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Establishment of a qPCR Assay for the Detection and Quantification of *Mycobacterium fortuitum* DNA

Application to Sputum Samples from Patients with Suspected Nontuberculous Mycobacterial Infection in Ghana

Dissertation

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Zusammenfassung

Nicht-tuberkulöse-Mykobakterien (NTM) können eine Lungenerkrankung auslösen, die der weltweit grassierenden Lungentuberkulose (TB) sehr ähnlich ist. Jährlich erkranken ca. 10 Millionen Menschen an TB und 1.5 Millionen versterben. Die Prävalenz von NTM-Lungenerkrankungen ist zwar weit niedriger, dennoch ist sie in den letzten Jahrzehnten kontinuierlich angestiegen und stellt somit ein ernstzunehmendes Problem dar. Klinisch und diagnostisch sind Lungenerkrankungen mit TB und NTM schwer voneinander zu unterscheiden. Vor allem in Ländern mit hoher TB-Inzidenz und limitierten labordiagnostischen Ressourcen besteht somit das Risiko, dass NTM-Lungenerkrankungen übersehen und als TB fehldiagnostiziert werden. Eine korrekte Differenzierung beider Erkrankungen ist allerdings von hoher Relevanz für die Therapie und weitere Prognose, da die Erreger der NTM-Lungenerkrankungen häufig resistent gegen bei der TB eingesetzten Medikamente sind. Eine Pilotstudie unserer Arbeitsgruppe in Ghana kam zu dem Ergebnis, dass dort *M. fortuitum* das häufigste NTM bei Patienten mit TB-Verdacht ist. Ähnliche Befunde liegen aus anderen Studien in Afrika vor. Molekularbiologische Verfahren wie quantitative PCR (qPCR) können dazu beitragen, eine schnelle und sichere Diagnose von NTM z.B. aus Sputum oder Kultur, zu ermöglichen. Im Rahmen dieser Arbeit wurden ein quantitatives PCR Assay und DNA-Extraktionsverfahren entwickelt, um *M. fortuitum* aus Patientenproben nachweisen zu können und somit die Differenzierung zu TB zu erleichtern. Als Zielregion für die qPCR dienten das *rpoB Gen* bei *M. fortuitum* und das *Gen/Sequenz IS6110* bei *M. tuberculosis*. Wir verglichen zudem mehrere DNA-Extraktionsmethoden und identifizierten zwei aussichtsreiche Protokolle zur Extraktion mycobakterieller DNA. Die Schlüsselkomponenten der beiden Methoden waren die Nutzung von 1) Chelex® Resin und einem Ultraschallwasserbad, sowie 2) Tween Lyse Puffer, Proteinase K und Lysozym sowie mehreren Koch- und Frier-Zyklen. Andere getestete Methoden erwiesen sich als weniger effektiv. Beim Vergleich der Nachweismethoden wurde bei der Testung von Sputum Kulturen eine hohe Übereinstimmung/Kongruenz zwischen dem *M. fortuitum* qPCR Assay und der Referenzmethode GenoType CM LPA erzielt. Die Nachweisgrenze des *M. fortuitum* qPCR Assay wurde mit 10^2 bis 10^3 CFU/ml bestimmt. Bei der direkten Testung von Sputum Proben mittels unserem *M. tuberculosis* qPCR Assay und der Referenzmethode GeneXpert® Xpert® MTB/RIF Ultra legten erste Experimente eine hohe Sensitivität und Spezifität nahe. Zusammenfassend zeigt unsere Arbeit neue molekularbiologische Methoden zur Identifizierung von *M. fortuitum* auf. Zum genaueren Verständnis der Epidemiologie und pathogenetischen Relevanz von *M. fortuitum* in Ghana wäre eine Genomsequenzierung unserer Proben ein wichtiger nächster Schritt.

Summary

Non-tuberculous mycobacteria (NTM) can cause pulmonary disease, which is clinically very similar to pulmonary tuberculosis (TB). Worldwide, up to 10 million people are infected with TB and 1.5 million die annually. Although the prevalence of NTM infections is much lower, it has been rising continually over the past years. Pulmonary disease with NTM is difficult to distinguish diagnostically from TB. This poses an especial problem in regions with a high incidence of TB, such as the countries in sub-Saharan Africa. A pilot study conducted by our research group found *M. fortuitum* to be the most commonly isolated NTM in presumptive TB patients throughout Ghana, a finding supported by other literature. In contrast to TB, few options for diagnosing NTM infections exist. In resource-limited settings, even less methods are available for differential diagnosis of presumptive TB patients. However, correctly distinguishing between the two infections is crucial for patients, as therapeutic approaches differ significantly. Molecular biological methods such as qPCR may enable a rapid and reliable diagnosis of infections with NTM, for example from sputum or culture samples. We designed custom primers and probes for *M. fortuitum* and *M. tuberculosis* targeting the *rpoB gene* and the *IS6110* respectively. As part of this study several DNA extraction methods were compared and a quantitative PCR assay to identify *M. fortuitum* from culture samples was developed. Two promising methods for the extraction of mycobacterial DNA were identified. Key components of the protocols were the use of 1) Chelex® resin, thermal degradation and ultrasonication, 2) Tween lysis buffer, Proteinase K and lysozyme digestion and repeated thermal degradation. Detection methods achieved a high overall agreement between our *M. fortuitum* qPCR assay and the GenoType CM LPA reference method when testing DNA extracted from sputum culture samples. The limit of detection of our *M. fortuitum* qPCR assay was between 10^2 and 10^3 CFU/ml. When using the Chelex® DNA extraction protocol and our *IS6110* qPCR assay on sputum samples from presumptive TB patients, initial experiments suggested high sensitivity and specificity. To summarize, our results elucidate possible molecular biological methods for the identification of *M. fortuitum*. For a comprehensive understanding of the epidemiologic and pathogenetic relevance of *M. fortuitum* in Ghana, WGS of our samples would be an important next step.

List of Abbreviations

AFB: Acid-fast bacilli

AIDS: Acquired immune deficiency syndrome

ATS: American Thoracic Society

BU: Buruli ulcer disease

CFU: Colony forming units

COPD: Chronic obstructive pulmonary disease

CT: Cut-off threshold

DST: Drug sensitivity testing

HIV: Human immunodeficiency virus

IDSA: Infectious Disease Society of America

IS6110: Insertion Sequence 6110

KCCR: Kumasi Centre for Collaborative Research in Tropical Medicine

LPA: Line probe assay

MAC: *Mycobacterium avium* complex

MGIT: Mycobacteria growth indicator tube

MOTT: Mycobacteria other than Tuberculosis

NGS: Next generation sequencing

NTC: No template control

NTM: Non-tuberculous mycobacteria

NTM-PD: Non-tuberculous mycobacteria pulmonary disease

OADC: Oleic Acid-Dextrose-Catalase

OD_{600}: Optical density at 600 nm

PCR: Polymerase Chain Reaction

qPCR: Quantitative Polymerase Chain Reaction

RFU: relative fluorescent units

rpoB: Gene that encodes the ß-subunit of the bacterial RNA polymerase

TB: Tuberculosis

WHO: World Health Organization

WGS: Whole genome sequencing

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1.0 Introduction

1.1 Relevance of Mycobacteria as Human Pathogens

1.1.1 *M. tuberculosis complex*

According to estimates by the WHO, around 10 million people are sickened by Tuberculosis (TB) every year, 95 % of those live in developing nations. In spite of safe and effective treatment 1.5 million people die each year. TB is one of the most common and deadliest infectious diseases, especially in developing nations. The causative agent of TB are species belonging to the *Mycobacterium tuberculosis complex* [1].

1.1.2 Nontuberculous mycobacteria (NTM) and different species

Apart from *M. tuberculosis complex*, the genus/family of Mycobacteria include a heterogenous group of over 200 so called nontuberculous-mycobacteria or NTM for short, which are ubiquitous in the environment [2-4]. NTM are generally defined as Mycobacteria other than *M. tuberculosis complex* and *M. leprae*. They are synonymously referred to as atypical mycobacteria or MOTT (Mycobacteria other than Tuberculosis) [5]. These NTM can be further divided into slow and rapidly growing Mycobacteria. Several species of NTM have been shown to be pathogenic to humans [6, 7]. NTM constitute an increasingly relevant human pathogen and awareness for NTM diseases has risen recently, especially since the HIV/AIDS-Epidemic [7].

Slow growing NTM are defined as taking seven days or longer to grow in culture. The most commonly encountered slow growing NTM are *M. avium* complex (MAC), *M. kansasii* and *M. ulcerans* [7]. The MAC includes at least ten mycobacterial species, the most important being *M. avium, M. intracellulare* and *M. chimaera* [5, 8]. As with other NTM species, slow-growing Mycobacteria are found ubiquitously in the environment, primarily in contaminated water sources. MAC is the predominant cause of NTM pulmonary disease (NTM-PD) and should also be familiar as a cause of disseminated disease in HIV/AIDS-patients. Additionally, MAC can be a cause of NTM

lymphadenitis. The *M. chimaera* species, which is part of MAC, has specifically been implicated in a global outbreak of extrapulmonary infections during open heart surgery caused by contaminated operating room machinery [5-7, 9, 10]. *M. kansasii* is one of the most pathogenic NTM species and another common cause of NTM-PD, as well as disseminated disease in HIV/AIDS-patients. According to some sources, *M. ulcerans* is the third most common pathogenic mycobacterium worldwide after *M. tuberculosis* and *M. leprae*. It is known for causing the so-called "Buruli ulcer" (BU) disease. This disease is part of the group of neglected tropical skin diseases and characterized by a necrotizing, ulcerating skin lesion. BU disease can be found throughout Africa, Southeast Asia, South America and Australia [5].

Rapidly growing NTM are defined as taking less than seven days to grow in culture. They are also frequently found in the environment, especially in soil and sometimes in municipal water sources. The clinically most important species of rapidly growing NTM are *M. abscessus, M. chelonae* and *M. fortuitum*. The spectrum of disease that these three species cause is similar, ranging from pulmonary infections to skin and (post-traumatic or surgical) wound infections, to catheter infections or even corneal infections [5]. Out of this group of rapidly growing NTM, *M. abscessus* is a common cause of NTM-PD and often affects patients with cystic fibrosis [11]. The other rapidly growing NTM tend to affect patients with pre-existing conditions of the respiratory tract [5-7, 9, 12].

In summary, NTM comprise a group of bacteria that are rich in variety not only regarding their growth characteristics or environmental niches but also their pathogenicity in humans. The spectrum of disease caused by NTM ranges from cutaneous infections, nosocomial infections or pulmonary disease to severe disseminated disease.

1.2 Epidemiology of NTM in the context of TB: Globally and in Africa and Ghana specifically

Recent studies describe NTM as an emerging infectious disease of increasing relevance [10, 13, 14]. The prevalence of NTM-PD is increasing in North America and Europe. In some parts of the US, it may even surpass the prevalence of pulmonary TB [9, 13, 14]. Additionally, evidence of person-to-person transmission for at least *M. abscessus* has emerged. Previously it was assumed that NTM cannot spread directly via person-to-person transmission [15-17].

Several authors have reported on the impact of NTM-PD in the context of TB in Africa. A review and meta-analysis by *Okoi et. al* found a 7.5 % prevalence of pulmonary NTM colonization in patients with presumptive TB [18]. One should however note that, according to the 2007 ATS/IDSA statement on NTM "colonization without infection is an unproven condition" [7]. Out of the different NTM identified, *M. fortuitum* was among the most common species in several African countries. It was the third most common NTM in Kenya, Nigeria and Mali, whilst it was the most common NTM in Uganda [18]. Other more localized studies investigating NTM-PD among suspected TB cases have described a prevalence of up to 23 % in some regions of Africa [19].

Preliminary studies from Ghana suggest that the overall prevalence of NTM could be up to 10 % and that the most common NTM (35 %) in clinical isolates there is *M. fortuitum* [18, 20, 21]. Own unpublished data from a pilot study conducted at the Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR) in Kumasi, Ghana between 2018 and 2020 supports this finding. It identified 78 patients (15 %) infected with NTM out of 521 recruited presumptive TB patients. A total of 28 NTM patient isolates could be characterized as *M. fortuitum* (36 %), making it the most commonly isolated NTM in our pilot study in Ghana [22]. This is also evident in the distribution pattern of NTM cases throughout the different regions in Ghana (Fig. 1).

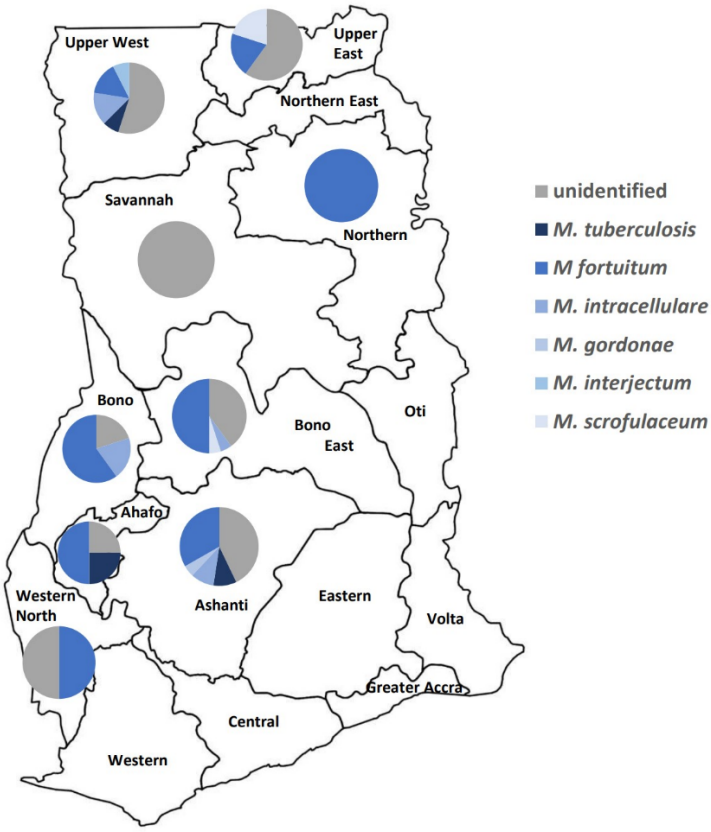


Fig. 1: *M. fortuitum* is most common NTM throughout Ghana.
Map of NTM species distribution in different regions of Ghana. According to unpublished data from a pilot study by our research group, data kindly provided by Rejoice Arthur of KCCR [22]. The pilot study was conducted between 2018 and 2020 at KCCR and recruited a total of 521 presumptive TB patients. It identified 78 patients (15%) as infected with NTM. The most commonly isolated NTM in our study was *M. fortuitum* throughout the different regions of Ghana. Out of the 78 NTM patients, 28 patient isolates could be characterized as *M. fortuitum* (36%). However, a high number of cases throughout could not be comprehensively identified and these are labeled as "unidentified". Figure modified from a map created using mapchart.net.

1.3 NTM pulmonary disease and similarities to TB infection

In 65-80 % of cases, NTM infections present in the form of a pulmonary disease [23]. On the basis of clinical signs and symptoms, a pulmonary infection with NTM is very similar to pulmonary TB. Patients usually present with symptoms such as chronic cough, hemoptysis, fatigue, night sweats and weight loss. Computed tomography or chest radiography imaging of patients with NTM-PD also shows similarities to pulmonary TB with findings such as nodular bronchiectasis or granuloma and cavitation. Currently, there are no methods available for an exact and specific distinction of these two diseases [9, 10, 14]. Several patient groups are especially at risk of NTM-PD. Predisposing factors include immunosuppression, caused either by a disease such as AIDS or pharmacologically by medication such as TNF-alpha-Inhibitors, and a history of underlying conditions and structural changes affecting the lungs such as cystic fibrosis, COPD, bronchiectasis or prior TB [9, 12, 14, 17].

Groups specifically vulnerable to *M. fortuitum* lung disease similarly include those with pre-existing conditions affecting the lungs or respiratory tract such as COPD, achalasia (an esophageal motility disorder) and previous TB infections [12, 24].

1.3.1 Diagnosis and ATS/IDSA-Criteria

Since 1990 the American Thoracic Society (ATS) and the Infectious Disease Society of America (IDSA) have published several joint statements and clinical practice guidelines on the diagnosis and treatment of NTM. The rationale being the rising awareness for NTM-PD in both the AIDS patient population and increasingly also the non-AIDS patient population, as well as improvements in the diagnosis of mycobacteria. Owing to controversy in the NTM field and a lack of sufficient empirical data however, most ATS/IDSA recommendations are based solely on expert opinion [6, 7, 25, 26].

In their 2007 statement, the most recent comprehensive criteria for the diagnosis of NTM-PD were put forth. Although with the limitation that, due to a lack of knowledge, the diagnostic criteria are not necessarily applicable to all NTM pathogens outside of the most common MAC*, M. kansasii* and *M. abscessus*. The diagnostic criteria are divided into two categories: clinical and microbiological. The clinical criteria consist of: “(1)

pulmonary symptoms, characteristic imaging findings such as cavitary opacities on chest x-ray or multifocal nodular bronchiectasis on a high-resolution computed tomography (HRCT) and (2) the appropriate exclusion of other diagnoses, especially TB. The main microbiologic criteria necessitate (1) two separate positive culture results from sputum samples or (2) at least one positive culture from a bronchial lavage/wash or (3) a biopsate of lung tissue showing histopathological features of mycobacterial infection in combination with a positive culture for NTM from either the biopsate, from sputum or from a bronchial lavage. Additionally, expert consultation for infrequently encountered NTM or NTM which might usually represent contamination is recommended. Suspected patients should be followed until a diagnosis can be definitively established or excluded, although a diagnosis of NTM-PD does not automatically necessitate initiation of treatment" [7].

Microbiological and molecular biological methods are necessary for the identification of NTM infections, because the clinical presentation does not allow for a definitive diagnosis or characterization to the species-level [5]. The following diagnostic methods are available. Mycobacteria can be cultured on several different types of medium for microbiological identification. Options include Loewenstein-Jensen Agar and Middlebrook solid or liquid growth medium [27]. The BACTEC™ MGIT™ 960 System manufactured by BD provides an automated approach to culturing and identifying mycobacteria in addition to resistance testing. The so-called mycobacteria growth indicator tubes (MGIT) also use liquid Middlebrook medium [28]. For species-level characterization of NTM after culturing, line probe assays (LPA) are available from several different manufacturers. The LPA technique is based on amplification using PCR followed by reverse-hybridization of the DNA target sequences, which bind to species-specific fragments on a nitrocellulose strip, leading to a color reaction forming a certain banding pattern. Available options include the INNO-LiPA® Mycobacteria v2 Kit (Fujirebio, Tokyo, Japan), the GenoType Mycobacterium CM/AS Kits (Hain Lifescience, Nehren, Germany) and the Genoscholar™ NTM+MDRTB II (Nipro, Osaka, Japan) [5, 29]. Several authors have also reported on using and validating qPCR-based assays for the species-level identification of NTM. Even though this approach is experimental, qPCR assays provide the opportunity for a timely, direct, *ex vivo* diagnosis, in case of a multiplexed assay even

for several different species at the same time [11, 30-35]. Whole or partial genome sequencing poses another alternative for definitive identification of mycobacteria, although the capacity for performing sequencing in low- and middle-income countries is limited. Several genes have been described for the species-level identification of NTM, among them *erm, hsp65, rpoB* and *16S rRNA gene* [5]. Options for deep-sequencing-based identification exist with innovative approaches such as the Deeplex® Myc-TB kit (GenoScreen, Lille, France). It combines PCR with deep sequencing of amplicons using next generation sequencing (NGS), with the data automatically analyzed by a corresponding web application and can identify many Mycobacteria as well as drug resistances simultaneously [36]. Mycobacteria can also be definitively identified to the species-level using matrix assisted laser desorption ionization time of flight spectrometry (MALDI-ToF MS), with relevant limitations to this method being the high cost of procuring a mass spectrometer and lacking public databases [5].

For identification of *M. tuberculosis* several commercial options are available, mainly the GeneXpert® (Cepheid®, Sunnyvale, CA, USA) system, the usage of which is supported by the WHO and many previous publications (Global Laboratory Initiative. GLI model TB diagnostic algorithms. www.stoptb.org/wg/gli/assets/documents/GLI_algorithms.pdf, accessed Jun. 2023) [37, 38]. Several authors have described developing custom qPCR assays for the identification of *M. tuberculosis* using different genetic target regions such as *SenX* or *IS6110* [33, 39-42].

1.3.2 Treatment

Treatment for NTM-PD differs from that of TB, since other substances have to be administered over a longer period of time, because NTM are often resistant to first line antituberculotic drugs [4, 8, 12, 16, 43]. In general, treatment for pulmonary infections with NTM is lengthy and multidrug regimens have to be administered. Key substances used include macrolides such as clarithromycin or azithromycin, amikacin, clofazimine, linezolid and doxycycline. The duration of therapy is usually 12 months and continually monitored with sputum cultures, with the goal being culture conversion [5-7, 12]. For some species of NTM, surgery may also be considered and improves the outcome if the

pulmonary infection is localized and drug resistance is present [5, 7]. Decisive treatment recommendations are also complicated by the fact that quality data on *in vitro-in vivo* correlations of drug sensitivity testing (DST) are not available for all antimycobacterial drugs used in the therapy of NTM infections. Predicting the *in vivo* effectivity of the drug and ultimately the treatment outcome of a multidrug regimen can thus be difficult [5, 8]. Additionally, the usefulness of DST for NTM is limited by the fact that many drug breakpoints have not been clinically validated, also due to a lack of quality study data [5, 44]. However, DST for NTM infections is part of the standard of care and should ideally be pursued before initiating treatment [5-8, 45].

Treatment of *M. fortuitum* consists of a combination of two or more drugs for mild to moderate disease and three or more drugs for severe disease. The following antibiotics are recommended: cefoxitin, imipenem, amikacin, moxifloxacin, clofazimine, linezolid, co-trimoxazole and doxycycline. The treatment should also be monitored with regular sputum cultures and continued for the duration of 12 months beyond sputum conversion [5-7, 12].

1.3.3 Importance of correctly diagnosing TB/NTM infection

Correctly differentiating between TB and NTM-PD in a suspected patient is crucial for therapeutic prognosis, especially in countries with a high prevalence of TB like Ghana. As previously described, the prevalence of NTM-PD among patients with suspected pulmonary TB is high. Because of the similarities, patients with NTM-PD could be easily overseen and the clinical picture misdiagnosed as TB [46-48]. Often times, due to a lack of diagnostic capabilities for definitive identification of NTM in resource limited settings, patients may be falsely labeled as “chronic TB” after failing empiric TB treatment [5, 46, 48, 49]. A study from Burkina Faso investigated all such “chronic TB” patients registered in the country, which were defined as having failed re-treatment for TB based on positive AFB smear results. These patients were started on a 21-month treatment regimen using second-line anti-TB drugs. However, the study identified NTM as the cause of infections in 20.6% of these “chronic TB” patients [49].

Mis- or underdiagnosis of atypical mycobacterial infections not only unnecessarily exposes patients to expensive and potentially toxic first-/second-line TB medication but could in turn also lead to a higher morbidity and mortality among NTM patients receiving ineffective treatment and thus to the selection of more drug resistant microorganisms [5, 48, 49]. Increasing awareness for these infections and their similarity to TB, especially in clinicians and vulnerable populations is important, as a large number of patients in these countries may be predisposed and at risk of acquiring an NTM infection [50, 51].

1.3.4 Diagnostic limitations and difficulties for NTM

Generally, due to characteristics such as a thick, acid-fast cell wall and long replication time the diagnosis of Mycobacteria is challenging. For TB, alternative means for supporting a diagnosis such as the IGRA or Tuberculin Skin Test exist [52]. This is not the case for NTM [7]. Although the microbiological culturing of NTM from samples is the gold standard for diagnosis at the moment and recommended by ATS/IDSA clinical guidelines, it is time consuming while also having a high risk of culture contamination due to the slow growth of these organisms [6, 7]. In contrast to TB-infected sputum, which may contain a large number of bacilli, the bacillary load of NTM in sputum is usually low (termed paucibacillary infection) [5, 7, 12]. Clinically, patients in high-TB-prevalence settings may often be diagnosed and treated for TB based purely on symptoms, exam findings, smear microscopy and x-ray even if GeneXpert and culture results are negative, as only limited diagnostic options are available in these settings for investigating a possible NTM infection [46, 48, 49, 53, 54]. Thus, the increasing emergence of NTM infections and disease poses a rising risk for health care systems in resource-limited, high-TB-prevalence settings due to challenging diagnosis and treatment [48, 55]. Alternative means for a rapid and specific diagnosis of NTM to the species-level are urgently needed. Molecular biological diagnostic approaches such as qPCR may offer a possible solution for reliable diagnosis of NTM infections and differentiation from TB.

