Challenges in Environmental Monitoring of Pathogens: Case Study in *Mycobacterium avium*

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Monitoring pathogens whose natural habitat is the environment, requires careful consideration of a variety of questions regarding the target organisms (species, sub-species or ecotypes), the detection level required and the methods to be employed (culture vs. molecular techniques). These questions, and others, are particularly crucial for monitoring *Mycobacterium avium*, an environmental opportunistic pathogen of humans, animals, and poultry. Herein is a discussion of challenges for *M. avium* detection and enumeration and guidance for the adaptation of clinical methods to environmental samples.

Keywords *Mycobacterium avium*; detection; enumeration; culture; PCR; environment; water; soil

Introduction

If the environment is a proven source for an infectious disease, it is necessary to develop monitoring protocols for these pathogens to perform risk analysis and estimate the impact of the environmental pathogens on humans and other susceptible hosts. For instance *Mycobacterium avium* is a species belonging to the non-tuberculous mycobacteria (NTM), and an environmental opportunistic pathogen that can be found in various terrestrial and aquatic habitats [1], including drinking water distribution systems and household plumbing [2]. *M. avium* detection techniques were mostly developed for clinical samples and often did not require abundance measurements [3-8]. Development of techniques for *M. avium* monitoring has required substantial modification of existing clinical microbiological techniques, because the patient microbial flora represents a reduced and selected population pool compared to natural and human-engineered environments [9]. Natural habitats for *M. avium* are highly heterogeneous and carry a high diversity of microbial species [10, 11] which may interfere with the detection of NTM. As a consequence, detecting and measuring *M. avium* in environmental samples requires modification of classical clinical laboratory protocols in order to study its distribution and ecology and to carry out risk analysis [12]. Such studies require quantitative results of temporal and spatial distribution in the different compartments of ecosystems [13]. For instance, the extremely high surface hydrophobicity of *M. avium* leads to its preferential attachment to surfaces. As a consequence, *M. avium* in drinking water distribution systems and households is growing on pipe surfaces and only present in low numbers in the bulk water [14]. Moreover, environmental populations of *M. avium* are highly diverse and may constitute genetic reservoirs for virulent genotypes which have been yet identified [15-17]. As a result, targeting only the population density (e.g. *M. avium* density) may not be a sufficient indicator for risk analysis.

1. Objectives

The monitoring of environmental pathogens requires three invariable elements: (1) a specific and sensitive detection tool in order to avoid false-positive and false-negative results, (2) a quantitative tool in order to determine the main natural sources, and (3) a typing tool in order to track them from environment to susceptible hosts. Traditionally *M. avium* has been detected in clinical and environmental samples using culture-based techniques; however these techniques may not be well suited for environmental samples. As *M. avium* is relatively slow growing compared to other microorganisms, decontamination of samples from patient or environmental samples have been successfully developed for *M. avium* cultivation and enumeration [7, 8, 18, 19]. However, some of these decontamination procedures are insufficient for environmental samples as non-target species may be resistant to decontamination and outcompete *M. avium* on non selective media [12]. Also, decontamination results in loss of mycobacteria [6-8]. Methods based on the polymerase chain reaction (PCR) were also developed for clinical samples. Although these molecular techniques provide rapid results and permit detection of unculturable cells [20], they are currently limited in that epidemiological tracing cannot be performed. Molecular techniques (e.g., PCR) that were developed for clinical samples may also not be adapted to environmental samples. Several biases may arise from the presence of interfering agents such as organic matter and microorganisms leading to false-negative or -positive results. For example, polysaccharides and humic and fulvic acids interfere with PCR, thus requiring their removal from samples before DNA amplification [21-23]. As the spectrum of microorganisms may be wider and more diverse in environmental than in patient samples, PCR primers for species-, subspecies-, or type-specific amplification reactions should be highly

specific. Our goal is to propose a guideline (in the form of a decision tree) to help selecting and developing appropriate tools (culture and non-culture methods) for *M. avium* monitoring in surface water, treatment plant, water distribution system, and in the household.

2. General considerations

Depending on the physiological, epidemiological and ecological characteristics of the waterborne microorganisms for which environmental monitoring studies must be conducted, several analytical points must be taken into account before development of new analytical methods or before application of previously described methods. Table 1 lists these considerations, which may be unique to the studied waterborne microorganism, and which integrate analytical points related to the study as the expected outcome, the favored method, the identification level or the investment and analytical points related to the nature of the studied matrix, the presence of potential inhibitors or the size of the samples.

Table 1: List of general considerations for environmental waterborne microorganism monitoring

Analytical points	Questions	Answers
Relative to the study	Outcome	Detection or enumeration
	Method	Cultivation or non-cultivation
	Identification level	Genus, species, subspecies or type
	Investment	Time, cost
Relative to the sample	Studied matrix	Suspension, biofilm, soil
	Sample size	Volume, concentration
	Interference	Competitors, inhibitors, pH, salinity

2.1 Analytical points related to the study

Concerning *M. avium* monitoring, studies have demonstrated that it is present in a variety of aquatic environments such as surface water [9, 24-26], water distribution systems [27], hospital plumbing [28], spas and hot tubs [29], nail salons [30], and household plumbing [31]. Consequently, measurement of *M. avium* densities is necessary to rank these potential sources on the basis of risk. Specifically for *M. avium* and other slowly growing pathogenic mycobacteria, non-cultural methods represent an alternative to avoid long cultivation times, especially molecular methods which could type at the subspecies level members of *M. avium* in complex matrix. As the *M. avium* complex consists of four subspecies, *avium*, *hominissuis*, *silvaticum*, and *paratuberculosis*, each with a unique ecology and epidemiology, and genetic variability [15-17], identification of major sources of each requires a quantitative tool which could be able to distinguish subspecies and type each isolate. Knowledge of the ecological niche of *M. avium* and its subspecies may be useful to determine optimal growth conditions and thus improve culture methods. The presence of *M. avium* in surface water is correlated with low pH level, presence of humic and fulvic acids, microaerobic conditions [32]. For example, coastal southeastern United States swamps and peatlands and boreal forest soils of northern Europe and North America yield high numbers of *M. avium* [2, 33]. Quite possibly, inclusion of humic and fulvic acids addition to culture media could stimulate and increase *M. avium* growth [34] and shorten the time of detection by culture methods. Knowledge of *M. avium* ecology could also assist in direction of the sampling strategy. For instance monitoring *M. avium* in watersheds should give priority to waters and soils of low pH and high humic and fulvic acid content. Moreover, the hydrophobicity NTM cell wall, favor their attachment to interfaces (water/solid surface or water/air) and hydrophobic compounds uptake (such as oil, polycyclic aromatic hydrocarbons). As a consequence it seems preferable to track *M. avium* in biofilms of water distribution systems and neuston of surface water, than in bulk water. Indeed, *M. avium* is able to form biofilms in water distribution system, particularly *M. avium* subsp. *hominissuis* [35] in the presence of Ca^{2+}, Mg^{2+} or Zn^{2+} ions and glucose or peptone as a carbon source [36]. *M. avium* biofilms in drinking water distribution systems also protect cells from chlorine exposure [37, 38].

