

Rapid Microscope Based Identification Method for Tuberculosis and Other Mycobacteria: Fluorescence *In Situ* Hybridization (FISH) - Current State of The Art and Future Research Needs

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ABSTRACT

Tuberculosis (TB) is one of the major increasing causes of illness and death worldwide, especially in Asia and Africa. Rapid and accurate diagnosis of mycobacteria is important in the prevention and effective treatment of tuberculosis. Today, conventional culture methods are still accepted as the gold standard for the identification of mycobacteria in routine mycobacteriology laboratories. However, even if these methods are highly efficient and useful, these methods are time-consuming and labor-laborious. In recent years, several novel DNA-based and non-invasive techniques, such as RAMAN spectroscopy and microcalorimetry have been developed for a more rapid and reliable identification. Unfortunately, these methods are not capable of visualizing the cells in their natural environment such as in tissues. Visualization of cells may however provide fundamental, complementary information for the overall understanding of the molecular and microbial ecology of mycobacteria in disease processes. Here, we present a current state of the art review of the Fluorescence *In Situ* Hybridization (FISH) methods which can be used to identify, visualize and quantify whole cells of different species of mycobacteria, especially the tuberculosis complex, and their associates in their natural environment without prior cultivation. Although this method also allows for an easy, rapid and cost-efficient identification (~1-3 hours)

and simultaneous *in situ* visualization of different microbial species, it has so far only been used to a limited extent. Here, we will discuss both the potentials as well as limitations of FISH for the detection of mycobacteria and other relevant associates, and suggest some future research needs.

Keywords: Mycobacteria; *Mycobacterium spp.*; Fluorescence *In Situ* Hybridization (**FISH**), Identification; Tuberculosis

INTRODUCTION

Although Tuberculosis (**TB**) is one of the oldest diseases of mankind, it still belongs to one of the major increasing causes of illness and death worldwide, especially in Asia and Africa. According to the World Health Organization (**WHO**) 2015 tuberculosis report, 9.6 million people were estimated to have fallen ill with TB in 2014 worldwide: 5.4 million men, 3.2 million women and 1.0 million children. Globally, 12% of the 9.6 million new TB cases in 2014 were HIV-positive and 1.5 million deaths from TB (1.1 million in HIV-negative and 0.45 million deaths in HIV-positive persons). Asia and Africa alone constitute 86% of all cases [1-3].

One of the major concerns with TB is the resistance of *Mycobacterium tuberculosis* to drugs. In 2014, there were an estimated 480.000 cases of Multidrug Resistant TB (**MDR-TB**) [1]. Drug resistance, especially multi-resistance against TB has caused high morbidity and mortality, particularly among immuno-compromised patients. The emergence of MDR-TB and, more recently, of Extensively Drug-Resistant (**XDR**)-TB present a major obstacle for a successful TB control and elimination [4,5]. In order to combat this, the development of rapid and accurate microbiological diagnosis of *Mycobacterium* species is crucial for the effective treatment of patients and further prevention of mycobacterial infection.

Although conventional methods are still accepted as the gold standard for identification of mycobacteria, they are laborious and time-consuming. Fortunately, several improvements have been made. For example, the recently developed culture methods based on radiometric BACTEC and non-radiometric Mycobacteria Growth Indicator Tube (**MGIT**) have reduced the time for the identification from weeks to days [6-9]. These methods are however not rapid enough. During the last years, different immunological [10,11], molecular methods and non-invasive methods, such as RAMAN spectroscopy [12-14] and microcalorimetry [15], have therefore been introduced. The most common molecular methods are based on nucleic acid amplification based tests (Polymerase Chain Reaction, **PCR**) and different hybridization methods, which employ nucleic acid probes for species determination [8,9,16-18]. Other advanced screening strategies are based on Single Nucleotide Polymorphism (**SNP**) analysis, or target other genes, proteins or even genomes and use sophisticated gene databases for advanced evaluations [19-22]. While PCR based methods and sequencing present several advantages (e.g. low detection limit, sequence based identification) there are several limitations, such as risk of contamination, chimera products, dependency on appropriate nucleic acid extraction protocols and appropriate primers and protocols for PCR or sequencing, susceptibility to inhibiting compounds (e.g. in blood), difficulties to find appropriate alternative gene targets [21] and failure to simultaneously visualize, identify and localize whole

cells in their natural environment, e.g. in a tissue. Visualization of cells may however provide fundamental, complementary information for the overall understanding of the molecular and microbial ecology of mycobacteria in disease processes. Some of these limits can be overcome by Fluorescence *In Situ* Hybridization (**FISH**), which has proven to be useful for many applications in almost all fields of microbiology for a quick and simultaneous visualization, identification, enumeration and localization of culturable as well as unculturable microorganisms in their natural environment [23].

HISTORY OF FLUORESCENCE *IN SITU* HYBRIDIZATION

The principle of the *In Situ* Hybridization (**ISH**) technique was independently developed in 1969 by Pardue and Gall [24] and John et al. [25]. This technique was originally based on autoradiographic labeling to map both repetitive as well as low copy DNA sequences. Because these techniques had some drawbacks, non-isotopic *in situ* hybridization was developed. The radioactive ³²phosphorus label, which was used in the first assays for *in situ* hybridization, was then replaced by different non-isotopic labels such as biotin, digoxigenine and fluorescein [26]. Following this, ISH was then adapted to a wide range of different medical research fields, such as chromosome analysis of tumors and leukaemia, and cytogenetics [27].

In Situ Hybridization was introduced into microbiology by Giovannoni et al in the late 1980s [28], where radioactively labeled rRNA-oligonucleotide probes were still used. DeLong et al [29] were the first to use multiple fluorescently labeled oligonucleotides for the simultaneous identification of different species of microorganisms without prior cultivation. This technique (oligonucleotide FISH) was then further developed and applied by Amann et al in the early 1990s on a variety of environmental samples [30]. Some of the first oligonucleotide FISH applications on medical samples were made by Moter and Gobel in 2000 [31]. Since then, the oligonucleotide FISH technique has become an invaluable tool for phylogenetic, diagnostic and environmental studies in different fields within microbiology, medical and environmental science and biotechnology. Along with the growing applications of oligonucleotide FISH in various research fields, several obstacles such as confounding background, low cell concentrations, low cell activities, impermeable cell wall packages and non-optimal probes have been encountered, depending on the nature of the sample, the targeted species and the detection method employed. Furthermore, it was realized that the main gene target, the ribosomal genes, in particular the 16S rRNA gene, often failed to reveal information about the true identity, function or activity of certain microbial species. Since then, several developments have been undertaken to solve these problems, e.g. by modifying the labelling strategy or the probe chemistry, combining FISH with other analytical methods or advanced microscopes, or targeting other genes [23,32,33]. Some examples of new FISH methods are Polynucleotide (**Poly**)-FISH, Helper-FISH, FISH-Microautoradiography (**MAR**), mRNA-FISH, Catalyzed Reporter Deposition (**CARD**)-FISH, Peptide Nucleic Acid (**PNA**)-FISH, Locked Nucleic Acid (**LNA**)-FISH, Recognition of Individual Genes (**RING**)-FISH, CLONE-FISH, Secondary Ion Mass Spectrometry (**SIMS**)-FISH, Raman Microspectroscopy (**RAMAN**)-FISH, Tyramide Signal

Amplification **(TSA)**-FISH, Double Labeling of Oligonucleotide Probes **(DOPE)**-FISH, Beacon-Based **(bb)**FISH, Cycling Primed *In Situ* Amplification **(CPRINS)**-FISH, EM-FISH/Gold FISH, Quantum Dot-Based **(QD)**-FISH, Combinatorial Labeling and Spectral Imaging **(CLASI)**-FISH, Gene FISH, and phage FISH [33-44].

Thus, today, it is now possible, at least in some well demonstrated cases, to identify, visualize and quantify both the phylogenetic affiliation as well as activity and function of some taxa in all three recognized domains, including also some viruses and bacteriophages. So far, approximately 3,000 oligonucleotide gene probes, targeting mainly the 16S rRNA or the 23S rRNA genes, have been developed against different taxa in different types of natural as well as anthropogenic ecosystems. Many of these probes can be found on the online database (probeBase) created by Loy et al. [45]. (see <http://probebase.csb.univie.ac.at/>). However, other types of probes such as PNA-FISH and CARD-FISH probes are not listed there. Furthermore, many of the oligonucleotide probes in the probeBase may need to be updated due to the constant expansion of our knowledge about the microbial diversity on Earth (see e.g. the ribosomal gene databases on www.silva-arb.de), or further adapted to novel FISH protocols that expand our current application possibilities.

GENERAL ADVANTAGES OF FISH

The FISH method offers several advantages over other molecular detection methods based on cell extracts such as nucleic acids, for example:

1. **Top to bottom identification:** Identification of microorganisms at different taxonomical levels (e.g. on the domain level or on lower levels such as the family, genus or species) without the need to determine traditional phenotypic characteristics, extract and amplify DNA, or to sequence. Both culturables (fast or slow-growing microorganisms) as well as unculturables can be identified.
2. **Identification of multiple species:** Several species of microorganisms can be simultaneously identified using gene probes with different types of fluorochromes, and the level of relatedness can be deduced both for known as well as for unknown species (Figure 1).
3. **Simple:** All steps of the protocol are performed at room temperature. The method is simple, easy and, except for certain advanced FISH protocols which are combined with expensive analytical equipment, inexpensive.
4. **Time:** The identification time is short (usually 0,5h-3h). However, some advanced FISH protocols, which depend on e.g. combination with other analytical methods, may require longer time (24h-96h).
5. **Several types of information:** With FISH it is possible to visualize, quantify and make three-dimensional reconstructions of the distribution, abundance and association of the target species and their associates in their natural environment, e.g. in a tissue or in a biofilm.
6. **Single cell microbiology:** Probe-targeted cells can be enriched specifically by e.g. flow cytometry, polynucleotide FISH or magnetoFISH, and thereafter further analysed, e.g. for single cell genomics [36,46].