1.4 Previous research from our group and relevance for this study

In 2015, AG Jacobsen was able to establish a method for direct, *ex-vivo* identification and quantification of *M. abscessus* by qPCR from sputum samples of cystic fibrosis patients. This was accomplished by targeting the *rpoB*-gene sequence of *M. abscessus* [11]. We aimed to adapt this technique to enable rapid detection of *M. fortuitum* in sputum samples from patients in Ghana as part of this study. Developing a direct, *ex-vivo* method of identifying *M. fortuitum* from patient samples by qPCR in Ghana could ideally bypass the microbiological culture as a means for NTM diagnosis.

1.5 Research Objectives

So far, there has been only little research into *M. fortuitum* lung disease both worldwide and in Ghana specifically. By testing sputum samples and sputum culture samples of suspected NTM-PD-patients in Ghana with a modified version of the established qPCR method [11], we want to gather novel data on the pathogenic relevance of *M. fortuitum*. The development of a more rapid NTM identification procedure would allow for an easy differentiation between TB and NTM patients, which is of clinical relevance, as there are meaningful implications for further therapy. We thus aim to establish and optimize a method for identification and quantification of *M. fortuitum* by qPCR. Further we aim to determine a DNA-extraction protocol that is optimal for isolation of mycobacterial DNA directly from sputum and from culture samples. After optimization using clinical isolates, these methods will be used to test patient samples in Kumasi, Ghana. Specifically, we aim to:

- Correctly classify patients by qPCR whose *M. fortuitum* infection was previously confirmed using other diagnostic tools or reference methods.
- Determine and apply a specific primer/probe combination yielding the best amplification result for *M. fortuitum,* as well as a primer/probe combination specific for *M. tuberculosis,* enabling a correct differentiation between infections with these two Mycobacteria. Additionally, determine a primer/probe combination specific for NTM in general to identify infections with other Mycobacteria (Pan-Mycobacteria primers and probe).

- Determine the limit of detection (LOD) of our qPCR assay.
- Compare DNA extraction methods to identify protocols that are optimal for sputum and for culture samples.

2.0 Materials and Methods

2.1 Materials

2.1.1 Reagents

Item	Manufacturer
Ampuwa®	Fresenius Kabi
NaCl 0,9%	Fresenius Kabi
DPBS Dulbecco's Phosphate-buffered Saline	Gibco™
PBS Phosphate-buffered Saline	Invitrogen™
DEPC-treated water	Invitrogen™
Trizma hydrochloride	Sigma Aldrich
Tween 20	Sigma Aldrich
$MgCl^{2+}$	Sigma Aldrich
KCl	Sigma Aldrich
NaOH	Sigma Aldrich
NALC	Sigma Aldrich
Sodium dodecyl sulfate (SDS)	Sigma Aldrich / VWR
Sodium acetate buffer solution	Sigma Aldrich
Tris-EDTA buffer solution	Sigma Aldrich
Lysozyme	Sigma Aldrich
Proteinase K	Sigma Aldrich
Ethanol, *non-denatured, molecular biology grade*	Sigma Aldrich
Ethanol, *absolute*	VWR / Sigma Aldrich
Isopropanol	Sigma Aldrich
UltraPure™ Agarose	Invitrogen
GelRed™ Nucleic Acid Gel Stain, 10,000X	Biotium
DNA Ladder TrackIt™ / GeneRuler	Invitrogen™ / ThermoScientific™l
TBE Running Buffer (5X)	Novex
Glycogen	Qiagen
Glycerin	Sigma Aldrich
Tween 80	Sigma Aldrich
Medium, 7H11	BD
Medium, 7H9	BD
OADC	BD
Wash Buffer BW	Macherey-Nagel
Buffer B5	Macherey-Nagel
Buffer BE	Macherey-Nagel
Cell lysis solution	Qiagen
Protein precipitation solution	Qiagen
DNA hydration solution	Qiagen

2.1.2 Clinical isolates

Isolate	Provided by
M. tuberculosis H37Rv	Prof. Rainer Kalscheuer, Institute of Pharmaceutical Biology and Biotechnology, HHU

M. fortuitum	Prof. Colin MacKenzie, Dept. of Microbiology, UKD
M. abscessus	AG Jacobsen, Mathis Steindor

2.1.3 Consumables

Item	Manufacturer
Petri Dish sterile	Greiner bio-one
Nunc™ sterile Inoculation Loops	ThermoFisher Scientific
Sterile Square Media Bottle, PETG	Nalgene
Hard-Shell PCR Plates	Bio-Rad
Microseal 'B' seal	Bio-Rad
PCR Reaction Tubes	ThermoScientific
Microdilution Tubes 0.5, 1 , 2 ml	Eppendorf & StarLab
Nunc™ Cryotubes	ThermoScientific
Spectrophotometry cuvettes	Sarstedt
Graduated Filter Tips sterile 10µl	StarLab
Graduated Filter Tips sterile 20µl	StarLab
Graduated Filter Tips sterile 200µl	StarLab
Graduated Filter Tips sterile 1000µl	StarLab
Corning Costar Stripette serological pipette 5ml	Corning
Corning Costar Stripette serological pipette 10ml	Corning
Corning Costar Stripette serological pipette 25ml	Corning
Falcon 15ml Conical Tubes	ThermoFisher Scientific
Falcon 50ml Conical Tubes	ThermoFisher Scientific
Sterican 20G Canula	Braun
Injekt Solo Luer 5ml, 10ml, 20ml Syringes	Braun
0,2µm syringe filter	VWR
Bottle top PES Vacuum Filtration System	VWR
MGIT Tubes	BD
NucleoSpin Blood Columns	Macherey-Nagel
Gloves Dermagrip	WRP
Tyvek Classic Xpert Overall	DuPont
Sterile disposable surgical gown	Braun
N95 Mask / KN95 Mask	Makrite / 1AK

2.1.4 Culture medium components

Medium type	Preparation
Middlebrook 7H9 liquid medium for Mycobacteria	2.35 g Middlebrook 7H9 powder, 450 ml Ampuwa, 50 ml OADC, 5 ml 50 % (w/v) Glycerol, 1.25 ml 20 % Tween 80.
Middlebrook 7H11 solid medium for Mycobacteria	10.5 g 7H11 powder, 450 ml Ampuwa, 50 ml OADC, 5 ml 50 % (w/v) glycerol
Freezing medium for Mycobacteria	500 µl Middlebrook 7H9 liquid medium, 500 µl of 50 % Glycerol (w/v)

2.1.5 Buffers

Buffer	Preparation
Tween 20 Lysis Buffer for DNA extraction	0.45 % Tween 20, 50 mM Tris–HCl, 50 mM KCl, 2.5 mM MgCl, all reagents from Sigma-Aldrich, prepared in Ampuwa® and sterilized by filtration through 0.2 µm filter
20% Chelex solution	20 % Chelex 100 Resin (Bio-Rad, 200-400 dry mesh size, 75-150 µm wet bead size) prepared in TE-Buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA)
1x TBE	200 ml 5 X TBE running in 800 ml Ampuwa

2.1.6 Kits

Item	Manufacturer
BBL MycoPrep Kit	BD
MGIT Growth Supplement Kit	BD
MGIT PANTA	BD
REDTaq® ReadyMix™ PCR Reaction Mix	Sigma-Aldrich, Merck KGaA
QuantiTect® Multiplex PCR NoROX Mix	Qiagen
Sputum DNA isolation kit	Norgen Biotek Corp.
QIAmp DNA Blood Mini Kit	Qiagen
GenoType CM	Hain Lifescience

2.1.7 Oligonucleotides

Oligos	Manufacturer
Forward Primer 2 (*M. fortuitum)*	metabion
Forward Primer 3 (*M. fortuitum)*	metabion
Reverse Primer 1 (*M. fortuitum)*	metabion
Reverse Primer 2 (*M. fortuitum)*	metabion
Reverse Primer 3 (*M. fortuitum)*	metabion
Reverse Primer 4 (*M. fortuitum)*	metabion
Reverse Primer 5 (*M. fortuitum)*	metabion
Reverse Primer 6 (*M. fortuitum)*	metabion
Probe FAM (*M. fortuitum)*	metabion
Probe HEX (*M. fortuitum)*	metabion
Forward Primer 1 (*MTBc*)	metabion
Reverse Primer 1 (*MTBc*)	metabion
Probe HEX (*MTBc*)	metabion
Forward Primer 1 (*PAN-Myco)*	Eurofins Genomics
Reverse Primer 1 (*PAN-Myco)*	Eurofins Genomics
Probe FAM (*PAN-Myco)*	Eurofins Genomics

2.1.8 Equipment

Item	Manufacturer
Research Pipette 0,5-10µl	Eppendorf
Research Pipette 10-100µl	Eppendorf
Research Pipette 20-200µl	Eppendorf

Research Pipette 100-1000µl	Eppendorf
ErgoOne Pipette 0,1-2,5µl	Starlab
ErgoOne Pipette 0,5-10µl	Starlab
ErgoOne Pipette 2,0-20µl	Starlab
ErgoOne Pipette 10-100µl	Starlab
Pipetboy acu 2	Integra
Centrifuge Rotina 420R	Hettich
Centrifuge 5415C	Eppendorf
Centrifuge Fresco 17	Heraeus
Refrigerator (4°C) and freezer (-20°C)	Liebherr/Whirlpool
TSX Series Freezer (-80°C)	Thermo Fisher Scientific
Systec VB-120 Autoclave	Systec
Biometra Power Pack P25	Biometra GmbH
Sonorex Super RK 31 H Ultrasonic Bath	Bandelin
Sonorex Super RK 100 H Ultrasonic Bath	Bandelin
HLC Heating Block	Ditabis
Shaking Incubator MaxQ 4450	Thermo Scientific
Incubator	Memmert
Incubator	Heraeus
BACTEC MGIT 960 System	BD
NanoDrop 1000	ThermoFisher
NanoDrop 2000	ThermoFisher
Spectrophotometer DS-11	Denovix
Spectrophotometer UV Mini 1240	Shimadzu
ChemiDoc™ Touch System	Bio-Rad
Biometra T3 Thermoblock PCR Thermal Cycler	Biometra GmbH
Analytik Jena Flex Cycler^2	Analytik Jena GmbH
Bio-Rad C1000 Touch™ Thermal Cycler CFX96™ Real-Time System	Bio-Rad Laboratories Inc.
GeneXpert	Cepheid
GT-Blot 48	Hain Lifescience
GenoScan®	Hain Lifescience

2.1.9 Software

Software	Manufacturer
NanoDrop 1000 Data Viewer	ThermoScientific
NanoDrop 2000 Data Viewer	ThermoScientific
Geneious Prime (Version 2023.0)	Biomatters
BLAST, pubmed, GenBank	NCBI, NLM, NIH
ImageLab	BioRad
CFX-Maestro	R&D Systems
OpenEvidence (2026)	OpenEvidence Inc. [AI medical information platform].https://www.openevidence.com/
MS Office (Word, Excel, PowerPoint)	Microsoft
Endnote™ 20	Clarivate™
SPSS® Statistics Version 29.0.1.0	IBM®

2.2 Methods

2.2.1 Mycobacterial strains from Düsseldorf

In Düsseldorf, testing was conducted using four different clinical isolates (ID 1/2/3/4) of *M. fortuitum* kindly provided to us by Prof. Colin MacKenzie at the Dept. of Microbiology at UKD. Pure cultures were obtained through subculturing by picking individual colonies from Löwenstein-Jensen medium, transferring them to Middlebrook 7H9 liquid medium and from there onto Middlebrook 7H11 solid medium. Pure *M. fortuitum* gDNA for testing was available and had previously been obtained by our research group through cetyltrimethylammonium bromide (CTAB) extraction [56]. The gDNA samples from the H37Rv strain of the *M. tuberculosis complex* used for qPCR, were kindly provided by Prof. Rainer Kalscheuer (Institute of Pharmaceutical Biology and Biotechnology at Heinrich-Heine-Universität).

2.2.2 Patient samples and microbiological diagnostics in Kumasi, Ghana

In Ghana, patient samples were collected at three study sites, Kumasi South Hospital and KNUST Hospital in Kumasi, Ashanti Region, Ghana as well as Sene West District Hospital in Kwame Danso, Bono East Region, Ghana. All recruited patients gave written and verbal informed consent. The study was approved by the Ethics Committee of the Medical Faculty at Heinrich-Heine-University Düsseldorf (Reference: 2022-2085) and the Committee on Human Research, Publication and Ethics at Kwame Nkrumah University of Science and Technology School of Medicine and Dentistry, Kumasi, Ghana (Reference: CHRPE/AP/031/21) pursuant to ICH-GCP guidelines.

Routine laboratory diagnostic workflow on sputum samples of patients with suspected TB was as follows.

Initially patients presented with suspicious history and clinical picture such as chronic cough, weight loss, night sweats and fatigue, possibly in addition to indicative imaging like an x-ray with cavitation, opacities or bronchiectasis [9]. Sputum was collected as part of routine TB screening and decontaminated for all further diagnostic applications, using either in-house prepared sodium hydroxide (NaOH, 4%), sodium citrate (NaCl 2.9%) and

N-acetyl-L-cysteine (NALC) solution with PBS or BD BBL™ MycoPrep™ Kit, which includes a prepared phosphate buffer according to the manufacturer's protocol (Becton Dickinson, Franklin Lakes NJ, USA; Cat. 2240862). Smear microscopy with Ziehl-Neelsen staining for acid-fast-bacilli (AFB) and GeneXpert® MTB/RIF Ultra (Cepheid, Sunnyvale, CA, USA) testing according to manufacturer's protocol was conducted from the decontaminated samples. Additionally, decontaminated sputum samples were cultured using the BD BACTEC™ MGIT™ 960 system (Becton Dickinson, Franklin Lakes NJ, USA). MGIT tubes were treated with BD BACTEC™ MGIT™ 960 Supplement Kit (Becton Dickinson, Franklin Lakes NJ, USA; Cat. 245124) and BD BBL™ MGIT™ PANTA™ (Becton Dickinson, Franklin Lakes NJ, USA; Cat. 245114) before inoculation with processed sputum.

Regarding standard incubation times of the samples, NTM may have a shorter time to positivity, whilst TB requires longer periods of incubation. As evident from the colony forming unit (CFU) experiments described in Fig. 12 (3.2.1) and Fig. 15 (3.2.2) for NTM of the rapid-growing type like *M. fortuitum*, it may take only a few days for liquid and solid cultures to grow and become positive. This is also clear from the literature on NTM [5, 7]. As *M. tuberculosis* generally grows slower, it may take several weeks for the MGIT culture to become positive. Thus, samples were generally incubated until positive or for a maximum duration of up to 42 days as recommended by the manufacturer.

An overview of the microbiological diagnostic workflow can be seen in Fig. 2 below.

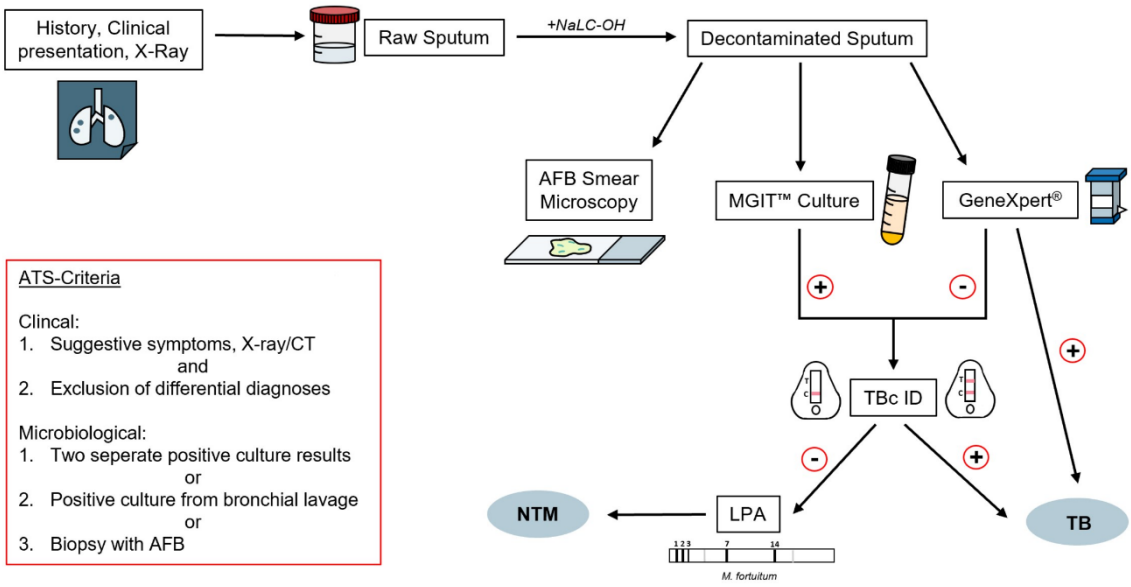


Fig. 2: Diagnostic workflow for sputum samples of suspected TB patients at KCCR as part of the "NoTe-Me Study", schematic overview.

Patients presenting with characteristic clinical signs and symptoms at cooperating hospitals gave sputum samples, which were then decontaminated for all further diagnostic applications. The decontaminated sputum was subsequently used to inoculate MGIT™ cultures, perform AFB smear microscopy and GeneXpert® testing. If a MGIT™ culture was positive, a TBc ID rapid antigen test was performed for GeneXpert® negative samples. If either the GeneXpert® or the TBc ID test of the culture were positive, an infection with TB would be likely. However, if the GeneXpert® and TBc ID were negative, whilst the AFB smear and culture were positive, an infection with NTM was considered likely and LPA performed. Clinical and microbiological criteria suggested by the ATS for definitive diagnosis of NTM-infection are displayed on the bottom left [7].

2.2.3 Screening of presumptive TB samples for NTM

Positive results in the GeneXpert® and AFB microscopy, were interpreted as indicative of a TB-infected sputum sample (Global Laboratory Initiative. GLI model TB diagnostic algorithms. www.stoptb.org/wg/gli/assets/documents/GLI_algorithms.pdf accessed Jun. 2023). However, a combination of GeneXpert® negative result and AFB microscopy/culture positive result, was deemed suggestive of infection with NTM [46, 57, 58]. For positive MGIT™ cultures with concomitant negative GeneXpert®, a BD MGIT™ TBc ID rapid test (Becton Dickinson, Franklin Lakes NJ, USA; Cat. 245159) was conducted (according to the manufacturer's instructions), which can detect the MPT64 antigen secreted specifically by *M. tuberculosis* in culture. If positive, it was counted as additional evidence supporting a TB infection. If negative, it was viewed as another possible indication of infection with NTM [59]. Further diagnosis and speciation of NTM

was conducted using a LPA (GenoType Mycobacterium CM kit; Bruker/Hain Lifescience GmbH, Nehren, Germany) according to the manufacturer's protocol.

2.2.4 Culturing of mycobacteria on liquid/solid medium

Protocols for culturing *M. fortuitum* were adapted from *Larsen et al.* [27].

For liquid culture, pure *M. fortuitum* colonies were transferred from Middlebrook 7H11 agar plates to a square, sterile 30 ml Nalgene® Media Bottle (Nalgene®, Rochester NY, USA; Cat. 2019-0030) containing 10 ml of Middlebrook 7H9 liquid medium. This was performed in a BSL-2 laboratory under sterile conditions. The liquid culture was then placed in a shaking incubator (Thermo Scientific MaxQ 4450, Thermo Scientific, Waltham Mass., USA) to grow at 37°C with mild agitation of about 90 rpm. To ensure similar conditions for the experiments described below, each liquid *M. fortuitum* culture used was grown for a duration of approx. 3 days and the optical density of the culture quantified by spectrophotometry before use. This process is described below in further detail.

Continuous subcultures were performed by transferring 100 µl of late-log-phase *M. fortuitum* liquid culture (growth time of approx. 3 days) into a new sterile media bottle containing 10 ml of 7H9, to ensure the viability of the cultured Mycobacteria. The 7H9 liquid medium consisted of 2.35 g Middlebrook 7H9 powder (BD Difco™ Middlebrook 7H9 Broth, Becton Dickinson, Franklin Lakes NJ, USA; Cat. 271310), 450 ml Ampuwa (Ampuwa Plastipur, Fresenius Kabi Deutschland GmbH, Bad Homburg, Cat. 1088811), 50 ml OADC (BD Difco™ BBL™ Middlebrook-OADC, Becton Dickinson, Franklin Lakes NJ, USA; Cat. 212351), 5 ml 50 % (w/v) Glycerol, 1.25 ml 20 % Tween 80. The medium was sterilized by vacuum pump filtration through a 0.22 µm membrane and subsequently stored at 4°C.

For preparing frozen *M. fortuitum* strain stock, liquid cultures of *M. fortuitum* were first transferred into a 15 ml falcon tube. The tubes were then centrifuged at 4500 rpm for 5 minutes to pellet the cells, after which the supernatant was carefully and completely decanted or removed by pipette. The pellet was then resuspended in 500 µl of fresh Middlebrook 7H9 liquid medium, before transferring the entire sample into a 1 ml

cryotube. Then, 500 µl of 50 % (w/v) Glycerol was added and the samples promptly transferred to storage at -80°C until further use.

For solid cultures, 60 µl of *M. fortuitum* liquid culture was inoculated onto Middlebrook 7H11 agar plates for the initial CFU analysis, subsequently switching to 50 µl for all following experiments. Stacks consisting of < 10 inoculated agar plates were inverted, wrapped tightly in aluminum foil to keep from drying out and incubated for 4-5 days at 37°C. The Middlebrook 7H11 Medium consisted of 10.5 g 7H11 powder (BD BBL™ 7H11 Agar Base, Becton Dickinson, Franklin Lakes NJ, USA; Cat. 212203) in 450 ml Ampuwa (Ampuwa Plastipur, Fresenius Kabi Deutschland GmbH, Bad Homburg, Cat. 1088811). Autoclaved borosilicate bottles and magnetic stirrer were used for this step of medium preparation. The mixture was then autoclaved and placed in 55°C water bath to cool but prevent from solidifying until further use. Then 50 ml OADC (BD Difco™ BBL™ Middlebrook-OADC, Becton Dickinson, Franklin Lakes NJ, USA; Cat. 212351) and 5 ml 50 % (w/v) glycerol were added and the mixture poured into petri dishes. After airdrying, plates were stored inverted at 4°C.

2.2.5 DNA extraction

The objective of the project was to establish a DNA extraction method suitable for the direct, *ex-vivo* identification and quantification of *M. fortuitum* from sputum samples. Additionally, a method for extraction of high purity gDNA suitable for subsequent genomic analyses from cultured *M. fortuitum* specimen was to be established. For this purpose, extraction methods were first tested on *M. fortuitum* liquid cultures serially diluted in PBS or sterile saline, after which the methods were to be established using sputum samples in Ghana.

Two commercially available kits, the Sputum DNA isolation kit (Norgen Biotek Corp., Thorold, ON, Canada) and QIAmp® DNA Blood Mini Kit (QIAGEN N.V., Venlo, Netherlands), were tested [60]. There were several previous publications reporting on the successful use of in-house resin- and/or sonication-based DNA extraction methods or the use of a Tween-based lysis buffer approach [39, 58, 61-64]. These methods were also tested

A condensed overview of the different DNA extraction methods used in this project and the key steps of each protocol can be seen in table 1 below.

Table 1: Overview of DNA Extraction Methods, Approaches I-VI.