2.2 Analytical points related to the sample

Primary consideration must be placed on estimates of required water sample volumes and whether a concentration step is required for detection and enumeration. For *M. avium* monitoring by either culture or molecular techniques, water sample volumes can range between 50 ml and 1000 ml, and water can be concentrated by centrifugation or filtration [29, 32, 39-46]. The method and sampling volume will differ according to the anticipated number of *M. avium* cells, to the turbidity of the sample that may limit the filtration step, and also to the quantity of non-targeted microorganisms or PCR inhibitors which may interfere with NTM detection using cultural methods or non-cultural methods, respectively. For example, *M. avium* cultivation from spas (hot tubs) [29] or footbaths [30] involved collection using swab samples in biofilms [30] or centrifugation of high water volumes (400 ml) [29]. PCR-based detection of *M. avium*

subspecies *paratuberculosis* (MAP) was conducted using centrifugation or tangential-flow-filtration of high sample volume from untreated water (1 liter) and from sewage treatment effluent (10 liter), or very high volume (100 liter) from surface water [24-26], because of the variability of numbers of MAP in each type of sample. As emphasized above, the sampling volume of molecular methods is also conditioned by the DNA extraction method to remove PCR inhibitors [47, 48]. The presence of suspended matter may also determine the choice of the concentration method, as a filter may rapidly be obstructed by particles [12]. In addition, it is known that *Mycobacterium* species are present outside but also inside the soil aggregates [49]. Consequently, isolation of mycobacteria from soil requires the use of pre-treatment of the sample, which could be homogenized using for example a blender or sonicator [49] before DNA extraction or decontamination procedures. Most of the time, as *M. avium* isolation, NTM isolation from water is performed using concentration procedure prior the decontamination procedure [12, 29, 32, 44-46], and seldom posterior [43]. Consequently, a future harmonization of sampling volume and concentration method is necessary in order to be able to compare results of different studies. The choice and improvement of detection methods should also take in consideration the presence of potential competitors and inhibitors in the sample.

3. Testing methods

3.1 Recovery rate of the targeted microorganism or its DNA

Both culture and molecular methods are dependent upon high percent recovery of the target. In fact, sampling volume is conditioned by the recovery rate of the targeted organisms, which is closely related to efficiency of the applied method. Because it is not yet possible to know with certainty the number of any pathogen in an environmental samples [12], recovery rate of both culture and molecular methods cannot be evaluated directly. However, parallel studies of *M. avium* isolation methods using different methods does allow identification of superior methods based on either number of *M. avium* or spectrum of types. Further, isolation methods from water or soil samples can be compared by measurement of the number of non-target contaminants [39, 40]. For *M. avium* and other mycobacteria, reducing the number of competitors, able to outgrow and cover mycobacterial colonies, is important. One way around this dilemma is to inoculate environmental samples in order to compare the efficiencies of isolation methods. One can measure the survival frequency of target and contaminant cells exposed to chemical disinfectants [41], measure the isolation frequency of target and non-target microorganisms [50], or independently measure recovery of target and interfering microorganisms [12] after artificial inoculation of the sample.

3.2 Models based on spiking and growing

When assessing the recovery of a particular pathogen using either a culture- or molecular-based methods for detection, isolation or enumeration by spiking a sample, an important consideration is the physiological state of the added microbial cells. Concerning *M. avium* detection, isolation, or enumeration, the spiking procedure is usually performed with a reference strain which is cultivated in nutrient-rich laboratory medium before being added into the sample [12, 41, 50]. Firstly, even if several reference strains are used, it can never be representative of all *M. avium* subspecies or types or mycobacteria [1]. Secondly, the physiologic state of target or non-target microorganisms from nutrient-rich laboratory-media into environmental samples is considerably different from the state of those targets in environment. The targeted NTM from environment are likely more resistant toward isolation procedures (that include disinfectants or antibiotics), than the spiked reference strain cells, and only a substantial part of the microbial community of environment is constituted of viable but nonculturable (VBNC) microorganism [51]. Thirdly, without knowing the recovery rate of the isolation methods, targeted microorganisms are often spiked into the sample using high level of cells in order to be able to compare methods to each other, which does not reflect the low numbers of environmental harmful microorganisms.

4. Adaptation of clinical laboratory techniques to environmental monitoring

The different problems which must be resolved in order to adapt cultural or molecular clinical laboratory techniques, to environmental monitoring of pathogenic microorganisms are listed in Table 2.

Table 2: List of the problems for adapting clinical laboratory techniques according to the method type

Method	Adaptation problems
Cultivation	- Selectivity of the media used to isolated microorganisms from clinical samples is not adapted to the environmental samples, which contain a wider diversity of microorganisms. - Efficiencies of decontamination procedures used to isolate microorganisms in clinical samples are low for the treatment of environmental samples, which contain higher densities and diversity of resistant non-target microorganisms.
Non-cultivation	- Specificity and sensitivity towards targets of clinical methods are not adapted to the environmental samples, because they are checked, most of the time, using microorganisms only isolated from human cases. - DNA extraction protocols, which were developed to treat medical samples, do not take into account of the environmental PCR inhibitors.

4.1 Case Study: Adaptation of cultural techniques for *M. avium*

The mycobacterial growth media [39-41], as Löwenstein-Jensen (LJ), Ogawa egg yolk medium (OEY), Ogawa medium ofloxacin ethambutol (OEOE) and Middlebrook medium (7H10 or 7H11), were developed for the cultivation of *Mycobacterium tuberculosis* from clinical samples. The media composition is directed by the nutritional requirements of *M. tuberculosis*, not other mycobacteria. As a consequence, those standard mycobacterial media do not provide optimal growth of mycobacteria that are not members of the *M. tuberculosis* group. Further, some of the constituents are there to neutralize the decontaminants used to process sputum; for example oxalic acid and cetylpyridinium chloride to kill other bacteria and mucolytic agents to break up sputum. However the decontaminating agents used to process sputum are inadequate for decontamination of environmental samples due, in part, to the great diversity of microorganisms in environmental samples [10, 11]. Removal of interfering microorganisms is a critical point of *M. avium* isolation from environmental samples because the target concentration level is very low. Consequently, selective *M. avium* isolation methods (i.e. ability to isolate the targeted microorganism avoiding interfering microorganism growth) must be improved, for example by antibiotics supplementation in the media [12, 52, 53]. But it is important to be careful with the choice of antibiotics, because certain can be harmful for the target [12]. Moreover, selectivity of the media could be improved optimizing pH, nutrient concentration, CO_2 and oxygen level that will be more adapted to *M. avium* growth. Using media which contain low concentrations of nutrients (including the carbon source) and using extracellular factors such as resuscitation promoting factor (Rpf), may be a good strategy for resuscitation of nonculturable dormant cells and thus improve the cultivation of mycobacteria from environmental samples [54].

4.2 Case Study: Adaptation of non-cultural techniques for *M. avium*

The specificity (i.e. the proportion of true negatives that are correctly identified by the test) is measured using strain collections of non-target microorganisms (negative control) [55-58]. The sensitivity (i.e. the proportion of true positives that are correctly identified by the test) of molecular methods is tested using populations of target microorganisms (positive control) [55-58]. Consequently, specificity and sensitivity (as defined above) of a molecular method originally developed for clinical samples must be checked before application to environmental samples. More precisely, as the spectrum of environmental microorganisms may be different than that found in clinical samples, measurements of specificity and sensitivity should include environmental strains and not only clinical strains. Moreover, the specificity and sensitivity of molecular methods should not be evaluated only using *in silico* analysis, but should always be confirmed *in vitro* using target and non-target strains [5, 33, 59-61].