GENERAL DISADVANTAGES OF FISH

The most common disadvantages of FISH are generally related to the nature of the sample type, cell concentration, activity and the activity/function detection possibilities. These can however often be solved with special measures, as outlined below:

1. **Cell concentration:** The cell detection limit for FISH is low (according to different studies, between 10^{3-6} CFU/ml) [33]. The identification of cells below this limit is a problem but this can often be overcome by appropriate concentration of the sample. Too concentrated samples can be extracted, diluted or sectioned.
2. **Unspecific signals:** Some microbial species such as fungi and phototrophs may be autofluorescent. In addition, minerals, artifacts and staining residues can cause a confounding background and thus interpretation problems during microscopy. However, these problems can often be overcome by using suitable controls, confocal laser scanning microscopy, spectral imaging, or employing special measures to dissolve disturbing substances [33,47].
3. **Low signal intensity:** Some microorganisms produce only low probe signal intensities. This may be caused by several parameters, such as low rRNA content due to low metabolism, or weak permeabilization of the cell so that the probe cannot penetrate into the cell. To increase the permeability in general, different types of chemical-physical treatments or enzymes (e.g. lysozyme, mutanolysin, proteinase K), or other types of probe chemistries (e.g. PNA, LNA or quantum dot [beacon FISH] probes) can be used. To increase the probe signal intensity in cells with low metabolism, other types of FISH protocols can be used that increase the access to the target site or enhance the fluorochrome intensity of the probes, either by multiple labelling of the probe (e.g. HELPER-FISH, prolonged hybridization time, polynucleotide FISH or DOPE-FISH), or by amplification (e.g. CARD-FISH) [23,33].
4. **Negative results:** Depending on the target species, the target gene region and quality of available gene sequences, the probes may have a too low resolving power or not work at all. Depending on the reason(s) for this, there are several types of solutions: i) similar to those suggested in step 3, ii) further sequencing (to expand the gene database and to produce sequences of better quality) in order to enable a more accurate probe design, or iii) change target gene [23,33].
5. **Too low information content:** FISH based on ribosomal gene probes can generally only provide limited information about the activity and function of the target species. This can be overcome by targeting other genes or expanding the standard FISH method with other labels employing other probe chemistries, or combining with other analytical methods, such as FISH-MAR, nano-SIMS or RAMAN spectroscopy [32,33,40].
6. No commercial diagnostic FISH test is for the time being available for the *Mycobacterium spp.*

PRINCIPLES OF THE OLIGONUCLEOTIDE FISH METHOD

The procedure of the oligonucleotide FISH method is based on several steps. Below a short summary based on the protocol in e.g. Amann 1995 [48]:

Step 1: Harvest cells and fixation. Fixation and permeabilization are done to preserve the cellular morphology but should also maximize the diffusion of the probe throughout the cytoplasmic matrix. Most of the cells, depending on their Gram stain character, are fixed either with aldehydes (mostly paraformaldehyde [PFA]) or alcohols (mostly ethanol). Depending on the sample type, the fixation time is usually around 0.5h-3h, but it may also range from 1 min (especially for mycobacteria) to up to 12h or even longer.

Step 2: Spot cells on microscope slides (coated or non-coated) and air-dry. Alternatively, FISH can also be performed in liquid solutions in eppendorf tubes.

[**Step 3:** Optional, depending on the cell wall package of the target species: pretreatment of fixed cells with e.g. enzymes or other mild denaturing methods to increase the permeability of the cell wall.]

Step 4: Dehydration step, in a successive EtOH series.

Step 5: Hybridization step, using e.g. fluorescently labeled probes. Common gene targets for prokaryotes are the 16S rRNA or the 23S rRNA genes, and for eukaryotes the 18S rRNA or the 28S rRNA genes, but other (non-ribosomal) gene targets are also possible. In some cases, also the Intergenic Spacer Region (**ISR**) can be useful for both prokaryotes as well as for eukaryotes. Several different gene probes with different fluorescent labels can be combined to target either different taxonomical ranks for one species, or different taxa [49-51], (see Figure 1). The hybridization is performed at a fixed temperature (usually 46° C), but with varying formamide concentrations depending on the probes employed. Depending on the sample type and FISH protocol, the hybridization time can be 0.5h to 96h long.

Step 6: Washing step, to remove unbound or non-specifically bound probe molecules.

[**Step 7:** Optional, counterstain with a universal nucleic acid stain such as DAPI, SYBR Green/Gold, or SYTO], or with some other reagent depending on the project goal.

Step 8: Evaluation, by e.g. epifluorescence microscopy, confocal laser scanning microscopy, flow cytometry or other methods such as nano-SIMS and RAMAN spectroscopy, or microarray technology [13,32,52]. Quantification can be made by several different kinds of digital image analytical softwares, for example the DAIME software [53, <http://dome.csb.univie.ac.at/daime>].

Comment: Principally the workflow is nearly the same for most of the different FISH protocols. The most common deviations are made for steps 3, 5 and 6.

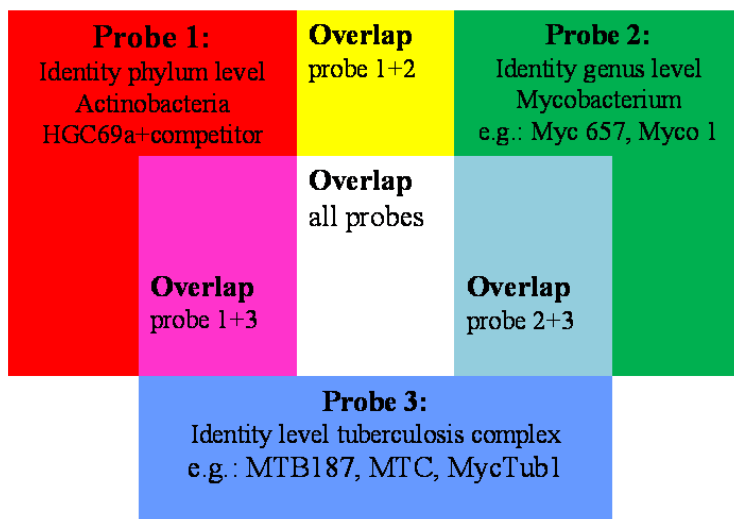


Figure 1: General overview of the interpretation possibilities of taxonomical relationships based on a hierarchical probe set, which will allow a more reliable identification than a single probe, especially in samples with unknown microbial composition. Example: probe 1 (red colour) targeting the phylum *Actinobacteria*; probe 2 (green color) targeting the genus *Mycobacterium*; probe 3 targeting only the *Mycobacterium tuberculosis* complex (blue colour) – see Tables 1 and 2. Overlap 1 and 2 (producing the yellow colour): the probe targeted species belongs to the genus *Mycobacterium* but not to the *Mycobacterium tuberculosis* complex. Overlap of all probes (producing the white colour): the targeted species belongs to the *Mycobacterium tuberculosis* complex. The overlap combinations 1 and 3, and 2 and 3, respectively might indicate inconsistent identities.

FISH PROBES AND PROTOCOLS FOR THE IDENTIFICATION OF *MYCOBACTERIUM SPP*

The first standard oligonucleotide probes (Myb736a, Myb736b, MLP), (Table 1) for FISH applications targeting mycobacteria and other filamentous actinobacteria in activated sludge in wastewater treatment plants were developed by De los Reyes et al. [54] and Schuppler et al. [55]. Both these studies as well as other studies reported problems (weak or no FISH probe signals) with the rigid, hydrophobic cell wall of mycobacteria and other species of actinobacteria. Different types of fixation protocols were therefore tested and successful FISH results could then be obtained with e.g. short (1 min.) PFA fixation, EtOH fixation, or post treatment with enzymes or chemicals like HCl of already fixed cells [54-57]. Furthermore, a range of other oligonucleotide probes were also developed, such as Myc657 and several other probes for both clinical (especially for the avium and the tuberculosis complexes) as well as for environmental applications (Table 1).

Another alternative solution was introduced by Stender et al. [58], who both designed new oligonucleotide probes (MTC and NTM, see Table 1) as well as started to employ another type of probe chemistry PNA-FISH. This initiated the further development of other specific PNA-FISH probes targeting different types of clinically relevant mycobacteria (Tables 1 and 2).

Table 1: FISH probes targeting the 16S rRNA gene for the identification of the *Mycobacterium* spp. – on the genus, species and complex levels. Probes were downloaded from the ProbeBase (<http://probebase.csb.univie.ac.at/>), or if not listed there, retrieved from publications. The parameters sensitivity and specificity are based on results from the quoted articles.