Protocol	Approach I		Approach II	Approach III		Approach IV	Approach V		Approach VI
	QIAmp DNA Blood mini kit	*Norgen Sputum DNA isolation kit*	*Modified Norgen Sputum Kit*	*TE-Buffer boil method*	*Tween 20 Method*	*Tween 20 + Macherey-Nagel spin columns*	*Chelex + Ultrasonication*	*Tween 20 freeze/boil method*	*Method previously used for M. ulcerans*
Pelleting	X	X	X	✓	✓	✓	✓	✓	X
Lysis	1) QIAGEN Protease 2) Lysis Buffer AL 3) Incubation 56°C, 10 min.	1) Slurry D 2) PK & LZ 3) Incubation 60°C, 20 min. 4) Solution WN	1) Slurry D 2) LZ 3) Incubation 37°C, 30 min. 4) TLS 5) Incubation 95°C, 20 min. 6) PK 7) Incubation 50°C, 30 min.	1) 150 µl TE-Buffer (10 mM Tris-HCl, 1 mM EDTA) 2) 15 µl Lysozyme 3) Overnight incubation 37°C 4) Boiling 30min 5) 20 µl Proteinase K 6) Incubation 55°C, 30 min.	1) Tween20 lysis buffer (0,45%) 2) 70 µl Lysozyme 3) Incubation 37°C, 1h 4) Proteinase K 5) 30 µl PK & 30 µl SDS (2%) 6) Incubation 56°C, 1h 7) Boiling 15 min.	1) Tween20 lysis buffer (0,45%) 2) Lysozyme (10mg/ml) 3) Incubation 37°C, 1 h 4) 30 µl PK & 30 µl SDS (2%) 5) Incubation 56°C, 1 h 6) Boiling, 15 min.	1) 20% Chelex 100 Resin 2) Boiling, 15min. 3) Ultrasonic Waterbath, 15 min. → After centrifugation, SN used for all further applications	1) Tween20 lysis buffer (0,45%) 2) Freeze -80°C, 7 min. 3) Boil 95°C, 7 min. 4) Repeat x3 total 5) PK (20 mg/ml) 6) Incubation 56°C overnight 7) Incubation 80°C, 15 min. 8) Repeat steps 2/3/4 9) Lysozyme (10 mg/ml) 10) Incubation 37°C, 30 min.	1) QIAGEN cell lysis solution 2) Lysozyme (10 mg/ml) 3) Incubation 37°C, 1h 4) PK (20 mg/ml) 5) Incubation 55°C, 4 h 6) Incubation 80°C, 15 min.
DNA Precipitation/ Binding	Spin columns	Spin columns	Spin-columns	Isopropanol (ice-cold)	3 M sodium acetate (pH 4.8-5.2), final concentration of 0.3 M and double-volume ice-cold ethanol (non-denatured), cooling of samples at -20°C, 30 min.	Spin columns	X	3 M sodium acetate (pH4.8-5.2), final concentration of 0.3M and double-volume ice-cold ethanol (non-denatured), cooling of samples at -20°C, 30 min.	QIAGEN protein precipitation solution, Ice bath, Isopropanol and Glycogen
Washing	Wash buffers AW1 and AW2	Wash solution BE and Ethanol absolute	Wash solution BE and Ethanol absolute	Ethanol (70%)	Ethanol (70%)	Wash Buffer BW and buffer B5	X	Ethanol (70%)	Ethanol (70%)
Elution	Buffer AE	Elution Buffer B	Elution Buffer B	X	X	Elution Buffer BE (preheated)	X	X	X
Drying/ DNA-Hydration	X	X	X	1) Airdry 2) 50 µl TE-Buffer	1) Airdry 2) 50µl TE-Buffer	X	X	1) Airdry 2) 50 µl TE-Buffer	1) Airdry 2) QIAGEN DNA hydration solution 3) Incubation 65°C, 1 h

Note: The DNA extraction protocols in the table above were shortened for clarity and steps such as centrifugation and vortex mixing left out. For exact protocols including the different volumes of reagents added, please refer to the detailed descriptions below.

2.2.5.1 Approach I: The Norgen Sputum DNA isolation kit and the QIAmp DNA Blood Mini kit

To extract DNA from sputum samples in a simple and efficient way, we initially focused on kit-based techniques, which pose many benefits. Previous authors had described the successful use of QIAGEN kits for DNA extraction from Mycobacteria, which is why we decided to initially use the QIAmp® DNA Blood Mini Kit [60, 61, 65]. In addition to the kit from QIAGEN (1), we decided to use the Norgen Sputum DNA isolation kit (2), which is specifically designed for extracting DNA from sputum samples. This kit had previously been successfully used for extracting *M. tuberculosis* from sputum samples in similar study settings [66, 67].

(1) The QIAGEN QIAmp DNA Blood mini kit protocol was performed as follows. Before starting, reagents were warmed to room temperature and the heating block was preheated. To start 20 µl of QIAGEN Protease was added into 1.5 ml Eppendorf tubes, to which 210 µl of sample consisting of 150 µl PBS mixed with 60 µl of serially diluted CFU was added. Then 200 µl of Buffer AL was added and samples vortexed for 15 seconds, followed by incubation at 56°C for 10 minutes. Samples were spun-down briefly before adding 200 µl ethanol (absolute), after which samples were again vortexed for 15 seconds and spun-down briefly. Samples were then transferred into QIAmp spin columns placed in collection tubes, which were centrifuged at 8000 rpm for 1 minute, discarding the filtrate afterwards. Spin columns were placed into a new collection tube and 500 µl Buffer AW1 was applied to the spin column before centrifuging at 8000 rpm for 1 minutes and again discarding the filtrate. Spin columns were then again placed into a new collection tube and 500 µl Buffer AW2 was added before centrifuging at 13.300 rpm for 3 minutes and discarding the filtrate. Samples were then centrifuged for 1 minute at full speed to dry. Following this, spin columns were placed in clean 1.5 ml tubes for elution, 200 µl Buffer AE was added and incubated at room temperature for 5 minutes. Samples were then centrifuged at 8000 rpm for 1 minute to release the DNA from the spin column. This step was repeated once to increase the yield of eluted DNA. The eluate was stored at -20°C as template DNA.

(2) The Norgen Biotek Corp. Sputum DNA isolation kit protocol was performed as follows. Before starting, reagents were warmed to room temperature and the heating block was

preheated. To start 210 µl of sample consisting of 150 µl PBS mixed with 60 µl of serially diluted CFU was aliquoted into a 2 ml Eppendorf tube. Then 800 µl of Slurry D was added and mixed by inversion. The Slurry D contained silicon carbide resin, making it necessary to continuously mix the sample and also the working solution of Slurry D before every use. Samples were then centrifuged for 5 minutes at 2000 rpm and the supernatant was discarded. Then 20 µL of Proteinase K and Lysozyme was added to the precipitated slurry pellet and this was vortex mixed for 10 seconds to resuspend, followed by incubation at 60°C for 20 minutes. After incubation, 260 µL of Solution WN was added and the samples mixed thoroughly by pipette. The samples were then transferred into a Norgen Mini Filter Spin column with collection tube and centrifuged for 1 minute at 13.300 rpm, discarding the flow-through. This was followed by a wash step, 500 µL of Wash Solution BE was added to the columns and they were centrifuged for 1 minute, discarding the flow-through. This step was repeated once. Then 500 µL of absolute ethanol was added, samples centrifuged for 1 minute and flow-through discarded. Columns were centrifuged for 2 minutes to dry, after which the columns were transferred to fresh collection tubes for DNA elution. Finally 100 µL of Elution Buffer B was applied to the column and samples were centrifuged for 2 minutes at 2000 rpm, followed by 1 minute at 13.300 rpm. The centrifugation was repeated to increase yield of eluted DNA. The eluate was stored at -20°C as template DNA.

2.2.5.2 Approach II: Modifications to Norgen Sputum DNA isolation kit with reference to innuPREP Mycobacteria DNA Kit

To possibly optimize the DNA extraction from *M. fortuitum*, the Norgen Sputum DNA isolation kit protocol was adapted as follows. To start 200 µl of serially diluted CFU was aliquoted into 1.5 ml Eppendorf tubes, 800 µl of Slurry D was added and the sample mixed by inversion. Cells in the sample were then pelleted by centrifugation for 15 minutes at 10.000 rpm, after which the supernatant was carefully and thoroughly discarded using a pipette. Cell pellets were resuspended by adding 200 µl of TE-buffer. For lysing the cells, 15 µl of lysozyme (20 mg/ml) was added and the samples incubated in a heating block for 30 minutes at 37°C. Then 200 µl TLS, a lysis solution formerly used as part of the innuPREP Mycobacteria DNA kit (Analytik Jena GmbH, Jena, Germany),

was added and the samples incubated for 20 minutes at 95°C. Afterwards, samples were left to cool before adding 20 µl of Proteinase K (component provided as part of Norgen kit) and incubating for 30 minutes at 50°C. Note that during the long incubation times, the Slurry D resin would form clumps at the bottom of the tubes, so samples were vortexed every 5 minutes during incubation to avoid clumping. To bind the DNA to the spin column membrane, 260 µl of Solution WN was added and samples mixed by pipette before transferring to Norgen Mini Filter spin columns with collection tubes. When transferring the samples to the spin columns, small black clumps/aggregates which could not be resuspended were observed. These clumps were thought to most likely be leftover Slurry D resin. Columns were then centrifuged for 2 minutes at 13.300 rpm and flow-through was discarded. Samples were then washed by applying 500 µL of Wash Solution BE to the columns and centrifuging for 1 minute at 12.000 rpm, discarding the flow-through. This step was repeated once, after which 500 µL of absolute ethanol were applied. Columns were then centrifuged for 1 minute at 12.000 rpm, discarding the flow-through, followed by 2 minutes of centrifugation at max speed to dry, before transferring the columns to fresh 1.5 ml Eppendorf tubes for elution. Finally 50 µL of Elution Buffer B was applied onto the membrane of the column and incubated at room temperature for 2 minutes before centrifuging at 2000 rpm for 2 minutes followed by 13.300 rpm for 1 minutes. Another 30 µl of Elution Buffer B was added to the column and the centrifugation repeated as above. The eluate was then stored at -20°C as template DNA.

2.2.5.3 Approach III: Chemical lysis-based extraction protocols using TE buffer and Tween 20 buffer

To extract DNA from mycobacteria in culture and in sputum, many different in-house chemical/thermal lysis and non-kit-based techniques have been reported on previously [39, 61]. Next, we tested a simple protocol, which originally only consisted of adding TE-Buffer to a spiked sputum pellet, boiling for 15 minutes, centrifuging and using the supernatant as template DNA [61]. We additionally incorporated steps of enzymatic lysis with lysozyme and proteinase K, as well as traditional ethanol precipitation, in hopes of increasing the amount and quality of eluted DNA.

(1) TE-buffer method: First 200 µl of serially diluted CFU/liquid culture was aliquoted into 1.5 ml Eppendorf tubes, pelleted at 13.300 rpm for 5 minutes and the supernatant discarded. Pellets were then resuspended in 150 µl TE-Buffer (10 mM Tris-HCl, 1 mM EDTA) and mixed by vortex. Then 15 µl of Lysozyme was added and incubated overnight at 37°C. The next day, samples were boiled for 30 minutes to deactivate the lysozyme and cooled before adding 20 µl Proteinase K and incubating at 55°C for 30 minutes. Samples were then pelleted for 5 minutes at 13.300 rpm before harvesting the supernatant. 200 µl of ice-cold isopropanol was added to the supernatant, samples were mixed thoroughly by inversion and left to stand at room temperature for 5 minutes. Following this, samples were centrifuged for 10 minutes at 10.000 rpm and the supernatant carefully discarded using a pipette. Then 1 ml of 70 % ethanol was added to the pellet to wash, before centrifuging again at 10.000 rpm for 10 minutes and discarding the supernatant. Samples were then left to airdry for approximately 30 minutes before adding 50 µl of TE-Buffer to hydrate the DNA.

(2) Tween 20 method: After aliquoting 200 µl of each sample into 1.5 ml Eppendorf tubes, the samples were centrifuged for 10 minutes at 13.300 rpm to pellet cells. The supernatant was discarded completely using a pipette and 200 µl of Tween 20 (0,45 %) lysis buffer solution (0.45 % Tween 20, 50 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl, all reagents from Sigma-Aldrich, prepared in Ampuwa and sterilized by filtration through 0.2 µm filter) was added to the pellet. Then 70 µl of Lysozyme (10 mg/ml) was added and the samples mixed thoroughly by vortex. This was followed by incubation at 37°C for one hour, after which 30 µl of Proteinase K and 30 µl of SDS (Sodium Dodecyl Sulfate, 2 %) was added. Samples were incubated for another hour at 56°C, before boiling them for 15 minutes to deactivate the Proteinase K. To pellet and remove any possible cell debris, samples were centrifuged at 13.300 rpm for 10 minutes before harvesting the supernatant and transferring it to fresh 1.5 ml Eppendorf tubes. To precipitate DNA contained in the supernatant, 30 µl of 3 M sodium acetate (pH 5.2) was added, so as to achieve a final concentration of 0.3 M in each sample. Double volume of ice-cold absolute ethanol was also added and samples placed in a freezer at -20°C to aid in precipitation. Precipitated DNA was then pelleted by centrifugation of samples at 13.300 rpm for 10 minutes, after which the supernatant was aspirated using a pipette

and discarded. Next, pellets were washed with 700 µl of ethanol (70 %) before centrifuging for another 10 minutes at 13.300 rpm and pouring off the ethanol. Samples were left to airdry until the remaining ethanol had completely evaporated before resuspending in 50 µl TE-Buffer.

2.2.5.4 Approach IV: Combination of Tween 20 method and spin columns

To increase the yield and DNA purity from the Tween 20 protocol described above, it was combined with purification and elution using NucleoSpin Blood Columns (Macherey-Nagel GmbH & Co. KG, Düren, Germany).

Samples were first centrifuged at 13.300 rpm for 10 minutes to pellet cells, discarding the supernatant afterwards. 200 µl of Tween 20 lysis buffer (0.45 % Tween 20, 50 mM Tris–HCl, 50 mM KCl, 2.5 mM MgCl, all reagents from Sigma-Aldrich, prepared in Ampuwa® and sterilized by filtration through 0.2 µm filter) was added to each sample, followed by 70 µl of Lysozyme (10 mg/ml) and samples were thoroughly vortex mixed to resuspend. The samples were then incubated at 37°C for 1 hour, after which 30 µl of Proteinase K and 30 µl of SDS (sodium dodecyl sulfate, 2 %) was added. Samples were incubated for another hour at 56°C and boiled for 15 minutes. To remove any cell debris following lysis, samples were centrifuged for 10 minutes at 13.300 rpm, the supernatant harvested and transferred to fresh 1.5 ml Eppendorf tubes. Before loading the samples on NucleoSpin Blood Columns, binding conditions were adjusted by adding 210 µl of ethanol (100 %) to each sample. After loading the samples on NucleoSpin Blood Columns, they were centrifuged for 1 minute at 13.300 rpm until having been completely drawn through the spin column matrix. The collection tube containing the flow-through was discarded and the spin columns were placed in new collection tubes. To wash the silica membrane of the spin columns, 500 µl of wash buffer BW was added, the columns centrifuged for 1 minute at 13.300 rpm and the flow-through discarded. Next, 600 µl of buffer B5 was added, samples again centrifuged at 13.300 rpm for 1 minute and flow-through discarded. To dry the spin column membrane, samples were centrifuged for another minute at 13.300 rpm. This step was repeated once. For elution, spin columns were placed in 1.5 ml collection tubes and 100 µl of buffer BE, which had been preheated

to 70°C, was added, dispensing the elution buffer directly onto the silica membrane. This was followed by a one-minute incubation at room temperature, after which the samples were centrifuged for 1 minute at 13.300 rpm. The eluate was then stored at -20°C as template DNA.

2.2.5.5 Approach V: Chelex resin and ultrasonication based extraction and chemical lysis-based extraction incorporating repeat temperature degradation

Two chemical lysis DNA-extraction protocols were adapted from a previous publication [39].

Prior to extraction, all samples were prepared as follows: 200 µl of liquid culture or serially diluted liquid culture was pelleted by centrifuging at 13.300 rpm for 10 minutes. and supernatant was discarded.

(1) Chelex Method: 200 µl of 20 % Chelex 100 Resin (Bio-Rad, 200-400 dry mesh size, 75-150 µm wet bead size) prepared in TE-Buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA, from Sigma-Aldrich, Merck KGaA, Darmstadt Germany, Cat. 93283) was added to the cell pellet. After vortexing, the sample was placed in a 100°C heating block to boil for 15 minutes and then transferred to an ultrasonic water bath (Sonorex Super RK 31 H and RK 100 H from Bandelin electronic GmbH & Co. KG, Berlin, Germany) for 15 minutes. Alternatively, a shaking heating block with agitation of 300 rpm was used. Samples were then centrifuged in a microcentrifuge for 15 minutes at 13.300 rpm to separate the Chelex beads. The supernatant was harvested for all subsequent applications. No further purification or washing steps were performed.

(2) Tween 20 freeze-boil method: This protocol was significantly altered from the version described by *Kolia-Diafouka et al.* and *Aldous et. al.* We included steps of repeat freezing at -80°C and boiling at 95°C that stemmed from a protocol used by the Department of Pathology at UKD to isolate mycobacterial DNA from tissue samples. After the pre-treatment described above, pellets were suspended in 200 µl of 0,45 % Tween 20 lysis buffer solution (0.45 % Tween 20, 50 mM Tris–HCl, 50 mM KCl, 2.5 mM MgCl, all reagents from Sigma-Aldrich, prepared in Ampuwa and sterilized by filtration through 0.2 µm filter) then frozen at -80°C for 7 minutes followed by boiling at 95°C for 7 minutes. This was repeated a total of three times. Samples were then left to cool to < 56°C for

approx. 10 minutes. Then 10 µl of 20 mg/ml Proteinase K was added and samples were incubated overnight at 56°C in a shaking heating block (300 rpm). The next day, Proteinase K digestion was deactivated by heating the samples to 80°C for 15 minutes. Then the temperature degradation steps were repeated with another three cycles of freezing at -80°C for 7 minutes followed by boiling at 95°C for 7 minutes. After this, the samples were cooled for approx. 10 minutes (< 37°C). Then 20 µl of 10 mg/ml Lysozyme was added, followed by short vortex mixing and incubation at 37°C for 30 minutes. To remove cell debris samples were centrifuged at 13.300 rpm for 10 minutes and the supernatant transferred to a fresh tube. DNA was then precipitated and purified using 3 M sodium acetate (pH 4.8-5.2) buffer to achieve a final concentration of 0.3 M as well as double volume ice-cold 100 % ethanol (molecular-biology grade, non-denatured). Samples were then placed in a freezer at -20°C for 30 minutes to enhance precipitation. DNA was then pelleted by centrifugation at 13.300 rpm for 10 minutes at 4°C. The supernatant was discarded and pellets washed with 70 % (w/v) ethanol, air dried and resuspended in 50 µl TE-Buffer.

The Chelex protocol was intended for DNA extraction from sputum samples. The short and simple protocol could more easily be implemented as part of routine microbiologic workflow at KCCR. For suspected TB sputum samples, the GenoLyse® (Bruker/Hain Lifescience GmbH, Nehren, Germany) protocol had been used previously, which also takes around 1h. The Tween freeze/boil extraction was intended for extracting high purity gDNA from culture samples for WGS.

2.2.5.6 Approach VI: Extraction protocol previously used on *M. ulcerans*

For testing of samples from patients with presumptive BU disease, a chemical lysis-based protocol incorporating several reagents from QIAGEN Puregene kits had previously been successfully used at KCCR to extract *M. ulcerans* DNA [68, 69]. This protocol was adapted and tested for extraction of *M. fortuitum* DNA.

First 200 µl of sample were transferred into an Eppendorf tube containing 200 µl of QIAGEN cell lysis solution. Then 15 µl of 10 mg/ml Lysozyme was added to each sample and incubated at 37°C in a thermomixer on moderate speed for 1 hour. After this, 10 µl of Proteinase K [20 mg/ml] was added and samples incubated at 55°C in a thermomixer

on moderate speed for 4 hours, followed by the inactivation of Proteinase K by heating the sample to 80°C for 15 minutes. Samples were then placed in an ice bath for 5 minutes to cool and 230 µl of Protein Precipitation Solution (QIAGEN N.V., Venlo, Netherlands) was added, after which samples were vortexed vigorously at high speed for 20 seconds. Samples were again placed in an ice bath for 5 minutes to aid in precipitation of proteins and centrifuged for 5 minutes at 13.300 rpm. If no tight protein pellet was visible, the ice bath and centrifugation were repeated. The supernatant containing the DNA was harvested and transferred to 2 ml Eppendorf tubes containing 700 µl of isopropanol and 2 µl glycogen. Samples were mixed by inverting them gently approx. 50 times to precipitate the DNA. Following this, samples were centrifuged at 13.300 rpm for 5 minutes to pellet the DNA and the supernatant was carefully discarded using a pipette. To wash the DNA 700 µl of ethanol (70 %) was added and tubes gently inverted to wash the DNA pellets. After centrifuging for 5 minutes at 13.300 rpm, the ethanol was carefully poured off with the pellets remaining in the tube. Tubes were inverted, drained and allowed to airdry until complete evaporation of remaining ethanol. DNA pellets were then hydrated and resuspended using 200 µl of DNA Hydration Solution (QIAGEN N.V., Venlo, Netherlands) and in a final step incubated for 60 minutes at 65°C.

2.2.6 CFU serial dilution and count

CFU analysis was performed using cultures of our *M. fortuitum* clinical isolate (ID1). The aim was to compare CFU with qPCR-based detection.

For CFU analysis *M. fortuitum* was grown in liquid culture as described below until late-log phase (approximately 3 days), which was confirmed by measuring the optical density of the culture at 600 nm (OD_{600}) using a spectrophotometer [70]. The target OD_{600} for late-log growth in liquid culture was 1.5 (+/- 0.3). Following this, 1 ml of liquid culture was pelleted in a microcentrifuge at 13.300 rpm for 10 minutes to pellet the cells. The supernatant was discarded entirely by pipette. Initially, pellets were then resuspended in 1 ml of PBS, later switching to sterile saline solution (NaCl) and a syringe with a small gauge needle (20G) was used to further separate the cells. Then eight serial dilution steps (1/10) were done. For each dilution step, 60 µl mycobacterial culture was inoculated on 7H11 agar plates for CFU analysis and was used for gDNA extraction with

different protocols concomitantly. Subsequently, the volume of mycobacterial culture used was reduced to 50 µl for all following experiments. For higher dilutions such as the $10^{-6}/10^{-7}$ steps, 3-4 plate repetitions were inoculated. For all other dilutions, only single or duplicate plates were inoculated. Plates were inverted, stacked, tightly wrapped in aluminum foil to prevent from drying out and incubated at 37°C for 4-5 days until the colonies could be counted. CFU in the original sample were then calculated using following formula: $CFU/mL = \frac{(n\ colonies \times dilution\ factor)}{volume\ per\ culture\ plate}$.

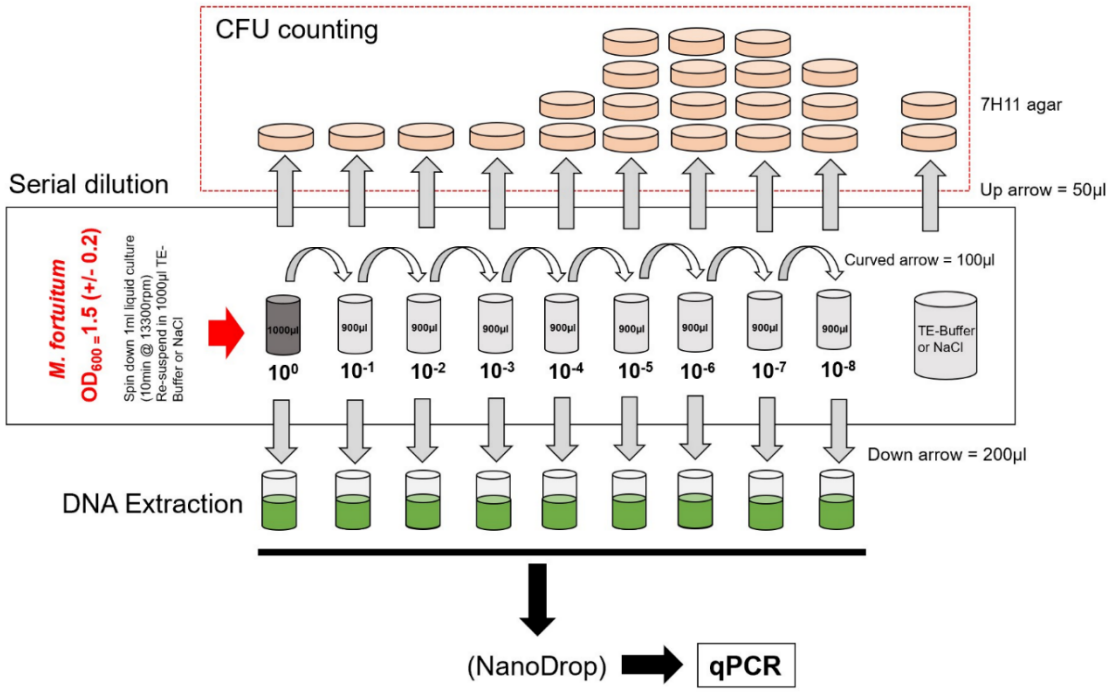


Fig. 3: CFU-Analysis, schematic overview.
This experiment was carried out to evaluate extraction protocols and qPCR analyses. Late-log-phase *M. fortuitum* liquid cultures were used. The growth stage was determined by measuring the optical density (OD) at 600nm (OD_{600}). Cells from the liquid culture were pelleted by centrifugation and eight tenfold serially diluted in TE-Buffer or sterile NaCl. Aliquots of each dilution were used to inoculate replicates of 7H11 solid medium plates for CFU counting. DNA extraction was performed from the serially diluted samples followed by qPCR and in some cases NanoDrop assessment of the eluted DNA.

2.2.7 Design of primers and probes

Primers and probes were designed using Geneious™ Prime® software (Geneious version 2023.0, created by Biomatters, available from https://www.geneious.com). The *M. fortuitum* and *M. tuberculosis* primers and probes were purchased from Metabion (Metabion international AG, Planegg, Germany). To allow for the identification of other NTM, so-called Pan-Mycobacteria primers and probe, designed to detect a genetic sequence present in all NTM, were purchased from Eurofins Genomics (Eurofins Genomics Europe Shared Services GmbH, Ebersberg, Germany).