Sensitivity, as defined above for diagnostic tests, should not be confused with the limit of detection (LOD) of a particular molecular method. Contrary to environmental samples whose volume can be modulated, the LOD of non-cultural methods applied to clinical samples must be taken into account. But, as clinical samples, the yield of DNA extraction from environmental samples must be high enough in order to avoid false-negative results. Moreover, as emphasized previously, DNA extraction method must be able to remove PCR inhibitors from environmental samples. Although different DNA extraction procedures have been developed for detection or enumeration of *M. avium* [47, 62] or other microorganisms from environmental samples [63, 64], their effectiveness in removal of PCR inhibitors have not been assessed against a wide variety of sample types [47, 62-64]. Concerning the LOD, colony counts are used as the reference or "gold standard" for determining the limit of detection of non-cultural methods for detection or enumeration. That reference is based on the assumption that every viable cell in a population can form a colony. However, that is not necessarily the case; witness the number of reports of VBNC microorganisms [20, 51, 65]. Further, as culture conditions for a substantial proportion of microorganisms in a population have not been identified, it would follow that molecular methods would be superior as potentially all microorganisms could be detected, identified, and enumerated.

5. Culture or non-culture methods?

Tables 3 and 4 sum the advantages and disadvantages of cultural and non-cultural methods. These tables could serve as a guide for selecting a method for monitoring an environmental pathogen.

5.1 Culture methods

As shows in Table 1, the main advantage of cultural methods resides in their low cost and the ability to extend the investigation to including identification, physiology profiling, or genetic analyses. For *M. avium* , identification can be performed by cultural and biochemical analysis [66], analysis of mycobacterial (mycolic acids by high performance liquid chromatography (HPLC) [67-78], or multilocus sequencing analysis (MLSA) of genes such as *rrs*, *gyr*A, *gyr*B, *hsp*65, *rec*A, *rpo*B, *sod*A or the 16S-23S internal transcribed spacer [79, 80]. Isolation of *M. avium* colonies allows analysis of such physiologic characteristics as growth requirements (e.g., auxotrophy, iron) and growth conditions (e.g., response of growth to different oxygen concentrations) [81]. Genetic analysis has led to knowledge of NTM complete genomes (e.g. *M. abscessus*, *M. avium*, *M. gilvum*, *M. marinum*, *M. smegmatis*, *M. ulcerans*, *M. vanbaalenii*) [82-84]. In order to carry out molecular epidemiology studies and library-dependant source tracking, several clinical and environmental NTM isolates can be now fingerprinted. Indeed, the typing methods of NTM strains were recently reviewed [85]. Today, *M. avium* and other slow growing NTM such as *M. kansasii*, *M. xenopi*, *M. malmoense*, *M. haemophilum* and *M. ulcerans*, and rapid growing mycobacteria as *M. fortuitum*, *M. abscessus* and *M. chelonae* can be characterized using serotyping, biotyping, multi-locus enzyme electrophoresis (MLEE), plasmid profiling, repetitive sequence typing (*rep*-PCR), arbitrary primed PCR (AP-PCR), pulsed field gel electrophoresis (PFGE), and insertion sequence restriction fragment length polymorphism (IS-RFLP) [85].

Nevertheless, as shown in Table 3, the choice of cultural methods poses several problems. Although the media and the culture conditions of *M. avium* appear to generally involve either primary plating on either Löwenstein-Jensen or Middlebrook agar media, not all *M. avium* colonies can be detected. Principally, the virulent transparent colony types are almost invisible on Löwenstein-Jensen medium. Although a temperature of 30°C can be used for growth, that is below the optimal temperature of growth for most mycobacterial species, including *M. avium*. However, higher temperatures exclude recovery of a number of pathogens; for example, *Mycobacterium haemophilum*, *and Mycobacterium marinum*.

Table 3: Advantages and disadvantages of cultivation methods

Advantages	Disadvantages
Identification	Choice of media
Physiological analysis	Choice of culture conditions
Genetic analysis	Growth of interfering microorganisms
Fingerprinting	Fast growing organisms are favored
Inexpensive, no special equipment required	Time consuming
	Bias of viable but non cultivable targets

5.2 Non-cultural methods

As in other fields of the microbiology, advances in molecular biology for 20 years have increased considerably the knowledge of mycobacteriology [86]. As shown in Table 4, non-cultural methods are of great interest because these high throughput methods allow to detection of unculturable microorganisms and are less time-consuming, particularly for NTM which grow very slowly. Moreover, contrary to the cultural methods, the non-cultural methods are able to detect specifically a target without other complementary investigations as identification, which saves also a considerable amount of time. The majority of mycobacterial detection methods are based on PCR [57, 59], PCR and probe hydrolysis [5], sandwich hybridization [61], real-time PCR [58, 60], PCR and dot blot [55, 56] and target the 16S rRNA gene [5, 55, 56, 59-61] or genes such as *rpo*B [57] or *hsp*65 [58]. Recently, some studies describe methods to detect and fingerprint mycobacterial assemblages using denaturing gradient gel electrophoresis (DGGE) and targeting the 16S rRNA gene [33, 87-91]. Nevertheless, no study has managed to compare specificity, sensitivity, and limit of detection of these methods which target different genes and also different loci in these genes.

A clear advantage of non-culture methods is their ability to detect VBNC. Unfortunately, non-cultural methods can amplify extracellular DNA and do not differentiate viable microorganisms from non-viable microorganisms. Indeed, DNA persists a relatively long time after cell death, and consequently molecular methods have been developed to detect viable cells by amplifying mRNA using reverse transcriptase PCR (RT-PCR or RT-qPCR). However, RT-PCR methods are difficult to implement due to the instability of RNA [92].

Recently, ethidium monoazide bromide (EMA) was proposed in order to differentiate viable of non-viable cells in environmental samples from wastewater treatment samples. EMA is a DNA/RNA intercalating dye that enters in cells with damaged membranes and covalently binds to DNA upon photoactivation which avoid other molecular applications as PCR [93]. This approach is of growing interest, but optimum conditions must still be determined for each sample

due to sample matrix effects [20]. Moreover, and this analytical bias is seldom taken into account, PCR reactions can also be inhibited by high DNA concentrations. Indeed, as in clinical samples, reactions for molecular detection or enumeration developed for environmental samples, require dilution of the samples if the total extracted DNA inhibits the PCR reaction. Such cases decrease the detectable quantities of the targets, and can lead to fault negative results.