In probe base	Probe name	Target goal (by author)	Target rRNA	Sequence (5'-3')	<i>E. coli</i> start position (59)*	<i>E. coli</i> start position in this study	Probe type	F (%)	Hits within the SSU 126 REF database (%)** - domain, phylum, order, family	Hits for the genus <i>Mycobacterium</i> * (%)	Hits for the tuberculosis complex** (%)	Sample type	Sensitivity (%)	Specificity (%)	Reference
Not	MAV	<i>M. avium</i>	16S	GACCTC AAGACG CAT	ND by author	183	PNA	30	<i>Bacteria</i> 0,02 (296 seq) <i>Actinobacteria</i> 0,13 (296 seq) <i>Corynebacteriales</i> 0,35 (296 seq) <i>Mycobacteriaceae</i> 5,1 (296 seq)	5,1 (296 seq)	0	Clinical	ND	ND	[60]
Not	MAV	<i>M. hemo philum</i>	16S	GACCTCA AGCGCAT	ND by author	183	PNA	30	<i>Bacteria</i> 0,0004 (6 seq) <i>Actinobacteria</i> 0,003 (6 seq) <i>Corynebacteriales</i> 0,01 (6 seq) <i>Mycobacteriaceae</i> 0,1 (6 seq)	0,1 % (6 seq)	0	Clinical	ND	ND	[60]
Not	MAV 148	<i>M. avium</i> , <i>M. avium</i> subsp. <i>avium</i> , <i>M. avium</i> subsp. <i>paratuberculosis</i> , <i>M. avium</i> subsp. <i>silvaticum</i>	16S	TGCGTCT TGAGG TCC	148	183	PNA	30	<i>Bacteria</i> 0,02 (295 seq) <i>Actinobacteria</i> 0,13 (295 seq) <i>Corynebacteriales</i> 0,35 (295 seq) <i>Mycobacteriaceae</i> 5,1 (295 seq)	5,1 (295 seq)	0	Environmental	ND	ND	[61]
Yes	MAVP 187	<i>Mycobacterium avium</i> subsp. <i>avium</i> ; <i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i>	16S	TGCGTCT TGAGGTC CTATCC	187	176	Oligo	ND	<i>Bacteria</i> 0,02 (294 seq) <i>Actinobacteria</i> 0,3 (294 seq) <i>Corynebacteriales</i> 0,35 (294 seq) <i>Mycobacteriaceae</i> 5,1 (294 seq)	5,1 (294 seq)	0	Clinical	ND	ND	[62]
Not	MKA	<i>M. kansasii</i>	16S	CTGCACA CCGGG ATA	ND by author	136	PNA	40	<i>Bacteria</i> 0,002 (30 seq) <i>Actinobacteria</i> 0,01 (30 seq) <i>Corynebacteriales</i> 0,04 (30 seq) <i>Mycobacteriaceae</i> 0,52 (30 seq)	0,52 (30 seq)	0	Clinical	ND	ND	[60]
Not	MKA	<i>M. intermedium</i>	16S	CTGCACA CTGGGA TA	ND by author	136	PNA	40	<i>Bacteria</i> 0,0009 (14 seq) <i>Actinobacteria</i> 0,01 (14 seq) <i>Corynebacteriales</i> 0,01 (9 seq) <i>Mycobacteriaceae</i> 0,14 (8 seq)	0,14 (8 seq)	0	Clinical	ND	ND	[60]
Not	MKA	<i>M. gordonae</i>	16S	CTGCACA TCGGG ATA	ND by author	136	PNA	40	<i>Bacteria</i> 0,004 (66 seq) <i>Actinobacteria</i> 0,03 (66 seq) <i>Corynebacteriales</i> 0,08 (64 seq) <i>Mycobacteriaceae</i> 1,0 (60 seq)	1,0 (60 seq)	0	Clinical	ND	ND	[60]

Not	MLEP	<i>M. leprae</i>	16S	TAGGACT TCAAG GCG	ND by author	179	PNA	30	<i>Bacteria</i> 0,0006 (10 seq) <i>Actinobacteria</i> 0,004 (10 seq) <i>Corynebacteriales</i> 0,01 (10 seq) <i>Mycobacteriaceae</i> 0,17 (10 seq)	0,17 (10 seq)	0	Clinical	ND	ND	[60]
Not	MLEP	<i>M. hemophilum</i>	16S	TAGGACC TCAAG GCG	ND by author	179	PNA	30	<i>Bacteria</i> 0,0004 (6 seq) <i>Actinobacteria</i> 0,003 (6 seq) <i>Corynebacteriales</i> 0,01 (6 seq) <i>Mycobacteriaceae</i> 0,1 (6 seq)	0,1 (6 seq)	0	Clinical	ND	ND	[60]
Yes	MLP	M. sp. SMKN 23, M. sp. SMKN 22, nocar dioform actino mycetes	16S	AACCCAT GCAGGC CGTAG TCC	182	183	Oligo	ND	<i>Bacteria</i> 0,003 (43 seq) <i>Actinobacteria</i> 0,02 (43 seq) <i>Corynebacteriales</i> 0,05 (43 seq) <i>Mycobacteriaceae</i> 0,74 (43 seq)	0,74 (43 seq)	0	Environ mental	ND	ND	[55]
Not	MNP1	Nocar dioform- actino mycete- affiliated sequences, <i>M. chelonae</i> , <i>M. farcino</i> <i>genes</i>	16S	TTAGACC CAGTTTC CCAGGCT	152	151	Oligo	50	<i>Bacteria</i> 5,4 (84,137 seq) <i>Actinobacteria</i> 37 (83,986 seq) <i>Corynebacteriales</i> 81,6 (68,814 seq) <i>Mycobacteriaceae</i> 95,0 (5,511 seq)	96,0 (5,509 seq)	99,9 (1,914 seq)	Environ mental	ND	ND	[55]
Yes	MTB 187	<i>M. tuberculosis</i> strains	16S	TGCATCC CGTGGTC CTATCC	187	176	Oligo	ND	<i>Bacteria</i> 0,13 (1,962 seq) <i>Actinobacteria</i> 0,85 (1,962 seq) <i>Corynebacteriales</i> 2,3 (1,962 seq) <i>Mycobacteriaceae</i> 34, (1,962 seq)	34 (1,962 seq)	99,8 (1,912 seq)	Clinical	ND	ND	[63]
No	MTB 223	<i>M. tuberculosis</i>	16S	CCCA CACC GCTA AAG CGC	196	197	DNA	50	<i>Bacteria</i> 0,12 (1,924 seq) <i>Actinobacteria</i> 0,84 (1,924 seq) <i>Corynebacteriales</i> 2,3 (1,924 seq) <i>Mycobacteriaceae</i> 33 (1,924 seq)	33 (1,924 seq)	99,8 (1,911 seq)	Clinical	ND	ND	[64]
Yes	MTB 226	<i>M. tuberculosis</i> strains	16S	CCACACC GCTAAAG	226	207	Oligo	20	<i>Bacteria</i> 0,12 (1,924 seq) <i>Actinobacteria</i> 0,84 (1,924 seq) <i>Corynebacteriales</i> 2,3 (1,924 seq) <i>Mycobacteriaceae</i> 33 (1,924 seq)	33 (1,924 seq)	99,8 (1,912 seq)	Clinical	ND	ND	[63]
Not	MTB 1284	<i>M. tuberculosis</i>	16S	GAGACCG GCTTTTAA GGATTTCG	1277	1276	DNA	50	<i>Bacteria</i> 0,13 (1,991 seq) <i>Actinobacteria</i> 0,84 (1,928 seq) <i>Corynebacteriales</i> 2,3 (1,927 seq) <i>Mycobacteriaceae</i> 33, 3 (1,927 seq)	33,3 (1,927 seq)	99,8 (1,912 seq)	Clinical	ND	ND	[64]
Not	MTBC	Tuberculosis complex	16S	AGGACCA CGGGA TGC	ND by author	180	PNA	50	<i>Bacteria</i> 0,13 (1965 seq) <i>Actinobacteria</i> 0,86 (1965 seq) <i>Corynebacteriales</i> 2,3 (1965 seq) <i>Mycobacteriaceae</i> 34,1 (1965 seq)	34,1 (1965 seq)	99,8 (1,912 seq)	Clinical	ND	ND	[60], [65]

