antigens in tissue sections using antibodies conjugated to an enzyme (mostly horseradish peroxidase or alkaline phosphatase) catalysing a colour-producing reaction or antibodies tagged with a fluorophore (Humphrey and Weiner 1987; Mustafa et al. 2006; Erokhina et al. 2016; Solomon et al. 2017).

The selection of a suitable method for mycobacteria detection and characterisation should be based on the aim of the study. It may be required the enumeration of either only live mycobacterial cells or live, nonculturable and dead cells together. The matter of study can also be the characterisation of species in a certain habitat or the assessment of the resistance to disinfectants or higher temperatures. For example, the aim of the determination of *M. avium* subsp. *paratuberculosis* (MAP), which causes paratuberculosis in cattle and affects the development of chronic inflammatory and autoimmune diseases in humans, may be the excretion of MAP in milk and faeces or the severity of meat contamination (Hruska and Pavlik 2014; Hruska and Cepica 2019). Interpretation of the obtained results should take into account the course of the disease and the possibility of an intermittent excretion of mycobacteria. In animals, paratuberculosis can last for a long time without any clinical symptoms, while mycobacteria at this time may not be excreted at all or may be excreted in huge numbers. It should be considered that mycobacteria, as a source of immuno-triggers of many human diseases, may have sensitised leukocytes to the production of proinflammatory cytokines long before the clinical manifestation of the disease, when mycobacteria may no longer be present in the patient's body. However, the disease can be activated by a similar trigger of non-mycobacterial origin as well. On the contrary, the detection of MAP in the intestinal tract of patients without any symptoms of Crohn's disease does not mean that mycobacteria cannot contribute to the development of the disease that clinically manifests later. Moreover, for the acquisition of valid results, it is necessary to understand the ecology of mycobacteria. According to the stated above, it is important to specify the aim of the study before its initiation in order to avoid financing projects that cannot provide correctly interpreted results.

The presence of mycobacteria in drinking water sources is not surprising (Hruska and Kaevska 2012). However, the number of mycobacteria and their distribution in water distribution systems is influenced by several factors. The most important factors are the hydrodynamics of the distribution systems, dead zones, the formation of biofilms and sediments. Not only free mycobacteria can be present in tap water, but also mycobacteria internalised in amoebas or contained in biofilm released from the pipe wall, hot water tank wall, faucet aerator, or showerhead. Therefore, the number of mycobacteria in drinking water is not possible to assess in the same manner as blood biochemistry parameters which fluctuate according to the course of the disease. Moreover, good reproducibility of the results obtained from repeated measurements cannot always be expected. Detection of high numbers of mycobacteria in drinking water confirms the possibility that the consumers may be exposed to triggers of chronic diseases. However, failure in the detection of mycobacteria in one or more of the repeatedly examined samples does not exclude the exposure.

Marquetoux et al. (2019) dealt in their recently published review with the complexity and difficulty of MAP enumeration in clinical samples and inoculum suspensions for both *in vivo* and *in vitro* studies. They described the enumeration of MAP using culture-based methods and considered the influence of storage, decontamination, dormancy, and clumping. They also informed about the application of quantitative PCR, turbidimetry, pelleted weights, and direct microscopic counts. Kotlarz et al. (2018) monitored the number of bacteria in drinking water after ozonation treatment with special regard to mycobacteria and legionella. They examined the water, biofilm, and sludge using heterotrophic plate counts, organic carbon analysis, and thermal gravimetric analysis.

Selected instrumental methods for mycobacteria detection and characterisation in this review include flow cytometry (FCM), matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS), spectroscopic techniques such as Raman spectroscopy and infrared (IR) spectroscopy, and biosensing.

In general, the appropriate method for assessing the importance of colonisation of water, air or soil by mycobacteria or for detecting mycobacteria in clinical samples should meet high sensitivity and specificity. According to the reason of determination, easy sampling and transport to the laboratory, large scale analysis, easy interpretation of results, and low cost of consumables may be important for a choice of the method.

FLOW CYTOMETRY

Flow cytometry (FCM) represents a method for microbial diagnostics which provides information re-

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garding cell number, cell morphology and other physiological properties applied in the whole cell method for microbial detection (Brehm-Stecher 2008). FCM has become a convenient option also for rapid bacterial detection and quantification mainly due to its high-speed multi-parametric data acquisition (Chang et al. 2005).

The identification of bacteria by FCM is based on nucleic acid staining and optical detection of intact cells. Liquid samples are taken up and hydrodynamically focused to form a laminar flow within a surrounding sheath fluid (Brehm-Stecher 2008). The cells form a single file line and pass through a sheath-fluid-focused flow cell, where incident light is introduced by a laser source (Chang et al. 2005). Owing to different size, shape, granularity, and internal complexity, particles exhibit distinct light-scattering characteristics. The signals produced by scattered light are amplified and recorded at high speed (Chang et al. 2005). Data collecting is mediated by detectors and filter sets, recording cellular responses, including forward-angle light scatter (providing information on cell size and number) and side-angle scatter relating to internal content or opacity of the particles (Gruden et al. 2004; Brehm-Stecher 2008).

The data generated by flow cytometers are in the form of single-dimensional histograms or two-dimensional dot plots. These outputs indicate what proportion of the cells in the sample are positive for a given marker. Numerous cell populations can be described in a single sample by performing complex sequential gating analyses based on applying multicoloured combinations of probes and attaining different fluorescence intensity. A typical example is the characterisation of leukocyte subpopulations in human blood (Fuller et al. 2016).

The stains frequently used to view bacteria by FCM are mostly specific for nucleic acids. However, fluorescein isothiocyanate (FITC) and SYPRO stain exceptionally proteins. Stains that bind to DNA and ribonucleic acid (RNA) either intercalate into the double-stranded helical structure [propidium iodide (PI)] or fit specific regions of the DNA [blue fluorescent nucleic acid stain (DAPI)]. Some of the stains are cell-permeant (SYTO) and can simply cross bacterial membranes or are cell-impermeant to live or intact membranes [red fluorescent nucleic acid stain (TOTO)]. The majority of the stains have selective binding to DNA [green fluorescent nucleic acid stain (SYBR) Green I, PicoGreen] and to RNA (SYBR Green II), with different fluorescence intensity (Gasol and Del Giorgio 2000). The most widely used stains in aquatic environments are SYTO 9, SYTO 13, SYBR Green I and PicoGreen (Gasol and Del Giorgio 2000; Wang et al. 2010). The majority of the recently named fluorochromes can be excited with the 488 nm line of an air-cooled argon-ion laser and result in a much higher quantum yield showing less interference with phototrophic pigments (Vives-Rego et al. 2000).

The visualisation of bacterial association with target host cells can be performed by two common methods. The first is labelling bacteria externally with antibodies against bacterial cell membrane components conjugated with fluorochromes or fluorescent dye. The second option is expressing the fluorescent protein in bacterial cells and analysing host cells infected with a fluorescently labelled pathogen. This latter method has been widely used to study host cell association with *M. bovis*, *M. tuberculosis* or *Chlamydia psittaci* (Haridas et al. 2017). A functional fluorescent protein frequently used in mycobacterial systems is, for example, mCherry belonging to the family of monomeric red fluorescent proteins derived from DsRed isolate of *Discosoma* sea anemones (Carroll et al. 2014).

In order to analyse complex samples in environmental or clinical applications, the combination of FCM and FISH is widely used. FISH involves the hybridisation of fluorochrome-labelled nucleic acid probes to target rRNA within morphologically intact cells (Lange et al. 1997). Furthermore, FISH can be performed in a solution, allowing the subsequent analysis of a large number of cells (Brehm-Stecher 2008). Despite that, the FISH sensitivity is limited to cells in an active growth phase; thus, quiescent cells may not hybridise at sufficient quantity to be detectable (Lange et al. 1997).

As most flow cytometers are not capable to directly measure the volume of an analysed sample, direct quantitation of the bacterial concentration in a sample is not possible. Therefore, the sample volume must be determined indirectly with the addition of a known concentration of fluorescent beads. The number of beads computed in such a sample will be proportional to sample volume, thereby allowing bacterial concentration to be calculated (Berney et al. 2007).

However, fluorescent beads are expensive and tend to attach to plastic tubing, which may lead to over-estimating the sample concentration. Sample application to the flow cytometer by a volumetric syringe does not require the use of beads for calibration. The maximum injection volume is usually limited to 500 µL. Moreover, a dilution effect can be observed due to the sheath injection at the beginning and end of the sample injection. In contrast, the peristaltic pump system al-

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lows to record unlimited sample volume (up to 4 mL) without any dilution effect, hence provides continuous and stable acquisition throughout the entire sample volume (Kuckuck et al. 2001; Grenot and Luche 2020).