If a NTM quantitative method is based on 16S rRNA analysis, a bias can occur due to the copy number of 16S rRNA operons [5, 55, 56, 59-61]. With the exception of the rapidly growing species *M. chelonae*, *M. abscessus* and *M. alvei* which contain one copy of 16S rRNA [82, 94], and the slowly growing species *M. terrae* and *M. celatum* which contain two copies of 16S rRNA [95, 96], the other rapid and slow growing mycobacteria (including *M. avium*), already studied, contain generally two and one copy of 16S rRNA, respectively. Consequently, the use of a mycobacterial strain DNA containing one 16S rRNA copy as standard, could overvalue a sample constituted of fast growing NTM containing two 16S rRNA copies, and in reverse, the use of strain DNA containing two 16S rRNA copies, could undervalue a sample mainly constituted of slow growing cells containing one 16S rRNA copy. The same problem occurs when the insertion sequences are used for measurement. For example, insertion sequence IS*900*, which is considered specific for MAP [24, 25] has 17 repeats in the reference genome of MAP strain K10 [83]. However, the copy number ranges between 14-20 times in other MAP strains [97, 98]. Consequently, real-time qPCR methods targeting the insertion sequence IS*900* can be used to detect MAP [62], but not to enumerate these subspecies [97].

Table 4: Advantages and disadvantages of non-cultivation methods

Advantages	Disadvantages
Rapid	Inhibition of amplification by PCR inhibitors
High throughput methods (many samples can be analyzed simultaneously)	Inhibition of amplification by high DNA concentration
Specifically identification of the targets without any other investigations	Bias of quantitative methods based on genes with variable number of copies
No bias of viable but non cultivable targets	Bias of non-viable targets and extracellular DNA
	Bias due to differential efficiency among species of DNA extraction and PCR amplification
	Expensive chemicals and equipment

6. Mycobacteria monitoring guideline

The possibilities for *M. avium* monitoring in environmental water are summed up in Figure 1. We do not propose an ideal method, but a tool box containing several methods which can be used in combination depending upon the objective of the exercise and the anticipated problems. Indeed, “what is our purpose?” is the first question which must be asked. This question will condition the choice of the analytical tools that can be used independently or in combination. Currently, as *M. avium* typing and source tracking still requires the isolation of strains, non-cultural typing methods must also be developed. Developments of cultural and non-cultural methods must be checked *in vitro* and not only *in silico*, and must take into account of sensitivity (i.e. ability to not detect the non-targeted microorganisms) and specificity (i.e. ability to detect all type of targeted microorganisms), using strain collections isolated from the studied matrix, and avoiding targets whose copy number is variable. Concerning cultural methods applied to environmental samples, quantitative tools could be developed using new inhibitors of interfering microorganisms and stimulating NTM growth based on better knowledge of their physiology and ecology. Typing methods of the isolated NTM could be developed for NTM species which cannot be currently characterized [85]. This decision tree of the adequate analytical strategy can be applied to environmental monitoring of other waterborne pathogens, but also to monitor NTM that can be useful for bioremediation and biotechnology purpose. For instance NTM species (not pathogenic and also pathogenic ones) are known to degrade recalcitrant organic pollutants such as polycyclic aromatic hydrocarbons (PAH) and other xenobiotic molecules [99-107]. The methods developed for pathogenic NTM monitoring in environment could be used for PAH degrader mining and monitoring in polluted environment or in bioreactors.

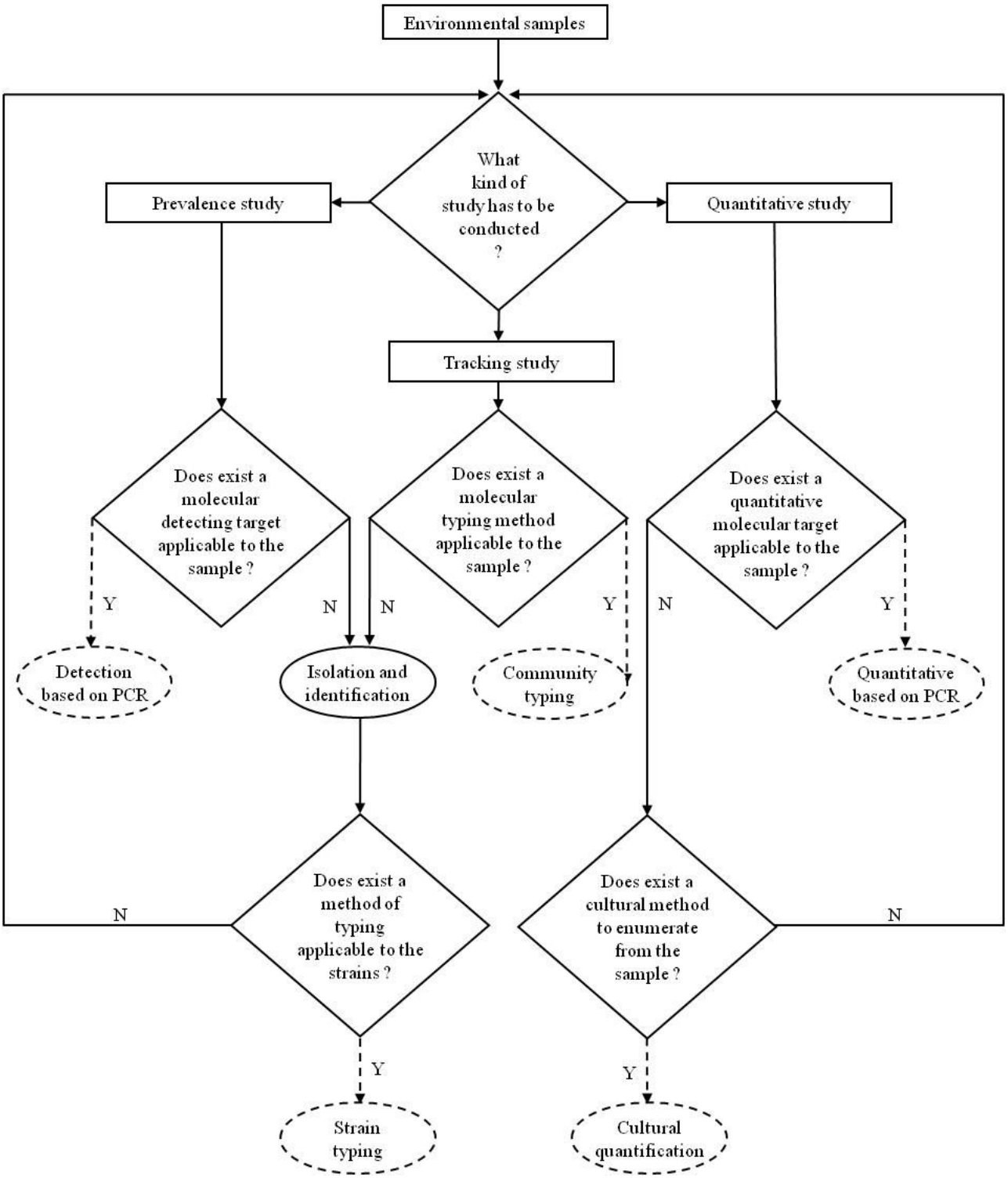

Fig. 1 Decision tree showing current possibilities (full lines) and future challenges (dotted lines) for environmental waterborne mycobacteria monitoring. Rectangles, circles and rhombuses correspond to the goals, to the questions which must be answered, and to the actions which must be conducted, respectively. PCR corresponds to polymerase chain reaction.

Acknowledgements The support by Paris-Est University and Virginia Polytechnic Institute and States University are gratefully acknowledged.

References

[1] Kazda J. The chronology of mycobacteria and the development of mycobacteiral ecology. In: Kazda J*, et al.*, eds. *The ecology of Mycobacteria: Impact on animal's and human's health.* Dordrecht Heidelberg London New York, Springer; 2009:1-11.