Not	MTBC	<i>M. marinum</i>	16S	AGGACCA CGGG ATTC	ND by author	180	PNA	50	<i>Bacteria</i> 0,004 (62 seq) <i>Actinobacteria</i> 0,03 (62 seq) <i>Corynebacteriales</i> 0,07 (62 seq) <i>Mycobacteriaceae</i> 1,1 (62 seq)	1,1(62 seq)	0	Clinical	ND	ND	[60]
Not	MTBC	<i>M. chubuense</i>	16S	AGGACCA CGGCA TGC	ND by author	180	PNA	50	<i>Bacteria</i> 0,0006 (10 seq) <i>Actinobacteria</i> 0,004 (10 seq) <i>Corynebacteriales</i> 0,01 (10 seq) <i>Mycobacteriaceae</i> 0,17 (10 seq)	10(17 seq)	0	Clinical	ND	ND	[60]
Not	MTBC	<i>M. celatum</i>	16S	AGGACCA TGGGA TGC	ND by author	180	PNA	50	<i>Bacteria</i> 0,003 (47 seq) <i>Actinobacteria</i> 0,02 (46 seq) <i>Corynebacteriales</i> 0,05 (45 seq) <i>Mycobacteriaceae</i> 0,64 (37 seq)	0,64(37 seq)	0	Clinical	ND	ND	[60]
Not	MTC (also named MTB)	<i>M. tuberculosis</i> complex	16S	CTTAGGA ATTTTCGG GAATCCTT AAAAGCC ***	1224	1277	PNA	30	<i>Bacteria</i> 0,13 (2,050 seq) <i>Actinobacteria</i> 0,84 (1,930 seq) <i>Corynebacteriales</i> 2,3 (1,927 seq) <i>Mycobacteriaceae</i> 33 (1,927 seq)	33,3 (1,927 seq)	99,8 (1,912 seq)	Clinical	84 80.2 98	97 100 100	[58] [66] [67]
Yes	Myb 736a Myb 736b	<i>Mycobacterium</i> complex	16S	CAGCGTC AGTTACT ACCCA GAG CAGCGTC AGTTACTx CCCAGAG X=base analog N5, 5-nitroindole	736	736	Oligo	30	<i>Bacteria</i> 0,5 (7,916 seq) <i>Actinobacteria</i> 3,4 7,915 seq) <i>Corynebacteriales</i> 9,4 (7,913 seq) Negative for <i>Mycobacteriaceae</i>	0	0	Environ mental	ND	ND	[54]
Yes	Myc 657	<i>Mycobacterium</i> subdivision (mycolic acid- containing actino mycetes)	16S	AGTCTC CCCTGY AGTA Translated: AGTCTCC CCTGCAG TAAGTCT CCCCTGT AGTA	657	656	Oligo	30	<i>Bacteria</i> 0,52; 0,51 (8,142; 7,881 seq) <i>Actinobacteria</i> 3,5; 3,4 (8,137; 7,881 seq) <i>Corynebacteriales</i> 9,5; 9,319 (7,982; 7,879 seq) <i>Mycobacteriaceae</i> 96,0 (5,556; 5,557 seq)	96,0; 96,0 (5,552; 5,553 seq)	100 (1,915 seq)	Environ mental Clinical	ND 96.6	ND 100	[56] [68]
Yes	MYCH	<i>M. chelonae</i>	16S	TTCAGTA GGGACGA AGCGA AAGT	ND by author	437	Oligo	ND	<i>Bacteria</i> 0,03 (515 seq) <i>Actinobacteria</i> 0,22 (515 seq) <i>Corynebacteriales</i> 0,53 (455 seq) <i>Mycobacteriaceae</i> 6,3 (366 seq)	6,3(366 seq)	0	Environ mental array studies	ND	ND	[69]
Yes	Mycoba1	<i>Mycobacterium</i>	16S	CGGCACG GATCCCA AGGAAG	839	839	Oligo	ND	<i>Bacteria</i> 0,32 (5,006 seq) <i>Actinobacteria</i> 2,2 (5,004 seq) <i>Corynebacteriales</i> 5,9 (4,993 seq) <i>Mycobacteriaceae</i> 86 (4,993 seq)	86 (4,993 seq)	99,8 (1,911 seq)	Environ mental array studies	ND	ND	[70]
Yes	Mycoba2	<i>Mycobacterium</i>	16S	ACGGCAC GGATCCC AAGGAA	840	840	Oligo	ND	<i>Bacteria</i> 0,35 (5,375 seq) <i>Actinobacteria</i> 2,3 (5,373 seq) <i>Corynebacteriales</i> 6,35,361 seq) <i>Mycobacteriaceae</i> 93 (5,361 seq)	93 (5,361 seq)	99,8 (1,911 seq)	Environ mental array studies	ND	ND	[70]

Yes	Mycoba3	<i>Mycobacterium</i>	16S	CACGGAT CCCAAGG AAGGAA	836	836	Oligo	ND	Bacteria 0,31 (4,874 seq) Actinobacteria 2,1 (4,871 seq) Corynebacteriales 5,8 (4,860 seq) Mycobacteriaceae 84,0 (4,860 seq)	84,0 (4,860 seq)	99,9 (1,913 seq)	Environ mental array studies	ND	ND	[70]
Yes	Mycoba4	<i>Mycobacterium</i>	16S	TCCCAAG GAAGGAA ACCCAC	831	831	Oligo	ND	Bacteria 0,31 (4,880 seq) Actinobacteria 2,1 (4,876 seq) Corynebacteriales 5,8 (4,865 seq) Mycobacteriaceae 84,0 (4,864 seq)	84,1 (4,864 seq)	99,9 (1,913 seq)	Environ mental array studies	ND	ND	[70]
Yes	MYFO	<i>M. fortuitum</i>	16S	TTCAATA GGGACGA AGCGC AAGT	ND by author	436	Oligo	ND	Bacteria 0,04 (569 seq) Actinobacteria 0,25 (569 seq) Corynebacteriales 0,67 (569 seq) Mycobacteriaceae 9,8 (569 seq)	9,8(569 seq)	0	Environ mental array studies	ND	ND	[69]
Yes	MYMA	<i>M. fortuitum</i>	16S	GACGAAG GTTCCGG TTTTTC TCG	ND by author	446	Oligo	ND	Bacteria 0,01 (120 seq) Actinobacteria 0,1 (120 seq) Corynebacteriales 0,1 (120 seq) Mycobacteriaceae 2,1 (120 seq)	2,1(120 seq)	0	Environ mental array studies	ND	ND	[69]
Not	NTM (OK623)	Nontuber culous myco bacterium	16S	CGGTCGCC CATTACGG CCAGCGG GTAATGC ***	1099	1126	PNA	30	Bacteria 0,71 (11,033 seq) Actinobacteria 1,2 (2,674 seq) Corynebacteriales 1,3 (1,104 seq) Mycobacteriaceae 19 (1,103 seq)	19 (1,103 seq)	0	Clinical	100 57 (LJ)- 100 (MGIT)	100 100	[58] (66) (71)
Not	"Unnamed"	<i>M. immuno genum</i>	16S	CATGCGG TCCTATC	ND by author	177	PNA	30	Bacteria 0,01 (164 seq) Actinobacteria 0,1 (164 seq) Corynebacteriales 0,19 (163 seq) Mycobacteriaceae 2,8 (163 seq)	2,8 (163 seq)	0	Clinical	ND	ND	(72)

F: Formamide, **LJ:** Lowenstein Jensen, **ND:** Not determined, **MTC:** *Mycobacterium tuberculosis* complex, **NTM:** Nontuberculous mycobacteria.

*The *E. coli* position listed by the publications may occasionally, due to different (outdated) alignment policies, differ from the ones observed in the Silva database that we used for the evaluation of these probes.

For example, the MTC probe with the *E. coli* position 1224-1262 according to reference [58] is in our database 1278-1292. Such shifts can be observed for several of the other probes listed in Tables 1 and 2. Updated *E.coli* positions based on our database is shown in the next column.

**The general probe specificity was determined online using the function test probe in the Silva database version 126 from 4th of April 2016 (<http://www.arb-silva.de/search/testprobe/>) containing quality checked near full length gene sequences (minimum length 1200 nuc.) within the SSU REF (1,553,095 SSU gene sequences from the domain Bacteria). The probe specificity of *Mycobacterium* was checked in the ARB software, using the downloaded databases of *Mycobacteriaceae*, after phylogenetic evaluation of the affiliations (odd sequences were excluded). Amount of sequences of the *Mycobacterium* sequences in the downloaded SSU REF database: 5,805. Amount of the *Mycobacterium tuberculosis* complex SSU REF data base: 1,915 sequences.

*** Probe match evaluations were done on the complementary version.

Table 2: FISH probes targeting either the 16S rRNA or the 23S rRNA genes for the identification of different taxonomical levels of *Mycobacterium* spp. – from all organisms, domain, phylum, order, family, genus, complex to species level. Probes were downloaded from the ProbeBase (<http://probebase.csb.univie.ac.at/>), or if not listed there, retrieved from publications.

In probe base	Taxonomical level	Probe name	Target rRNA	Sequence (5'-3')	<i>E. coli</i> position (59)'	<i>E. coli</i> start position in this study	Probe type	F (%)	Hits within the SSU or the LSU 126 REF database** (%)	Hits within the genus <i>Mycobacterium</i> ** (%)	Hits within the tuberculosis complex** (%)	Reference
Yes	All	Univer 1390	16S	GACGGG CGGTGT GTACAA	1390	1389	Oligo	0	All organisms 18 (874,979 seq) <i>Bacteria</i> 49 (768,276 seq) <i>Actinobacteria</i> 28 (64,348 seq) <i>Corynebacteriales</i> 13 (10,637 seq) <i>Mycobacteriaceae</i> 66 (3,841 seq)	66 (3,837 seq)	99,8 (1,911 seq)	[73]
Yes	Domain bacteria	EUB 338	16S	GCTGCC TCCCGT AGGAGT PNA: CTG CCTCCC GTAGGA	338	337	Oligo/ PNA	0-50	<i>Bacteria</i> 92 (1,432,610 seq) <i>Actinobacteria</i> 96 (221,318 seq) <i>Corynebacteriales</i> 98 (82,438 seq) <i>Mycobacteriaceae</i> 98 (5,682 seq)	98 (5,678 seq)	100 (1,915 seq)	[60] [74]
Yes	Phylum <i>Planctomycetales</i> not targeted by EUB	EUB 338_II	16S	GCAGCC ACCCGT AGGTGT	338	337	Oligo	0-50	<i>Bacteria</i> 0,5 (7,742 seq) <i>Actinobacteria</i> 0,002 (5 seq) <i>Corynebacteriales</i> and <i>Mycobacteriaceae</i> 0	0	0	[75]
Yes	Phylum <i>Verrucomicrobiales</i> not targeted by EUB	EUB 338_III	16S	GCTGCC ACCCGT AGGTGT	338	337	Oligo	0-50	<i>Bacteria</i> 1,0 (16,124 seq) <i>Actinobacteria</i> 0,003 (7 seq) <i>Corynebacteriales</i> and <i>Mycobacteriaceae</i> 0	0	0	[75]
Yes	Phylum <i>Actinobacteria</i>	HGC69a HGC69a competitor	23S	TATAGT TACCAC CGCCGT TATAGT TACGGC CGCCGT	1901	1900	Oligo	25	<i>Bacteria</i> 7,3 (4,211 seq) <i>Actinobacteria</i> 97,0 (4,211 seq) <i>Corynebacteriales</i> 99,8 (2,521 seq) and <i>Mycobacteriaceae</i> 99,8 (2,155 seq)	99,9 (2,154 seq)	99,9 (1,872 seq)	[76]
Not	Genus <i>Mycobacterium</i>	Myco 1	23S	TGTCCC TGACTC GCAGGC	579	578	Oligo	0**	<i>Bacteria</i> 3,7 (2,130 seq) <i>Actinobacteria</i> 49 (2,130 seq) <i>Corynebacteriales</i> 50 (2,130 seq) <i>Mycobacteriaceae</i> 99 (2,130 seq)	99 (2,157 seq)	99,7 (1,869 seq)	This study, based on [77]