Microscopy and image analysis can be used for the enumeration of bacterial cells in suspensions (Selinummi et al. 2005). In order to increase flow cytometric spatial resolution to analyse more cellular properties, imaging flow cytometry (IFCM) was developed. IFCM combines single-cell identification and high-throughput capabilities of conventional FCM with fluorescence microscopy's cell image acquisition (Han et al. 2016). IFCM allows direct visualisation of fluorescently labelled pathogens and produces thousands of multi-spectral cell images per second. Furthermore, IFCM makes it possible to correlate acquired cellular images with events on bivariate dot plots (Han et al. 2016; Haridas et al. 2017). This technique enables simultaneously both – analysis of morphological features and phenotypic characterisation of single cells within an enormous and heterogeneous population (Han et al. 2016). Therefore, IFCM appears to be a suitable tool for researching intracellular pathogens and for studying the host-pathogen interaction heterogeneity on the single-cell level. For example, Haridas et al. (2017), using the IFCM technique, revealed that mCherry labelled *M. tuberculosis* internalised bacilli had a tendency to particularly co-localise at late endosome/lysosome cluster of differentiation 107 (CD107) and late endosome Rab7 (small GTPase belonging to the Rab family and controlling the transport to late endocytic compartments such as late endosomes and lysosomes) compartment (Haridas et al. 2017). Automated image analysis software was developed and validated for the quantification of bacterial cells from digital microscope images by Selinummi et al. (2005).

In the last few years, FCM has become a useful tool for the rapid identification and enumeration of bacteria from aquatic environments (Lange et al. 1997). Unlike the time-consuming microscopic method and heterotrophic plate count, the results from FCM can be obtained rapidly and with the highest relative accuracy, less than 5% (Wang et al. 2010). Flow cytometric analyses are commonly performed at a flow rate of 10–100 μL min^{-1} and detection of up to 10 000 events s^{-1} when the microbial concentration in the processed sample is sufficiently high (Vives-Rego et al. 2000). It has been demonstrated that FCM poses as a powerful substitute for counting the total number of microorganisms since the FCM measurements are reproducible with small relative standard deviations, provide quantification of the entire bacterial community and can be available within 15 min, unlike heterotrophic plate count, which requires days. Moreover, FCM analysis is, in comparison with heterotrophic plate count, more cost-effective with the possibility of complete automation (Wang et al. 2010; Van Nevel et al. 2017b). FCM, when combined with an appropriate molecular probe, can be applied in testing for specific microbial cells (Brehm-Stecher 2008) which might be slow-growing microorganisms, such as mycobacteria (Alvarez-Barrientos et al. 2000).

Due to its safety, reliability and rapid data acquisition, FCM proved to be an important technique in research and diagnostics (Bownds et al. 1996; Pina-Vaz et al. 2005; Hendon-Dunn et al. 2016). Bownds et al. (1996) employed FCM in susceptibility testing of NTM. The assay suspensions containing *M. avium*, *M. fortuitum*, *M. gordonae*, or *M. marinum* were incubated with various mycobacteriostatics by using fluorescein diacetate staining. The use of FCM was based on measuring the differences in the amounts of accumulated fluorescein between susceptible and resistant mycobacteria. Only viable mycobacteria could hydrolyse fluorescein diacetate to fluorescein by the intrinsic esterase and therefore be detected by FCM analysis. In contrast, in mycobacteria inhibited by the antimycobacterial agents, the hydrolysis was significantly lower. Consequently, the decrease in the viability of mycobacteria correlated with a decrease in the mean fluorescence channel. It was demonstrated that the flow cytometric assay can be completed in 24 h or less after the initiation of testing, therefore providing rapid, simple and reproducible measurement. Moreover, this technique did not require the multiplication of mycobacteria for accurate results (Bownds et al. 1996). Similarly, Hendon-Dunn et al. (2016) assessed the susceptibility of *M. tuberculosis* to antimycobacterial drugs with the use of rapid dual-fluorescence FCM. Mycobacterial cells were exposed to isoniazid or rifampin at different concentrations over time and dual stained with calcein violet with an acetoxy-methyl ester group and SYTOX green. While the calcein violet stained bacteria at different growth rates and was indicative of a viable population, the SYTOX green permeated damaged bacteria and confirmed loss of bacterial viability. The differential patterns of fluorescence provided insights into the mode of action of the drugs since the agents targeting the cell wall gave a FCM profile distinct from those inhibiting intracellular processes (Box A). Consequently, this FCM technique could contribute to the development of more effective antimycobacterial agents (Hendon-

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Staining of cells at different growth rates sampled from chemostats: One millilitre of cells at either growth rate was adjusted to a turbidity of 0.5 (at 540 nm) using growth medium. Five microliters of the CV-AM stock solution (Invitrogen, Life Technologies) in 25 µL of DMSO was then added to 1 mL of the cells incubated at 37 °C for 1 h while shaking at 220 rpm. Then the samples were spun at 6 000 rpm for 5 min, the supernatant was removed, and the resulting pellet was suspended again in 1 mL of HBSS containing 4% (v/v) formaldehyde to kill viable mycobacteria. The cells were left overnight to fix them prior to FCM.

Staining of antibiotic-exposed cells: One hundred microliters of mycobacteria in each well of a 96-well microtiter plate was stained with 0.5 µl CV-AM and 1 µl SYBR Green (20 µM solution in DMSO, Invitrogen, Life Technologies), and the plate was incubated at 37 °C for 1 h in the dark. An unstained sample was treated similarly to provide a control. Controls containing zero levels of antibiotic were stained with CV-AM only, as well as dual stained. After staining, the bacteria were spun by centrifugation at 2 885 × g for 2 min and resuspended in HBSS. A fixation time of 30 min was sufficient (at an OD_{540} of 0.5).

Stained samples were examined using a CyAn ADP (9-color) analyser (Beckman Coulter) with an attached Cytek plate loader. Two lasers with excitatory wavelengths of 488 nm and 405 nm were used. Ten thousand events were collected at a set standard low event rate. Summit software (version 4.3) was used to analyse the acquired data to create one-parameter fluorescence histogram overlays and two-parameter dot plots.

Box A. Staining protocol according to Hendon-Dunn et al. (2016)

CV-AM – calcein violet with an acetoxy-methyl ester group; DMSO – dimethyl sulfoxide; FCM – flow cytometry; SYBR – green fluorescent nucleic acid stain (Applied Biosystems^{TM}); HBSS – Hanks' balanced salt solution buffer; OD_{540} – optical density at 540 nm; CyAn ADP – type of flow cytometer produced by Beckman Coulter

-Dunn et al. 2016). The susceptibility of *M. tuberculosis* to antimycobacterial agents was also tested by Pina-Vaz et al. (2005). Isolates of 16 mycobacterial strains were grown for 72 h in the absence or presence of selected drugs, then heat-killed, stained with SYTO 16, and analysed by FCM. The sensitive phenotypes were determined by comparing the fluorescence intensity of mycobacterial cells because SYTO 16 penetrated only the damaged membrane of sensitive strains.

A significant example of total cell counting using FCM is the enumeration of bacterial cells in treatment processes for drinking water and wastewater (Wang et al. 2010). FCM showed to be a powerful tool for indicating the changes in water quality after maintenance and various treatments, including ultrafiltration and ozonation.

Hammes et al. (2008) analysed total bacterial cell concentrations in water samples from a drinking water pilot plant in Zürich, Switzerland, using SYBR Green I staining coupled with quantitative FCM. It was demonstrated that the impact of the three major treatment processes (namely sequential ozonation, granular active carbon filtration, and membrane ultrafiltration) on the microbiology in the system could be accurately described with total cell counting measured with FCM. The raw water contained about 1×10^6 cells mL^{-1}, which was after ozonation reduced to circa 1×10^3 cells mL^{-1}. During granular active carbon filtration, regrowth of bacterial cells was observed as the total cell concentration increased to about 1×10^5 cells mL^{-1}. Finally, the membrane ultrafiltration caused the total cell concentrations to decrease to or below circa 200 cells mL^{-1}. In comparison with heterotrophic plate count performed on the same water samples, the FCM detected 1–2 log units more and therefore proved to be a more sensitive method. Moreover, heterotrophic plate count results had a standard error of 30% and more, while FCM data showed a standard error of less than 5% (Hammes and Egli 2005). Hammes and Egli (2005) also employed the combination of fluorescence staining and FCM for quantification of microbial growth during the assimilable organic carbon (AOC) measurement instead of conventional plate count [or adenosine triphosphate (ATP) analysis]. The AOC is readily assimilated by microorganisms, resulting in microbial regrowth; hence, its concentration is a critical parameter for drinking water treatment. It was demonstrated that the FCM method compared to plating provides a more realistic interpretation of a natural microbial consortium growing on AOC as FCM additionally al-

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lowed also the detection of inactive and/or unculturable microorganisms.