[2] Falkinham JO. Surrounded by mycobacteria: nontuberculous mycobacteria in the human environment. *Journal of Applied Microbiology*. 2009;107:356-367.
[3] Brunello F, Favari F, and Fontana R. Comparison of the MB/BacT and BACTEC 460 TB Systems for Recovery of Mycobacteria from Various Clinical Specimens. *Journal of Clinical Microbiology*. 1999;37:1206-1209.
[4] Buijtels PCAM and Petit PLC. Comparison of NaOH-N-acetyl cysteine and sulfuric acid decontamination methods for recovery of mycobacteria from clinical specimens. *Journal of Microbiological Methods*. 2005;62:83-88.
[5] Garcia-Quintanilla A, Gonzalez-Martin J, Tudo G, Espasa M, and Jiménez de Anta MT. Simultaneous identification of *Mycobacterium* genus and *Mycobacterium tuberculosis* complex in clinical samples by 5'-exonuclease fluorogenic PCR. *Journal of Clinical Microbiology*. 2002;40:4646-4651.
[6] Kubica GP, Kaufmann AJ, and Dye WE. Comments on the use of the new mucolytic agent, N-acetyl-L-cysteine, as a sputum digestant for the isolation of mycobacteria. *American Review of Respiratory Diseases* 1964;87:775-779.
[7] Petroff SA. A new and rapid method for the isolation and cultivation of the tubercle bacillus directly from sputum and feces. *Journal of Experimental Medecine*. 1915;XXI:38.
[8] Tacquet A and Tison F. Nouvelle technique d'isolement des mycobactéries par le lauryl sulfate de sodium. *Annales de l Institut Pasteur*. 1961;100:676-680.
[9] Bland CS, Ireland JM, Lozano E, Alvarez ME, and Primm TP. Mycobacterial ecology of the Rio Grande. *Applied and Environmental Microbiology*. 2005;71:5719-5727.
[10] Kenzaka T, Yamaguchi N, Tani K, and Nasu M. rRNA-targeted fluorescent in situ hybridization analysis of bacterial community structure in river water. *Microbiology*. 1998;144:2085-2093.
[11] Winter C, Hein T, Kavka G, Mach RL, and Farnleitner AH. Longitudinal Changes in the Bacterial Community Composition of the Danube River: a Whole-River Approach. *Applied and Environmental Microbiology*. 2007;79:421-431.
[12] Radomski N, Cambau E, Moulin L, Haenn S, Moilleron R, and Lucas FS. Comparison of culture methods for isolation of nontuberculous mycobacteria from surface waters. *Applied and Environmental Microbiology*. 2010;76:3514–3520.
[13] Garcia-Armisen T and Servais P. Respective contributions of point and non-point sources of *E. coli* and enterococci in a large urbanized watershed (the Seine river, France). *Journal of Environmental Management*. 2007;82:512-518.
[14] Falkinham JO, Norton CD, and LeChevallier MW. Factors influencing numbers of *Mycobacterium avium*, *Mycobacterium intracellulare*, and other Mycobacteria in drinking water distribution systems. *Applied and Environmental Microbiology*. 2001;67:1225-1231.
[15] Mijs W, de Haas P, Rossau R, van der Laan T, Rigouts L, Portaels F, and van Soolingen D. Molecular evidence to support a proposal to reserve the designation *Mycobacterium avium* subsp. *avium* for bird-type isolates and *M. avium* subsp. *hominissuis* for the human/porcine type of *M. avium*. *International Journal of Systematic and Evolutionary Microbiology*. 2002;52:1505-1518.
[16] Radomski N, Thibault VC, Karoui C, de Cruz K, Cochard T, Gutiérrez C, Supply P, Biet F, and Boschiroli ML. Genotypic diversity of *Mycobacterium avium* subspecies from human and animal origins, studied by MIRU-VNTR and IS*1311* RFLP typing methods. *Journal of Clinical Microbiology*. 2010;48:1026–1034.
[17] Thorel MF, Krichevsky M, and Lévy-Frébault VV. Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium*, and description of *Mycobacterium avium* subsp. *avium* subsp. nov., *Mycobacterium avium* subsp. *paratuberculosis* subsp. nov., *Mycobacterium avium* subsp. *silvaticum* subsp. nov. *International Journal of Systematic Bacteriology*. 1990;40:254-260.
[18] Kubica GP, Baess I, Gordon RE, Jenkins PA, Kwapinski JB, McDurmont C, Pattyn SR, Saito H, Silcox V, Stanford JL, Takeya K, and Tsukamura M. A co-operative numerical analysis of rapidly growing mycobacteria. *Journal of General Microbiology*. 1972;73:55-70.
[19] Soulier Majidi M, Ciupek C, Malbrunot C, and Baufine Ducrocq H. Etude comparative du système BACTEC 9000 MB et du milieu de Lowenstein Jensen pour la culture des mycobactéries. *Pathologie-biologie*. 2001;49:799-807.
[20] Gedalanga PB and Olson BH. Development of a quantitative PCR method to differentiate between viable and nonviable bacteria in environmental water samples. *Applied Microbiology and Biotechnology*. 2009;82:587-596.
[21] Lloyd KG, MacGregor BJ, and Teske A. Quantitative PCR methods for RNA and DNA in marine sediments: maximizing yield while overcoming inhibition. *FEMS Microbiology Ecology*. 2009:1-9.
[22] Robeson MS, Costello EK, Freeman KR, Whiting J, Adams B, Martin AP, and Schmidt SK. Environmental DNA sequencing primers for eutardigrades and bdelloid rotifers. *BMC Ecology*. 2009;9:1-10.
[23] Sagova-Mareckova M, Cermak L, Novotna J, Plhackova K, Forstova J, and Kopecky J. Innovative methods for soil DNA purification tested in soils with widely differing characteristics. *Applied and Environmental Microbiology*. 2008;74:2902-2907.
[24] Pickup RW, Rhodes G, Arnott S, Sidi-Boumedine K, Bull TJ, Weightman A, Hurley M, and Hermon-Taylor J. *Mycobacterium avium* subsp. *paratuberculosis* in the catchment area and water of the River Taff in South Wales, United Kingdom, and Its potential relationship to clustering of Crohn's disease cases in the city of Cardiff. *Applied and Environmental Microbiology*. 2005;71:2130-2139.
[25] Pickup RW, Rhodes G, Bull J, Arnott S, Sidi-Boumedine K, Hurley M, and Hermon-Taylor J. *Mycobacterium avium* subsp. *paratuberculosis* in lake catchments, in river water abstracted for domestic use, and in effluent from domestic sewage treatment works: Diverse opportunities for environmental cycling and human exposure. *Applied and Environmental Microbiology*. 2006;72:4067-4077.
[26] Whan L, Ball HJ, Grant IR, and Rowe MT. Occurrence of *Mycobacterium avium* subsp. *paratuberculosis* in Untreated Water in Northern Ireland. *Applied and Environmental Microbiology*. 2005;71:7107-7112.
[27] Vaerewijck MJM, Huys G, Palomino JC, Swings J, and Portaels F. Mycobacteria in drinking water distribution systems; ecology and significance for human health. *FEMS Microbiology Reviews*. 2005;29:911-934.
[28] Primm T, Lucero CA, and Falkinham JO. Health impacts of environmental mycobacteria. *Clinical Microbiology Reviews*. 2004;17:98-106.