Yes	<i>M. tuberculosis</i> strains	MTB770	16S	CACT ATTG ACAC GCG CGT	770	652	Oligo	20	<i>Bacteria</i> 3,1 (3,704 seq) <i>Actinobacteria</i> 47,0 (3,704 seq) <i>Corynebacteriales</i> 74,6 (3,704 seq) <i>Mycobacteriaceae</i> 85,1 (1,871 seq)	85,1 (1,871 seq)	99,8 (1,871 seq)	[63]
Yes	<i>M. avium</i> complex	MAC2543	23S	ACGCCA CTCAC CCAAA	2543	651	Oligo	ND	<i>Bacteria</i> 0,1 (86 seq) <i>Actinobacteria</i> 1,7 (86 seq) <i>Corynebacteriales</i> 2,6 (86 seq) <i>Mycobacteriaceae</i> 3,0 (86 seq)	3,0 (86 seq)	0	[62]
Yes	<i>M. avium</i> complex	MAVP515	23S	TGTCCA TGCATG CGGTTT	515	268	Oligo	ND	<i>Bacteria</i> 0,12 (68 seq) <i>Actinobacteria</i> 1,6 (68 seq) <i>Corynebacteriales</i> 2,7 (68 seq) <i>Mycobacteriaceae</i> 3,2 (68 seq)	3,2 (69 seq)	0	[62]
Yes	<i>M. intracellulare</i>	MIN351	23S	AGGT AGAG CTGA GATG TAT CCT	351	268	Oligo	ND	<i>Bacteria</i> 0,02 (14 seq) <i>Actinobacteria</i> 0,32 (14 seq) <i>Corynebacteriales</i> 0,55 (14 seq) <i>Mycobacteriaceae</i> 0,65 (14 seq)	0,65 (14 seq)	0	[62]
Yes	<i>M. avium</i> complex	MIN1586	23S	CCCCGA AACTCC ATGCC	1586	1419	Oligo	ND	<i>Bacteria</i> 0,03 (16 seq) <i>Actinobacteria</i> 0,37 (16 seq) <i>Corynebacteriales</i> 0,63 (16 seq) <i>Mycobacteriaceae</i> 0,74 (16 seq)	0,74 (16 seq)	0,05 (1 seq)	[62]
Not	<i>M. tuberculosis</i> complex	MycTub1	23S	GCCCCA GAAGTC CACACC	1419	1419	Oligo	0***	<i>Bacteria</i> 3,2 (1,832 seq) <i>Actinobacteria</i> 42 (1,832 seq) <i>Corynebacteriales</i> 72 (1,832 seq) <i>Mycobacteriaceae</i> 85, (1,832 seq)	85 (1,832 seq)	97,8 (1,832 seq)	This study, based on [77]
Yes	Non-Bacteria	Non-EUB	16S	ACTCCTA CGGGAG GCAGC	-	337	Oligo	ND	Opposite results to EUB 338			[78]
Not	<i>M. tuberculosis</i> complex	OKG682	23S	CGTAGA TTGGAG CTTGCA TCTAACC TCG AA****	ND by author	2191	PNA	30	<i>Bacteria</i> 3,2 (1,866 seq) <i>Actinobacteria</i> 43 (1,866 seq) <i>Corynebacteriales</i> 74 (1,866 seq) <i>Mycobacteriaceae</i> 86 (1,866 seq)	87 (1,866 seq)	100 (1,784 seq)	[71]

Abbreviations: F=formamide. ND=not determined. seq=sequence.

*The *E. coli* position listed by the publications may occasionally, due to different (outdated) alignment policies, differ from the ones observed in the Silva database that we used for the evaluation of these probes. For example, the MTC probe with the *E. coli* position 1224-1262 according to reference [58] is in our database 1278-1292. Such shifts can be observed for several of the other probes listed in tables 1 and 2.

***The general probe specificity was determined online using the function test probe in the Silva database version 126 from 4th of April 2016 (<http://www.arb-silva.de/search/testprobe/>) containing quality checked near full length gene sequences (minimum length 1200 nuc.) within the SSU REF (with 1,553,095 sequences of *Bacteria*, 229,484 sequences of *Actinobacteria*, 84,442 sequences of *Corynebacteriales*, 5,784 sequences of *Mycobacteriaceae*) and the LSU REF (with 57,473 sequences of *Bacteria*, 4,347 sequences of *Actinobacteria*, 2,527 sequences of *Corynebacteriales*, 2,158 sequences of *Mycobacteriaceae*) database, respectively. The probe specificity of the *Mycobacterium tuberculosis* complex was checked in the ARB software, using the downloaded databases of *Mycobacteriaceae*, after phylogenetic evaluation of the affiliations (odd sequences with unoptimal alignments were excluded). Amount of sequences of the *Mycobacterium tuberculosis* complex in the downloaded SSU REF database: 1,915; in the downloaded LSU REF database 1,874.

***The probe was only tested at 0% formamide concentration.

**** Probematch evaluations were done on the complementary version.

There are several benefits with PNA probes, such as that they are uncharged and have a high conformational flexibility, which will make it easy for them to diffuse through rigid, hydrophobic cell walls, such as those of mycobacteria. Both the sensitivity and specificity of the PNA FISH method is high. Although some new studies have reported that PNA-FISH did not always produce better probe signals than DNA-FISH [79], PNA-FISH has nevertheless been used with great success as a rapid, reliable and easy screening method in clinical applications on different kinds of samples (e.g. sputum, lymph nodes, blood, Lowenstein-Jensen positive cultures and MGIT positive culture) (60,61,65,66,68,72,80-82). Figure 2 shows examples of images produced by both oligonucleotide- and PNA-FISH, respectively, of *Mycobacterium* spp. from MGIT positive culture [68].

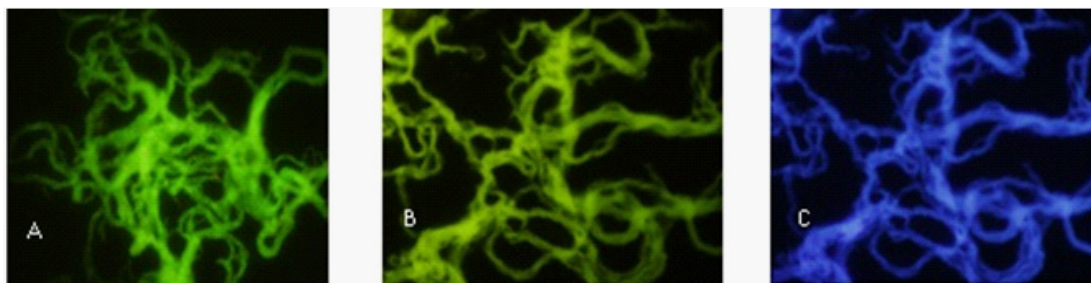


Figure 2: Images by Oligo- and PNA-FISH of *Mycobacterium* spp. from MGIT positive culture (A). PNA-FISH with MTC specific probe labeled with FITC (1000X magnification), (B). *Mycobacterium* spp. by Oligo-FISH with Myc657 oligonucleotide probe labeled with FITC (1000X magnification), (C). DAPI-positive sample (1000X magnification).

Although PNA FISH has in most cases been used with such success, this method has also some few drawbacks. For example, compared to oligonucleotide FISH, PNA FISH is more expensive and the probes are shorter (and may thus in certain cases become less unspecific). Furthermore, the amount of different PNA probes is rather low compared to the relatively high amount of oligonucleotide rRNA probes that have been developed since the late 1980s (<http://probase.csb.univie.ac.at/>). Thus, there are less options for employing multiple FISH probes on a wide range of different species. Furthermore, neither negative probe controls (“nonsense” probes like the non-EUB probe [78], used in oligonucleotide FISH to check for unspecific binding of the fluorochrome (“false-positive”) to the sample), are available as a PNA probe, nor have proper hierarchic probe sets been developed. The latter, a hierarchic probe set based on e.g. three different probes targeting different taxonomical levels (e.g. phylum, genus, species) of the target organism enables a reliable top to bottom down identification, which is crucial due to the vast, still largely unknown genetic diversity of all organisms on Earth. In addition, the pattern of probe overlaps identified via colour overlaps can be used to deduce different taxonomical relationships in a sample with unknown microbial composition (Figure 1). So far, extensive hierarchic probe sets for the *Mycobacterium tuberculosis* complex have only been developed for standard oligonucleotide FISH probes targeting either the 16S rRNA or the 23S rRNA gene.