An overview of all primers and probes described below can be found in table 2.

2.2.7.1 Selection of *M. fortuitum* target and design of primers/probe

To establish a qPCR assay for the detection of *M. fortuitum*, we focused on *M. fortuitum*-specific *rpoB gene* as a target sequence. The single copy *rpoB gene* encodes the beta-subunit of the bacterial RNA polymerase and has previously been used as a sequencing-based tool for the identification of NTM in several studies [71-74]. Our research group had also used this gene in 2015 to successfully establish a qPCR assay for the detection of *M. abscessus* complex [11].

Literature research on *M. fortuitum rpoB* primers using PubMed® was conducted and a paper by *Rocchetti et. al* from 2017 identified which reported on a *rpoB* primer/probe combination for *M. fortuitum* [31]. Alignment of these primers with the *M. fortuitum rpoB gene* was confirmed using NCBI BLAST® search. These previously identified primers were tested as forward primer 2 and reverse primer 1 with an amplicon length of 162 bp. Several other primers and probes binding in the same region of the *rpoB gene* were also designed using Geneious™ Prime® software and NCBI BLAST®. Subsequently and based on the previously reported primers, forward primer 3 and reverse primers 2, 3 and 4 were designed with a similar amplicon length. Additionally, reverse primers 5 and 6 were designed at a later point in time with a shorter overall amplicon length of about 70 bp. The *M. fortuitum*-specific probe consisted of an oligonucleotide labeled with a fluorescent FAM-dye at the 5‘ end and a nonfluorescent BHQ1 quencher molecule at the 3‘ end.

2.2.7.2 Selection of *M. tuberculosis* target and design of primers/probe

To be able to correctly differentiate between infections with *M. fortuitum* and *M. tuberculosis* among our study participants, we sought to additionally establish a qPCR assay for *M. tuberculosis.* We searched for primers from literature targeting regions of the *M. tuberculosis* specific *IS6110*, which could also possibly be integrated into our qPCR assay as a multiplex solution. To identify a feasible primer/probe combination for *M. tuberculosis,* focused literature research was conducted using PubMed®, with special emphasis being placed on primers which had previously been tested in Africa/Sub-Saharan Africa. Several papers from South Africa [40, 75], Uganda [76, 77], Kenya [78], Morocco [79] and Burkina Faso [80] were identified, which all reported on the use of the *IS6110* gene sequence as a target for PCR.

The so-called insertion sequence *IS6110*, which was first described in 1990, is a multicopy, 1.36 kb mobile genetic element specific and exclusive to the *M. tuberculosis* complex. It has been extensively used as a tool for DNA fingerprinting and epidemiological studies due to its polymorphism between different *M. tuberculosis* strains [81-86].

We again mapped the primer/probe combinations using Geneious™ Prime® Software and chose a primer/probe combination first reported by *Kolia-Diafouka et al.* in 2018 [39].

The *M. tuberculosis*-specific probe consisted of an oligonucleotide labeled with a fluorescent HEX-dye at the 5‘ end and a nonfluorescent BHQ1 quencher molecule at the 3‘ end.

2.2.7.3 Selection of Pan-Mycobacteria target and design of primers/probe

To allow detection of other mycobacterial infections, a so-called Pan-Mycobacteria primer was selected.

Several authors previously reported on different targets used for the identification of mycobacterial species by PCR such as 16s rRNA [32, 33] or the mycobacterial internal

transcribed spacer (ITS) [34]. We used Pan-Mycobacteria primers previously reported by *Rocchetti et al.* in 2016 [32].

The probe consisted of an oligonucleotide labeled with a fluorescent HEX-dye at the 5‘ end and a nonfluorescent BHQ1 quencher molecule at the 3‘ end.

Table 2: Overview Primers and Probes.

Target:	Primers:	Sequence (5‘ to 3‘)	BP	GC	Tm	Gene:	Ref.
M. fortuitum	Forward 2	TCACCTGATCTGCACATAATGT	22	41	58	rpoB	[31]
	Forward 3	CCCTCACCTGATCTGCACAT	20	55	60		
	Reverse 1	AGCACCTCATGCGACTT	17	53	52		
	Reverse 2	CACCTCATGCGACTTTTGAA	20	45	56		
	Reverse 3	GACTTTTGAAAAGACGAGCGA	21	43	57		
	Reverse 4	CAGCACCTCATGCGACTTTT	20	50	58		
	Reverse 5	ATACGCACGGCACAGGACTA	20	55	61.3		
	Reverse 6	ACCTGTCCAGAACCCCATAC	20	55	58.7		
	Probe	FAM-CTAGCCTGAGCRTTGCTCAGCA-BHQ1					
M. tuberculosis	Forward 1	GGCTGTGGGTAGCAGACCT	19	63.2	61.3	IS6110	[39]
	Reverse 1	GTAGGCGTCGGTGACAAAG	19	57.9	58.6		
	Probe	HEX-GGGCAGGGTTCGCCTACGT-BHQ1					
Pan-Mycobacteria	Forward 1	CGAACGGAAAGGYCYCTTCG	20	60	61.3	16s rRNA	[32]
	Reverse 1	CCGTCGTCGCCTTGGTAG	18	67	54.9		
	Probe	HEX-TTTWGCGGTGTGGGATGRGCCCG-BHQ1					

Note: BP refers to number of base pairs in each oligonucleotide, GC to the content of the nucleotides guanine and cytosine, Tm to the melting temperature.

2.2.8 Primer testing and assay parameters

Initial screening of the different primer combinations was conducted using conventional PCR and gel electrophoresis. Additionally, a temperature gradient PCR was performed. In a final step, primers were tested by qPCR using clinical isolates.

2.2.8.1 Screening of primers using conventional PCR

Initial performance testing of the different primer combinations for *M. fortuitum* and *M. tuberculosis* was conducted using conventional PCR and gel electrophoresis. Mastermixes were prepared using 12.5 µl REDTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich, Merck KGaA, Darmstadt Germany, Cat. R2523), 7.25 µl PCR-grade Water, 0.125 µl forward and reverse primer (final concentration 0.5 µM) and 5 µl of template DNA for a final volume of 25 µl per reaction. Alternatively, a master-mix consisting of

12.5 µl REDTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich, Merck KGaA, Darmstadt Germany, Cat. R2523), 5 µl PCR-grade Water, 1.25 µl 1:10 dilution of each forward and reverse primer (final concentration 0.5 µM) and 5 µl of template DNA for a final volume of 25 µl per reaction was used. The alternative protocol using a 1:10 dilution of primers was established to simplify workflow. Cycling conditions were as follows: 5 minutes of initial denaturing at 94°C, followed by 30 cycles of 60 seconds at 94°C, 60 seconds at 60°C and 60 seconds at 72°C. A T3 Thermoblock PCR Thermal Cycler (Biometra GmbH, Göttingen, Germany) was used. PCR products were then loaded on an Agarose gel (1 %) (UltraPure™ Agarose, Invitrogen, Thermo Fisher Scientific Inc., Waltham, Mass., USA) prepared with 1 X TBE Buffer (200 ml 5 X TBE running buffer (Novex™, Thermo Fisher Scientific, Waltham, Mass., USA) in 800 ml Ampuwa (Ampuwa Plastipur, Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany Fresenius Kabi)) and gel red (2 µl in 100 ml gel) (Biotium, Fremont, Cal., USA), which was then run at 120 mV using a 100 bp standard (Invitrogen™, Thermo Fischer Scientific Inc., Waltham, Mass., USA). Different dilutions (10^{-5}/10^{-6}/10^{-7}) of *M. fortuitum* clinical isolate (ID1, ID4) were used as template DNA. For the testing of the *IS6110* primers four dilutions (10^{-1}/10^{-4}/10^{-5}/10^{-6}) of *M. tuberculosis* H37Rv DNA were used as template DNA.

2.2.8.2 Temperature gradient PCR

Subsequently, an 8-step temperature gradient PCR was performed with selected primer combinations on Analytik Jena Flex Cycler^2 (Analytik Jena GmbH, Jena, Germany) to determine optimal annealing temperature. The master mix was the same as above and 5 µl of *M. fortuitum* clinical isolate DNA diluted to 10^{-6} was used as template. The temperature gradient was as follows: Step 1 at 54°C, Step 2 at 54.7°C, Step 3 at 55.9°C, Step 4 at 57.8°C, Step 5 at 60.2°C, Step 6 at 62.1°C, Step 7 at 63.3°C, Step 8 at 64°C for a total run time of approx. 2 hours and 20 minutes. The PCR product was again used for gel electrophoresis as described above.

2.2.8.3 Establishment of qPCR assay for *M. fortuitum*

Following the initial testing with conventional PCR and gradient PCR, primers were tested using qPCR.

Before preparing the master-mix, all reagents were taken out of storage at -20°C and left to thaw at room temperature. After thawing, all reagents were briefly vortexed to ensure even distribution and then shortly spun-down using a microcentrifuge to avoid residual reagent from contaminating the tube cap. When preparing a PCR or qPCR run, laboratory work was conducted in adherence to the "3-room-prinicple" in order to avoid contamination. Reagents, consumables and pipettes used for processing of PCR/qPCR material were strictly separated from the reagents, consumables and pipettes used for processing of samples. All laboratory surfaces and pipettes were cleaned thoroughly before/after each run and at the beginning/end of the workday using alcohol-based disinfectant.

After thawing of the reagents, the master mix was prepared according to the following calculation. For the qPCR assay the master mix was calculated with 2.5 µl of PCR water per reaction, 10 µl of QuantiTect® Multiplex PCR NoROX Master Mix (QIAGEN N.V., Venlo, Netherlands), 1 µl of 1:10 dilution forward primer (final conc. 0.5 µM), 1 µl of 1:10 dilution reverse primer (final conc. 0.5 µM), 0.5 µl of 1:10 dilution probe (final conc. 0.25 µM) and 5 µl of DNA sample for a final volume of 20 µl per reaction. Cycling conditions were set at 95°C for 15 minutes, followed by 40 cycles of 94°C denaturation for 1 minute and an annealing and extension step at 60°C for 1 minute, but increased to 50 cycles at a later point in time, as only linear qPCR curves could be observed initially. The assay was run on Bio-Rad C1000 Touch™ Thermal Cycler CFX96™ Real-Time System and designed/analyzed using Bio-Rad CFX Maestro Software (Bio-Rad Laboratories Inc., Hercules, CA, USA). To account for pipetting error/inaccuracy, the number of reactions was increased by 1-2 per master mix.

2.2.9 Statistical Analysis

Analysis and interpretation of qPCR results such as the calculation of mean values, overall concordance between methods and sensitivity was carried out using Microsoft® Excel® 2016. Figures and Tables were created using Microsoft® Word® and Microsoft®

PowerPoint® and SPSS®. Analysis CFUs were calculated according to the formula described under point 2.2.5 above.

3.0 Results

3.1 Screening of primers and establishment of qPCR assay

To establish our qPCR assay, we conducted testing of our custom primers using conventional PCR with gel electrophoresis, temperature gradient PCR and finally qPCR.

3.1.1 Screening of *M. fortuitum* primers using conventional PCR

Initially, we tested a set of primers using conventional PCR and gel electrophoresis, to evaluate whether the custom primers amplified their target. *M. fortuitum* forward primers 2 and 3 were tested in combination with reverse primers 1-4. The master mix was prepared as stated in the methods section under point 2.2.7.1 and the cycling conditions were as stated ibidem. The primer concentration per reaction was 0.5 µM and the annealing temperature was set at 60°C.

Several different dilutions of *M. fortuitum* clinical isolate (ID4) were used as template DNA, Fig. 4 A shows the results using a 10^{-5} dilution of *M. fortuitum* DNA, Fig. 4 B shows the results using a 10^{-6} dilution of *M. fortuitum* DNA and Fig. 4 C shows the results using a 10^{-7} dilution of *M. fortuitum* DNA.

As shown by the gel electrophoresis analysis (Fig. 4 A-C), all eight *M. fortuitum* primer combinations amplified the target DNA from our *M. fortuitum* clinical isolate at the determined dilutions. The highest band intensity for the PCR products was observed at the lowest dilution of target DNA in Fig. 4 A. At the lowest dilution of *M. fortuitum* DNA (Fig. 4 A), which equates to a higher amount of target per sample, the band intensity of the PCR products was increased when compared to the higher dilutions, which equate to lower concentrations of target per sample (Fig. 4 B and C). This inverse correlation between target concentration and band intensity of the PCR product was expected. The bands seem to fade out and disappear almost completely at the highest dilution of *M. fortuitum* template DNA (Fig. 4 C), a possible explanation being a lack of amplifiable target. When referencing the bands to the 100 bp standard, it can be observed that the PCR products are located between 100 bp and 200 bp marks on the gel. This matches the amplicon size calculated when designing the primers, which ranges from 151 bp to

167 bp. A slight formation of primer dimers can be observed in the background (Fig. 4 A), increasing in intensity with the higher dilutions (Fig. 4 B and C). The gel electrophoresis for the eight different primer combinations shows that the *M. fortuitum* primers seem to work almost equally well, with the exception of the combination forward primer 2 and reverse primer 1. The band intensity for the product of this primer combination is lower in all 3 runs when compared to the other primer combinations (Fig. 4 A-C).

Upon comparing the gel electrophoresis bands of the different primer combinations with special regard to background intensity and primer dimers, the combinations of forward primer 2 and reverse primer 2, forward primer 2 and reverse primer 4, forward primer 3 and reverse primer 2, forward primer 3 and reverse primer 4 were chosen for further testing.

At a later point in time, two additional reverse primers were designed for our *M. fortuitum* PCR assay. These reverse primers had a shorter amplicon length when combined with our existing forward primers. They were termed reverse primer 5 and reverse primer 6.

To determine if these primers worked and before integrating them into our qPCR assay for *M. fortuitum*, they were also tested using conventional PCR and gel electrophoresis. The preparation and setup of this PCR and gel electrophoresis were as previously described and DNA from a *M. fortuitum* clinical isolate (ID4) at the dilution 10^{-6} was used as a template. The results can be seen in Fig. 5 below and suggest that reverse primers 5 and 6 work as well. However, slightly more primer dimers can be observed and the band of the product is not as clear when compared to the product of forward primer 3 with reverse primer 2 (Fig. 5). When referencing the bands to the 100 bp standard, it can be observed that the PCR products are located close to, but below the 100 bp mark on the gel (Fig. 5). This matches the amplicon size calculated when designing the primers, which is around 70 bp.

In short, all of our custom primers amplified their target, the *M. fortuitum*-specific *rpoB* gene, when using gDNA from *M. fortuitum* clinical isolates on a conventional PCR assay with gel electrophoresis analysis.

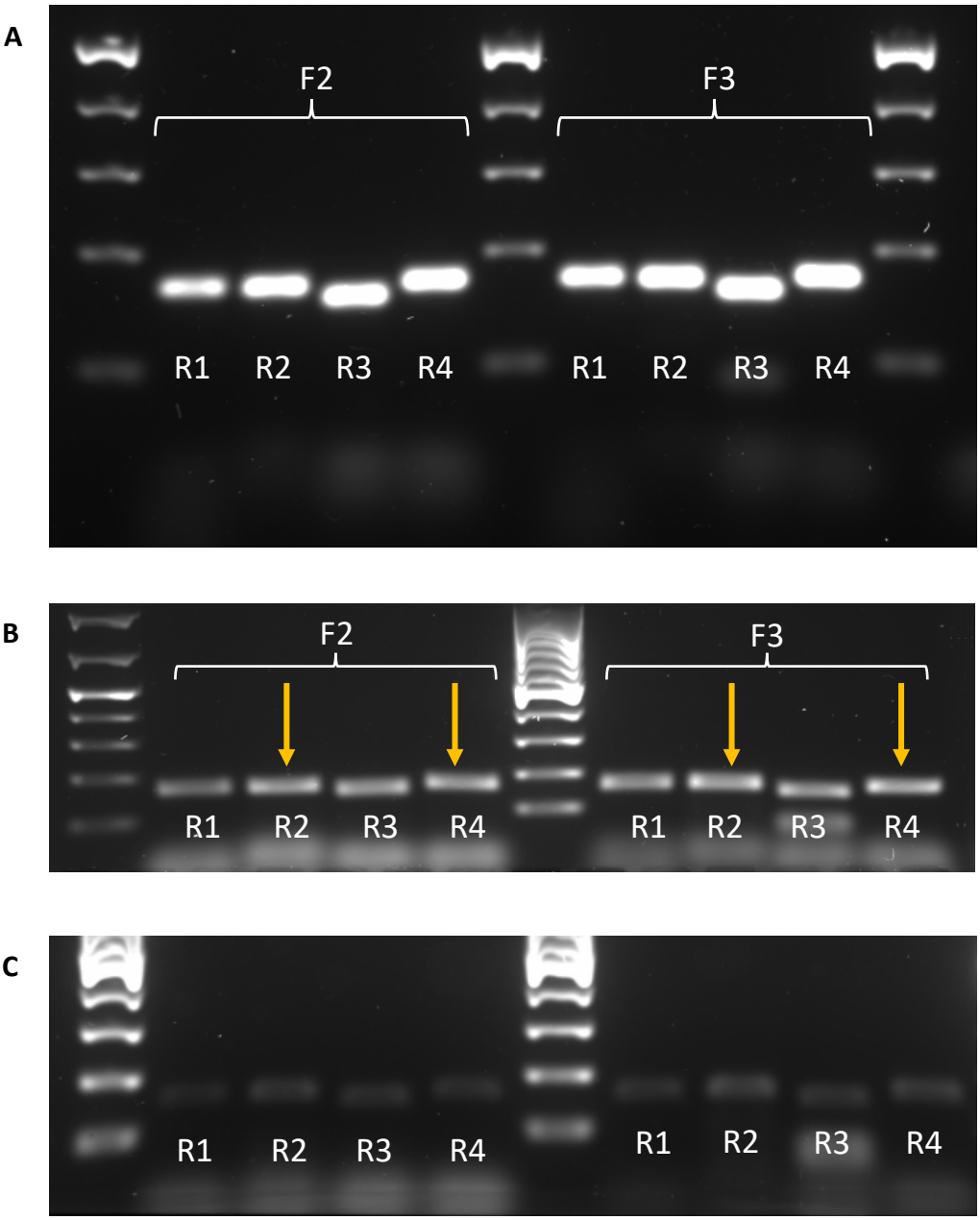


Fig. 4 A-C: Custom *rpoB* primers amplify *M. fortuitum* in conventional PCR.
Gel electrophoresis analysis of conventional PCR results using *M. fortuitum* forward primers 2 and 3 with reverse primers 1-4. Gel electrophoresis showing the PCR product amplified by our *M. fortuitum* primers. Note that "F2/F3" refers to forward primers 2/3 and "R1-4" refers to the reverse primers 1-4. A 100 bp standard can be seen on both sides and in the middle as a reference. Diluted gDNA from a *M. fortuitum* clinical isolate (ID4) was used as a template for PCR. **A** depicts the results for the 10^{-5} dilution of *M. fortuitum* gDNA, **B** depicts the results for the 10^{-6} dilution of *M. fortuitum* gDNA and **C** depicts the results for the 10^{-7} dilution of *M. fortuitum* gDNA. The combinations labeled with a yellow arrow were chosen for further testing.

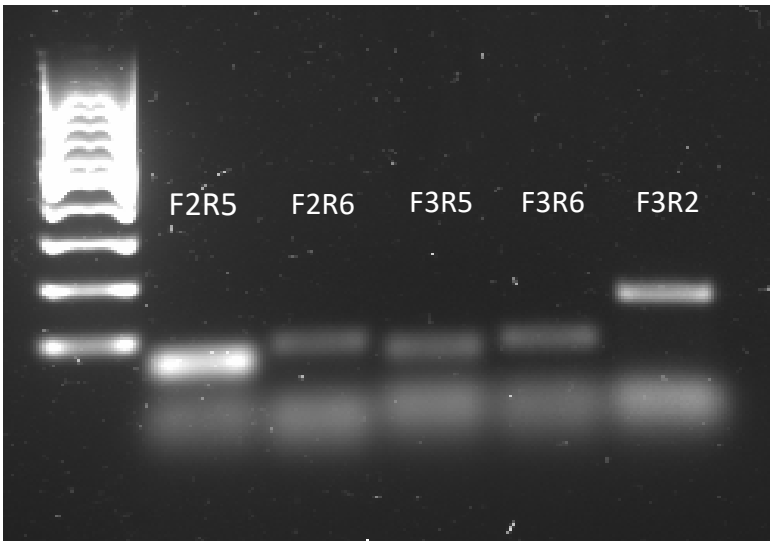


Fig. 5: Reverse primers with reduced amplicon length amplify *M. fortuitum* clinical isolate in conventional PCR.

Results of conventional PCR using *M. fortuitum* forward primers 2 and 3 with reverse primers 5 and 6. Gel electrophoresis image showing the testing of *M. fortuitum* reverse primers 5 and 6 in combination with the forward primers 2 and 3. As a reference and for comparison, one of the previous primer combinations was included and can be seen on the right side (F3R2). Note that "F2/F3" refers to forward primers 2/3 and "R5/R6" refers to the reverse primers 5/6. The primers were tested using a *M. fortuitum* gDNA from a clinical isolate (ID4) at the dilution 10^{-6} as template. A 100 bp standard can be seen on the left side for reference.

3.1.2 Primer dependent *M. fortuitum* gDNA amplification using gradient PCR

The protocol used for the temperature gradient PCR was described in the methods section under point 2.2.7.2 and the same master mix as for the conventional PCR was used. Background intensity and primer dimers were evaluated. The results of the temperature gradient PCR can be seen in Fig. 6 below. Of the four primer combinations tested, the combinations of forward primer 3 with reverse primer 2 and forward primer 3 with reverse primer 4 seem to have the best overall performance at the different temperature steps (Fig. 6, bottom). When comparing all four combinations, one can deduce that an annealing temperature between roughly 60-63°C seems to be optimal overall (Fig. 6) with individual variations. The primer combination forward 2 with reverse 4 (Fig. 6, top right) seems to have an optimal annealing temperature at around 60°C, whilst forward 3 with reverse 2 (Fig. 6, bottom left) seems to have an optimal annealing temperature at around 62°C and forward 3 with reverse 4 (Fig. 6, bottom right) at around 63°C.

In summary, the temperature gradient PCR we performed suggested an optimal annealing temperature in the range between 60°C and 63°C, with individual variations for the four primer combinations we tested. Based on these results the annealing temperature for initial qPCR testing was set at 60°C.

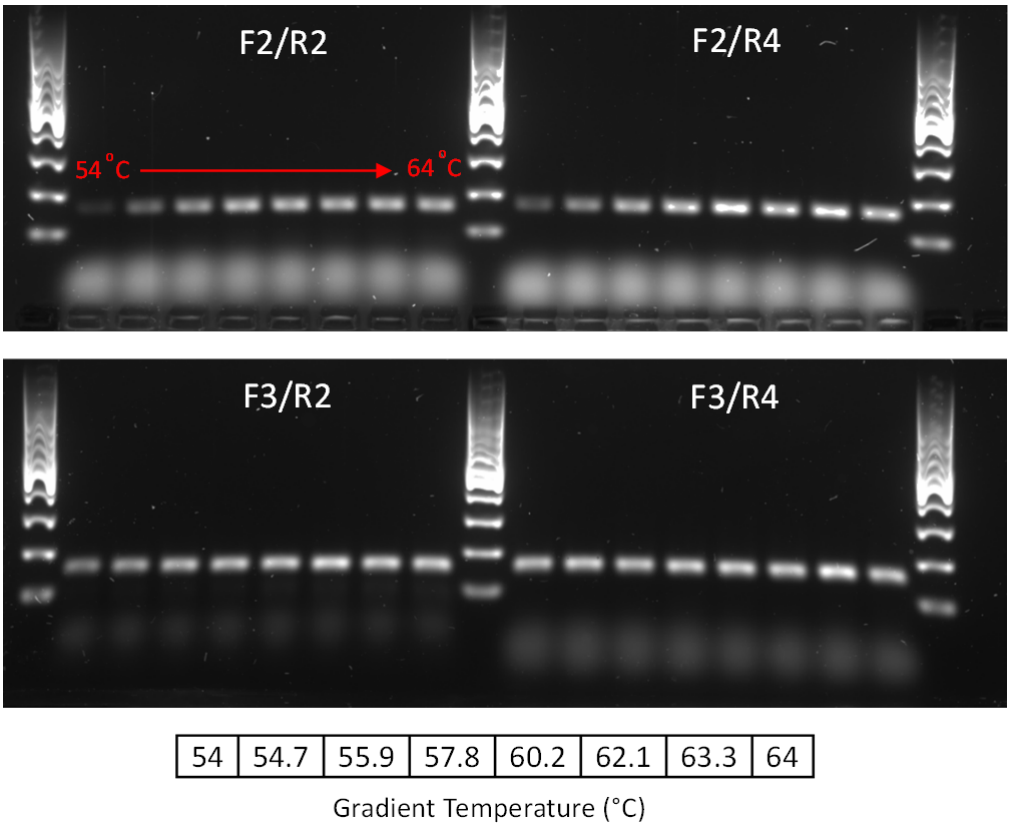


Fig. 6: Temperature gradient PCR, annealing temperature 60-63°C optimal for custom *M. fortuitum* primers.