[29] Lumb R, Stapledon R, Scroop A, Bond P, Cunliffe D, Goodwin A, Doyle R, and Bastian I. Investigation of spa pools associated with lung disorders caused by *Mycobacterium avium* complex in immunocompetent adults. *Applied and Environmental Microbiology*. 2004;70:4906-4910.
[30] Vugia DJ, Jang Y, Zizek C, Ely J, Winthrop KL, and Desmond E. Mycobacteria in nail salon whirlpool footbaths, California. *Emerging Infectious Diseases*. 2005;11:616-618.
[31] Marras TK, Wallace RJ, Koth LL, Stulbarg MS, Cowl CT, and Daley CL. Hypersensitivity pneumonitis reaction to *Mycobacterium avium* in household water. *Chest*. 2005;127:664-671.
[32] Iivanainen EK, Martikainen PJ, Vaananen PK, and Katila ML. Environmental factors affecting the occurrence of mycobacteria in brook waters. *Applied and Environmental Microbiology*. 1993;59:398-404.
[33] Niva M, Hernesmaa A, Haahtela K, Salkinoja-Salonen M, Sivonen K, and Haukka K. Actinobacteria communities of borel forest soil and lake water are rich in mycobacteria. *Boreal Environment Research*. 2006;11:45-53.
[34] Kirschner RA, Parker BC, and Falkinham JO. Humic and fulvic acids stimulate the growth of *Mycobacterium avium. FEMS Microbiology Ecology*. 1999;30:327-332.
[35] Johansen TB, Agdestein A, Olsen I, Nilsen SF, Holstad G, and Djønne B. Biofilm formation by *Mycobacterium avium* isolates originating from humans, swine and birds. *BMC Microbiology*. 2009;9:1-10.
[36] Carter G, Wu M, Drummond DC, and Bermudez LE. Characterization of biofilm formation by clinical isolates of *Mycobacterium avium. Journal of Medical Microbiology*. 2003;52:747-752.
[37] Steed KA and Falkinham JO. Effect of growth in biofilms on chlorine susceptibility of *Mycobacterium avium* and *Mycobacterium intracellulare. Applied and Environmental Microbiology*. 2006;72:4007-4011.
[38] Lehtola MJ, Torvinen E, Kusnetsov J, Pitkänen T, Maunula L, von Bonsdorff CH, Martikainen PJ, Wilks SA, Keevil CW, and Miettinen IT. Survival of *Mycobacterium avium, Legionella pneumophila, Escherichia coli*, and *Caliciviruses* in Drinking Water-Associated Biofilms Grown under High-Shear Turbulent Flow. *Applied and Environmental Microbiology*. 2007;73:2854-2859.
[39] Neumann M, Schulze-Robbecke R, Hagenau C, and Behringer K. Comparison of methods for isolation of mycobacteria from water. *Applied and Environmental Microbiology*. 1997;63:547-552.
[40] Kamala T, Paramasivan CN, Herbert D, Venkatesan P, and Prabhakar R. Evaluation of procedures for isolation of nontuberculous mycobacteria from soil and water. *Applied and Environmental Microbiology*. 1994;60:1021-1024.
[41] Carson LA, Cusick LB, Bland LA, and Favero MS. Efficacy of chemical dosing methods for isolating nontuberculous mycobacteria from water supplies of dialysis centers. *Applied and Environmental Microbiology*. 1988;54:1756-1760.
[42] Parashar D, Chauhan DS, Sharma VD, Chauhan A, Chauhan SV, and Katoch VM. Optimization of procedures for isolation of mycobacteria from soil and water samples obtained in northern India. *Applied and Environmental Microbiology*. 2004;70:3751-3753.
[43] Hilborn ED, Covert TC, Yakrus MA, Harris SI, Donnelly SF, Rice EW, Toney S, Bailey SA, and Stelma GN. Persistence of Nontuberculous Mycobacteria in a Drinking Water System after Addition of Filtration Treatment. *Applied and Environmental Microbiology*. 2006;72:5864–5869.
[44] Torvinen E, Suomalainen S, Lehtola MJ, Miettinen IT, Zacheus O, Paulin L, Katila ML, and Martikainen PJ. Mycobacteria in water and loose deposits of drinking water distribution systems in Finland. *Applied and Environmental Microbiology*. 2004;70:1973-1981.
[45] Torkko P, Suomalainen S, Iivanainen E, Suutari M, Paulin L, Rudbäck E, Tortoli E, Vincent V, Mattila R, and Katila ML. Characterization of *Mycobacterium bohemicum* isolated from human, veterinary, and environmental sources. *Journal of Clinical Microbiology*. 2001;39:207-211.
[46] Le Dantec C, Duguet JP, Montiel A, Dumoutier N, Dubrou S, and Vincent V. Occurrence of mycobacteria in water treatment lines and in water distribution systems. *Applied and Environmental Microbiology*. 2002;68:5318-5325.
[47] Käser M, Ruf MT, Hauser J, Marsollier L, and Pluschke G. Optimized method for preparation of DNA from pathogenic and environmental mycobacteria. *Applied and Environmental Microbiology*. 2009;75:414-418.
[48] Queiroz APS, Santos FM, Sassaroli A, Harsi CM, Monezi TA, and Mehnert DU. Electropositive filter membrane as an alternative for the elimination of PCR inhibitors from sewage and water samples. *Applied and Environmental Microbiology*. 2001;67:4614-4618.
[49] Matsumoto A, Takahashi Y, Iwai Y, and Ômura S. Isolation of Gram-positive bacteria with high G+C from inside soil aggregates. *Actinomycetologica*. 2006;20:30-34.
[50] Thomson R, Carter R, Gilpin C, Coulter C, and Hargreaves M. Comparison of methods for processing drinking water samples for the isolation of *Mycobacterium avium* and *Mycobacterium intracellulare. Applied and Environmental Microbiology*. 2008;74:3094-3098.
[51] Lleo MM, Bonato B, Tafi MC, Signoretto C, Pruzzo C, and Canepari P. Molecular vs culture methods for the detection of bacterial faecal indicators in groundwater for human use. *Letters in Applied Microbiology*. 2005;40:289-294.
[52] Donaghy JA, Totton NL, and Rowe MT. Evaluation of culture media for the recovery of *Mycobacterium avium* subsp. *paratuberculosis* from Cheddar cheese. *Letters in Applied Microbiology*. 2003;37:285-291.
[53] Stephan R, Schumacher S, Tasara T, and Grant IR. Prevalence of *Mycobacterium avium* subspecies *paratuberculosis* in Swiss raw milk cheeses collected at the retail Level. *Journal of Dairy Science*. 2007;90:3590-3595.
[54] Shleeva MO, Salina EG, and Kaprelyants AS. Dormant forms of mycobacteria. *Microbiology*. 2010;79:1-12.
[55] Böddinghaus B, Rogall T, Flohr T, Blocker H, and Bottger EC. Detection and identification of mycobacteria by amplification of rRNA. *Journal of Clinical Microbiology*. 1990;28:1751-1759.
[56] Zolg JW and Philippi-Schulz S. The superoxide dismutase gene, a target for detection and identification of mycobacteria by PCR. *Journal of Clinical Microbiology*. 1994;32:2801-2812.
[57] Kim BJ, Hong SK, Lee KH, Yun YJ, Kim EC, Park YG, Bai GH, and Kook YH. Differential identification of *Mycobacterium tuberculosis* complex and nontuberculous mycobacteria by duplex PCR assay using the RNA polymerase gene (*rpo*B). *Journal of Clinical Microbiology*. 2004;42:1308-1312.