Although a range of different probes have been developed, it is difficult to evaluate them systematically, since the different studies use occasionally different fixation procedures and FISH protocols. For some cases, it is not even obvious if all proper controls against false-positives and false-negatives have been used, if the used formamide concentration is optimal, and in other cases it has simply not even been determined. Furthermore, some of the 16S and 23S rRNA mycobacteria and other general probes were designed over 10-20 years ago when the amount of recognized mycobacterial taxa and ribosomal gene sequences was considerably lower compared to today. We therefore performed updated phylogenetic analyses and probematches of both the 16S and the 23S rRNA genes for the genus *Mycobacterium*, the *Mycobacterium tuberculosis* complex and for some of the higher taxonomical levels within its phylum *Actinobacteria*. For this we evaluated the SSU (small subunit, the 16S and the 18S rRNA genes) and the LSU (large subunit, the 23S and the 28S rRNA genes) gene sequences from the curated ribosomal Silva database (version 126, [83], <https://www.silva-arb.de>), in order to perform a critical comparative study. This was made possible with the Silva database since it is based on only high quality sequences from the NCBI database, thus several sequences of rather low quality have been excluded (<http://www.ncbi.nlm.nih.gov/>). Another benefit with the Silva database is that it includes not only its own classification systems, Silva and LTP (the Living-Tree_Project, <http://www.arb-silva.de/projects/living-tree/>), but also the other commonly used taxonomical systems in other gene databases such as EMBL (<http://www.embl.de/>), green genes (<http://greengenes.lbl.gov/cgi-bin/nph-index.cgi>), and RDP (<https://rdp.cme.msu.edu/>). The sequence data were evaluated online as well as downloaded and further evaluated by the bioinformatic software package ARB, where both the alignment was checked and different reconstructions of phylogenetic trees were performed ([84, www.arb-home.de]). Despite the larger amount of gene sequences available today, there are several obstacles for a proper evaluation of these sequence data:

- 1) Different gene databases e.g. Silva (<https://www.silva-arb.de>), EMBL (<http://www.embl.de/>), RDP (<https://rdp.cme.msu.edu/>), green genes (<http://greengenes.lbl.gov/cgi-bin/nph-index.cgi>), NCBI (<http://www.ncbi.nlm.nih.gov/>) employ different criteria to classify the various gene sequences, thus the amount of sequences classified as mycobacteria may differ between these gene databases (Table 3);
- 2) The amount of ribosomal gene sequences is relatively high for the *Mycobacterium tuberculosis* complex, but low for several other mycobacterial taxa-several cultured taxa as well as uncultured species are unfortunately only represented by one or a few partial gene sequences.
- 3) A large amount of the gene sequences are retrieved from unculturable organisms and are of a low quality (they are either too short or contain too many unambiguities).

Table 3: Summary of amount of SSU (16S/18S rRNA) and LSU (23S/28S rRNA) gene sequences on different taxonomical levels - from the domain of *Bacteria* down to the species level -for different types of online gene databases. The data are based on the online databases Silva SSU and Silva LSU (version 126, of 4th of April 2016), respectively, and on NCBI (1st of April 2016).

The ribosomal gene sequence data for the family *Mycobacteriaceae* were downloaded and evaluated by the bioinformatic software package arb (www.arb-home.de).

Type of gene	Database & classification	Domain	Phylum	Order	Family	Genus	Complex	Species
		<i>Bacteria</i>	<i>Actino bacteria</i>	<i>Coryne bacteriales</i>	<i>Mycobacte riaceae</i>	<i>Mycobacterium</i>	Tuberculosis ¹	<i>M. tuberculosis</i>
SSU	Silva tax ²	4,385,526	467,761	112,320	19,836	19,828	2,013	1,916
	Silva REF tax ³	1,553,095	229,484	84,442	5,811	5,805	1,915	1,830
	LTP tax ⁴	11,135	2,804	nd	166	163	5	1
	EMBL tax ³	4,454,827	139,859	21,866	10,112	9,997	3,835	3,713
	RDP tax ³	3,018,550	344,385	nd	12,043	11,998	127	94
	Green genes ³	1,130,575	171,685	nd	2,838	2,740	83	64
LSU	Silva tax ⁵	120,306	9,247	5,067	4,431	4,428	3,736	3,362
	Silva tax REF ⁵	57,473	4347	2,527	2,181	2,178	1,874	1,798
	EBML tax REF ⁵	102,113	8523	4,977	4,392	4,389	3,752	3,647
Full genomes	NCBI	nd	nd	nd	nd	406	48 ⁵	25

Abbreviations and weblinks: EMBL (<http://www.embl.de/>), LSU=large subunit, LTP (<http://www.arb-silva.de/projects/living-tree/>), nd=not determined, NCBI (<http://www.ncbi.nlm.nih.gov/>), RDP (<https://rdp.cme.msu.edu/>), green genes (<http://greengenes.lbl.gov/cgi-bin/nph-index.cgi>), Silva (www.silva-arb.de), SSU=small subunit, tax=taxonomy.

¹ Consisting of *M. africanum*, *M. bovis*, *M. bovis BCG*, *M. canetti*, *M. caprae*, *M. microti*, *M. mungi*, *M. orygis*, *M. pinnipedii*, *M. suricattae*, *M. tuberculosis*.

²The full Silva database (called PARC online) including full as well as partial gene sequences.

³The SSU REF database- a database that contains both culturable as well as unculturable organisms with gene sequences of good quality and over 1200 nuc. long (for Bacteria):

Mycobacterium africanum, (28 sequences), *M.bovis+bovis BCG* (43 sequences), *M. canetti* (9 sequences), *M. caprae* (2 sequences), *M. microti* (1 sequence), *M. orygis* (1 sequence), *M. pinnipedii* (1 sequence), *M. tuberculosis* (1830 sequences). 16S rRNA gene similarity within this group is 99-100% (for some most likely misidentified or badly sequenced species, the similarity was around or lower than 96 or higher % but these were excluded from the calculations. This database includes several different taxonomical classifications –based on Silva, LTP, EMBL, RDP and green genes.

⁴The LTP SSU database– a database extracted from the Silva database (<http://www.arb-silva.de/projects/living-tree/>) which contains only culturable organisms with gene sequences of good quality: *M. africanum*, *M. caprae*, *M. microti*, *M. pinnipedii*, *M. tuberculosis* (one sequence of each).

⁵The LSU REF database - a database that contains both culturable as well as unculturable organisms with gene sequences of good quality and over 1900 nuc. long: *M. africanum* (27 sequences), *M. bovis+bovis BCG* (39 sequences), *M. canetti* (9 sequences), *M. orygis* (1 sequence), *M. tuberculosis* (1,798 sequences). 23S rRNA gene similarity within this group is 99-100 %. This database includes several different taxonomical classifications –based on Silva, LTP, EMBL, RDP and green genes.

⁶Full genome sequences – from the genome database of 1st of April 2016, from the NCBI website www.ncbi.nlm.nih.gov/: *M. bovis+bovis BCG* (10 sequences), *M. canetti* (9 sequences), *M. caprae* (1 sequence), *M. microti* (1 sequence), *M. mungi* (1 sequence), *M. orygis* (1 sequence), *M. tuberculosis* (25 sequences).

Since it is well known that the gene sequences of some of the clinically important clusters such as the *Mycobacterium tuberculosis* cluster are difficult to classify and to resolve, especially on the strain and substrain level [19,21,85-87], it is therefore obvious that further improved sequencing of ribosomal genes, other genes and/or genomes, of different mycobacteria is strongly needed. This will enable the discovery of more suitable gene targets and design of more accurate probes.

Thus, for the time being, no distinction can be made for different strains within e.g. the *Mycobacterium tuberculosis* complex, by neither oligonucleotide FISH nor PNA-FISH. Along with expanded sequencing efforts, this limit can eventually most likely be overcome by either: i) targeting other genes more relevant for a further resolution of e.g. different strains and substrains, and/or ii) employing other FISH-protocols such as mRNA-CARD-FISH, RING-FISH, gene-FISH, LNA-FISH or CPRINS-FISH (32,33,40). Although some of these protocols may be tedious and expensive in the beginning, we postulate that the further development of such advanced FISH methods (or even novel FISH protocols) will become easier, cheaper and open up new application possibilities. These will allow more specific identifications and provide additional information about the activity level and function– which is for the time being not possible with neither the oligonucleotide FISH nor the PNA-FISH developed for mycobacteria. Furthermore, if these new

FISH methods and probes are combined with other methods based on e.g. radioactive compounds, stable isotopes or RAMAN spectroscopy, and with FISH probes targeting other associated species (e.g. other prokaryotes, eukaryotic host cells like amoebae, or even bacteriophages), it will then become possible to retrieve systems ecological information on a single cell basis. This will then in turn enable a total new approach to explore the molecular and microbial ecology of mycobacteria in disease processes and how they survive in the environment in general.

Summary: Principally, the FISH method is easy, quick and inexpensive and can be used to visualize, identify and quantify different taxonomical levels of mycobacteria, including several clinical clusters such as the *Mycobacterium tuberculosis* and the *avium* cluster in their natural environment. The main limits today is the lack of more specific probes (e.g. on the strain level within the clusters) that can also reveal activity status and function (e.g. pathogenic potential or antibiotic resistance). Current obstacles can be overcome through: i) improved screening and classification of mycobacteria so that more informative gene targets can be identified and even more efficient and accurate gene probes can be developed; ii) develop other FISH protocols in combination with other analytical methods to retrieve more information about activity, function, *in situ* physiology and association to other species. Together with other molecular cell extract (“black box”) based and chemical approaches, this combined approach may then open up new, interesting ways to explore the *in situ* molecular and microbial ecology of mycobacteria in different environments.