Results of temperature gradient PCR for selected primer combinations. Gel electrophoresis image of temperature gradient PCR using *M. fortuitum* primers. A 100 bp standard can be seen on both sides and in the middle as a reference. As a template, 5 µl of *M. fortuitum* clinical isolate (ID4) DNA diluted to 10^{-6} was used. The temperature gradient from left to right was as follows: Step 1 at 54°C, Step 2 at 54.7°C, Step 3 at 55.9°C, Step 4 at 57.8°C, Step 5 at 60.2°C, Step 6 at 62.1°C, Step 7 at 63.3°C, Step 8 at 64°C. Note that "F2/F3" refers to forward primers 2/3 and "R2/R4" refers to the reverse primers 2/4. Out of the four primer combinations tested, the combinations of forward primer 3 with reverse primer 2 and forward primer 3 with reverse primer 4 seem to have the best overall performance at the different temperature steps (Fig. 6, bottom). An annealing temperature between roughly 60-63°C seems to be optimal overall (Fig. 6) with individual variations. Combination F2R4 (top right) seems to have an optimal annealing temperature around 60°C, whilst F3R2 (bottom left) seems to have an optimal annealing temperature around 62°C and F3R4 (bottom right) at around 63°C.

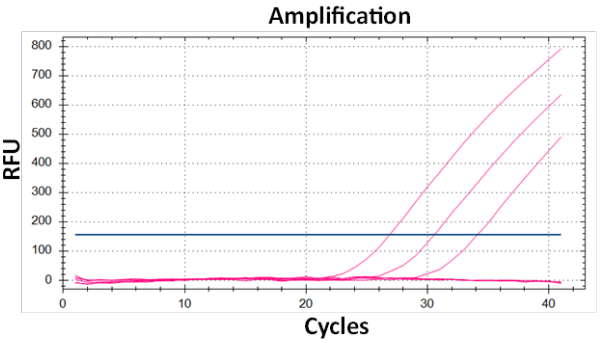
3.1.3 *M. fortuitum* specific primer usage in a qPCR assay

To test the performance of our selected primers and observe possible concentration-dependent titration, three dilutions of *M. fortuitum* DNA at 10^{-4}, 10^{-5} and 10^{-6} were used in our initial qPCR. The cycling conditions were based in part on the recommendations of the qPCR kits manufacturer (QIAGEN), with 15 minutes of initial denaturation at 95°C followed by 40 cycles of 60 seconds denaturation at 95°C and 60 seconds of annealing and extension at 60°C. The probe we used was a FAM fluorophore and the threshold was set at 155 relative fluorescent units (RFU).

The results of our qPCR test-run using *M. fortuitum* primers can be seen in Fig. 7 A-D below. All primer combinations amplified the three dilutions of *M. fortuitum* DNA above the cut-off threshold (Fig. 7 A-D), thus indicating a positive reaction. The amplification curves of our selected primers followed the titration of template DNA concentration. This is also evident when comparing the CT values, which are all separated by about three cycles (Fig. 7 A-D). No sigmodal qPCR standard curve was achieved, possibly suggesting issues with our assay. The selected primers appear to be specific for *M. fortuitum,* since the DNA extracts of *M. abscessus* and *M. tuberculosis* were not amplified (Fig. 7 A-D).

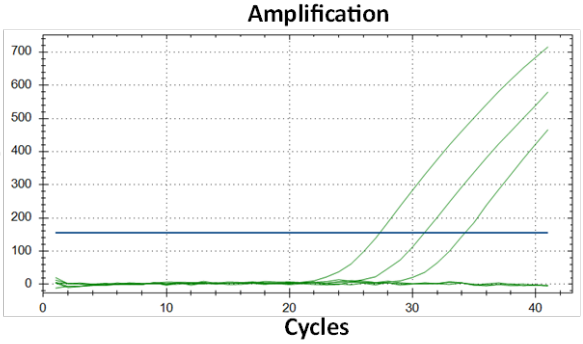
Reverse primer 5 and 6 were designed and tested at a later time-point in combination with the previously tested forward primers. The results of this qPCR test can be seen in Fig. 7 below. The cycling conditions were as described above, the probe was a FAM fluorophore and the threshold was set at 95 relative fluorescent units (RFU). All primer combinations amplified the two dilutions of *M. fortuitum* DNA above the cut-off threshold (Fig. 8 A-D), thus indicating a positive reaction. The amplification curves of our selected primers again followed the titration of template DNA concentration, as evident by comparing the CT values, which are all separated by about three cycles (Fig. 8 A-D).

A



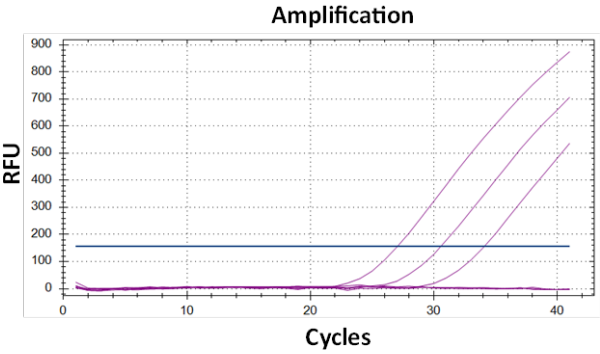
Primer pair: Fwd. 2 Rev. 2	
Samples	**CT**
M. fortuitum DNA 10^{-4}	26,85
M. fortuitum DNA 10^{-5}	30,55
M. fortuitum DNA 10^{-6}	34,09
M. abscessus DNA	N/A
M. tuberculosis H37Rv	N/A
NTC	N/A

B



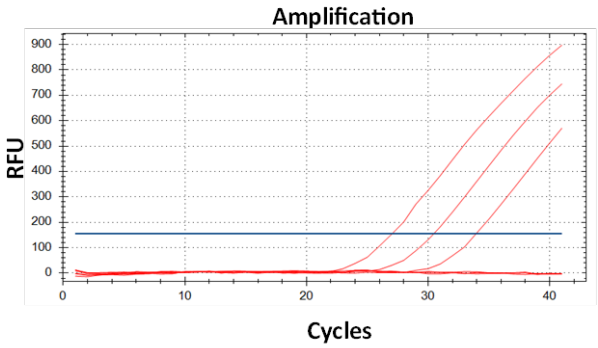
Primer pair: Fwd. 2 Rev. 4	
Samples	**CT**
M. fortuitum DNA 10^{-4}	27,34
M. fortuitum DNA 10^{-5}	30,96
M. fortuitum DNA 10^{-6}	34,26
M. abscessus DNA	N/A
M. tuberculosis H37Rv	N/A
NTC	N/A

C



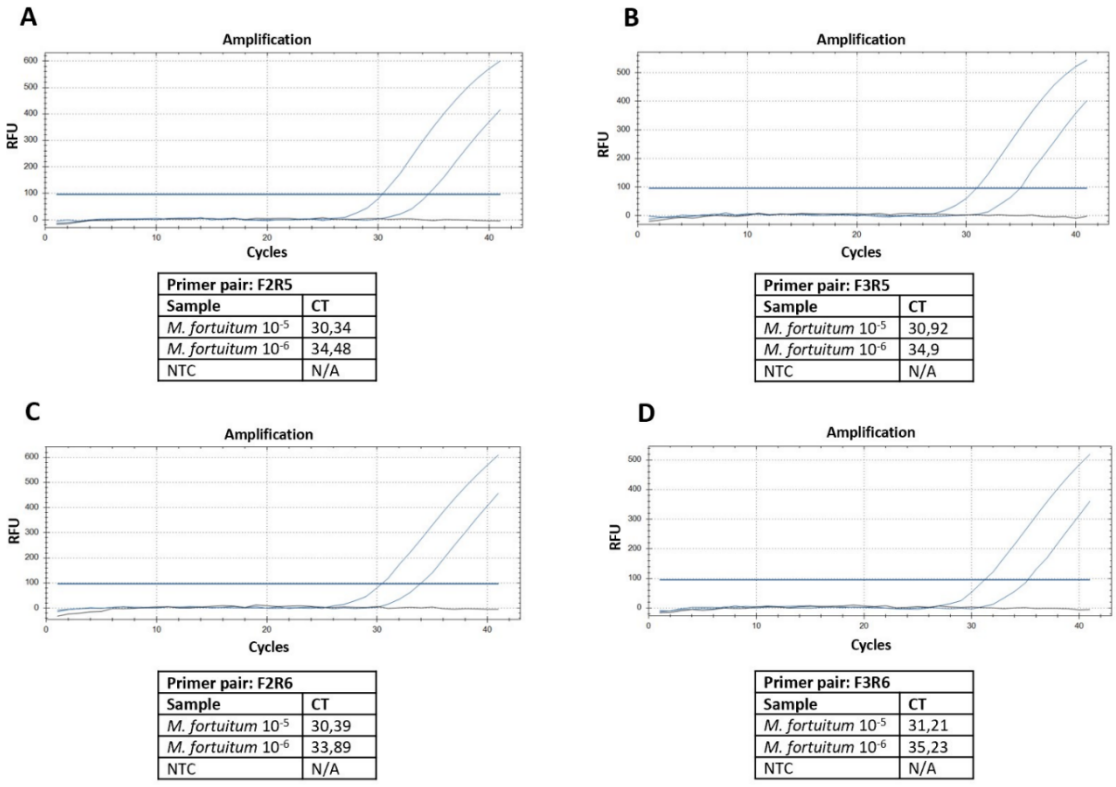
Primer pair: Fwd. 3 Rev. 2	
Samples	**CT**
M. fortuitum DNA 10^{-4}	27,04
M. fortuitum DNA 10^{-5}	30,55
M. fortuitum DNA 10^{-6}	34,08
M. abscessus DNA	N/A
M. tuberculosis H37Rv	N/A
NTC	N/A

D



Primer pair: Fwd. 3 Rev. 4	
Samples	**CT**
M. fortuitum DNA 10^{-4}	27,04
M. fortuitum DNA 10^{-5}	30,46
M. fortuitum DNA 10^{-6}	33,95
M. abscessus DNA	N/A
M. tuberculosis H37Rv	N/A
NTC	N/A

Fig. 7: Primers and probe for *M. fortuitum* correctly identify clinical isolate in test qPCR.
Results of qPCR test run using *M. fortuitum* forward primers 2/3, reverse primers 2/4 and FAM-probe. qPCR amplification of *M. fortuitum* DNA from a clinical isolate at dilutions 10^{-4} to 10^{-6}. Neither the NTC, *M. tuberculosis* DNA or *M. abscessus* DNA show amplification above the cut-off threshold. **A** shows the results of forward primer 2 and reverse primer 2, **B** shows the results of forward primer 2 and reverse primer 4, **C** shows the results of forward primer 3 and reverse primer 2, **D** shows the results of forward primer 3 and reverse primer 4.



Primer pair: F2R5	
Sample	CT
M. fortuitum 10^{-5}	30,34
M. fortuitum 10^{-6}	34,48
NTC	N/A

Primer pair: F3R5	
Sample	CT
M. fortuitum 10^{-5}	30,92
M. fortuitum 10^{-6}	34,9
NTC	N/A

Primer pair: F2R6	
Sample	CT
M. fortuitum 10^{-5}	30,39
M. fortuitum 10^{-6}	33,89
NTC	N/A

Primer pair: F3R6	
Sample	CT
M. fortuitum 10^{-5}	31,21
M. fortuitum 10^{-6}	35,23
NTC	N/A

Fig. 8 A-D: Reduced amplicon length reverse primers for *M. fortuitum* correctly identify clinical isolate in test qPCR.
Results of qPCR test run using *M. fortuitum* forward primers 2/3, reverse primers 5/6 and FAM-probe. To test the new reverse primers 5 and 6, *M. fortuitum* DNA from a clinical isolate at dilutions 10^{-5} and 10^{-6} was used. **A** shows the results of forward primer 2 and reverse primer 5, **B** shows the results of forward primer 3 and reverse primer 5, **C** shows the results of forward primer 2 and reverse primer 6, **D** shows the results of forward primer 3 and reverse primer 6. The NTC did show amplification above the cut-off threshold.

On the basis of the results in Figs. 4-8 above, we concluded that our custom *M. fortuitum* primers showed sufficient performance and specificity for further implementation of the qPCR assay for laboratory experiments in Düsseldorf and field work in Kumasi. The parameters for the cycling conditions of our *M. fortuitum* qPCR assay were set. We established the assay and continued using it for further experiments based on the following master mix recipe and cycling conditions.

3.1.4 *M. tuberculosis* specific primer usage in a qPCR assay

We initially tested our *M. tuberculosis IS6110* forward and reverse primers using conventional PCR and gel electrophoresis as described in the methods section under point 2.2.7.1 above. The results can be seen in Fig. 9 below and indicate that the primers do bind and amplify the *M. tuberculosis* specific *IS6110* target region when using our *M. tuberculosis* H37Rv strain as template. At higher dilutions of *M. tuberculosis* DNA, the band intensity of the PCR product fades, suggesting a concentration-dependent reaction.

Next, we tested the *M. tuberculosis* primers with our previously established qPCR assay. Three dilutions of *M. tuberculosis* H37Rv DNA (10^{-4}/10^{-5}/10^{-6}) were used as target DNA, as well as *M. fortuitum* and *M. abscessus* DNA extracts from clinical isolates. These were included to test the specificity of the primers. The master-mix calculation and cycling conditions were as previously described, the probe was a HEX fluorophore and the threshold was set at 195 relative fluorescent units (RFU). The results of this qPCR test run can be seen in Fig. 10 below. The *IS6110* primers successfully amplified all three dilutions of *M. tuberculosis* DNA above the cut-off threshold, thus indicating a positive reaction. Additionally, CT values consistent with the titration of diluted DNA can be observed (each about 3 cycles apart), which supports the performance of our primers. The DNA extracts of *M. fortuitum* and *M. abscessus* were not amplified, suggesting that our *IS6110* primers are specific to *M. tuberculosis.* The NTC also did not amplify.

On the basis of the results in Fig. 9 and Fig. 10, we concluded that our custom *M. tuberculosis* primers showed sufficient performance and specificity for further implementation in the qPCR assay and use in experiments.

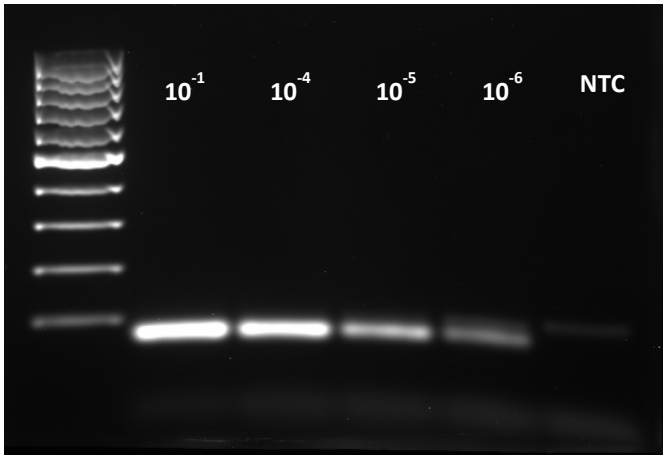
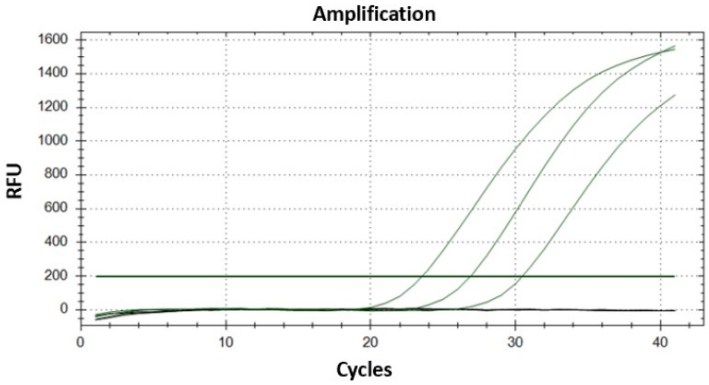


Fig. 9: *IS6110* primers amplify *M. tuberculosis* H37Rv in conventional PCR.
Gel electrophoresis image showing the testing of our custom *M. tuberculosis IS6110* primers. Four dilutions of *M. tuberculosis* H37Rv DNA were used as template DNA (10^{-1}/10^{-4}/10^{-5}/10^{-6}). A 100 bp standard can be seen on the left side for reference. The NTC did not amplify.



Sample	CT
M. tuberculosis H37Rv 10^{-4}	23,49
M. tuberculosis H37Rv 10^{-5}	26,84
M. tuberculosis H37Rv 10^{-6}	30,41
M. abscessus	N/A
M. fortuitum	N/A
NTC	N/A

Fig. 10: *IS6110* primers and probe correctly identify *M. tuberculosis* H37Rv in test qPCR.
Results of qPCR test run using *IS6110* primers and HEX-fluorophore probe. qPCR amplification of *M. tuberculosis* H37Rv DNA using custom *IS6110* specific primers. All three dilutions of *M. tuberculosis* H37Rv DNA amplified above the cut-off threshold, indicating a positive reaction. Neither the NTC, *M. fortuitum* DNA or *M. abscessus* DNA show amplification above the cut-off threshold.

3.1.5 Troubleshooting sigmoidal qPCR curve

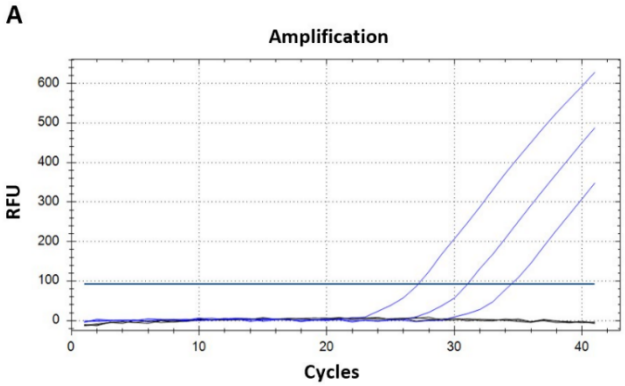
We initially interpreted that the lack of a sigmoidal qPCR curve in our assay constituted suboptimal performance of our primers. This was the reason we designed reverse primers 5 and 6 with a shorter amplicon length. However, these primers with a shorter amplicon length did not solve this perceived problem and sigmoidal qPCR curves still could not be observed.

Next, we tried changing the qPCR protocol, specifically the master mix and the concentration of our qPCR reagents per reaction to investigate if this had any effect on the qPCR curve. The assay was run with increased primer concentration of 1 µM per reaction and a decreased probe concentration of 0.15 µM per reaction. The amount of PCR-water per reaction was adjusted accordingly to reach a final volume of 20 µl per reaction (including 5 µl of sample). For this purpose, we used three dilutions (10^{-4}-10^{-6}) of *M. fortuitum* clinical isolate (ID1) DNA as a template, along with DNA from our *M. tuberculosis* H37Rv strain and DNA from *M. abscessus.* An NTC was also included. The assay was run using forward primer 3 and reverse primer 2, as well as our FAM-fluorophore probe. The cut-off threshold was set at 92 RFU.

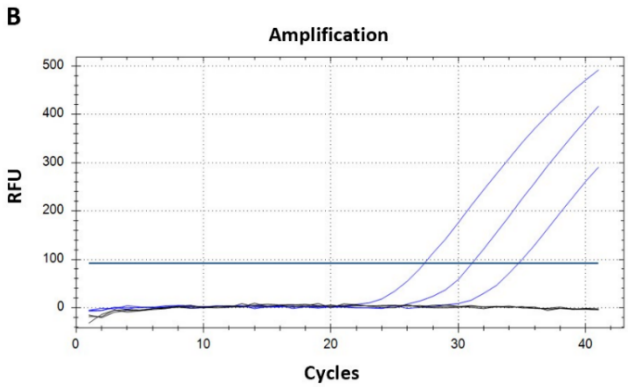
The results can be seen in Fig. 11 A-B below. The results for the increased primer concentration at 1 µM per reaction are depicted in Fig. 11 A and the results for the decreased probe concentration at 0.15 µM are depicted in Fig. 11 B. Although all dilutions of *M. fortuitum* DNA were amplified above the cut-off threshold, indicating a positive reaction, no sigmoidal qPCR curve could be observed for either run. The NTC and the DNA from *M. tuberculosis* and *M. abscessus* were not amplified.

Neither an increase in the primer concentration, nor a decrease in the probe concentration led to a sigmoidal curve in our qPCR assay. Regardless of the change in primer or probe concentration, the CT-values for each sample were similar (Fig. 11 A-B).

More or less by accident we discovered that in our case, the solution to the sigmoidal curve problem was an extension of the number of cycles of our assay. By increasing the number of cycles in our protocol to 50 from 40, sigmoidal qPCR curves could finally be observed (see Fig. 23 A-B below).



Sample	CT
M. fortuitum clinical isolate gDNA 10^{-4}	27,15
M. fortuitum clinical isolate gDNA 10^{-5}	31,02
M. fortuitum clinical isolate gDNA 10^{-6}	34,45
M. tuberculosis H37Rv gDNA	N/A
M. abscessus gDNA	N/A
NTC	N/A



Sample	CT
M. fortuitum clinical isolate gDNA 10^{-4}	27,34
M. fortuitum clinical isolate gDNA 10^{-5}	31,08
M. fortuitum clinical isolate gDNA 10^{-6}	34,73
M. tuberculosis H37Rv gDNA	N/A
M. abscessus gDNA	N/A
NTC	N/A

Fig. 11 A-B: No sigmoidal qPCR curve with increased primer concentration or decreased probe concentration.

M. fortuitum qPCR assay run with increased primer concentration and decreased probe concentration. qPCR analysis of *M. fortuitum* clinical isolate gDNA using forward primer 3, reverse primer 2 and FAM-fluorophore probe with increased primer concentration (**A**) and decreased probe concentration (**B**). The cut-off threshold was set at 92 RFU. Three dilutions (10^{-4}–10^{-6}) of *M. fortuitum* gDNA from a clinical isolate were tested, gDNA from *M. tuberculosis* strain H37Rv and from *M. abscessus* was also included. The primer concentration was increased to 1 µM per reaction in **A** and the probe concentration was decreased to 0.15 µM per reaction in **B**, to account for these changes the PCR water was adjusted accordingly to reach a final volume of 20 µl per reaction. All three dilutions of *M. fortuitum* gDNA amplified above the cut-off threshold, indicating a positive reaction. Regardless of primer or probe concentration, the CT-values of each sample were similar and no sigmoidal qPCR curve could be observed. Neither the NTC, nor the DNA of the other mycobacterial species was amplified.

3.2 Comparison of the different DNA extraction methods

To compare and evaluate DNA extraction methods in combination with our qPCR assay, we used a CFU dilution and count experiment that was described in detail in the methods section under point 2.2.5 and in Fig. 3 above.

3.2.1 Approach I: QIAmp DNA Blood Mini Kit & Norgen Sputum DNA isolation kit

The extraction methods that we initially compared were two spin-column based kits, the QIAmp DNA Blood Mini Kit (QIAGEN N.V., Venlo, Netherlands) and the Norgen Sputum DNA isolation kit (Norgen Biotek Corp., Thorold, ON, Canada). For this purpose, a CFU count was set up as described above. The protocols for the DNA extraction methods were also described under point 2.2.5.1 above.

The eluted DNA from both protocols was subsequently used for qPCR analyses, the protocol for which has also been described previously. In this case, forward primer 3 and reverse primer 2 were used together with our FAM-probe. The cut-off threshold was set at 124 RFU.

The eluted DNA was also measured using a NanoDrop 1000/2000 (Thermo Fischer Scientific Inc., Waltham, Mass., USA) to ascertain the concentration and purity, as another means of evaluating the efficiency of each extraction protocol (Table 3). However, the purity values fall in a wide and varied range and are mostly outside of the 1.8-2.2 target range. Also, no titration of the DNA concentration values in accordance with their degree of dilution could be observed.