[58] Tobler NE, Pfunder M, Herzog K, Frey JE, and Altwegg M. Rapid detection and species identification of *Mycobacterium* spp. using real-time PCR and DNA-Microarray. *Journal of Microbiological Methods*. 2006;66:116-124.
[59] Mendum TA, Chilima BZ, and Hirsch PR. The PCR amplification of non-tuberculous mycobacterial 16S rRNA sequences from soil. *FEMS Microbiology letters*. 2000;185:189-192.
[60] Dutil S, Veillette M, Mériaux A, Lazure L, Barbeau J, and Duchaine C. Aerosolization of mycobacteria and legionellae during dental treatment: low exposure despite dental unit contamination. *Environmental Microbiology*. 2007;9:2836-2843.
[61] Nieminen T, Pakarinen J, Tsitko I, Salkinoja-Salonen M, Breitenstein A, Ali-Vehmas T, and Neubauer P. 16S rRNA targeted sandwich hybridization method for direct quantification of mycobacteria in soils. *Journal of Microbiological Methods*. 2006;67:44-55.
[62] Cook KL and Britt JS. Optimization of methods for detecting *Mycobacterium avium* subsp. *paratuberculosis* in environmental samples using quantitative, real-time PCR. *Journal of Microbiological Methods*. 2007;69:154-160.
[63] Jara C, Mateo E, Guillamón JM, Torija MJ, and Mas A. Analysis of several methods for the extraction of high quality DNA from acetic acid bacteria in wine and vinegar for characterization by PCR-based methods. *International Journal of Food Microbiology*. 2008;128:336-341.
[64] Mitchell KR and Takacs-Vesbach CD. A comparison of methods for total community DNA preservation and extraction from various thermal environments. *Journal of Industrial Microbiology and Biotechnology*. 2008;35:1139–1147.
[65] Sharma R, Ranjan R, Kapardar RK, and Grover A. 'Unculturable' bacterial diversity: An untapped resource. *Current Science*. 2005;89:72-77.
[66] Timpe A and Runyon EH. The relationship of atypical acid-fast bacteria to human disease; a preliminary report. *The Journal of laboratory and clinical medicine*. 1954;44:202-209.
[67] Butler WR, Jost KC, and Kilburn JO. Identification of mycobacteria by high-performance liquid chromatography. *Journal of Clinical Microbiology*. 1991;29:2468-2472.
[68] Butler WR and Kilburn JO. High-performance liquid chromatography patterns of mycolic acids as criteria for identification of *Mycobacterium chelonae*, *Mycobacterium fortuitum*, and *Mycobacterium smegmatis*. *Journal of Clinical Microbiology*. 1990;28:2094-2098.
[69] Butler WR, Thibert L, and Kilburn JO. Identification of *Mycobacterium avium* complex strains and some similar species by high-performance liquid chromatography. *Journal of Clinical Microbiology*. 1992;30:2698-2704.
[70] Cage GD. High-performance liquid chromatography patterns of *Mycobacterium gordonae* mycolic acids. *Journal of Clinical Microbiology*. 1992;30:2402-2407.
[71] Cage GD. Direct Identification of *Mycobacterium* Species in BACTEC 7H12B Medium by High-Performance Liquid Chromatography. *Journal of Clinical Microbiology*. 1994;32:521-524.
[72] Duffey PS and Guthertz LS. *Mycobacterium avium* and *Mycobacterium intracellulare* chromatotypes defined by curvilinear gradient HPLC of mycolic acids. *FEMS Microbiology Letters*. 1992;95:27-36.
[73] Duffey PS, Guthertz LS, and Evans GC. Improved rapid detection of mycobacteria by combining solid-phase extraction with high-performance liquid chromatography analysis of BACTEC cultures. *Journal of Clinical Microbiology*. 1996;34:1939-1943.
[74] Guthertz LS, Lim SD, Jang Y, and Duffey PS. Curvilinear-gradient high-performance liquid chromatography for identification of mycobacteria. *Journal of Clinical Microbiology*. 1993;31:1876-1881.
[75] Raffi F, Butler WR, and Cerniglia CE. Differentiation of a rapidly growing, scotochromogenic, polycyclic-aromatic-hydrocarbonmetabolizing strain of *Mycobacterium* sp. from other known *Mycobacterium* species. *Archives of Microbiology*. 1992;157:512-520.
[76] Ridderhof JC, Wallace RJ, Kilburn JO, Butler WR, Warren N, Wong ES, Tsukamura M, and Steele LC. Chronic tenosynovitis of the hand due to *Mycobacterium nonchromogenicum*: Use of high-performance liquid chromatography for identification of isolates. *Reviews of Infectious Diseases*. 1991;13:857-864.
[77] Steck PA, Schwartz BA, Rosendahl MS, and Gray GR. Mycolic acids - a reinvestigation. *The Journal of Biological Chemistry*. 1978;253:5625-5629.
[78] Thibert L and Lapierre S. Routine application of high-performance liquid chromatography for identification of mycobacteria. *Journal of Clinical Microbiology*. 1993;31:1759-1763.
[79] Adékambi T and Drancourt M. Dissection of phylogenetic relationships among 19 rapidly growing *Mycobacterium* species by 16S rRNA, *hsp*65, *sod*A, *rec*A and *rpo*B gene sequencing. *International Journal of Systematic and Evolutionary Microbiology*. 2004;54:2095-2105.
[80] Gomila M, Ramirez A, and Lalucat J. Diversity of environmental *Mycobacterium* isolates from hemodialysis water as shown by a multigene sequencing approach. *Applied and Environmental Microbiology*. 2007;73:3787-3797.
[81] Cook GM, Berney M, Gebhard S, Heinemann M, Cox RA, Danilchanka O, and Niederweis M. Physiology of mycobacteria. *Advances in Microbial Physiology*. 2009;55:81-184.
[82] Ripoll F, Pasek S, Schenowitz C, Dossat C, Barbe V, Rottman M, Macheras E, Heym B, Herrmann JL, Daffé M, Brosch R, Risler J, and Gaillard JL. Non mycobacterial virulence genes in the genome of the emerging pathogen *Mycobacterium abscessus*. *PLoS One*. 2009;4:1-12.
[83] Li L, Bannantine J, Zhang Q, Amonsin A, May B, Alt D, Banerji N, Kanjilal S, and Kapur V. The complete genome sequence of *Mycobacterium avium* subspecies *paratuberculosis*. *Proceeding of the National Academy of Science of the United States of America*. 2005;102:12344-13349.
[84] Stinear TP, Seemann T, Harrison PF, Jenkin GA, Davies JK, Johnson PDR, Abdellah Z, Arrowsmith C, Chillingworth T, Churcher C, Clarke K, Cronin A, Davis P, Goodhead I, Holroyd N, Jagels K, Lord A, Moule S, Mungall K, Norbertczak H, Quail MA, Rabbinowitsch E, Walker D, White B, Whitehead S, Small PLC, Brosch R, Ramakrishnan L, Fischbach MA, Parkhill J, and Cole ST. Insights from the complete genome sequence of *Mycobacterium marinum* on the evolution of *Mycobacterium tuberculosis*. *Genome Research*. 2010;18:729-741.
[85] Behr MA and Falkinham JO. Molecular epidemiology of nontuberculous mycobacteria. *Future Microbiology*. 2009;4:1009-1020.