Wang et al. (2007) applied the combination of fluorescence staining and FCM to quantify the filterability of bacteria from different freshwater samples through 0.45, 0.22, and 0.10 μm pore size filters. The samples included both surface and drinking water. It was revealed that on average, 50% of the natural aquatic bacterial communities passed through 0.45 μm pore size filters, 0.03–3% was able to pass through 0.22 μm, and a small percentage from 0.003% to 0.2% could pass through 0.10 μm pore size filters. The original total cell concentrations in the surface water samples ranged from 2 to 4×10^6 cells mL^{-1} which implied a total cell concentration of as high as 10 000 cells mL^{-1} in a 0.22 μm filtrate and 1 000 cells mL^{-1} in a 0.10 μm filtrate. For the drinking water samples, similar passage percentages were observed with lower original cell concentrations around 0.3×10^6 cells mL^{-1}.

Van Nevel et al. (2017a) proposed a combination of FCM bacterial cell count with FCM fingerprinting technique for quality control of drinking water networks after maintenance. The study examined three full-scale cases where major supply pipes were emptied, disinfected and flushed with large amounts of clean drinking water. Samples were taken at the reference and flushing points before and after the maintenance works. FCM measurements revealed that after 2–4 h of flushing, both clean reference and the discharged flushing waters had similar intact cell concentrations ranging from 2.1×10^3 cells mL^{-1} to more than 10^4 cells mL^{-1}, which was considered as the endpoint. The FCM fingerprinting was used as an additional technique to verify the similarity between samples and yielded analogous results. To conclude, FCM proved to be a reliable additional technique, which is able to obtain results faster than routinely applied heterotrophic plate count.

Liu et al. (2016) and Van Nevel et al. (2017a) assessed microbial differences in drinking water distribution systems in Beijing, China. In 12 water samples obtained from different water sources, the FCM total cell concentrations varied from 7.3×10^3 cells mL^{-1} to 2.6×10^5 cells mL^{-1}. The combined use of FCM and 16S rRNA gene sequencing to determine the relative abundance of pathogenic bacteria allowed the estimation of viable *Mycobacterium* between samples. The highest determined *Mycobacterium* concentration within a sample was 500 cells mL^{-1} (Liu et al. 2016). Chan et al. (2019) examined bacterial release from pipe biofilm in a full-scale drinking water distribution system in Varberg, Sweden. Following the installation of ultrafiltration at full-scale to remove the high background cell count, the bacteria entering drinking water from a pipe biofilm could be both quantitated and described using FCM. It was found that 58% of the bacteria in the distributed water originated from pipe biofilm. The number of cells released from this biofilm was $2.1 \times 10^3 \pm 1.3 \times 10^3$ cells mL^{-1} included *Mycobacterium,* among identified species determined by 16S rRNA analysis.

Chan et al. (2019) applied FCM analysis according to Prest et al. (2013). The staining procedure is summarised in Box B.

Viable bacteria concentrations were monitored across multi-chamber ozone contactors in a full-scale

Water samples were stained according to the standardised protocol proposed in the SLMB (2012). In short, samples (500 μL) were preheated to 35 °C (5 min) and then stained with 10 μL mL^{-1} SYBR Green I (1 : 100 dilution in DMSO; Molecular Probes), and incubated in the dark for 10 min at 35 °C before measurement. Where necessary, samples were diluted just before measurement in filtered (0.22 μm; Millex-GP, Millipore) bottled mineral water (EVIAN, France), so that the bacterial concentration measured with the flow cytometer was always less than 2×10^5 cells mL^{-1}. Unless stated otherwise, this staining protocol was strictly applied in order to achieve comparable data.

Stained samples (50 μL of the 500 μL) were measured using a BD Accuri C6 flow cytometer equipped with a 50 mW laser emitting at a fixed wavelength of 488 nm. A threshold value of 500 arbitrary units was set up on the green fluorescence channel.

Box B. Staining protocol according to Prest et al. (2013)

SLMB – Swiss guideline for drinking water analysis; SYBR – green fluorescent nucleic acid stain; DMSO – dimethyl sulfoxide; BD Accuri C6 – type of flow cytometer produced by BD Biosciences™

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drinking water treatment plant using FCM by Kotlarz et al. (2018). It was found that intact cell concentrations in water samples from ozone contactor effluents ranged from 1 200 cells mL^{-1} to 3 750 cells mL^{-1}, gradually increasing in subsequent chambers despite the growing ozone exposure. The explanation was a microbial detachment from biofilms on contactor surfaces and from biomass present in a hydraulic dead zone. A higher relative abundance of *Mycobacterium* spp. was detected in the biofilm and sludge samples using 16S rRNA sequencing, which implicated the possibility of mycobacterial ozone resistance.

A study conducted by Chang et al. (2005) was aimed at comparing recovering efficiency of immunomagnetic separation and centrifugation of *M. immunogenum* in a semisynthetic metal working fluid (MWF). Immunomagnetic separation was found to be compatible with FCM, thus coupling together increased cell detection and enumeration speed. This approach enabled much higher cell recovery efficiency and fluorescent light intensities in comparison to the centrifugation technique.

Chang and Adriaens (2007) compared the labelling efficiency of nano-immunomagnetic particles (NIMP) and free antibody to detect mycobacteria in a mixed community in a semisynthetic MWF by using FCM. Pure culture samples of six different bacterial strains were tested, and two mycobacterial strains (*M. immunogenum* and *M. parafortuitum*) showed distinct higher light intensity on the green fluorescence detector than others. The results support the applicability of NIMP and free antibody to detect mycobacteria in semisynthetic MWF by using both traditional visualisation analysis and cluster analysis-aided visualisation analysis (CAAVA). For quantitative enumeration, Trucount tubes containing dye-coated beads were utilised.

The antibacterial potential of MWF formulations independent of bacterial growth was evaluated by Vanhauteghem et al. (2019). The study was based on monitoring the viability of *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Proteus mirabilis* determined by the degree of membrane damage using FCM analysis of SYTO 9/PI dual-stained cells. The FCM technique was in comparison with a biochallenge test used in MWF industry and with traditional plate counts substantially faster since it could reliably predict the antibacterial capacity of MWF already within one day of incubation.

Vives-Rego et al. (2000) summarised the advantages of FCM as follows: 'In flow cytometry, several parameters can be simultaneously measured for several thousand cells per second with high precision. The detection limit 100–1 000 cells mL^{-1} is dependent on electronic and fluorescence background, the sample heterogeneity, the fluorescence distribution and on the fluorescence signal-to-noise ratio. However, even at a high flow rate of 100 µL min^{-1}, the detection of 100 targeted cells present in a sample at a concentration of 10 cells mL^{-1} requires 100 min. Therefore, this technique may require physical or biological preenrichment to detect rare events in aquatic samples. Despite this limitation, FCM appears to have high potential for studying the microbiology of aquatic systems as it obtains and processes multiparametric data at high speed and enables the transfer of specific populations or even single cells to a determined location, thus allowing further analysis.' Hence, FMC seems to be a priority choice for the enumeration of NTM in water. Furthermore, if 1 000 mycobacteria in 1 ml of water are assumed to be a threshold, no centrifugation of a sample should be needed to achieve a short measuring time and high throughput of samples.

MALDI-TOF MASS SPECTROMETRY

Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) poses a valuable tool for rapid and accurate microbial identification and characterisation, mainly due to its high throughput and speed associated with complete automation (Singhal et al. 2015; Di Gaudio et al. 2018). It represents an analytical technique based on an ionisation process and the conversion of neutral compounds into charged ions, allowing the measurement of their mass-to-charge (m/z) ratio (Di Gaudio et al. 2018). In general, the characterisation or identification of bacteria is targeted to peptides and proteins or to lipids after the disruption of bacteria. For the determination of species-specific substances in the bacterial cell wall, the whole cells without disintegration can be used.

In MALDI-TOF MS a 'soft ionisation' method was introduced where ion formation does not lead to a significant loss of sample integrity. The analysed sample is mixed or coated with a solution of an energy-absorbent, organic compound called the matrix [usually an ultraviolet (UV)-absorbing organic acid], which crystallises on drying. The sample co-crystallises within the matrix and is ionised with a laser beam. After desorption and ionisation, singly protonated ions are generated from analytes in the sample, then accelerated at a fixed potential and separated from each other according to their m/z ratio. The charged analytes are

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detected using a time-of-flight (TOF) mass analyser, measuring the m/z ratio of an ion by determining the mass-dependent time required for it to pass through the length of the flight tube (Singhal et al. 2015; Jang and Kim 2018). The output is in the form of a characteristic spectrum called mass fingerprint (Singhal et al. 2015). The mass spectrometry (MS) spectra of peptides and proteins extracted from microorganisms of interest are contained in searching databases, which are utilised for microbial identification using scoring algorithms to match analysed spectra with reference spectra (Jang and Kim 2018).