References

1. WHO: Global Tuberculosis Report. 2015.
2. WHO. Global Health Observatory Data. Tuberculosis. 2016.
3. Zaman K. Tuberculosis: a global health problem. J Health Popul Nutr. 2010; 28: 111-113.
4. Matteelli A, Migliori GB, Cirillo D, Centis R, Girard E, et al. Multidrug-resistant and extensively drug-resistant *Mycobacterium tuberculosis*: epidemiology and control. Expert Rev Anti Infect Ther. 2007; 5: 857-871.
5. Sheno S, Heysell S, Moll A, Friedland G. Multidrug-resistant and extensively drug-resistant tuberculosis: consequences for the global HIV community. Curr Opin Infect Dis. 2009; 22: 11-17.
6. Metchock BG, Nolte FS, Wallace RJ Jr. *Mycobacterium*. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Tenover RH, editors. Manual of Clinical Microbiology. Washington, 1999; 399-437.
7. Forbes BA, Sahm DF, Weissfeld AS. Bailey and Scott's Diagnostic Microbiology, 2002; 538-571.
8. Neonakis IK, Gitti Z, Krambovitis E, Spandidos DA. Molecular diagnostic tools in mycobacteriology. J Microbiol Methods. 2008; 75: 1-11.
9. Palomino JC. Nonconventional and new methods in the diagnosis of tuberculosis: feasibility and applicability in the field. Eur Respir J. 2005; 26: 339-350.
10. Kim JH, Lee KH, Cangelosi GA, Chung JH. Immunofluorescence microtip sensor for point-of-care tuberculosis (TB) diagnosis. Methods Mol Biol. 2015; 1256: 57-69.
11. Said HM, Ismail N, Osman A, Velsman C, Hoosen AA. Evaluation of TBc identification immunochromatographic assay for rapid identification of *Mycobacterium tuberculosis* complex in samples from broth cultures. J Clin Microbiol. 2011; 49: 1939-1942.
12. Tang M, McEwen GD, Wu Y, Miller CD, Zhou A. Characterization and analysis of mycobacteria and Gram-negative bacteria and co-culture mixtures by Raman microspectroscopy, FTIR, and atomic force microscopy. Anal Bioanal Chem. 2013; 405: 1577-1591.
13. Stöckel S, Stanca AS, Helbig J, Rösch P, Popp J. Raman spectroscopic monitoring of the growth of pigmented and non-pigmented mycobacteria. Anal Bioanal Chem. 2015; 407: 8919-8923.

14. Mühlig A, Bocklitz TW, Labugger I, Dees S, Henk S, et al. LOC-SERS: A promising closed system for the identification of mycobacteria. *Anal Chem*. 2016; 88: 7998-8004.
15. Rodríguez D, Daniels AU, Urrusti JL, Wirz D, Braissant O. Evaluation of a low-cost calorimetric approach for rapid detection of tuberculosis and other mycobacteria in culture. *J Appl Microbiol*. 2011; 111: 1016-1024.
16. Fredricks DN, Relman DA. Improved amplification of microbial DNA from blood cultures by removal of the PCR inhibitor sodium polyanetholesulfonate. *J Clin Microbiol*. 1998; 36: 2810-2816.
17. Soini H, Musser JM. Molecular diagnosis of mycobacteria. *Clin Chem*. 2001; 47: 809-814.
18. Wolff A, Perch-Nielsen IR, Poulsen CR, El-Ali J, Bang DD. Removal of PCR inhibitors using dielectrophoresis for sample preparation in a microfabricated system. 7th. International Conference on Miniaturized Chemical and Biological Analysis Systems, Squaw Valley, California USA. 2003; 1137-1140.
19. Filliol I, Motiwala AS, Cavatore M, Qi W, Hazbón MH, et al. Global phylogeny of *Mycobacterium tuberculosis* based on single nucleotide polymorphism analysis: insights into tuberculosis evolution, phylogenetic accuracy of other DNA fingerprinting systems, and recommendations for a minimal standard SNP set. *J Bacteriol*. 2006; 188: 759-772.
20. Midha M, Prasad NK, Vindal V. MycoRRdb: a database of computationally identified regulatory regions within intergenic sequences in mycobacterial genomes. *PLoS One*. 2012; 7: e36094.
21. Rodríguez-Campos S, Smith NH, Boniotti MB, Aranaz A. Overview and phylogeny of *Mycobacterium tuberculosis* complex organisms: implications for diagnostics and legislation of bovine tuberculosis. *Res Vet Sci*. 2014; 97: S5-S19.
22. Zhang H, Li D, Zhao L, Fleming J, Lin N, et al. Genome sequencing of 161 *Mycobacterium tuberculosis* isolates from China identifies genes and intergenic regions associated with drug resistance. *Nat Genet*. 2013; 45: 1255-1260.
23. Amann R, Fuchs BM. Single-cell identification in microbial communities by improved fluorescence in situ hybridization techniques. *Nat Rev Microbiol*. 2008; 6: 339-348.
24. Gall JG, Pardue ML. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proc Natl Acad USA*. 1969; 63:378-383.
25. John HA, Birnstiel ML, Jones KW. RNA-DNA hybrids at the cytological level. *Nature*. 1969; 223: 582-587.
26. Kricka LJ. Nucleic Acid Hybridization Test Formats: Strategies and Applications. In: Kricka LJ, editor. *Nonisotopic DNA Probe Techniques*. Academic Press, Inc. California. 1992; 3-28.
27. Döhner H, Pohl S, Bulgay-Mörschel M, Stiglenbauer S, Bentz M, et al. Trisomy 12 in chronic lymphoid leukemias--a metaphase and interphase cytogenetic analysis. *Leukemia*. 1993; 7: 516-520.
28. Giovannoni SJ, DeLong EF, Olsen GJ, Pace NR. Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. *J Bacteriol*. 1988; 170: 720-726.
29. DeLong EF, Wickham GS, Pace NR. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. *Science*. 1989; 243: 1360-1363.
30. Amann RI, Krumholz L, Stahl DA. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J Bacteriol*. 1990; 172: 762-770.
31. Møter A, Göbel UB. Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. *J Microbiol Methods*. 2000; 41: 85-112.
32. Wagner M, Haider S. New trends in fluorescence in situ hybridization for identification and functional analyses of microbes. *Curr Opin Biotechnol*. 2012; 23: 96-102.
33. Lee NM, Meisinger DB, Schmid M, Rothballer M, Löffler FE. Fluorescence in situ hybridization in geomicrobiology. In: Reitner HJ, Thiel V, editors. *Encyclopedia in Geobiology*. Berlin: Springer Verlag. 2011; 854.
34. Fuchs BM, Glöckner FO, Wulf J, Amann R. Unlabeled helper oligonucleotides increase the in situ accessibility to 16S rRNA of fluorescently labeled oligonucleotide probes. *Appl Environ Microbiol*. 2000; 66: 3603-3607.
35. Wagner M, Horn M, Daims H. Fluorescence in situ hybridisation for the identification and characterisation of prokaryotes. *Curr Opin Microbiol*. 2003; 6: 302-309.
36. Zwirgmaier K. Fluorescence in situ hybridisation (FISH)--the next generation. *FEMS Microbiol Lett*. 2005; 246: 151-158.
37. Kenzaka T, Tamaki S, Yamaguchi N, Tani K, Nasu M. Recognition of individual genes in diverse microorganisms by cycling primed in situ amplification. *Appl Environ Microbiol*. 2005; 71: 7236-7244.
38. Bentolila LA, Weiss S. Single-step multicolor fluorescence in situ hybridization using semiconductor quantum dot-DNA conjugates. *Cell Biochem Biophys*. 2006; 45: 59-70.