The results of the CFU count are displayed in Fig. 12 below. After 7 days of incubation at 37°C the colonies on each plate were counted to calculate the amount of CFU in the original sample. The incubation control plates that had been inoculated with the PBS used for the serial dilution showed no growth (Fig. 12). The plates (n=4) inoculated with 10^{-8} diluted sample also showed no growth (Fig. 12). The plates that had been inoculated with the dilutions 10^{0}–10^{-4} all had too many colonies to count. The mean number of colonies counted at the 10^{-5} dilution was 359, at the 10^{-6} dilution the mean was 56,8 and at the 10^{-7} dilution the mean number of colonies counted was 5 (Fig. 12). We used the mean number of colonies at the 10^{-6} dilution to calculate the amount of colony forming

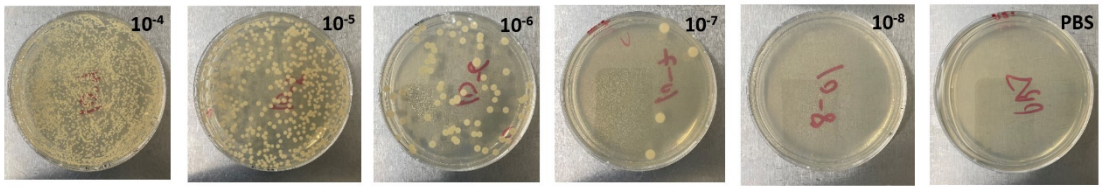
units per milliliter in the original sample using the formula described under point 2.2.5 above. The amount of colony forming units per milliliter in the original sample was calculated to be 9.5×10^8 CFU/ml (Fig. 12).

The qPCR results of both DNA extraction methods can be seen below in Fig. 13 (Qiagen) and Fig. 14 (Norgen).

For the extraction using QIAGEN QIAmp DNA Blood mini kit, all of the serially diluted samples (10^0-10^{-8}) amplified above the cut-off threshold at 124 RFU, indicating a positive reaction (Fig. 13). Together with this extraction protocol, our assay was able to identify *M. fortuitum* at a concentration of 9.5 CFU/ml (10^{-8} dilution), which had a CT-value of 39,87. Even though the first three dilutions (10^0-10^{-2}) show a clear titration in the qPCR with an approximate difference of 2-3 cycles in between, this cannot be said for all dilutions. The difference between the CT-values the dilutions 10^{-2} and 10^{-3} is almost 5 cycles whilst there is only a minute difference in CT-values when comparing 10^{-6} and 10^{-8} dilutions at a CT of 39,47 and 39,87 respectively (Fig. 13).

In contrast to the extraction using the QIAGEN QIAmp DNA Blood mini kit, for the extraction using the Norgen Biotek Sputum DNA isolation kit some of the serially diluted samples did not amplify above the cutoff threshold. The dilutions of 10^0, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-7} and 10^{-8} amplified above the cut-off threshold, indicating a positive reaction. The samples at dilutions 10^{-2} and 10^{-5} and 10^{-6} did not amplify above the cut-off threshold. The NTC was not amplified. The assay was able to detect 9.5 CFU/ml at the highest dilution. Another difference when comparing the results of this extraction with the results of the QIAmp DNA Blood mini kit extraction is that no titration of CT values according to the sample concentration could be observed (Fig. 14).

Whilst we were able to identify 9.5 CFU/ml at the lowest sample dilution with either extraction method and our assay, the QIAmp DNA Blood mini kit did show more promising results regarding overall performance, as all dilutions 10^0-10^{-8} of *M. fortuitum* could be identified (Figs. 13 and 14).



Dilution factor	Nr. of colonies					Mean
	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	
10^{-5}	377	389	360	329	340	359
10^{-6}	40	71	58	55	60	**56,8**
10^{-7}	4	5	9	2		5
10^{-8}	No growth					N/A
PBS ctrl.	No growth					N/A

Note: 10^{-0}, 10^{-1} to 10^{-4} had too many colonies to count

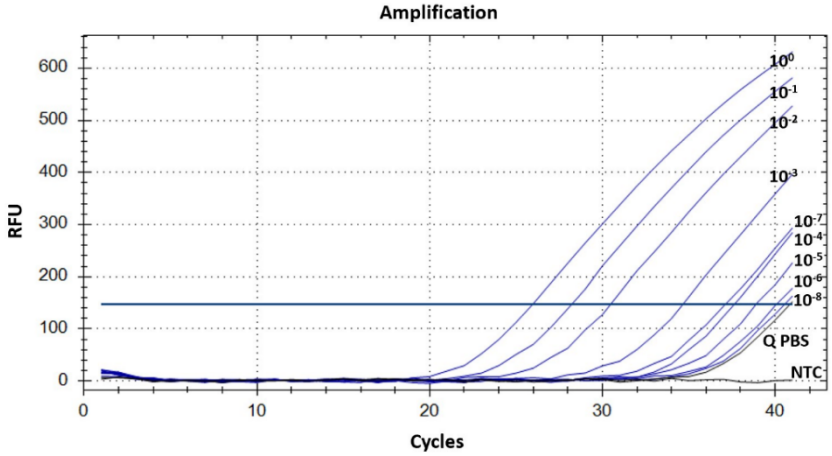
CFU/mL in original sample (10^0) was calculated at **9.5×10^8 CFU/ml**

Fig. 12: First CFU analysis determines 9.5×10^8 CFU/ml in *M. fortuitum* subculture.
At the top, pictures of *M. fortuitum* colonies on 7H11 solid media plates and the PBS incubation control after 7 days of incubation at 37°C can be observed. The dilution factor of each respective plate can be found in the top right corner. The chart below shows the number of colonies that were counted per plate repetition.

Table 3: NanoDrop indicates insufficient purity and concentration of DNA extracted with QIAGEN and Norgen kits.
NanoDrop results of the DNA extracted with the QIAGEN and the Norgen protocols. To further evaluate the DNA extraction protocols, eluted DNA was quantified with regard to its concentration and purity using a NanoDrop 1000 (Thermo Fischer Scientific Inc., Waltham, Mass., USA). No titration of the DNA concentration values in accordance to their degree of dilution can be observed. The purity values fall in a wide and varied range and are mostly outside of the 1.8–2.2 target range.

CFU dilutions	CFU/mL	QIAGEN QIAamp DNA mini kit		NORGEN BIOTEK sputum DNA kit	
		Concentration (ng/µl)	Purity (260/280)	Concentration (ng/µl)	Purity (260/280)
10^0	9.5×10^8	7,94	1,27	2,75	3,43
10^{-1}	9.5×10^7	5,99	1,56	6,05	1,08
10^{-2}	9.5×10^6	8,03	1,24	1,88	1,26
10^{-3}	9.5×10^5	4,27	1,34	2,58	1,06
10^{-4}	9.5×10^4	7,04	1,24	1,57	1,73
10^{-5}	9.5×10^3	5,76	1,23	4,26	1,40
10^{-6}	9.5×10^2	5,85	1,11	3,02	0,84
10^{-7}	9.5×10^1	5,51	1,37	3,37	1,42
10^{-8}	9.5	3,48	1,10	3,22	3,10

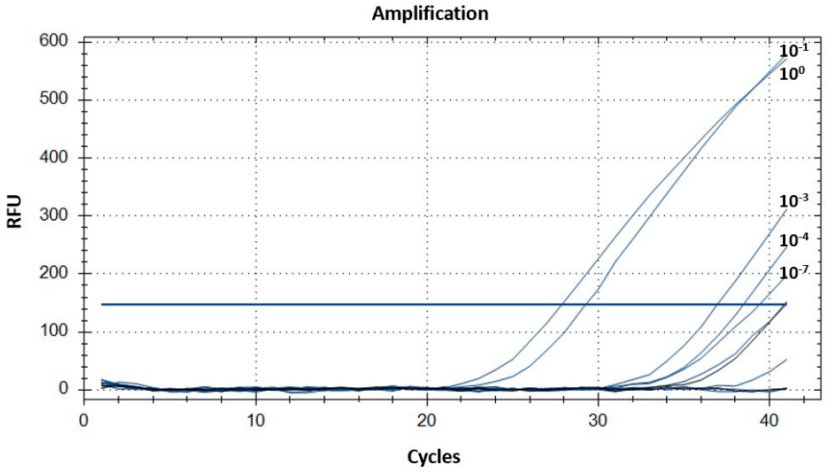
QIAGEN QIAamp DNA mini kit



Sample	CFU/ml	CT
10^{0}	9.5×10^{8}	25,32
10^{-1}	9.5×10^{7}	27,60
10^{-2}	9.5×10^{6}	29,89
10^{-3}	9.5×10^{5}	34,11
10^{-4}	9.5×10^{4}	37,02
10^{-5}	9.5×10^{3}	38,35
10^{-6}	9.5×10^{2}	39,47
10^{-7}	9.5×10^{1}	36,47
10^{-8}	9.5	39,87
PBS	-	40,21
NTC	-	N/A

Fig. 13: qPCR assay detects 9.5 CFU/ml in samples extracted with QIAmp kit.
Results of qPCR using DNA extracted from serial dilution with QIAmp DNA blood mini kit. Forward primer 3, reverse primer 2 and FAM-fluorophore probe were used. The cut-off threshold was set at 124 RFU. All dilutions 10^{0}–10^{-8} amplified above the cut-off threshold, indicating a positive reaction. The NTC was not amplified. The assay was able to detect ≥ 9.5 CFU/ml.

NORGEN BIOTEK Sputum DNA isolation kit



Sample	CFU/ml	CT
10^{0}	9.5×10^{8}	27,24
10^{-1}	9.5×10^{7}	28,66
10^{-2}	9.5×10^{6}	N/A
10^{-3}	9.5×10^{5}	36,38
10^{-4}	9.5×10^{4}	37,87
10^{-5}	9.5×10^{3}	N/A
10^{-6}	9.5×10^{2}	N/A
10^{-7}	9.5×10^{1}	38,59
10^{-8}	9.5	40,21
NTC	-	N/A

Fig. 14: Norgen extraction protocol unsuccessful.
Results of qPCR using DNA extracted from serial dilution with Norgen Biotek Sputum DNA isolation kit. Forward primer 3, reverse primer 2 and FAM-fluorophore probe were used. The cut-off threshold was set at 124 RFU. The dilutions 10^{0}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-7} and 10^{-8} amplified above the cut-off threshold, indicating a positive reaction. The NTC was not amplified. The assay was able to detect 9.5 CFU/ml at the highest dilution.

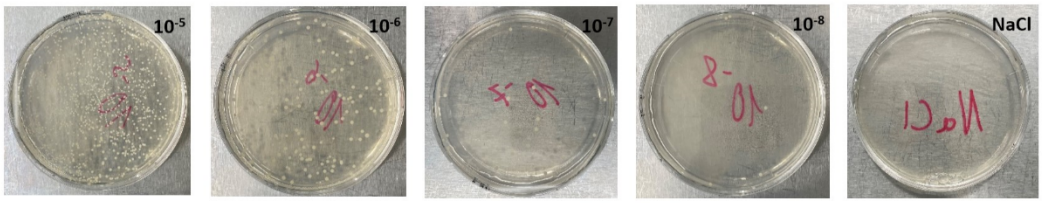
3.2.2 Approach II: Modifications to Norgen Sputum DNA isolation kit

Next, we made modifications to the protocol of the Norgen Biotek Sputum DNA isolation kit. We compared the manufacturer's protocol to the protocol of the innuPrep Mycobacteria DNA kit (Analytik Jena GmbH, Jena, Germany), which had previously been used by our research group but is no longer commercially available. We made several changes to the duration of chemical and thermal lysis in the Norgen extraction protocol. The full protocol is described under point 2.2.5.2 above.

The eluted DNA was subsequently used for qPCR analyses according to the previously described qPCR protocol. In this case, forward primer 3 and reverse primer 2 were used together with our FAM-probe. The cut-off threshold was set at 95 RFU.

A new serial dilution and CFU count experiment was also set up. The results of the CFU count are displayed in Fig. 15 below. After four days of incubation at 37°C, the colonies on all plate repetitions were counted. While the plates inoculated with the dilutions 10^{0}-10^{-4} had too many colonies to count, all other repetitions were counted and a mean number of colonies per plate calculated for each dilution. The incubation controls, which had been inoculated with just the NaCl used for the serial dilution did not show any growth, suggesting that no contamination had taken place. Using the mean number of colonies per plate at the 10^{-7} dilution (23,8), we calculated the amount of CFU/ml in the original sample. The number of CFU per milliliter of original culture sample was calculated at $4{,}76 \times 10^{9}$ (Fig. 15).

The results of the modified Norgen extraction protocol can be seen in Fig. 16 below. All samples that were extracted using this protocol and subsequently analyzed by our qPCR assay amplified above the cut-off threshold, indicating a positive reaction. The NTC did not amplify. Although a correlation between the CT-values and concentration titration of the samples can be observed for the dilutions 10^{0}-10^{2}, with a difference of 2-3 cycles in between, this is not the case for all samples tested. Our assay was able to detect the highest diluted sample (10^{-8}), containing approximately $4{,}76 \times 10^{1}$ CFU/ml at a CT of 37,61 cycles (Fig. 16).

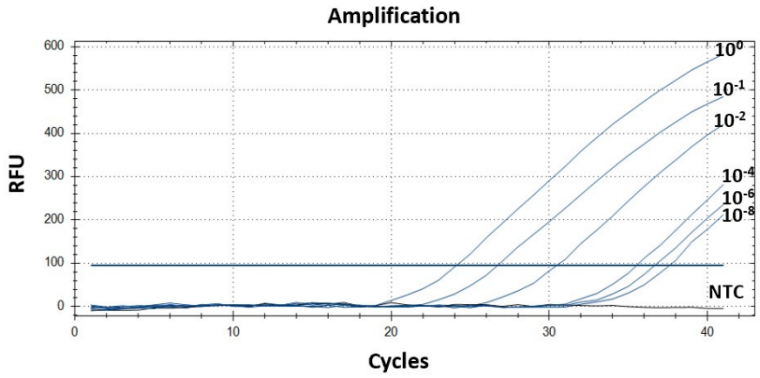


Dilution factor	Nr. of colonies					Mean
	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	
10^{-5}	657	695	790	885	863	778
10^{-6}	118	143	158	148	114	136,2
10^{-7}	13	26	19	13	48	**23,8**
10^{-8}	0	3	7	/		3,33
NaCl	No growth		/			N/A

Note: 10^{-0}, 10^{-1} to 10^{-4} had too many colonies to count

CFU/mL in original sample (10^{0}) was calculated at **$4{,}76 \times 10^{9}$ CFU/mL**

Fig. 15: Second CFU analysis determines $4{,}76 \times 10^{9}$ CFU/ml in *M. fortuitum* subculture.
At the top, pictures of *M. fortuitum* colonies on 7H11 solid media plates and the NaCl incubation control after 4 days of incubation at 37°C can be observed. The dilution factor of each respective plate can be found in the top right corner. The chart below shows the number of colonies that were counted per plate repetition and the mean number of colonies counted. On the basis of the mean value at the 10^{-7} dilution, the amount of CFU/ml in the original sample was calculated. The formula for this calculation has been described previously. The number of colony-forming-units per milliliter of original culture sample was calculated at $4{,}76 \times 10^{9}$. The incubation controls, which had been inoculated with the NaCl used for the serial dilution did not show any growth.



Sample	CFU/ml	CT
MF 10^{0}	$4{,}76 \times 10^{9}$	24,13
MF 10^{-1}	$4{,}76 \times 10^{8}$	26,73
MF 10^{-2}	$4{,}76 \times 10^{7}$	30,48
MF 10^{-4}	$4{,}76 \times 10^{5}$	35,53
MF 10^{-6}	$4{,}76 \times 10^{3}$	36,68
MF 10^{-8}	$4{,}76 \times 10^{1}$	37,61
NTC	-	N/A

Fig. 16: Improved qPCR assay results after changes to Norgen extraction protocol.
The qPCR results of DNA from serially diluted *M. fortuitum* culture extracted using modified Norgen Sputum DNA isolation kit. Forward primer 3, reverse primer 2 and FAM-fluorophore probe were used. The cut-off threshold was set at 95 RFU. All six dilutions 10^{0}–10^{-2} and 10^{-4}, 10^{-6}, 10^{-8} amplified above the cut-off threshold, indicating a positive reaction. The NTC was not amplified. The assay was able to detect *M. fortuitum* at $\geq 4.76 \times 10^{1}$ CFU/ml.

3.2.3 Approach III: TE-Buffer and Tween20 extraction methods

The use of a DNA extraction protocol for Mycobacteria based on a Tris-EDTA lysis buffer combined with thermal lysis and Lysozyme and Proteinase K digestion, was previously reported by *Aldous et al.* in 2005 and adapted from this paper [61]. The protocol is described in detail in the methods section under point 2.2.5.3 above. It was tested using aliquots of the serial dilution and CFU count described under point 3.3.2 and in Figure 15 above.

Six 200 µl samples from each of the dilutions 10^{0}-10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} were used to test the extraction protocol. The eluted DNA was then used for qPCR analysis. Forward primer 3, reverse primer 2 and our FAM-fluorophore probe were used for the assay. The cut-off threshold was set at 95 RFU. The results of the qPCR can be seen in Fig. 17 below. None of the six samples amplified, suggesting insufficient extraction of mycobacterial DNA.

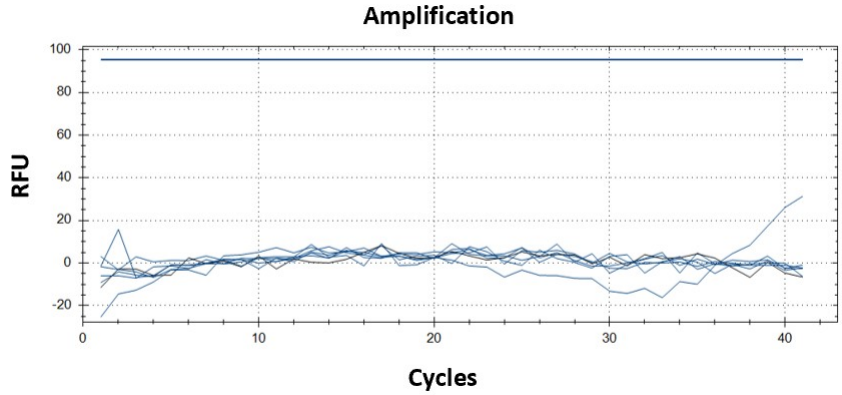


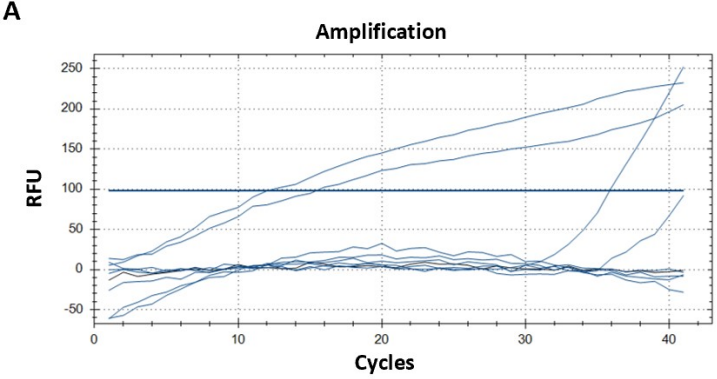
Fig. 17: TE-Buffer extraction unsuccessful.
qPCR of M. fortuitum DNA extracted with Tris-EDTA-Buffer protocol. The assay was run using forward primer 3, reverse primer 2 and the FAM-probe. The cut-off threshold was set at 95 RFU. None of the six samples extracted from the dilutions 10^{0}–10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} amplified above the cut-off threshold. This suggests that no mycobacterial DNA was extracted from the samples.

Another, similar DNA extraction protocol using a 0.45 % Tween 20 lysis buffer combined with thermal lysis and lysozyme and Proteinase K digestion was reported on previously by *Kolia-Diafouka et al.* in 2018 and adapted from this paper [39]. The protocol is described in detail in the methods section under point 2.2.5.3 above. It was tested on aliquots of the serial dilution and CFU count described under point 3.3.2 and in figure 15 above, as well as on aliquots of the serial dilution and CFU count described under point 3.3.4 and in table 4 below. Both times, DNA was extracted from eight samples, one of each dilution 10^{-1}-10^{-8}. The eluted DNA was subsequently used for qPCR, which was run using forward primer 3, reverse primer 2 and our FAM-probe.

The results of the qPCR runs can be seen in Fig. 18 A-B below. Fig. 18 A depicts the results for the dilutions 10^{-1}-10^{-8} from the CFU experiment described under 3.3.2. with 4,76 x 10^9 CFU/ml in the original sample. Fig. 18 B depicts the results for the dilutions 10^{-1}-10^{-8} from the CFU experiment described under 3.3.4. with 3,6 x 10^8 CFU/ml in the original sample. In both Fig. 18 A and Fig. 18 B no clear amplification of the extracted samples above the cut-off threshold can be observed. Although the samples tested in Fig. 18 A came from a different culture and CFU count than the samples tested in Fig. 18 B, neither set of samples delivered results in the qPCR. Individual samples did amplify above the cut-off threshold, for example the 10^{-1} dilution with 4,76 x 10^8 CFU/ml and a CT of 35,86 (Fig. 18 A); however, this was not a consistent but rather incidental observation, suggesting a failure of the extraction protocol.

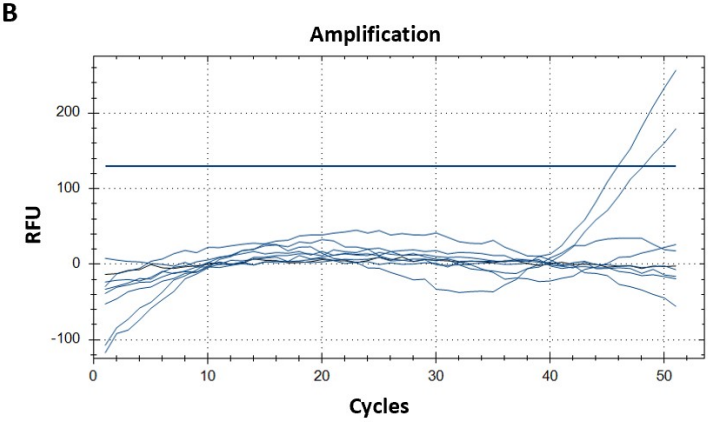
All in all, neither the TE-Buffer extraction protocol, nor the Tween 20 extraction protocol showed any consistent amplification of *M. fortuitum* DNA in the subsequent qPCR. It was concluded that no sufficient extraction of Mycobacterial DNA was achieved with either protocol and that another DNA extraction protocol should be used going forward.

A



Sample	CFU/ml	CT
MF 10^{-1}	4,76 x 10^{8}	35,86
MF 10^{-2}	4,76 x 10^{7}	N/A
MF 10^{-3}	4,76 x 10^{6}	15,44
MF 10^{-4}	4,76 x 10^{5}	N/A
MF 10^{-5}	4,76 x 10^{4}	N/A
MF 10^{-6}	4,76 x 10^{3}	N/A
MF 10^{-7}	4,76 x 10^{2}	12,02
MF 10^{-8}	4,76 x 10^{1}	N/A
NTC	-	N/A

B



Sample	CFU/ml	CT
MF 10^{-1}	3,6 x 10^{7}	N/A
MF 10^{-2}	3,6 x 10^{6}	N/A
MF 10^{-3}	3,6 x 10^{5}	48,11
MF 10^{-4}	3,6 x 10^{4}	45,89
MF 10^{-5}	3,6 x 10^{3}	N/A
MF 10^{-6}	3,6 x 10^{2}	N/A
MF 10^{-7}	3,6 x 10^{1}	N/A
MF 10^{-8}	3,6	N/A
NTC	-	N/A

Fig. 18 A-B: Tween 20 extraction protocol unsuccessful.

qPCR results of *M. fortuitum* DNA extracted using the Tween 20 protocol. The eight samples tested in **A** were aliquots of serially diluted *M. fortuitum* liquid culture (10^{-1}-10^{-8}) with 4,76 x 10^{9} CFU/ml in the original culture. After extraction with the Tween 20 protocol, samples were used for qPCR, which was run using forward primer 3, reverse primer 2 and the FAM-probe with the cut-off threshold set at 98 RFU. Only individual samples amplified above the cut-off threshold, most clearly the 10^{-1} dilution with 4,76 x 10^{8} CFU/ml and a CT of 35,86. The NTC did not amplify. The eight samples tested in **B** were aliquots of serially diluted *M. fortuitum* liquid culture (10^{-1}-10^{-8}) with 3,6 x 10^{8} CFU/ml in the original culture. After extraction with the Tween 20 protocol, samples were used for qPCR, which was run using forward primer 3, reverse primer 2 and the FAM-probe with the cut-off threshold set at 129,5 RFU. Only individual samples amplified above the cut-off threshold, most clearly the 10^{-4} dilution with 3,6 x 10^{4} CFU/ml and a CT of 45,89. The NTC did not amplify.

3.2.4 Approach IV: Tween20 method with Macherey-Nagel spin columns

After unsuccessfully testing the Tween 20 extraction method described by *Kolia-Diafouka et al.*, we decided to try and combine the protocol using the Tween 20 lysis buffer with spin-column-based DNA precipitation/elution [39]. We used the NucleoSpin blood columns by Macherey-Nagel (Macherey-Nagel GmbH & Co. KG, Düren, Germany) with their corresponding wash and elution buffers (wash buffer BW, buffer B5, buffer BE). The protocol was described in detail in the methods section under point 2.2.5.4 above.