[86] Spratt DA. Significance of bacterial identification by molecular biology methods. *Endodontic Topics*. 2004;9:5-14.
[87] Pryor M, Springthorpe S, Riffard S, Brooks T, Huo Y, Davis G, and Sattar SA. Investigation of opportunistic pathogens in municipal drinking water under different supply and treatment regimes. *Water Science & Technology*. 2004;50:83-90.
[88] Leys NM, Ryngaert A, Bastiaens L, Wattiau P, Top EM, Verstraete W, and Springael D. Occurrence and community composition of fast-growing *Mycobacterium* in soils contaminated with polycyclic aromatic hydrocarbons. *FEMS Microbiology Ecology*. 2005;51:375-388.
[89] Uyttebroek M, Vermeir S, Wattiau P, Ryngaert A, and Springael D. Characterization of cultures enriched from acidic Polycyclic Aromatic Hydrocarbon-contaminated soil for growth on pyrene at Low pH. *Applied and Environmental Microbiology*. 2007;73:3159-3164.
[90] Uyttebroek M, Breugelmans P, Janssen M, Wattiau P, Joffe B, Karlson U, Ortega-Calvo JJ, Bastiaens L, Ryngaert A, Hausner M, and Springael D. *Mycobacterium* community and polycyclic aromatic hydrocarbons (PAHs) among different size fractions of a long-term PAH-contaminated soil. *Environmental Microbiology*. 2006;8:836–847.
[91] Uyttebroek M, Spoden A, Ortega-Calvo JJ, Wouters K, Wattiau P, Bastiaens L, and Springael D. Differential responses of *Eubacterial*, *Mycobacterium*, and *Sphingomonas* communities in Polycyclic Aromatic Hydrocarbon (PAH)-contaminated soil to artificially induced changes in PAH profile. *Journal of Environmental Quality*. 2007;36:1403-1411.
[92] Sheridan GEC, Masters CI, Shallcross JA, and Mackey BM. Detection of mRNA by reverse transcription-PCR as an indicator of viability in *Escherichia coli* cells. *Applied and Environmental Microbiology*. 1998;64:1313-1318.
[93] Nogva HK, Dromtorp SM, Nissen H, and Rudi K. Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5′-nuclease PCR. *BioTechniques*. 2003;34:804-813.
[94] Menendez MC, Garcia MJ, Navarro MC, Gonzalez-y-Merchand JA, Rivera-Gutierrez S, Garcia-Sanchez L, and Cox A. Characterization of an rRNA Operon (*rrn*B) of *Mycobacterium fortuitum* and other mycobacterial species: Implications for the classification of mycobacteria. *Journal of Bacteriology*. 2002;184:1078-1088.
[95] Ninet B, Monod M, Emler S, Pawlowski J, Metral C, Rohner P, Auckenthaler R, and Hirschel B. Two different 16S rRNA genes in a mycobacterial strain. *Journal of Clinical Microbiology*. 1996;34:2531-2536.
[96] Reischl U, Feldmann K, Naumann L, Gaugler BJ, Ninet B, Hirschel B, and Emler S. 16S rRNA sequence diversity in *Mycobacterium celatum* strains caused by presence of two different copies of 16S rRNA gene. *Journal of Clinical Microbiology*. 1998;36:1761-1764.
[97] O'Mahony J and Colin H. A real time PCR assay for the detection and quantitation of *Mycobacterium avium* subsp. *paratuberculosis* using SYBR Green and the Light Cycler. *Journal of Microbiological Methods*. 2002;51:283-293.
[98] Doran T, Tizard M, Millar D, Ford J, Sumar N, Loughlin M, and Hermon-Taylor J. IS*900* targets translation initiation signals in *Mycobacterium avium* subsp. *paratuberculosis* to facilitate expression of its hed gene. *Microbiology*. 1997;143:547-552.
[99] Derz K, Klinner U, Schphan I, Stackebrandt E, and Kroppenstedt RM. *Mycobacterium pyrenivorans* sp. nov., a novel polycyclic-aromatic-hydrocarbon-degrading species. *International Journal of Systematic and Evolutionary Microbiology*. 2004;54:2313-2317.
[100] Hennessee CT, Seo JS, Alvarez AM, and Li QX. Polycyclic aromatic hydrocarbon-degrading species isolated from Hawaiian soils: *Mycobacterium crocinum* sp. nov., *Mycobacterium pallens* sp. nov., *Mycobacterium rutilum* sp. nov., *Mycobacterium rufum* sp. nov. and *Mycobacterium aromaticivorans* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*. 2009;59:378-387.
[101] Hormisch D, Brost I, Kohring GW, Giffhorn F, Kroppenstedt RM, Stackebrandt E, Färber P, and Holzapfel WH. *Mycobacterium fluoranthenivorans* sp. nov., a fluoranthene and aflatoxin B1 degrading bacterium from contaminated soil of a former coal gas plant. *International Journal of Systematic and Evolutionary Microbiology*. 2004;27:653-660.
[102] Khan AA, Kim SJ, Paine DD, and Cerniglia CE. Classification of a polycyclic aromatic hydrocarbon-metabolizing bacterium, *Mycobacterium* sp. strain PYR-1, as *Mycobacterium vanbaalenii* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*. 2002;52:1997-2002.
[103] Kleespies M, Kroppenstedt RM, Rainey FA, Webb LE, and Stackebrandt E. *Mycobacterium hodleri* sp. nov., a new member of the fast-growing mycobacteria capable of degrading polycyclic aromatic hydrocarbons. *International Journal of Systematic Bacteriology*. 1996;46:683-687.
[104] Rockne KJ, Liang W, Young LY, and Taghon GL. Toxicity of density separation media to *Escherichia coli* and *Mycobacterium* strain PC01: implication for density-separation of soils and sediments. *FEMS Microbiology Ecology*. 2003;43:185-189.
[105] Schneider J, Grosser R, Jayasimhulu K, Xue W, and Warshawsky D. Degradation of pyrene, benz[a]anthracene, and benzo[a]pyrene by *Mycobacterium* sp. strain RJGII-135, isolated from a former coal gasification site. *Applied and Environmental Microbiology*. 1996;62:13-19.
[106] Shor L, Liang W, Rockne KJ, Young LY, Taghon G, and Kosson D. Intra-aggregate mass transport-limited bioavailability of Polycyclic Aromatic Hydrocarbons to *Mycobacterium* strain PC01. *Environmental Science & Technology*. 2003;37:1545-1552.
[107] Willumsen P, Karlson U, Stackebrandt E, and Kroppenstedt RM. *Mycobacterium frederiksbergense* sp. nov., a novel polycyclic aromatic hydrocarbon-degrading *Mycobacterium* species. *International Journal of Systematic and Evolutionary Microbiology*. 2001;51:1715-1722.