Recently, MALDI-TOF MS is being successfully applied in mycobacteriology as improvements in database development and workflow make its use for the identification of mycobacteria imminent (Bryson et al. 2019). Established *Mycobacterium* databases, either National Institutes of Health (NIH) mycobacterial database or Bruker Daltonics database, comprise numbers of spectrum profiles of each *Mycobacterium* species (Lin et al. 2015). Consequently, MALDI-TOF MS has accelerated the process of identifying these microorganisms beyond what can be achieved with antimicrobial susceptibility testing (Bryson et al. 2019). Microbial ribosomal proteins are most often the main contributors to the generated mass spectrum, which is unique to individual organism-types, with peaks specific to genera, species, and strains. The original Bruker's Mycobacteria protein extract method (MycoEX) was validated for liquid or colonies taken from solid samples. Several modifications have been developed, most of them based on cell disruption, adding sonication or bead-beating steps for liberating mycobacterial protein for analysis (Alcolea-Medina et al. 2019). Recently, Bacanelli et al. (2019) modified the previously mentioned MycoEx method and introduced the MycoLyser method, which includes homogenisation in tissue macerator rather than vortex. In comparison, MycoLyser showed higher scores and identification consistency with the Biotyper system (Bacanelli et al. 2019). However, mass spectra can also be obtained from ionised lipid molecules after lipid extraction (Kim et al. 2018). In addition, MALDI-TOF MS technique is able to identify relevant envelope lipids directly from intact mycobacteria. Several groups of glycolipids have been shown to be biomarkers of both *M. tuberculosis* strains and NTM (Larrouy-Maumus and Puzo 2015). Moreover, MALDI-TOF MS provides highly resolved mass spectra of various types of mycolic acids. Laval et al. (2001) proved its capability of rapid structural analysis of mycolic acids and revealed that the chain lengths of the various mycolates correlated with the growth rate of mycobacterial strains (Laval et al. 2001).

In the last few years, the development of MALDI imaging mass spectrometry (MALDI IMS) has notably contributed to the detection of proteins and lipids in clinical pathology (Walch et al. 2008). This technique is capable of visualising the spatial distribution of molecules in biological samples, enabling label-free identification of analytes directly from tissue sections (Blanc et al. 2018; Yang et al. 2020). With high accuracy and sensitivity, MALDI IMS can quickly analyse high molecular weight substances with no or few fragments, while a spatial resolution of 5–10 μm is usually achieved (Michno et al. 2019; Yang et al. 2020). MALDI IMS can also provide the quantitative amounts of target compounds in the examined tissue if proper calibration techniques are performed (Rzagalinski and Volmer 2017). De Macedo et al. (2015) recommend MALDI IMS for a better understanding of lipid functions in leprosy pathogenesis.

MALDI-TOF MS showed to be an accurate and rapid method for identifying mycobacteria from solid culture media (Machen et al. 2013). Due to elevated biohazard, cell inactivation is necessary; therefore, organic solvents, short heat inactivation or mechanical lysis and protein extraction are often used. For direct analysis of liquid cultures or liquid clinical specimens such as positive blood cultures and urine samples, a prepurification is required. This involves short pre-culture on rich media, filtration, or combined centrifugation and washing steps (Welker et al. 2019). Using MALDI-TOF MS, it is also possible to analyse human skeletal remains by preparing a trypsin digested protein extract from a grounded bone powder (Boros-Major et al. 2011). For mycobacteria identification, another possibility is to prepare an extract from bone powder with a chloroform-methanol mixture in an ultrasonic bath in order to detect specific mycolic acids (Mark et al. 2010). The detection limit is standardly around 10^5 cells per sample, although it could be lowered with automation (Welker et al. 2019). Usually, the processing of 10^6 bacterial cells to 10^7 bacterial cells generates excellent mass spectra (Kliem and Sauer 2012). For urine samples, sufficient bacterial concentration is usually more than 10^3 cells mL^{-1}, which can be further concentrated from volumes larger than 10 mL (Angeletti and Ciccozzi 2019).

Another option is to analyse the surface of two-dimensional biological tissue slices *in situ*, which can be performed with MALDI IMS (Walch et al. 2008). In MALDI IMS, tissue sections are placed on glass

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or stainless steel and pre-coated with UV-light absorbing matrix before systematic scanning with a laser probe. Each pixel in the image represents a mass spectrum with defined *x*- and *y*-coordinates (Himmel et al. 2018; Michno et al. 2019). For instance, Blanc et al. (2018) developed a MALDI IMS method to visualise specific glycolipids of mycobacteria within tuberculosis lesions. There were clearly detected bacterial clusters heterogeneously distributed throughout the lesions (Blanc et al. 2018).

MALDI-TOF MS technology typically employs reaction volume up to 50 µL and sample deposition volume up to 2 µL in a 96- or 384-well format, with a sampling rate of approximately 5 s. Haslam et al. (2016) described the MALDI-TOF process applicable to high-throughput screening workflow as the analysis speed of 1.2 s per sample gave a read time of about 30 min/1536-well plate. The reaction volume minimised to 1–2 µL and the deposition volume to 100 nL, thereby reducing the further cost (Haslam et al. 2016). Studies conducted to analyse and identify mycobacteria usually employed sample deposition volume from 1 µL to 2 µL and matrix volume 1 µL (Alcaide et al. 2018; Epperson et al. 2018; Moreno et al. 2018; Rodriguez-Temporal et al. 2018; Bacanelli et al. 2019).

Automated processing and identification of positive blood cultures by MALDI-TOF MS has been described and evaluated in routine practice. There exist commercial products automating the matrix dispensing for MALDI-TOF MS analysis or ensuring correct manual spotting and traceability of target slides (Broyer et al. 2018). Two U.S. food and drug administration (FDA)-cleared automated MALDI-TOF MS systems are available, namely the VITEK MS system (BioMérieux Inc.) and the MALDI Biotyper CA system (Bruker Daltonics Inc.), differing in instrumentation and in algorithms and databases used to identify microorganisms (Patel 2015). Broyer et al. (2018) presented a prototype instrument for automated preparation of VITEK MS slides directly from positive blood culture broth in less than 25 min. This prototype allows full automation of all pre-analytical steps (extraction, spotting, drying, matrix addition), thereby reducing their duration and the process variability (Broyer et al. 2018).

The key advantages of MALDI-TOF MS are the comparatively low cost per analysis and a uniform sample preparation procedure suitable for many different types of microorganisms (Welker et al. 2019). According to a study conducted by Costa-Alcalde et al. (2019), MALDI-TOF MS methodology was associated with significantly lower consumable costs than the commercial molecular system GenoType® (Costa-Alcalde et al. 2019). It has also been suggested that MALDI-TOF MS may prove a higher yield than molecular methods (Bryson et al. 2019). In comparison with electrospray ionisation, MALDI is more practical for rapid bacteria identification due to faster sample preparation and its tolerance to impurities. Simultaneously, TOF mass spectrometers are simple, accurate, and inexpensive, achieving a high scan rate and acquisition of mass spectra in microseconds (Hayes et al. 2011). Several hundred samples can be analysed per hour with one MALDI mass spectrometer. There are pipetting robots, tracing systems and automated mass spectrometry analysis devices available (Kliem and Sauer 2012). Besides, direct identification from clinical specimens such as blood culture bottles and urine using MALDI-TOF MS is also possible as convenient methods have now been developed (Bryson et al. 2019).

The concrete examples of using the MALDI-TOF MS technique in mycobacteriology are summarised in Table 1. This method proved to be a convenient tool for the identification and typing of mycobacteria at the group, species, and even subspecies level, mostly from human and bovine clinical isolates. In addition, one case of species identification came from an aquarium fish sample. Using the MALDI-TOF MS technique, it was also possible to differentiate between individual species and subspecies and to distinguish growth states of a certain mycobacterial species. Furthermore, drug sensitivity testing in tuberculous and NTM was conducted as well. As for paleobacteriology, mycobacterial proteins and mycolic acids were identified by MALDI-TOF MS analysis in infected archaeological skeletal remains.

Target compounds used for the identification and characterisation of mycobacteria are listed in Table 2. The most commonly applied method was the detection of extracted proteins after cell disruption. In a few cases, mycolic acids or specific lipid molecules were used as targets instead of proteins. However, some references also demonstrated the possibility of utilising whole mycobacterial cells without disintegration to identify envelope lipid structures, mycolic acids or other non-covalently bound surface molecules.