As before, we set up another serial dilution and CFU count experiment. The results of the CFU count can be seen in table 4 below. After four days of incubation at 37°C, the colonies on each plate were counted and a mean number of colonies per plate calculated. Using the mean number of colonies per plate at the 10^{-6} dilution, we calculated the CFU/ml in the original sample at $3{,}6 \times 10^{8}$ (table 4).

The eluted DNA was analyzed by NanoDrop and the results of the DNA quantification using NanoDrop can be seen in table 5 below. Although the purity as indicated by the 260/280 index markedly improved compared to previous extraction protocols and mostly falls into the 1.8-2.2 target range, no titration of DNA concentration values along with the serial dilution could be observed (table 5).

The eluted DNA was also analyzed using our qPCR assay and these results can be seen in Fig. 19 below. Forward primer 3, reverse primer 2 and our FAM-fluorophore probe were used. The cut-off threshold was set at 100 RFU. Out of the samples 10^{-1}-10^{-8} only 10^{-1}-10^{-3} amplified above the cut-off threshold, indicating a positive reaction. The sample at the 10^{-1} dilution had a CT of 30.73, the sample at the 10^{-2} dilution had a CT of 33.92 and the sample at the 10^{-3} dilution had a CT of 38.03 (Fig. 19). The NTC did not amplify.

Table 4: Third CFU analysis determines 3,6 × 10^8 CFU/ml in *M. fortuitum* subculture.

A total of 21 plates were inoculated with 50 µl each of the serial dilution. 2 plates were inoculated with the NaCl used for the serial dilution and acted as incubation controls. All plates were incubated for four days at 37°C, after which the colonies on each plate were counted and the mean number of colonies per plate calculated. The amount of colony forming units in the original sample was calculated using the mean number of colonies per plate at the 10^{-6} dilution. It was determined to be 3,6 × 10^8 CFU/ml.

Dilution factor	Nr. of colonies				Mean
	Rep 1	Rep 2	Rep 3	Rep 4	
10^{-5}	146	147	161	162	154
10^{-6}	16	16	21	19	**18**
10^{-7}	2	1	0	1	1
10^{-8}	0	0	1	/	0,33
NaCl	No growth		/		N/A

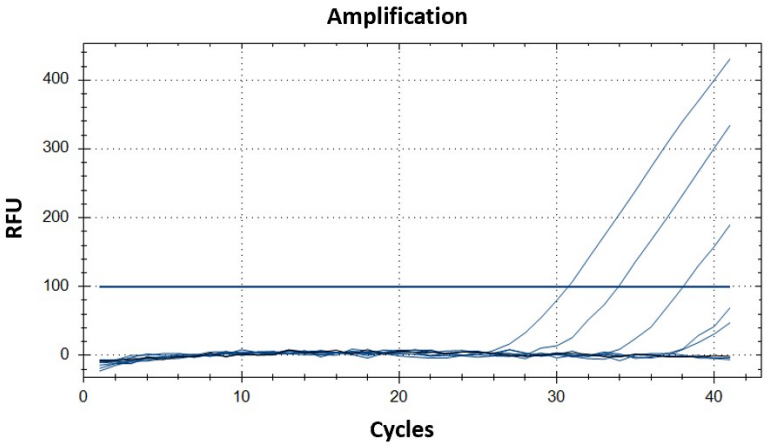
Note: 10^{-0}, 10^{-1} to 10^{-4} had too many colonies to count

CFU/mL in original sample (10^0) was calculated at 3,6 × 10^8 CFU/ml

Table 5: NanoDrop indicates improved purity of DNA extracted with combination of Macherey-Nagel spin columns and Tween 20.

NanoDrop 2000 (Thermo Fischer Scientific Inc., Waltham, Mass., USA) results Tween 20 Protocol with Macherey-Nagel spin columns. DNA was extracted using the Tween 20 protocol from Kolia-Diafouka et al. in combination with spin columns from Macherey-Nagel. Although no titration of DNA concentration values along the dilution gradient can be observed, the purity values mostly fall into the 1.8-2.2 target range.

CFU dilutions	CFU/ml	Tween20 + MN spin columns	
		Concentration (ng/µl)	Purity (260/280)
10^{-1}	3,6 x 10^7	2,5	1,94
10^{-2}	3,6 x 10^6	1,8	1,65
10^{-3}	3,6 x 10^5	2,6	1,93
10^{-4}	3,6 x 10^4	3,0	1,84
10^{-5}	3,6 x 10^3	1,8	2,08
10^{-6}	3,6 x 10^2	1,8	2,31
10^{-7}	3,6 x 10^1	1,6	2,23
10^{-8}	3,6	1,9	1,77



Sample	CFU/ml	CT
MF 10^{-1}	3,6 x 10^{7}	30,73
MF 10^{-2}	3,6 x 10^{6}	33,92
MF 10^{-3}	3,6 x 10^{5}	38,03
MF 10^{-4}	3,6 x 10^{4}	N/A
MF 10^{-5}	3,6 x 10^{3}	N/A
MF 10^{-6}	3,6 x 10^{2}	N/A
MF 10^{-7}	3,6 x 10^{1}	N/A
MF 10^{-8}	3,6	N/A
NTC	-	N/A

Fig. 19: Extraction protocol combining Tween 20 and Macherey-Nagel spin columns unsuccessful. Results of qPCR analysis with serial dilution samples extracted using Tween 20 method combined with Macherey-Nagel spin columns. The assay was run using forward primer 3, reverse primer 2 and our FAM-fluorophore probe. The cut-off threshold was set at 100 RFU. Only the dilutions 10^{-1}-10^{-3} amplified above the cut-off threshold, indicating a positive reaction. The NTC did not amplify.

3.2.5 Approach V: Chelex/Ultrasonication

As the DNA extraction methods described and tested previously were time consuming, work intensive and did not achieve satisfying results, we decided to try a short and simple DNA extraction protocol. The key steps of the protocol being thermal lysis, addition of Chelex resin solution and ultrasonication of the samples. Although we were guided by the protocol described by *Kolia-Diafouka et al.*, several other authors have described using an ultrasonication based DNA extraction protocol for mycobacteria in the past [39, 62-64]. The extraction protocol is described in detail under point 2.2.5.5 in the methods section above.

Due to time constraints, this extraction protocol was not tested on an entirely new serial dilution and CFU count, but on aliquots of the previous serial dilutions.

Initially, the Chelex and ultrasonication protocol was tested on five aliquots of the serial dilution and CFU count described under point 3.3.2 and Fig. 15 above. Aliquots of all serial dilutions had been stored frozen at -20°C. In this case, the dilutions 10^{-1}-10^{-3} and 10^{-5} and 10^{-6} were used. The extraction protocol was carried out as previously described, the only difference being a shorter centrifugation at the end of the protocol, in this case 10 minutes at 13.300 rpm. We used a Sonorex Super RK 100 H Ultrasonic Bath (Bandelin electronic GmbH & Co. KG, Berlin, Germany). The eluted DNA was analyzed with our qPCR assay using forward primer 3, reverse primer 2 and our FAM-fluorophore probe. The cut-off threshold was set at 100 RFU.

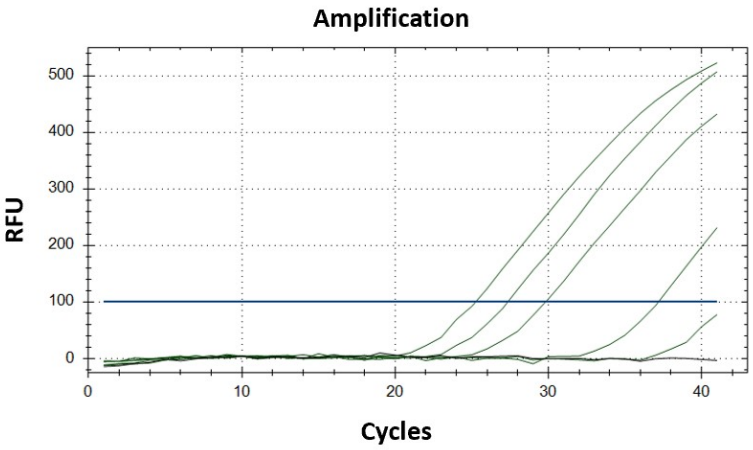
The results of the qPCR can be seen in Fig. 20 below. All samples except the 10^{-6} dilution amplified above the cut-off threshold, indicating a positive reaction. The highest dilution detected by our assay was 10^{-5}, with $4{,}76 \times 10^4$ CFU/ml and a CT of 37,18. (Fig. 20)

Additionally, the eluted DNA was quantified using a NanoDrop to assess the effectivity of the extraction protocol. The measured DNA concentration and purity are displayed in table 6 below. No titration of DNA concentration according to the respective sample dilution could be observed. The purity values exceed the 1.8-2.2 target range, possibly due to residual Chelex beads in the supernatant.

Table 6: NanoDrop indicates insufficient purity and concentration of DNA extracted with Chelex protocol.

NanoDrop measurements of DNA extracted with Chelex and ultrasonication protocol. The DNA extracted using the Chelex and ultrasonication protocol was quantified using a NanoDrop 2000 (Thermo Fischer Scientific Inc., Waltham, Mass., USA). The results can be seen in this table. No titration of DNA concentration values along with the dilution of the sample can be observed. Although the purity values are higher, they exceed the 1.8-2.2 target range. This may be due to Chelex beads remaining in the supernatant. Thus, a very careful harvesting of the supernatant after the extraction should be considered as important.

CFU dilutions	CFU/mL	Chelex 100 + USWB	
		Concentration (ng/µl)	Purity (260/280)
10^{-1}	4,76 x 10^{8}	3,2	2,58
10^{-2}	4,76 x 10^{7}	2,3	2,88
10^{-3}	4,76 x 10^{6}	2,7	2,90
10^{-5}	4,76 x 10^{4}	2,5	2,08
10^{-6}	4,76 x 10^{3}	2,6	4,22



Sample	CFU/ml	CT
MF 10^{-1}	4,76 × 10^{8}	25,24
MF 10^{-2}	4,76 × 10^{7}	27,34
MF 10^{-3}	4,76 × 10^{6}	29,81
MF 10^{-5}	4,76 × 10^{4}	37,18
MF 10^{-6}	4,76 × 10^{3}	N/A
NTC	-	N/A

Fig. 20: Trial of Chelex extraction protocol.

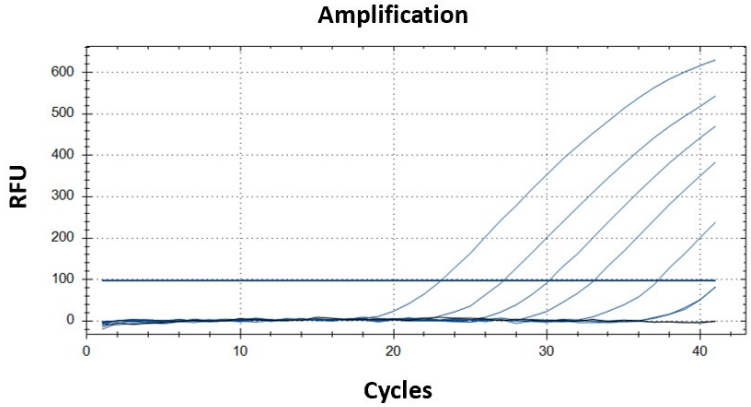
qPCR analysis of *M. fortuitum* DNA extracted with Chelex and ultrasonication protocol. Five samples from a previous serial dilution and CFU count were used for the testing of this DNA extraction protocol. We used the dilutions 10^{-1}–10^{-3}, 10^{-5} and 10^{-6}. The qPCR assay was run using our forward primer 3, reverse primer 2 and the FAM-fluorophore probe. The cut-off threshold was set at 100 RFU. All samples except the 10^{-6} dilution amplified above the cut-off threshold, indicating a positive reaction. The NTC did not amplify.

Next, we expanded the test of the Chelex extraction protocol to include more samples. We again used aliquots from the serial dilution and CFU count described previously under point 3.3.2 and in Fig. 15. Samples from all dilutions 10^{-1}-10^{-8} were now included. Additionally, we tested how a change in the protocol's incubation time (longer) or a change in the volume of Chelex used (less than the original 200 µl) might affect the outcome of the DNA extraction.

Some of the results can be seen in Fig. 21 below. Here we used the Chelex and ultrasonication protocol to extract from aliquots of all eight dilutions 10^{-1}-10^{-8}. The key difference to before being that both the boiling step and the ultrasonication step were increased in length to 20 minutes each. The centrifugation at the end of the extraction protocol was also increased to 15 minutes at 13.300 rpm. This was done to avoid having any residual Chelex beads in the supernatant. The eluted DNA was then analyzed using our *M. fortuitum* qPCR assay. The qPCR assay was run using forward primer 3, reverse primer 2 and the FAM-fluorophore probe, with the cut-off threshold set at 98 RFU. All samples except the 10^{-6}-10^{-8} dilutions amplified above the cut-off threshold, indicating a positive reaction. The 10^{-1} diluted sample with $4{,}76 \times 10^{8}$ CFU/ml had a CT of 23,11. The 10^{-2} diluted sample with $4{,}76 \times 10^{7}$ CFU/ml had a CT of 27,2. The 10^{-3} diluted sample with $4{,}76 \times 10^{6}$ CFU/ml had a CT of 30,16. The 10^{-4} diluted sample with $4{,}76 \times 10^{5}$ CFU/ml had a CT of 33,09. The highest dilution our assay was able to detect was again the 10^{-5} sample with $4{,}76 \times 10^{4}$ CFU/ml and a CT of 37,26. Titration of CT-values in accordance to the sample's concentration could be observed with a difference of 3-4 cycles. The NTC did not amplify (Fig. 21).

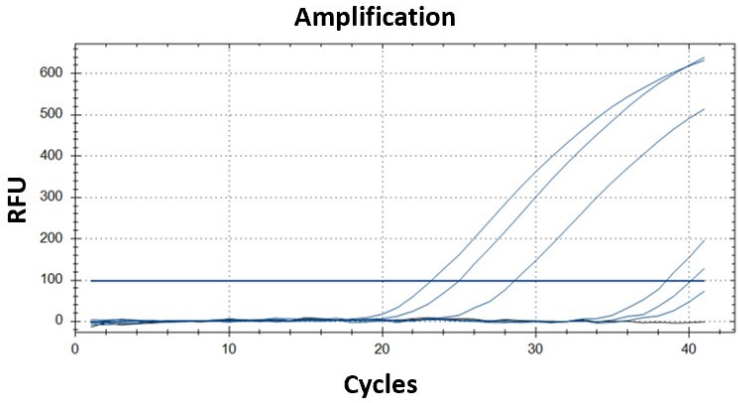
We also tested the extraction protocol with a decreased volume of Chelex, at 50 µl instead of the 200 µl. Otherwise, with the exception of the increased centrifugation time at the end of the protocol (15 minutes at 13.300 rpm), the protocol was carried out as detailed previously. Here we used six aliquots at the dilutions 10^{-1}-10^{-3} and 10^{-6}-10^{-8}. After extracting from these samples using the modified Chelex and ultrasonication protocol, we carried out qPCR analysis of the eluted DNA. The results can be seen in Fig. 22 below. The qPCR assay was run using our forward primer 3, reverse primer 2 and the FAM-fluorophore probe. The cut-off threshold was set at 98 RFU. All samples except the 10^{-8} dilution amplified above the cut-off threshold, indicating a positive reaction. The

sample diluted 10^{-1} with 4,76 × 10^{8} CFU/ml had a CT of 23,17. The 10^{-2} diluted sample with 4,76 × 10^{7} CFU/ml had a CT of 25,01. The 10^{-3} diluted sample with 4,76 × 10^{6} CFU/ml had a CT of 28,61. The 10^{-6} diluted sample with 4,76 × 10^{3} CFU/ml had a CT of 38,52. Titration of CT-values in accordance to the sample's concentration can be observed with a difference of 2-3 cycles. The NTC did not amplify. The highest dilution our assay was able to detect was the 10^{-7} sample with 4,76 × 10^{2} CFU/ml and a CT of 40,13 (Fig. 22).



Sample	CFU/ml	CT
MF 10^{-1}	4,76 × 10^{8}	23,11
MF 10^{-2}	4,76 × 10^{7}	27,2
MF 10^{-3}	4,76 × 10^{6}	30,16
MF 10^{-4}	4,76 × 10^{5}	33,09
MF 10^{-5}	4,76 × 10^{4}	37,26
MF 10^{-6}	4,76 × 10^{3}	N/A
MF 10^{-7}	4,76 × 10^{2}	N/A
MF 10^{-8}	4,76 × 10^{1}	N/A
NTC	-	N/A

Fig. 21: Chelex extraction successful in qPCR.
qPCR analysis of *M. fortuitum* DNA extracted with Chelex and ultrasonication protocol. Eight samples from a previous serial dilution and CFU count were used for the testing of this DNA extraction protocol. Here, longer incubation times than previously were used. We used the dilutions 10^{-1}-10^{-8}. The qPCR assay was run using our forward primer 3, reverse primer 2 and the FAM-fluorophore probe. The cut-off threshold was set at 98 RFU. All samples except the 10^{-6}-10^{-8} dilutions amplified above the cut-off threshold, indicating a positive reaction. Titration of CT-values in accordance to the sample's concentration can be observed with a difference of 3-4 cycles. The NTC did not amplify.



Sample	CFU/ml	CT
MF 10^{-1}	$4{,}76 \times 10^{8}$	23,17
MF 10^{-2}	$4{,}76 \times 10^{7}$	25,01
MF 10^{-3}	$4{,}76 \times 10^{6}$	28,61
MF 10^{-6}	$4{,}76 \times 10^{3}$	38,52
MF 10^{-7}	$4{,}76 \times 10^{2}$	40,13
MF 10^{-8}	$4{,}76 \times 10^{1}$	N/A
NTC	-	N/A

Fig. 22: Reducing Chelex volume does not improve DNA extraction.
qPCR analysis of *M. fortuitum* DNA extracted with reduced volume Chelex and ultrasonication protocol. Here we used a decreased volume of Chelex at just 50 µl instead of the original 200 µl. Six samples from a previous serial dilution and CFU count were used (dilutions 10^{-1}-10^{-3} and 10^{-6}-10^{-8}). The qPCR assay was run using our forward primer 3, reverse primer 2 and the FAM-fluorophore probe. The cut-off threshold was set at 98 RFU. All samples except the 10^{-8} dilution amplified above the cut-off threshold, indicating a positive reaction. Titration of CT-values in accordance to the sample's concentration can be observed with a difference of 2-3 cycles. The NTC did not amplify. The highest dilution our assay was able to detect was the 10^{-7} sample with $4{,}76 \times 10^{2}$ CFU/ml and a CT of 40,13.

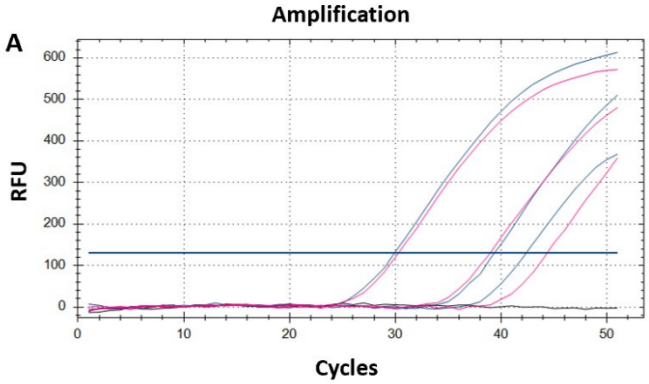
We chose to expand this test of the Chelex protocol to further compare possible effects of a change in incubation duration and Chelex volume on the extracted DNA. For this reason, we used 3 samples with 4 aliquots each that were extracted with 1) 50 µl Chelex and 15 minutes of incubation and sonication, 2) 50 µl Chelex and 30 minutes of incubation and sonication, 3) 200 µl of Chelex and 15 minutes of incubation and sonication, 4) 200 µl of Chelex and 30 minutes of incubation and sonication respectively.

The aliquots we used for this extraction came from a previous serial dilution and count described under point 3.3.4 and in table 4 above. They had been stored frozen at -20°C and were thawed before using for this extraction.

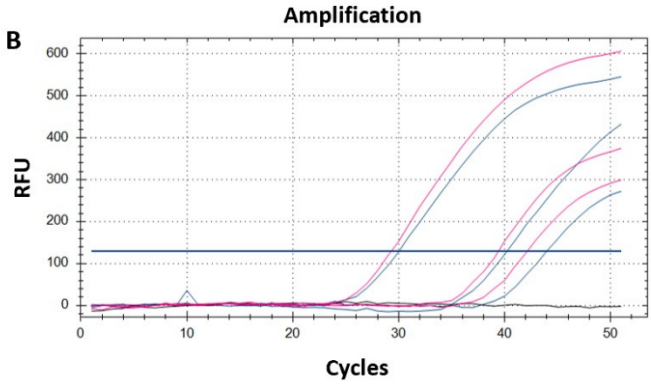
Three samples (n=3) were used for this extraction at the dilutions 10^{-2}, 10^{-5} and 10^{-6}. Four repetitions of the extraction were carried out as described above. The extraction protocol has been detailed previously. After extraction, the samples were run on our qPCR assay using forward primer 3, reverse primer 2 and our FAM-fluorophore probe. The cut-off threshold was set at 129.5 RFU. It is important to note that the run-time of

the assay was increased to 50 cycles, at which point a sigmoidal qPCR curve could be observed.

The results can be seen in Fig. 23 A-B below. Fig. 23 A depicts the results for extraction with 50 µl of Chelex (pink) and 200 µl of Chelex (blue) at 15 minutes of boiling and sonication, whilst Fig. 23 B depicts the results for the extraction with both 50 µl of Chelex (pink) and 200 µl of Chelex (blue) at 30 minutes of boiling and sonication. All samples we extracted amplified above the cut-off threshold, indicating a positive reaction. The CT-values of the samples regardless of the differences in the extraction protocol are similar. For the 10^{-2} dilution with approximately 3,6 x 10^{6} CFU/ml, the CT-value is around 30. For the 10^{-5} dilution with approximately 3,6 x 10^{6} CFU/ml, the CT-value is around 39,5. The highest dilution detected by our assay was 10^{-6}, with approximately 3,6 x 10^{2} CFU/ml and a mean CT-value of around 43 (Fig. 23 A-B).



Sample	CFU/ml	CT
200µl Chelex MF 10^{-2}	3,6 x 10^{6}	29,89
50µl Chelex MF 10^{-2}	3,6 x 10^{6}	30,27
200µl Chelex MF 10^{-5}	3,6 x 10^{3}	39,35
50µl Chelex MF 10^{-5}	3,6 x 10^{3}	38,96
200µl Chelex MF 10^{-6}	3,6 x 10^{2}	42,36
50µl Chelex MF 10^{-6}	3,6 x 10^{2}	44,34
NTC	-	N/A



Sample	CFU/ml	CT
200µl Chelex MF 10^{-2}	3,6 x 10^{6}	30,03
50µl Chelex MF 10^{-2}	3,6 x 10^{6}	29,29
200µl Chelex MF 10^{-5}	3,6 x 10^{3}	40,22
50µl Chelex MF 10^{-5}	3,6 x 10^{3}	39,42
200µl Chelex MF 10^{-6}	3,6 x 10^{2}	43,97
50µl Chelex MF 10^{-6}	3,6 x 10^{2}	42,14
NTC	-	N/A

Fig. 23 A-B: Increased incubation time and decreased Chelex volume show negligible effect on DNA extraction, good performance of qPCR assay and Chelex protocol.

qPCR results of serial dilution samples extracted using decreased Chelex volume vs. original Chelex volume and increased incubation time vs. original incubation time. Samples at three different dilutions (10^{-2}, 10^{-5}, 10^{-6}) were tested under four different conditions. Samples were extracted with either a 15-minute incubation (A) or a 30-minute incubation (B) and with either 200 µl of Chelex (blue curves, A and B) or 50 µl of Chelex (pink curves, A and B). The assay was run using forward primer 3, reverse primer 2 and our FAM-fluorophore probe. The number of cycles in the assay protocol was increased to 50. The cut-off threshold was set at 129.5 RFU. All 12 samples regardless of incubation time or volume of Chelex used, amplified above the cut-off threshold, indicating a positive reaction. The NTC did not amplify. Our assay was able to continually detect the highest diluted sample (10^{-6}), with 3,6 x 10^{2} CFU/ml and a mean CT of 43.