39. Stoecker K, Dorninger C, Daims H, Wagner M. Double labeling of oligonucleotide probes for fluorescence in situ hybridization (DOPE-FISH) improves signal intensity and increases rRNA accessibility. *Appl Environ Microbiol.* 2010; 76: 922-926.
40. Moraru C, Lam P, Fuchs BM, Kuypers MM, Amann R. GeneFISH--an in situ technique for linking gene presence and cell identity in environmental microorganisms. *Environ Microbiol.* 2010; 12: 3057-3073.
41. Valm AM, Mark Welch JL, Rieken CW, Hasegawa Y, Sogin ML, et al. Systems-level analysis of microbial community organization through combinatorial labeling and spectral imaging. *Proc Natl Acad Sci U S A* 2011; 108: 4152-4157.
42. Schmidt H, Eickhorst T, Musmann M. Gold-FISH: a new approach for the in situ detection of single microbial cells combining fluorescence and scanning electron microscopy. *Syst Appl Microbiol.* 2012; 35: 518-525.
43. Allers E, Moraru C, Duhaime MB, Beneze E, Solonenko N, et al. Single-cell and population level viral infection dynamics revealed by phageFISH, a method to visualize intracellular and free viruses. *Environ Microbiol.* 2013; 15: 2306-2318.
44. Sakarikou C, Parisato M, Lo Cascio G, Fontana C. Beacon-based (bbFISH®) technology for rapid pathogens identification in blood cultures. *BMC Microbiol.* 2014; 14: 99.
45. Loy A, Maixner F, Wagner M, Horn M. probeBase--an online resource for rRNA-targeted oligonucleotide probes: new features 2007. *Nucleic Acids Res.* 2007; 35: D800-804.
46. Pernthaler A, Dekas AE, Brown CT, Goffredi SK, Embaye T, et al. Diverse syntrophic partnerships from deep-sea methane vents revealed by direct cell capture and metagenomics. *Proc Natl Acad Sci U S A.* 2008; 105: 7052-7057.
47. Zeller P, Ploux O, Méjean A. A simple protocol for attenuating the auto-fluorescence of cyanobacteria for optimized fluorescence in situ hybridization (FISH) imaging. *J Microbiol Methods.* 2016; 122: 16-19.
48. Amann RI. In situ identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes, In: Akkermans ADL, van Elsas JD, De Bruijn FJ (editor), *Molecular Microbial Ecology Manual* 3.3.6. Kluwer Academic Publishers, Dordrecht, The Netherlands, 1995; 1-15.
49. Amann R, Snaird J, Wagner M, Ludwig W, Schleifer KH. In situ visualization of high genetic diversity in a natural microbial community. *J Bacteriol.* 1996; 178: 3496-3500.
50. Pernthaler A, Pernthaler J, Amann R. Sensitive multi-color fluorescence in situ hybridization for the identification of environmental microorganisms. *Molecular Microbial Ecology Manual*, Kluwer Academic Publishers: Netherlands, 2004; 711-726.
51. Mikš-Krajník M, Babuchowski A. 16S rRNA-targeted oligonucleotide probes for direct detection of *Propionibacterium freudenreichii* in presence of *Lactococcus lactis* with multicolour fluorescence in situ hybridization. *Lett Appl Microbiol.* 2014; 59: 320-327.
52. Schatz MC, Phillippy AM, Gajer P, DeSantis TZ, Andersen GL, et al. Integrated microbial survey analysis of prokaryotic communities for the PhyloChip microarray. *Appl Environ Microbiol.* 2010; 76: 5636-5638.
53. Daims H, Lückner S, Wagner M. daime, a novel image analysis program for microbial ecology and biofilm research. *Environ Microbiol.* 2006; 8: 200-213.
54. de los Reyes FL, Ritter W, Raskin L. Group-specific small-subunit rRNA hybridization probes to characterize filamentous foaming in activated sludge systems. *Appl Environ Microbiol.* 1997; 63: 1107-1117.
55. Schuppler M, Wagner M, Schön G, Göbel UB. In situ identification of nocardioform actinomycetes in activated sludge using fluorescent rRNA-targeted oligonucleotide probes. *Microbiology.* 1998; 144 : 249-259.
56. Davenport RJ, Curtis TP, Goodfellow M, Stainsby FM, Bingley M. Quantitative use of fluorescent in situ hybridization to examine relationships between mycolic acid-containing actinomycetes and foaming in activated sludge plants. *Appl Environ Microbiol.* 2000; 66: 1158-1166.
57. Carr EL, Eales K, Soddell J, Seviour RJ. Improved permeabilization protocols for fluorescence in situ hybridization (FISH) of mycolic-acid-containing bacteria found in foams. *J Microbiol Methods.* 2005; 61: 47-54.
58. Stender H, Lund K, Petersen KH, Rasmussen OF, Hongmanee P, et al. Fluorescence In situ hybridization assay using peptide nucleic acid probes for differentiation between tuberculous and nontuberculous mycobacterium species in smears of mycobacterium cultures. *J Clin Microbiol.* 1999; 37: 2760-2765.
59. Brosius J, Dull TL, Sletter DD, Noller HF. Gene organization and primary structure of a ribosomal operon from *Escherichia coli*. *J Mol Biol.* 1981; 148: 107-127.
60. Lefmann M, Schweickert B, Buchholz P, Göbel UB, Ulrichs T, et al. Evaluation of peptide nucleic acid-fluorescence in situ hybridization for identification of clinically relevant mycobacteria in clinical specimens and tissue sections. *J Clin Microbiol.* 2006; 44: 3760-3767.
61. Lehtola MJ, Torvinen E, Miettinen IT, Keevil CW. Fluorescence in situ hybridization using peptide nucleic acid probes for rapid detection of *Mycobacterium avium* subsp. *avium* and *Mycobacterium avium* subsp. *paratuberculosis* in potable-water biofilms. *Appl Environ Microbiol.* 2006; 72: 848-853.

62. St Amand AL, Frank DN, De Groote MA, Pace NR. Use of specific rRNA oligonucleotide probes for microscopic detection of *Mycobacterium avium* complex organisms in tissue. *J Clin Microbiol*. 2005; 43: 1505-1514.
63. St Amand AL, Frank DN, De Groote MA, Basaraba RJ, Orme IM, et al. Use of specific rRNA oligonucleotide probes for microscopic detection of *Mycobacterium tuberculosis* in culture and tissue specimens. *J Clin Microbiol*. 2005; 43: 5369-5371.
64. Ryan GJ, Hoff DR, Driver ER, Voskuil MI, Gonzalez-Juarrero M, et al. Multiple *M. tuberculosis* phenotypes in mouse and guinea pig lung tissue revealed by a dual-staining approach. 2010; 5: e11108.
65. Rodriguez-Nuñez J, Avelar FJ, Marquez F, Rivas-Santiago B, Quiñones C, et al. *Mycobacterium tuberculosis* complex detected by modified fluorescent in situ hybridization in lymph nodes of clinical samples. *J Infect Dev Ctries*, 2012; 6: 58-66.
66. Hongmanee P, Stender H, Rasmussen OF. Evaluation of a fluorescence in situ hybridization assay for differentiation between tuberculous and nontuberculous *Mycobacterium* species in smears of Lowenstein-Jensen and *Mycobacteria* Growth Indicator Tube cultures using peptide nucleic acid probes. *J Clin Microbiol*. 2001; 39: 1032-1035.
67. Kim N, Lee SH, Yi J, Chang CL. Evaluation of dual-color fluorescence in situ hybridization with peptide nucleic acid probes for the detection of *Mycobacterium tuberculosis* and non-tuberculous mycobacteria in clinical specimens. *Ann Lab Med*. 2015; 35: 500-505.
68. Borekci G, Aslan G, Aydin E, Fiandaca MJ, Stender H, et al. [Identification of *Mycobacterium* species from BACTEC MGIT™ positive cultures with Oligo-FISH and PNA-FISH methods]. *Mikrobiyol Bul*. 2014; 48: 385-401.
69. Warsen AE, Krug MJ, LaFrentz S, Stanek DR, Loge FJ, et al. Simultaneous discrimination between 15 fish pathogens by using 16S ribosomal DNA PCR and DNA microarrays. *Appl Environ Microbiol*. 2004; 70: 4216-4221.
70. Kyselková M, Kopecký J, Felföldi T, Cermák L, Omelka M, et al. Development of a 16S rRNA gene-based prototype microarray for the detection of selected actinomycetes genera. *Antonie Van Leeuwenhoek*. 2008; 94: 439-453.
71. Stender H, Mollerup TA, Lund K, Petersen KH, Hongmanee P, et al. Direct detection and identification of *Mycobacterium tuberculosis* in smear-positive sputum samples by fluorescence in situ hybridization (FISH) using peptide nucleic acid (PNA) probes. *Int J Tuberc Lung Dis*. 1999; 3: 830-837.
72. Selvaraju SB, Kapoor R, Yadav JS. Peptide nucleic acid-fluorescence in situ hybridization (PNA-FISH) assay for specific detection of *Mycobacterium immunogenum* and DNA-FISH assay for analysis of pseudomonads in metalworking fluids and sputum. *Mol Cell Probes*. 2008; 22: 273-280.
73. Zheng D, Alm EW, Stahl DA, Raskin L. Characterization of universal small-subunit rRNA hybridization probes for quantitative molecular microbial ecology studies. *Appl Environ Microbiol*. 1996; 62: 4504-4513.
74. Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, et al. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol*. 1990; 56: 1919-1925.
75. Daims H, Brühl A, Amann R, Schleifer KH, Wagner M. The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol*. 1999; 22: 434-444.
76. Roller C, Wagner M, Amann R, Ludwig W, Schleifer KH. In situ probing of gram-positive bacteria with high DNA G + C content using 23S rRNA-targeted oligonucleotides. *Microbiology*. 1994; 140: 2849-2458.
77. Grabowski B. [Nachweis und Identifizierung von Mykobakterien mittels FISH in einem hierarchischen Ansatz]. Supervisor Natuschka Lee and Wolfgang Liebl. Department of Microbiology, TU Muenchen, Germany. 2011.
78. Wallner G, Amann R, Beisker W. Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry*. 1993; 14: 136-143.
79. Rodriguez-Nuñez J, Avelar FJ, Marquez F, Rivas-Santiago B, Quiñones C, et al. *Mycobacterium tuberculosis* complex detected by modified fluorescent in situ hybridization in lymph nodes of clinical samples. *J Infect Dev Ctries*, 2012; 6:58-66.
80. Harris DM, Hata DJ. Rapid identification of bacteria and *Candida* using PNA-FISH from blood and peritoneal fluid cultures: a retrospective clinical study. *Ann Clin Microbiol Antimicrob*, 2013;12: 2.
81. Stender H. PNA FISH: an intelligent stain for rapid diagnosis of infectious diseases. *Expert Rev Mol Diagn*. 2003; 3: 649-655.
82. Yuan L, Ke Z, Ma J, Liu L, Li Y. The fluorescence in situ hybridization for diagnosis of *Mycobacterium tuberculosis* complex in sputum samples. *Ann Clin Lab Sci*. 2015; 45: 631-638.
83. Quast C, Priesse E, Yilmaz P, Gerken J, Schweer T, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res*. 2013; 41: 590-596.
84. Westram R, Bader K, Prüße E, Kumar Y, Meier H, et al. ARB: a software environment for sequence data. In: de Bruijn FJ, editor, *Handbook of Molecular Microbial Ecology I: Metagenomics and Complementary Approaches*, Wiley-Blackwell, 2011.

85. Devulder G, Pérouse de Montclos M, Flandrois JP. A multigene approach to phylogenetic analysis using the genus *Mycobacterium* as a model. *Int J Syst Evol Microbiol*. 2005; 55: 293-302.
86. Djelouadji Z, Raoult D, Daffé M, Drancourt M. A single-step sequencing method for the identification of *Mycobacterium tuberculosis* complex species. *PLoS Negl Trop Dis*. 2008; 2: 253.
87. Tortoli E. Phylogeny of the genus *Mycobacterium*: many doubts, few certainties. *Infect Genet Evol*. 2012; 12: 827-831.