In Table 3, it is clearly showed what can be used for MALDI-TOF MS analysis of mycobacteria. The colonies grown on a solid culture media were the most prevailing matter used. Another presented option was a mycobacterial suspension in the liquid culture medium. Frequently used solid culture media were Löwenstein–Jensen medium, Middlebrook 7H10, and

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Table 1. MALDI-TOF MS application for differentiation of mycobacteria

Purpose	Taxonomic unit or marker	Sample matter	References
Group identification	*M. avium* complex, *M. fortuitum-parafortuitum* complex	bovine, human and environmental isolates	(Ravva et al. 2017)
	M. tuberculosis complex, *M. abscessus* complex, *M. fortuitum* complex	clinical isolates	(Body et al. 2018)
	M. tuberculosis complex, *M. avium-intracellulare* complex and *M. chelonae-M. abscessus* complex	clinical and external quality control isolates	(Akyar et al. 2018)
Species and subspecies identification	*M. avium*	human clinical isolates	(Lin et al. 2015)
	M. avium subsp. *paratuberculosis*	bovine, human and environmental isolates	(Ravva et al. 2017)
	M. abscessus, *M. fortuitum*, *M. marinum*, *M. peregrinum*	aquarium fish	(Puk et al. 2018)
	M. bovis	bovine/bubaline clinical samples	(Bacanelli et al. 2019)
	M. chelonae subsp. *gwanakae* subsp. nov.	human sputum isolates	(Kim et al. 2018)
	NTM species	human clinical isolates (mainly respiratory specimens) clinical isolates	(Mediavilla-Gradolph et al. 2015) (Alcaide et al. 2018)
	Rapid growing NTM species and slow growing NTM species	human clinical isolates	(Alcolea-Medina et al. 2019) (Rodriguez-Temporal et al. 2018)
Species and subspecies differentiation	*M. abscessus* and *M. massiliense*	human clinical isolates	(Tseng et al. 2013)
	M. abscessus subsp. *abscessus*, *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii*	human clinical isolates (sputum, blood-culture, bronchial aspiration samples)	(Fangous et al. 2014)
	M. intracellulare and *M. chimaera*	clinical isolates (sputum, respiratory aspirates), isolates from heater-cooler units	(Pranada et al. 2017)
		clinical isolates (bronchoalveolar lavage, sputum, or tissue)	(Epperson et al. 2018)
Resistance	*M. tuberculosis* (rifampin, isoniazid, linezolid, ethambutol) and NTM (clarithromycin and rifabutin)	clinical isolates	(Ceyssens et al. 2017)
Growth states differentiation	dormant and exponential growth state of *M. smegmatis*	model-organism cultures	(Neumann et al. 2019)
Paleobacteriology	mycobacterial proteins	archaeological human skeletal remains	(Boros-Major et al. 2011)
	M. tuberculosis mycolic acids	archaeological human skeletal remains	(Mark et al. 2010)
	mycobacterial proteins and mycolic acids	archaeological human skeletal remains	(Hajdu et al. 2012)

MALDI-TOF MS – matrix-assisted laser desorption ionisation time-of-flight mass spectrometry; NTM – nontuberculous mycobacteria

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Table 2. Frequently used targets for identification and characterisation of mycobacteria

Target	Object	Sample origin	References
Lipids	isolates	human (sputum)	(Kim et al. 2018)
		human	(Larrouy-Maumus and Puzo 2015)
	tissue sections	mouse and rabbit lungs	(Blanc et al. 2018)
Mycolic acids	archaeological skeletal remains	human (complete skeleton)	(Hajdu et al. 2012)
		human (ribs and vertebrae)	(Mark et al. 2010)
	purified extracts from isolate	laboratory culture collection	(Laval et al. 2001)
Proteins or peptides	archaeological skeletal remains	human (vertebrae)	(Boros-Major et al. 2011)
	isolates	human; laboratory culture collection	(Akyar et al. 2018)
		human	(Alcaide et al. 2018)
		human	(Alcolea-Medina et al. 2019)
		bovine/bubaline (lymph nodes, lung, mammary, spleen and liver tissue); laboratory culture collection	(Bacanelli et al. 2019)
		human; environmental water sources; laboratory culture collection	(Balada-Llasat et al. 2013)
		human	(Body et al. 2018)
		human	(Costa-Alcalde et al. 2019)
		human; laboratory culture collection	(El Khechine et al. 2011)
		human (bronchoalveolar lavage, sputum, tissue)	(Epperson et al. 2018)
		human (sputum, blood-culture, bronchial aspiration samples)	(Fangous et al. 2014)
		human (bronchoalveolar and lavage, peritoneal fluid, pus, sputum, urine)	(Genc et al. 2018)
		laboratory culture collection	(Hettick et al. 2004)
		human; laboratory culture collection	(Lin et al. 2015)
		laboratory culture collection	(Mather et al. 2014)
		human	(Machen et al. 2013)
		human (respiratory specimens)	(Mediavilla-Gradolph et al. 2015)
		laboratory culture collection	(Moreno et al. 2018)
		laboratory culture collection	(Neumann et al. 2019)
		human	(Park et al. 2016)
		human (sputum, respiratory aspirates); heater-cooler units; laboratory culture collection	(Pranada et al. 2017)
		aquarium fish	(Puk et al. 2018)
		bovine, chicken, human, swine, wood pigeon; dairy water trough, soil	(Ravva et al. 2017)
		human	(Rodriguez-Temporal et al. 2018)
		human	(Rotcheewaphan et al. 2019)
		human	(Tseng et al. 2013)
Whole cells	isolates	laboratory culture collection	(Hettick et al. 2004)
		human	(Larrouy-Maumus and Puzo 2015)
	tissue sections	mouse and rabbit lungs	(Blanc et al. 2018)

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Table 3. What can be used for MALDI-TOF mass spectrometry of mycobacteria

Matter for analysis	Target	References
Bone powder	mycolic acids	(Mark et al. 2010)
	mycolic acids and proteins	(Hajdu et al. 2012)
	proteins	(Boros-Major et al. 2011)
Colonies grown on solid culture medium	lipids	(Larrouy-Maumus and Puzo 2015) (Kim et al. 2018)
	proteins	(El Khechine et al. 2011) (Balada-Llasat et al. 2013) (Machen et al. 2013) (Tseng et al. 2013) (Fangous et al. 2014) (Mather et al. 2014) (Mediavilla-Gradolph et al. 2015) (Lin et al. 2015) (Alcolea-Medina et al. 2019) (Alcaide et al. 2018) (Moreno et al. 2018) (Pranada et al. 2017) (Puk et al. 2018) (Rodriguez-Temporal et al. 2018) (Akyar et al. 2018) (Body et al. 2018) (Costa-Alcalde et al. 2019) (Epperson et al. 2018) (Bacanelli et al. 2019) (Rotcheewaphan et al. 2019)
Histological specimen	lipids	(Blanc et al. 2018)
Suspension in liquid culture medium	lipids	(Larrouy-Maumus and Puzo 2015)
	mycolic acids	(Laval et al. 2001)
	proteins	(Hettick et al. 2004) (El Khechine et al. 2011) (Balada-Llasat et al. 2013) (Ceyssens et al. 2017) (Park et al. 2016) (Pranada et al. 2017) (Ravva et al. 2017) (Rodriguez-Temporal et al. 2018) (Genc et al. 2018) (Neumann et al. 2019)

MALDI-TOF – matrix-assisted laser desorption ionisation time-of-flight

7H11 agar, while the most common liquid culture media were Middlebrook 7H9 broth, Sauton's medium or liquid BD BACTEC mycobacteria growth indicator tubes. The bone powder obtained from archaeological human skeletal remains proved to be also suitable for mycobacteria detection in a few cases. Furthermore, using MALDI IMS was possible to analyse animal lung-tissue sections directly *in situ*.

RAMAN SPECTROSCOPY

Raman spectroscopy is a vibrational technique widely used for the analysis of bacteria. Raman spectroscopy is typically considered as a label-free method, although it is possible to apply specific tags (Dina et al. 2017). The Raman effect is a result of the interaction between electromagnetic radiation and a molecule. When a molecule is subjected to radiation with a certain frequency, most of the photons are scattered from the molecule without a change in frequency. However, a small fraction of the photons (circa 1 in 10^7 photons) are scattered by losing or gaining a quantum of vibrational energy, termed Stokes and anti-Stokes Raman scattering, which occurs in 10^{-14} s or less. Owing to the weak nature of the anti-Stokes lines in the spectrum of the scattered light, only Stokes lines are considered in most applications (Egawa and Yeh 2005; Lu et al. 2008).

Generally, the Raman system consists of components, which can be categorised into excitation and detection branches. Excitation is achieved by delivering the light from a given laser source to the sample, usually by means of a fibre-optic probe or an articulated light delivery arm. The Raman scattered light is then collected, often through the same delivery system, and directed to a spectrograph and detector (Pence and Mahadevan-Jansen 2016).

Raman spectrophotometers can be sorted into two broad classes. The first is lab-based spectrophotometers, while the second class comprises in-field, *in situ*, or down-field use devices, including portable and hand-held spectrophotometers or remote or stand-off systems (Bumbrah and Sharma 2016). Two kinds of Raman instruments are mostly used: dispersive spectrophotometer, utilising prism or grating, and non-dispersive Fourier transform (FT) spectrophotometer, using a near-infrared (NIR) laser source (Bumbrah and Sharma 2016; Abbas et al. 2020).

Raman spectra provide selective and sensitive information on the vibrational bands of key functional groups within molecules. By following the variations in proportions of vibrational bands, it is possible to dis-

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tinguish bacterial strains since bacteria are composed of characteristic combinations of macromolecules. Raman spectra offer information on molecular interactions inside the cell; hence, they enable investigations of cellular metabolic changes, growth culture conditions, and cell interactions with drugs (Fernandez et al. 2017). The advantage of Raman spectroscopy over IR absorption spectroscopy is that the Raman signal is not interfered by water which, therefore, very little contributes to the absorption spectra (Tang et al. 2013; Van Belkum et al. 2013).

Buijtels et al. (2008) compared in their study Raman spectroscopy to 16S rRNA sequencing for the identification of *M. tuberculosis* complex (MTC) and NTM strains. The sensitivity of Raman spectroscopy was 95.2%, and the differentiation between MTC and NTM was invariably correct for all strains. Moreover, the spectra of the heat-inactivated and the viable mycobacteria showed minimal differences. In conclusion, Raman spectroscopy appeared to be rapid, accurate, easy-to-use, and therefore a suitable alternative for the routine identification of *Mycobacterium* species. The 16S rRNA sequencing was less appropriate due to its complexity and high costs (Buijtels et al. 2008).

Resonance Raman spectroscopy. The Raman intensity can be strongly enhanced (usually by a factor of 10^2 to 10^6) when the incident photon energy coincides with the electronic transition energy of the molecule, which is termed the resonance Raman effect. Metalloporphyrins in heme proteins have strong electronic transitions in the Soret (390–450 nm) and visible (500–600 nm) regions. After excitation with a laser that has an output coincident with these electronic transitions, the Raman spectrum of the heme moiety is resonantly enhanced. This allows the probe of the heme active centre without spectral interference from the surrounding protein matrix (Egawa and Yeh 2005; Lu et al. 2008).

Lu et al. (2008) and Egawa and Yeh (2005) reviewed properties of microbial haemoglobins from *M. tuberculosis*, *M. avium*, and *M. bovis* on the basis of resonance Raman spectroscopy (RRS) studies. They summarised that *M. tuberculosis* and *M. bovis* contains two types of truncated haemoglobins (trHbN, trHbO), while in *M. avium* are expressed all three types (trHbN, trHbO, trCtb). Besides, in aerobic cultures of *M. bovis* Bacillus Calmette-Guérin (BCG), a steady level of membrane-associated trHbO is detected throughout the growth phase, whereas cytoplasmic trHbN is only detectable during the stationary phase (Egawa and Yeh 2005; Lu et al. 2008).

Surface-enhanced Raman spectroscopy. Surface-enhanced Raman spectroscopy (SERS) represents a faster and ultrasensitive alternative to normal Raman spectroscopy capable of single-cell or even single-molecule detection (Dina et al. 2017). SERS deals with the increase of the weak Raman scattering intensity, thereby enabling the chemical characterisation and detection of various analytes at low concentrations directly from an aqueous sample (Alula et al. 2017). In the SERS technique, the target analyte approaches or adsorbs certain rough metal (gold, silver, etc.) nanoparticle surfaces, enhancing the signals by several orders of magnitude compared to normal Raman spectroscopy (Sivanesan et al. 2014). Three approaches are used in general. The first is to form colloidal silver directly on or inside the individual bacteria. In the second approach, the bacteria are placed directly on a SERS active surface. For the third approach, bacteria and colloid are mixed together, and the mixture is placed upon a flat surface (Mosier-Boss 2017). The SERS advantage is a non-destructive and molecular specific analysis, without the need of using labels or specific receptors (Dina et al. 2017). Moreover, the SERS spectra of intact bacteria reflect the cell wall structure, reporting on the physiological state of the bacteria cell (Alula et al. 2017).

Alula et al. (2017) demonstrated the SERS applicability to the detection of hydrophobic bacteria such as *M. smegmatis*, *M. bovis* BCG and *M. tuberculosis*. Their method was based on the formation of silver nanoparticles directly on the bacterial surface via the silver mirror reaction. A limit of detection below 100 bacilli was determined, requiring around 1 000 bacilli for routine detection and identification of *M. smegmatis* (Alula et al. 2017).

A study conducted by Muhlig et al. (2016) was aimed at the differentiation of six species of both MTC and NTM using the lab-on-a-chip SERS. This technique combined the high sensitivity of SERS with the high sample throughput of a microfluidic platform in a closed system. Without extraction or further treatment of the sample, the obtained SERS spectra were dominated by the vibrational signals of mycolic acids (Muhlig et al. 2016).

Rivera-Betancourt et al. (2013) employed silver nanorod-based SERS to detect the vibrational bands of mycolic acids extracted from the mycobacterial cell envelopes. It was demonstrated that multivariate statistical analysis of SERS spectra can be used for species and strain discrimination in mycobacteria. This method showed the ability to differentiate NTM from one another with 100% accuracy and was sensitive

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enough to distinguish clinically isolated *M. tuberculosis* strains that differed only by the presence or absence of a single extracytoplasmic sigma factor (Rivera-Betancourt et al. 2013).

Raman microspectroscopy. To combine spectral and spatial data, Raman analysis of biological samples can be assessed by using a confocal microscope (Dina et al. 2017). Merging Raman spectroscopy with microscopy [Raman microspectroscopy (RMS)], it is possible to measure single bacterial cells due to a laser focus of approximately 1 μm. This technique is non-destructive, cultivation-independent, and it does not require any special sample processing as only a few hundred single bacteria per sample are needed. The utilisation of RMS in clinical settings could, therefore, provide faster diagnosis since only isolation of the bacteria is necessary (Lorenz et al. 2017).

Tang et al. (2013) applied RMS to distinguish mycobacteria and Gram-negative bacteria by analysing characteristic spectral features. Distinct spectral patterns were observed as mycobacteria, and Gram-negative bacteria possess differences in biochemical components of the outer membrane and cell wall. Moreover, bacteria concentration at different locations of the sample could be determined according to peak intensities from RMS distribution maps (Tang et al. 2013).

Stockel et al. (2017) utilised RMS to identify MTC strains and to differentiate between pathogenic and commensal NTM. Cultivation independency was confirmed as only 50–100 cells were required for one sample to determine the result. The discrimination of MTC samples showed an accuracy of 94% (Stockel et al. 2017). Another study conducted by Stockel et al. (2015) employed RMS in monitoring the growth of pigmented *M. aurum* and non-pigmented *M. smegmatis*. It was proved that spectral profiles were affected by the cultivation time and a principal component analysis thus classified the spectra according to the cultivation age. Spectral profiles of *M. aurum* exhibited intense signals of carotenoid-like molecules at 72 h of growth, while the spectra of *M. smegmatis* were dominated by lipid signals after the late stationary growth phase was reached (after 48 h) (Stockel et al. 2015).

Major advantages of Raman spectroscopy are rapid and non-destructive sample analysis, high specificity, minimal sample preparation, small volume requirement, compatibility with physiological measurements due to weak water interferences and suitability for classification, quantification, and imaging of biological samples (Eberhardt et al. 2015). The disadvantage is low sensitivity due to weak Raman scattering. However, sensitivity can be enhanced using RRS and SERS. The combination of RRS and SERS techniques can amplify the sensitivity up to ten orders of magnitude as compared to Raman spectroscopy (Bumbrah and Sharma 2016).

There are also medical devices based on Raman spectroscopy which could be used at the point-of-care (POC) testing for *M. tuberculosis* in non-laboratory settings. The testing is rapid, is performed at the time and place of patient care and requires minimal manipulation of the sample (McNerney and Daley 2011).

INFRARED SPECTROSCOPY

Infrared (IR) spectroscopy is the absorption measurement of defined IR frequencies by a sample positioned in the path of an IR beam (NIR and middle-IR beams) (Abbas et al. 2020). The absorption of the IR radiation by a given bacterial sample causes excitation and vibration of the different chemical compounds of the cell. Different functional groups absorb radiation at different wavenumber ranges leading to the formation of characteristic spectral peaks (Novais et al. 2019).

IR instruments collect spectra by detecting changes in the absorption or transmittance intensity at different frequencies. NIR spectrometers generally feature a radiation source, wavelength selector, sampling accessories, and detector. Mid-IR spectrometers can be classified into two groups. Historically first were dispersive spectrometers consisting of a radiation source, a monochromator, and a detector. Those were, however, replaced by FT spectrometers, where an interferometer was introduced instead of a monochromator (Abbas et al. 2020). Typically, the Michelson interferometer is used to split one beam of light into two beams with different paths; then, it recombines them and conducts them into the detector (Bond et al. 2016).

IR spectra provide a specific fingerprint reflecting bacterial cell composition in nucleic acids, proteins, lipids, and carbohydrates. For acquisition of bacterial FT-IR spectra are commonly used wavenumbers between 4 000–400 cm^{-1} which corresponds to the mid-IR region of the electromagnetic spectrum (Novais et al. 2019). However, the FT-IR signals of bacteria are disturbed by the absorbance of water from aqueous systems. According to Shi et al. (2020), the polysaccharide and nucleic acid region (900–1 200 cm^{-1}) are the most reliable for bacterial typing as the absorbance of water has little impact on those regions (Shi et al. 2020). Another possibility is to remove water absorption bands completely by employing an attenuated

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total reflectance technique using a diamond or zinc selenide crystal (Parikh et al. 2014; Quintelas et al. 2018). A different alternative to overcome the problem of water is the use of NIR spectroscopy. Since the absorptivity of water in the NIR region (750–2 500 nm) is low, NIR spectroscopy has been used for the study of bacteria in water-based systems (Santos et al. 2015).

In the last century, Hamid et al. (1993) identified three characteristic glycolipids of *M. fortuitum* using IR spectroscopy. It was found that two of these lipids correspond to mycoside F, a family of glycolipids limited in distribution to *M. fortuitum* (Hamid et al. 1993). Smith et al. (1960) employed IR spectroscopy to demonstrate the presence of group-specific lipids or glycolipids in mycobacteria, including *M. tuberculosis*, *M. bovis* and *M. avium* (Smith et al. 1960).

Winder et al. (2006) described the use of FT-IR spectroscopy (FT-IRS) for the discrimination of *M. bovis* to the subspecies level. FT-IRS provided an insight into phenotype-genotype links in *M. bovis* clones as the metabolic fingerprints were generated and coupled with multivariate cluster analysis.

Nagy et al. (2008) analysed tuberculosis-infected bone samples from different burial environments by FT-IRS. In comparison with non-pathological bone samples, the tuberculotic remains showed more intensive IR bands at 2 800–3 000 cm^{-1}, which could be explained by the presence of mycolic acids, having very long alkyl chains (Nagy et al. 2008).

Ahmed et al. (2009) recorded IR spectra of *M. smegmatis* and five different bacteria using attenuated total reflection FT-IRS technique. Compared to other studied microorganisms, obtained mycobacterial spectra exhibited more pronounced peaks around 3 200–2 900 cm^{-1} and 1 450 cm^{-1}, presumably due to the CH stretching vibrations and CH bending modes of lipids (Ahmed et al. 2009).

A study conducted by Pesala et al. (2012) aimed at the detection of *M. tuberculosis* using FT-IR and NIR spectroscopy. Spectra of *M. tuberculosis* were compared with spectral profiles of *E. coli*, *M. smegmatis* and BCG bacteria and could be clearly distinguished as differences in cellular constituents gave rise to distinct IR and NIR fingerprints. However, fingerprints in the NIR region were not as strong as those obtained by the FT-IR technique (Pesala et al. 2012).

The FT-IR microspectroscopy combines an FT-IR spectrometer with a microscope. The advantage is that the spectra can be obtained from a single point or from a two-dimensional image of the sample (Carlos et al. 2011). This technique allows to detect and identify microbial cells within single microbial microcolonies; thus, reducing significantly the cultivation time needed for identification. The potential of FT-IR microspectroscopy to rapidly differentiate NTM at the species and strain level. It was possible to correctly discriminate between the 28 different mycobacterial strains and to group them into nine distinct clusters. Furthermore, species difficult to differentiate by other methods, for instance, *M. avium* from *M. intracellulare*, were successfully separated (Rebuffo-Scheer et al. 2007).

As a non-destructive, low cost and high-throughput technique, FT-IRS requires minimal sample preparation (Novais et al. 2019). It has been increasingly used for typing and classifying microorganisms below the species level due to the rapid and reliable acquisition of high-quality data (Wenning and Scherer 2013).

BIOSENSORS

Biosensors are analytical devices applied for the detection of biological and chemical samples (Pourakbari et al. 2019). A biosensor can be defined as a probe that integrates a biological analyte recognition system with an electronic transducer; thus, converting a biochemical signal into a measurable electrical or optical signal (Mobed et al. 2019). The structure of biosensors usually consists of a bioreceptor or biological recognition component, a signal transducer, and an amplifier (Mobed et al. 2019; Pourakbari et al. 2019). The interaction between the analyte and the bioreceptor is transformed into quantifiable signals. Various bioreceptors are typically utilised, such as enzymes, antigens, nucleic acids, antibodies, tissues, and whole cells (Mobed et al. 2019). The biosensors are divided into categories based upon the modality used for detection. The most commonly employed are optical, electrochemical, magnetic, and colorimetric modalities. Moreover, biosensors are often based on nano- or micro-technology platforms (Pashchenko et al. 2018).

Point-of-care devices. Biosensors are key devices used in many forms of point-of-care (POC) tests, which require minimal handling steps and can be operated by users with minimal laboratory or medical training at the point of need. POC tests can rapidly provide statistically significant results, do not demand specialised equipment and are cost-effective. Testing requires only small whole-blood or saliva samples and employs ready-to-use reagents. It can be applied for the early detection of life-threatening conditions and therefore remove the need for central laboratory testing (McPartlin and O'Kennedy 2014).

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The biosensor application expanded to be a powerful tool for the detection of difficult-to-culture bacteria such as *Legionella pneumophila*, *Brucella*, *Borrelia burgdorferi*, *M. tuberculosis*, or *Mycoplasma* (Mobed et al. 2019). Furthermore, numerous platforms have been constructed for the detection of viruses (Lee et al. 2019). It is possible to use electrochemical biosensors to detect *Flaviviruses* such as dengue and zika, influenza or human immunodeficiency virus (HIV) (Pashchenko et al. 2018). The rapid diagnosis of pathogens is crucial in the early stages of the treatment of diseases. The advantages compared to the conventional techniques are more rapid diagnosis, lower consumption of patient samples and valuable reagents, easy application, and high reproducibility (Nasseri et al. 2018).

Colorimetric biosensors. Another possibility for biosensing involves detection using colorimetric techniques. An exogenously added substrate is required that can be chosen on the basis of a user-desired colorimetric endpoint (Smartt and Ripp 2011). Besides lateral-flow assays, where labelled antibodies are bound to the membrane, and the antigen sample is allowed to diffuse along the membrane, aggregation and sedimentation-based diagnostic techniques are also being utilised. These methods often employ particles that change in colour when aggregated or the use of natural coagulants, which result in visual sedimentation (Pashchenko et al. 2018). The reading of the results can be usually achieved by either direct observation or by spectrophotometry (Soo et al. 2009). Several types of nanomaterials can be used as colorimetric labels. Gold nanoparticles are the most reported nanomaterial due to their stability, biocompatibility, easy manipulation, and intense red colour. Other alternatives are silver or carbon nanoparticles, which exhibit higher contrast against the background than gold nanoparticles (Quesada-Gonzalez and Merkoci 2015).

Electrochemical biosensors. Electrochemical biosensors are highly valued by biomedical and environmental research due to their simplicity and sensitivity. The main electrochemical methods involved in biosensor designing include voltammetry/amperometry, potentiometry, impedance, and conductometry (Mobed et al. 2019; Pourakbari et al. 2019). Highly conductive nanomaterials are widely used in biosensor constructions, whether organic carbon or inorganic metal nanoparticles. Both labelled and label-free methods are utilised (Pourakbari et al. 2019).

More digested references are presented in Table 4.

Magnetic biosensors. Magnetic nanoparticle-coupled detectors for biosensing can be used for signal amplification with the advantage of their application in solution phase sandwich assays such as diagnostic magnetic resonance. With diagnostic magnetic resonance, both the capture and detection agents are in solution and linked to magnetic particles. In the presence of the analyte, the magnetic particles cluster as the antibodies bind the analyte, resulting in a quantifiable signal (Sin et al. 2014).

More categorised references are presented in Table 5.

Optical biosensors. In optical biosensors are often utilised phenomena such as surface plasmon changes, scattering and interferometry for label-free methods. Examples of sensortag interactions include optical sensors used to detect fluorescent, colorimetric or luminescent tags. Based on the unique optical properties, gold and silver nanoparticles commonly serve as detection amplifiers (Sin et al. 2014).

Surface plasmon resonance (SPR)-based biosensing is one of the most advanced detection technologies. SPR occurs when polarised light interacts with the free electrons (surface plasmons) present at the interface of two media (metal-dielectric) with different refractive indices. The subsequent reflection is then detected (Abbas et al. 2020). Similar to SPR, surface-enhanced Raman scattering can be used to detect biomolecular interactions on the surface of metal nanoparticles via monitoring of the shift in the SERS spectra (Gopinath et al. 2014).

More selected references are presented in Table 6.

Immunosensors. Biosensors using antibodies as ligands are called immunosensors (Dupont 2011). Immunosensors employ antibodies as the recognition element and a transducer that converts the antibody-antigen interaction to a measurable physical signal. The types of immunosensors used for clinical and environmental applications are optical, evanescent wave, surface-plasmon resonance, fluorescence, and chemiluminescence (Van Emon 2011). Another way of sensing antibody-antigen interaction is micro-gravimetric quartz crystal microbalance analysis, which possesses the advantage of the ability to detect whole microorganisms without any extraction or purification (Mobed et al. 2019). The important characteristics of immunosensors are high specificity, sensitivity, and rapid acquisition of results enabling continuous and selective detection of an analyte, yielding a response in real time (Dupont 2011; Van Emon 2011).

The concrete examples of biosensors used for the detection of mycobacteria are summarised in the Tables 4–7. Colorimetric, electrochemical, magnetic, optical and immuno- biosensors are included.

More selected references are presented in Table 7.

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Table 4. Electrochemical biosensors for mycobacteria detection

Pathogen	Analytical technique	Target	Sample	Modifications	Reference
Mycobacterium sp.	EIS	genomic DNA	sputum	AuNP-modified electrode	(Thiruppathiraja et al. 2011)
M. avium subsp. *paratuberculosis*	voltammetry	genomic DNA	clinical and laboratory isolate	integrated silicon-based tri-modal nucleic acid transducer (TriSilix)	(Nunez-Bajo et al. 2020)
M. tuberculosis	EIS	genomic DNA	laboratory isolate	mercaptobenzoic acid and MNPs on bare gold electrode	(Costa et al. 2014)
		rpoB gene	oligonucleotides from IBA Gmbh	6-mercapto-1-hexanol passivated gold electrode	(Matsishin et al. 2016)
		rpoB gene	clinical isolates	composite carbon nanomaterials on gold electrode	(Zribi et al. 2016)
	impedimetric determination	16 kDa heat shock protein	tuberculosis heat shock protein from CalBioreagents	nanogapped dielectric surface coated with ZnO/gold thin film	(Gopinath et al. 2016)
	voltammetry	ESAT-6	laboratory isolate	anti-ESAT-6 antibodies covalently immobilised on the surface of a gold screen-printed electrode	(Diouani et al. 2017)
		IS6110 gene sequence	sputum	reduced graphene oxide-AuNP-modified electrode	(Liu et al. 2014)
		rpoB gene	oligonucleotides from Eurogentec	composite carbon nanomaterials on gold electrode	(Miodek et al. 2015)
		Ag85B	recombinant Ag85B protein from Abcam	crosslinking of Ag85B antibodies onto AuNP-modified carbon electrodes	(Murphy and Dempsey 2020)
		genomic DNA	laboratory isolate	colloidal AuNPs as labels; screen-printed carbon electrode	(Ng et al. 2015)
		DNA	oligonucleotides from First Base Asia	gold electrode modified by self-assembled monolayer of thiol	(Nurmalasari et al. 2015)
		IS6110 gene sequence	oligonucleotides from IDT	dextrin coated AuNP and amine-terminated MP functionalizing with DNA probes; screen printed carbon electrode chip	(Torres-Chavolla and Alocilja 2011)

EIS – electrochemical impedance spectroscopy; DNA – deoxyribonucleic acid; rpoB – gene encoding the beta subunit of ribonucleic acid (RNA) polymerase; ESAT-6 - early secreted antigenic target 6 kDa protein; Ag85B – antigen 85B; IDT – Integrated DNA Technologies, Inc; AuNP – golden nanoparticle; MNPs – magnetic nanoparticles; MP – magnetic particle; ; ZnO – zinc oxide

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Table 5. Colorimetric or optical biosensors for mycobacteria detection

Pathogen	Analytical technique	Target	Sample	Modifications	Reference
MTC	UV/Vis spectrophotometry	gyrB gene sequence	gene sequences from GenBank	Au-nanoprobes specific to gyrB gene sequences	(Silva et al. 2011)
	paper-based detection using smartphone	rpoB gene	gene sequences from GenBank	AuNP-probes; wells filled with $MgCl_2$	(Veigas et al. 2012)
MTC, MAC	SPR	genomic DNA	laboratory isolate	AuNPs-embedded surface	(Xiang et al. 2015)
MTC, non-MTC	UV-Vis spectroscopy	gyrB gene sequence	laboratory and clinical isolates	AuNP-probes	(Costa et al. 2010)
MTC, *M. tuberculosis*	direct observation and spectrophotometry	IS6110 and Rv3618 gene sequence	sputum; laboratory culture collection	AuNP-probes	(Soo et al. 2009)
M. avium subsp. *paratuberculosis*	scanning electron microscopy, UV-Vis spectroscopy	genomic DNA	oligonucleotides from the Biosafety and Environment Section	AuNP-probes	(Ganareal et al. 2018)
	visual reading and reflectometry	IS900 gene sequence	faecal samples	DNA-probe coupled to liposomes encapsulating sulforhodamine B	(Kumanan et al. 2009)
M. tuberculosis	direct observation and spectrophotometry	genomic DNA	laboratory isolate	catalytic oxidation of 3,3',5,5'-tetramethylbenzidine with horseradish peroxidase	(Ng et al. 2016)
	interferometry	CFP-10 antigen	urine samples	immobilised CFP-10 antibodies on a fibre tip surface	(Kim et al. 2015)
	SPR	IS6110 gene sequence	sputum, clinical isolate	anti-digoxigenin for detection of digoxigenin labelled PCR products	(Prabowo et al. 2018)
		genomic DNA	sputum, urine, clinical isolates	AuNP-modified probes	(Xiang et al. 2013)

MTC – *Mycobacterium tuberculosis* complex; MAC – *Mycobacterium avium* complex; UV/Vis – ultraviolet/visible; SPR – surface plasmon resonance; DNA – deoxyribonucleic acid; gyrB – gene encoding for the B subunit of the DNA gyrase; rpoB – gene encoding the beta subunit of ribonucleic acid (RNA) polymerase; Rv3618 – gene encoding a flavin-dependent monooxygenase which hydroxylates alkyl moieties of cyclic terpenes; AuNP – golden nanoparticle; CFP-10 – 10 kDa culture filtrate protein; PCR – polymerase chain reaction

Table 6. Magnetic biosensors for mycobacteria detection

Pathogen	Analytical technique	Target	Sample	Modifications	Reference
BCG	nuclear magnetic resonance	bacterial surface	laboratory isolate	MNPs	(Lee et al. 2009)
M. tuberculosis	nuclear magnetic resonance	genomic DNA	sputum	MNP – labelled beads	(Liong et al. 2013)
	optomagnetic detection	katG peroxidase gene	synthetic gene sequence	streptavidin coated magnetic microbeads and MNPs	(Minero et al. 2020)

BCG – Bacillus Calmette-Guérin; DNA – deoxyribonucleic acid; MNPs – magnetic nanoparticles

Table 7. Immunosensors for mycobacteria detection

Pathogen	Analytical technique	Target	Sample	Modifications	Reference
MTC, NTM, *M. smegmatis*	fluorescence microscopy	surface antigens	sputum	microtip with labelling wells containing fluorescent antibodies	(Inoue et al. 2014)
M. tuberculosis	amperometry, fluorescence microscopy	whole cell antigens	sputum	fluorescent labelled polyclonal IgY antibodies immobilised on the microtip surface	(Hiraiwa et al. 2015)
	evanescent wave fluorimetry	Ag85B	sputum	prism coated with antituberculosis antibodies primed with fluorescent labelled analogue molecules	(McNerney et al. 2010)
	interferometry	CFP-10 antigen	urine samples	immobilised CFP-10 antibodies on a fibre tip surface	(Kim et al. 2015)
	piezoelectric immunosensor	38 kDA antigen	laboratory isolate	chromatographic column with agarose beads coupled with Myc31 antibodies	(Marin et al. 2015)
	quartz crystal microbalance	surface antigen (lipoarabino-mannan) and whole cells	laboratory isolate	α-LAM and antiH37Rv antibodies immobilised on the crystal surface	(Hiatt and Cliffel 2012)
	voltammetry	ESAT-6	laboratory isolate	anti-ESAT-6 antibodies covalently immobilised on the surface of a gold screen-printed electrode	(Diouani et al. 2017)
		Ag85B	recombinant Ag85B protein from Abcam	crosslinking of Ag85B antibodies onto AuNP-modified carbon electrodes	(Murphy and Dempsey 2020)

MTC – *Mycobacterium tuberculosis* complex; NTM – non-tuberculous mycobacteria; Ag85B – antigen 85B; CFP-10 – 10 kDa culture filtrate protein; 38 kDA – 38 kDa protein antigen from *M. tuberculosis*; ESAT-6 – early secreted antigenic target 6 kDa protein; AuNP – golden nanoparticle; α-LAM – immobilised lipoarabinomannan; H37Rv – reference strain of *M. tuberculosis*; IgY – immunoglobulin Y

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CONCLUSION

Non-tuberculous mycobacteria pose a global public health risk, and their control can contribute to a decrease in the incidence of some diseases of civilisation. Protecting people from exposure to mycobacteria requires the examination of a large number of samples and the participation of professionals who are able to use sophisticated instrumental analytical techniques. The study of mycobacteria in potable water and beverages, in water plumbing and reservoirs, can use efficient analytical techniques for rapid determination of the presence of mycobacteria and for their characterisation as an alternative to classical time-consuming mycobacteria cultivation and a method of determination of specific nucleic acid sequences by PCR. Currently used methods can be complemented by FCM, MALDI-TOF mass spectrometry, Raman and IR spectrometry, and biosensors. Large series of samples can be analysed quickly and at a low cost.

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