Finally, we compared the results of the Chelex extraction with the other extraction protocols that we tested. A visualization of can be seen in Fig. 24 below. All DNA extraction protocols that we tested in Düsseldorf, except the repeated freeze/boil method, are depicted in the dot plot. The Chelex protocol (Fig. 24, light blue dots) was identified as the most promising protocol for mycobacterial DNA extraction because of its consistent performance when compared to the other methods tested

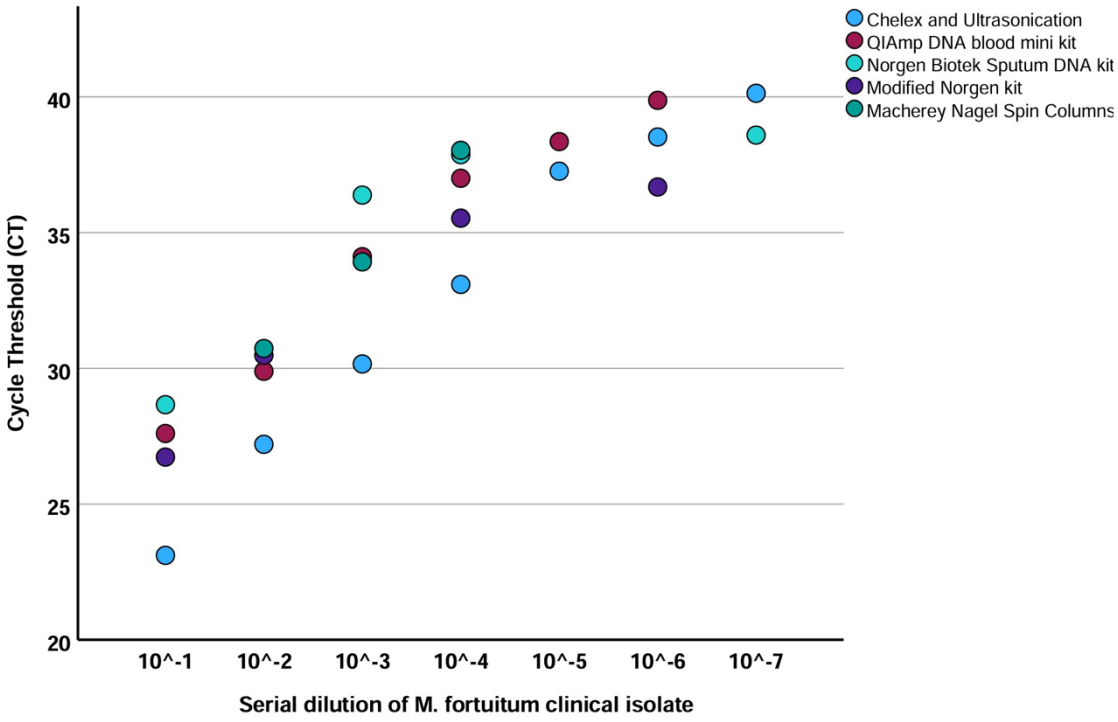


Fig. 24 Comparison of selected DNA extraction protocols.
Dot plot visualizing the direct comparison of selected DNA extraction protocols tested for *M. fortuitum.* Methods are indicated by colors (top left): Chelex and Ultrasonication method (light blue), QIAmp DNA blood mini kit (bordeaux), Norgen Biotek Sputum DNA kit. (turquoise), Modified Norgen kit (purple), Macherey Nagel Spin Columns (green). All extraction protocols were tested using serially diluted *M. fortuitum* cultures. For the QIAmp and Norgen extraction protocols the 10^{-1} serial dilution (written as 10^-1 in the diagram) represents 9.5 x 10^7 CFU/ml, whilst for the Chelex and Modified Norgen protocol the 10^{-1} serial dilution is equal to 4.76 x 10^8 CFU/ml and for the Macherey Nagel protocol the 10^{-1} serial dilution corresponds to 3.6 x 10^7 CFU/ml. The Chelex and Ultrasonication protocol (light blue) was identified as a promising protocol for mycobacterial DNA extraction because of its consistent performance when compared to the other methods tested.

Based on the results in Fig. 21, we concluded that the Chelex DNA extraction protocol showed promise as part of our *M. fortuitum* qPCR assay. This conclusion was bolstered by the results in Fig. 23 A-B and Fig. 24 above. The limit of detection (LOD) of our *M. fortuitum* qPCR assay in conjunction with Chelex extraction was determined to be $3{,}6 \times 10^2$ CFU/ml (n=4). It was decided to implement this short and simple protocol as part of routine microbiologic workflow at KCCR for further laboratory experiments.

3.2.6 Approach V: Tween20 with thermal degradation

Due to resource and time constraints, this extraction protocol was not tested with the serial dilution and CFU count experiment described above but directly on *M. fortuitum* MGIT culture samples at KCCR in Kumasi, Ghana.

The DNA extraction protocol has been described in detail previously (under point 2.2.5.5 above). Two *M. fortuitum* MGIT culture samples were used for testing this extraction method along with a positive control, which was a *M. fortuitum* clinical isolate. Once the extraction was completed, the eluate was used as template DNA in our *M. fortuitum* qPCR assay. Due to challenges with implementing our qPCR assay at KCCR, forward primer 3, reverse primer 5 and our FAM-fluorophore probe were used. The threshold was set at 136 RFU.

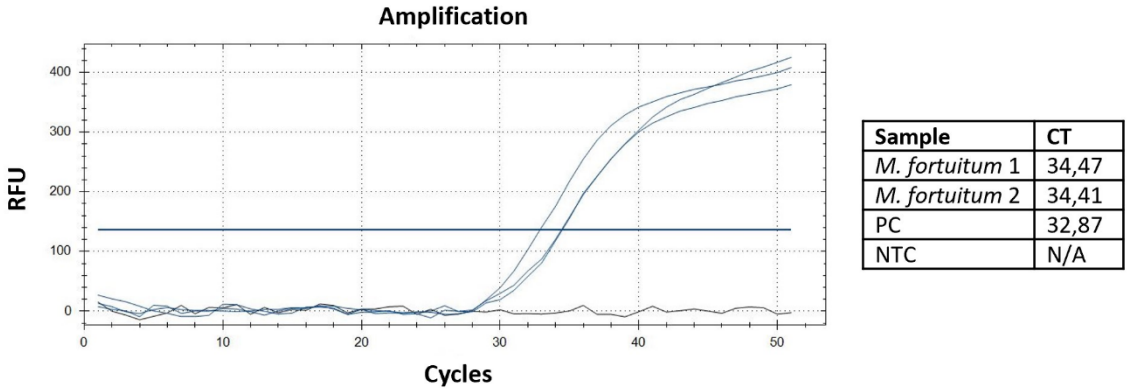
The results of this preliminary test can be seen in Fig. 25 below. Both culture samples as well as the positive control amplified above the cut-off threshold, indicating a positive reaction. The CT value of the culture samples was around 34 cycles, whilst the CT value of the positive control was around 33 cycles (Fig. 25).

To further evaluate the extraction protocol, the purity and concentration of eluted DNA were quantified by means of spectrophotometry using a DeNovix DS-11 Spectrophotometer (DeNovix Inc., Wilmington, DE, USA). The results of this test can be seen below (table 7). For two out of three samples, the purity of eluted DNA as indicated by the 260/280 index was in the 1.8-2.2 target range, indicating sufficient purity. The third sample was also close to this target range, with a purity value of 1.68.

Because of the promising results of the qPCR analysis and purity measurements, we decided to pursue this extraction protocol for the analysis of all other *M. fortuitum* MGIT cultures at KCCR. Those results are described further down under point 3.4 below.

Table 7: Spectrophotometry indicates sufficient purity of DNA extracted with Tween 20 and thermal degradation protocol.
Quantification of DNA quality from eluate extracted using Tween 20 method combined with repeated thermal degradation. Extracted DNA was measured using Denovix DS-11 spectrophotometer with regard to concentration and purity. The purity of one sample and the positive control is within the 1.8-2.2 target range and the purity of the third sample is close to this target range (1.68).

Sample	**Quality of eluted DNA**	
	Concentration (ng/µl)	Purity (260/280)
M. fortuitum MGIT 1	1.575	2.01
M. fortuitum MGIT 2	1.539	1.68
Pos. Ctrl.	8.265	2.04



Sample	CT
M. fortuitum 1	34,47
M. fortuitum 2	34,41
PC	32,87
NTC	N/A

Fig. 25: Custom primers and probe for *M. fortuitum* correctly identify clinical samples extracted from patient cultures with Tween 20 and thermal degradation method in test qPCR.
qPCR analysis of eluate obtained from Tween20 and thermal degradation extraction protocol test. Two *M. fortuitum* MGIT cultures were used for the initial extraction, they had previously been speciated using the GenoType CM LPA (Hain Lifescience, Nehren, Germany). A positive control for the extraction in form of a clinical isolate and a NTC was also included. The assay was run with forward primer 3, reverse primer 5 and our FAM-fluorophore probe. The cut-off threshold was set at 136 RFU. Both samples amplified above the cut-off threshold with a CT of around 34, indicating a positive reaction. The positive control also amplified above the cut-off threshold with a CT-value of around 33, indicating a positive reaction.

In short, our *M. fortuitum* qPCR assay together with this DNA extraction protocol was able to correctly identify *M. fortuitum* in sputum cultures from patients. Although the extraction protocol is lengthy and work intensive, we decided to implement the protocol for the testing of other suspected *M. fortuitum* sputum cultures at KCCR, with the hopes of obtaining high-quality gDNA for WGS.

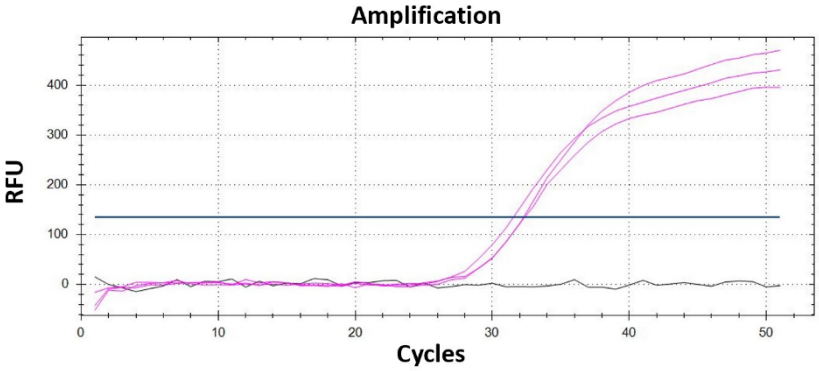
3.2.7 Approach VI: Extraction method previously used for *M. ulcerans*

This extraction method had been used previously by the research group "Skin NTDs" at KCCR to isolate *M. ulcerans* from tissue samples. It used some reagents from QIAGEN's Puregene extraction kit with chemical and thermal lysis methods.

Due to resource and time constraints, this extraction protocol was not tested with the serial dilution and CFU count experiment described above but directly on *M. fortuitum* MGIT culture samples at KCCR in Kumasi, Ghana.

Two MGIT cultures that had previously been identified as *M. fortuitum* using LPA (Hain Lifescience, Nehren, Germany) were tested along with a positive control, which was a *M. fortuitum* clinical isolate. The detailed protocol for this extraction method is described in the methods section under point 2.2.5.6 above. Once the extraction was completed, the eluate was used as template DNA in our *M. fortuitum* qPCR assay. Forward primer 3, reverse primer 5 and our FAM-fluorophore probe were used. The threshold was set at 136 RFU.

Fig. 26 shows the results of the DNA extracted from the MGIT cultures with this protocol and analyzed using our *rpoB* qPCR assay. Both culture samples as well as the positive control amplified above the cut-off threshold, indicating a positive reaction. The CT value of the culture samples and the positive control was around 32 cycles.



Sample	CT
M. fortuitum 1	32,39
M. fortuitum 2	31,56
Pos. ctrl.	32,31
NTC	N/A

Fig. 26: Custom primers and probe for *M. fortuitum* correctly identify clinical samples extracted from patient cultures with BU method in test qPCR.

qPCR analysis of eluate obtained with extraction protocol previously used for *M. ulcerans*. Two *M. fortuitum* MGIT cultures were used for the initial extraction, they had previously been speciated using the GenoType CM LPA (Hain Lifescience, Nehren, Germany). A positive control for the extraction in form of a clinical isolate and a NTC was also included. The assay was run with forward primer 3, reverse primer 5 and our FAM-fluorophore probe. The cut-off threshold was set at 136 RFU. Both samples amplified above the cut-off threshold with a CT of around 32, indicating a positive reaction. The positive control also amplified above the cut-off threshold with a CT-value of around 32, indicating a positive reaction.

3.3 Testing of presumptive TB-sputum samples

To investigate the feasibility of our *M. tuberculosis* qPCR assay and our DNA extraction protocol as part of routine clinical diagnostic testing, we performed both protocols using actual patient samples.

At KCCR six (n=6) fresh sputum samples (approx. 3 days old, stored in a freezer at -80°C) from presumptive TB patients were tested. All six sputa were split so that half could be tested raw and the other half decontaminated, to compare the effects of sample decontamination on the final qPCR result. Decontamination was performed using the protocol described under point 2.2.2 with NaCI-NaOH-NALC solution and phosphate buffer.

GeneXpert® (Cepheid®, Sunnyvale, CA, USA) testing was performed in addition for all samples according to manufacturer protocol. Two of the samples, sputum 4 and sputum 5 were GeneXpert® positive for *M. tuberculosis* (2/6), the remainder could not be confirmed as *M. tuberculosis* infections.

DNA was extracted from all samples (n=6 decontaminated, n=6 raw) using the Chelex and ultrasonication protocol (described under point 2.2.5.5 above), the only difference being that a shaking incubator was used for the boiling step (100°C, 300 rpm, 15 minutes). At KCCR, we used the smaller Sonorex Super RK 31 H ultrasonic water bath (Bandelin electronic GmbH & Co. KG, Berlin, Germany). This was due to restrictions in weight and size, as the water bath was transported to Kumasi from Düsseldorf.

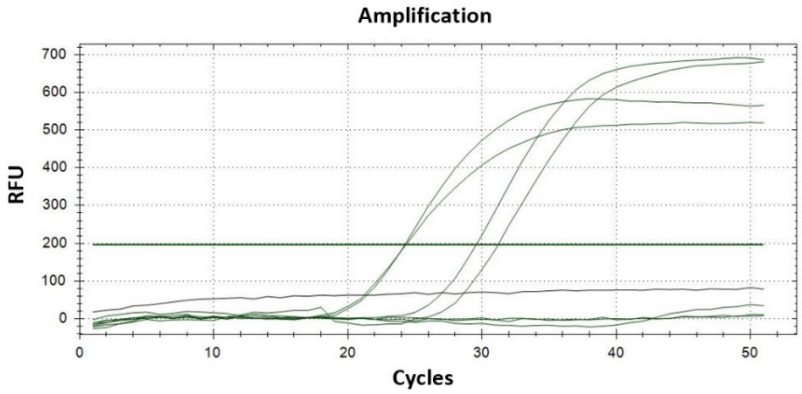
From each of the n=12 DNA extracts (n=6 from decontaminated sputa and n=6 from raw sputa), 5 µl were used as template DNA for the qPCR. DNA from *M. tuberculosis* H37Rv was used as a positive control and a NTC was also included. All samples were tested using the *M. tuberculosis IS6110* primers and HEX-probe, with the threshold set at 195 RFU. The qPCR master mix was prepared according to the calculations described previously for a total of 15 reactions to account for pipetting error.

The results are displayed in Fig. 27 A and B below. A total of 3/6 samples from both the decontaminated and raw sputa amplified above the cut-off threshold, indicating a positive reaction (Fig. 27 A-B). For the decontaminated samples Sputum 1 had a CT-value of 31.19, Sputum 4 had a CT of 24.31 and Sputum 5 had a CT of 29.55. For the raw

samples Sputum 1 had a CT-value of 36.07, Sputum 4 had a CT of 27.71 and Sputum 5 had a CT of 29.58. The sputa that had previously been tested positive using the GeneXpert® reference method (sputum 4 and sputum 5) were concomitantly positive in our qPCR assay. Additionally, one sample that had not been recognized by the GeneXpert® was clearly positive in our assay. Thus, for this small set of samples, 100 % could subsequently be confirmed using our qPCR assay. Although concordance between the positive results could be observed for both raw and decontaminated sputum, two of the samples from raw sputum have a higher CT value suggesting possible interference when using non-decontaminated samples.

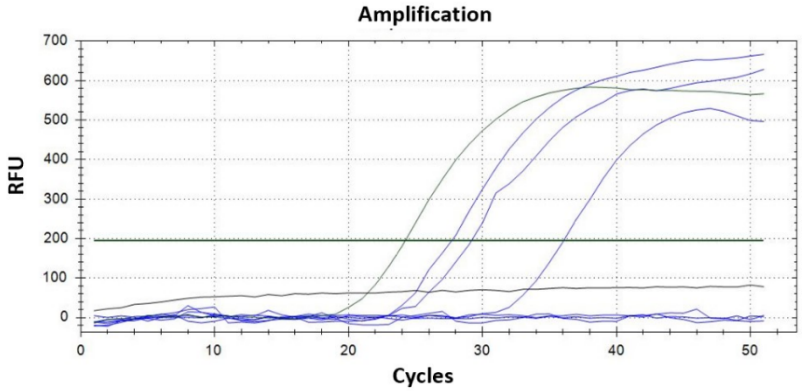
In short, when testing the Chelex and ultrasonication based DNA extraction protocol together with our *M. tuberculosis IS6110* qPCR assay, we were able to identify *M. tuberculosis* directly, *ex-vivo,* from the sputa of presumptive TB patients. These initial experiments suggested a high sensitivity and specificity.

A



Sample	CT
Sputum 1	31,19
Sputum 2	N/A
Sputum 3	N/A
Sputum 4	24,31
Sputum 5	29,55
Sputum 6	N/A
Tb H37Rv	24,24
NTC	N/A

B



Sample	CT
Sputum 1	36,07
Sputum 2	N/A
Sputum 3	N/A
Sputum 4	27,71
Sputum 5	29,18
Sputum 6	N/A
Tb H37Rv	24,24
NTC	N/A

Fig. 27 A-B: *IS6110* primers and probe correctly identify *M. tuberculosis* from raw and decontaminated sputum in test qPCR using Chelex DNA extraction protocol.

Testing of *M. tuberculosis*-*IS6110*-qPCR assay with Chelex and ultrasonication based DNA extraction on both raw and decontaminated sputum samples from presumptive TB patients. The results for the decontaminated sputum samples are depicted under **A**, the results for the raw sputum samples are depicted under **B**. All samples were tested using the *M. tuberculosis IS6110* primers and HEX-probe, with the threshold set at 195 RFU. Sputum was decontaminated using NaCl-NaOH-NALC solution and phosphate buffer according to previously described protocol. The NTC did not amplify above the cut-off threshold, whilst our positive control using *M. tuberculosis* H37Rv gDNA did. Both the raw and decontaminated samples from Sputa 1, 4 and 5 were tested positive for TB by our assay. Sputum 4 and 5 had previously been tested positive for TB by GeneXpert®.

3.4 Testing of *M. fortuitum* MGIT cultures from patient samples

Twenty-four TBc ID negative LPA-speciated *M. fortuitum* MGIT culture samples were used for DNA extraction (n=24) and qPCR analyses. DNA extraction was performed using the Tween 20 lysis buffer method with repeated heat degradation described under point 2.2.5.5 above and the extracted gDNA was measured using our qPCR assay.

The bar graph below (Fig. 28) shows the results of all twenty-four (n=24) samples we tested. Out of the 24 samples speciated as *M. fortuitum* using GenoType CM LPA kit (Hain Lifescience, Nehren, Germany), 22 could positively be identified as *M. fortuitum* using our qPCR assay. Initially, 4 out of the 24 culture samples could not be identified as *M. fortuitum* by our qPCR assay, as they did not amplify above the cut-off threshold. These four culture samples were re-extracted with the same DNA extraction method and again tested on our qPCR. Two out of the four previously negative samples were subsequently identified as *M. fortuitum* by our assay following the repeat extraction, thus bringing the total to 22 positively identified samples. The average CT-value of the samples was 37,35. Based on these results, the overall agreement between our *M. fortuitum* qPCR assay and the GenoType CM LPA kit used as a reference method was calculated to be 91.6 % (22/24) and the sensitivity 79 %.

The cycling conditions and the parameters for the master mix were as previously described. Forward primer 3 and reverse primer 5, as well as our *rpoB* specific FAM-fluorophore probe were used. One sample was previously tested positive using our assay as part of the experiments described under 3.2.6 and 3.2.7 above. The other samples were tested in two batches. Batch one consisted of the first 10 (n=10) samples and batch two consisted of the remaining 13 samples (n=13). This was done mostly due to workflow reasons, as the DNA extraction process was lengthy. The cut-off threshold was set at 132 RFU for batch one and at 135 RFU for batch two.

An excerpt of the qPCR results can be seen in Fig. 29 below. Here, gDNA extracted from nine *M. fortuitum* MGIT culture samples was re-run on our qPCR assay. The samples had already been positively identified as *M. fortuitum* by our assay and the LPA. They were re-run on our qPCR with double volume of probe per reaction. Seven out of nine samples amplified above the cut-off threshold, indicating a positive reaction for *M. fortuitum*. The

positive control, gDNA from a *M. fortuitum* clinical isolate, also amplified above the cut-off threshold (PC, red curve). The NTC did not amplify (Fig. 29).

In summary, our *M. fortuitum* qPCR assay combined with the described extraction method was able to correctly identify 22 out of 24 culture samples as *M. fortuitum.* The samples in question were MGIT cultures that had previously been tested negative for *M. tuberculosis* using the BD MGIT™ TBc ID rapid test (Becton Dickinson, Franklin Lakes NJ, USA; Cat. 245159) and speciated as *M. fortuitum* using the GenoType CM LPA kit. The overall agreement between our *M. fortuitum* qPCR assay and the LPA reference method was calculated to be 91.6 % (22/24) and the sensitivity 79 %.

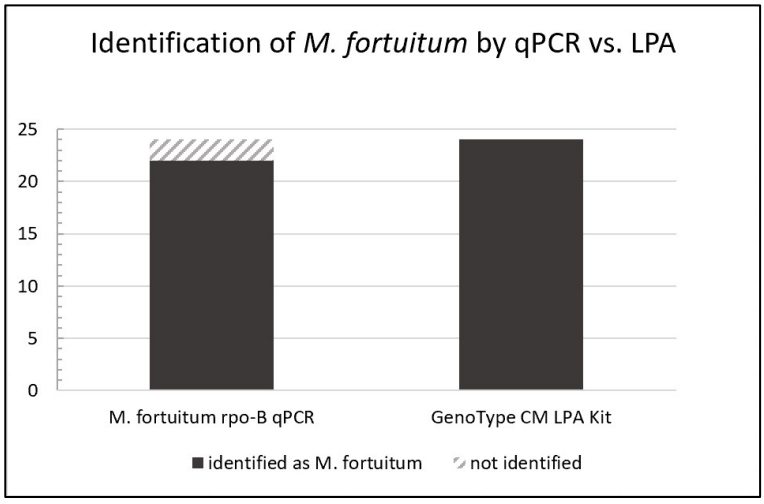
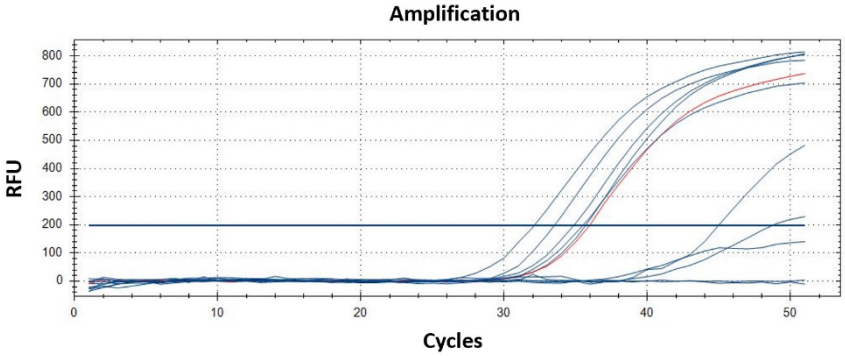


Fig. 28: High concordance of *M. fortuitum* qPCR vs. LPA reference method.
Bar graph comparing the identification of *M. fortuitum* MGIT culture samples using our *rpoB* targeting *M. fortuitum* qPCR assay and the GenoType CM LPA Kit. Out of 24 MGIT culture samples that had previously been identified as *M. fortuitum* using LPA, our qPCR assay was able to positively identify 91.6 % of tested sampled (22/24).



Sample	CT
M. fortuitum 12	34,83
M. fortuitum 13	32,08
M. fortuitum 14	33,48
M. fortuitum 15	35,46
M. fortuitum 17	N/A
M. fortuitum 18	44,94
M. fortuitum 20	48,67
M. fortuitum 21	N/A
M. fortuitum 22	35,66
PC	35,94
NTC	N/A

Fig. 29: qPCR assay for *M. fortuitum* correctly identifies clinical samples extracted from NTM patient's cultures.

Results of qPCR analyses for nine culture samples that had previously been identified as *M. fortuitum* using LPA. DNA was extracted using a chemical and thermal lysis protocol, previously described under point 2.2.5.5 above. Forward primer 3 and reverse primer 5 were used, as well as double volume of our probe per reaction. Seven out of nine samples amplified above the cut-off threshold, indicating a positive reaction for *M. fortuitum*. The positive control, gDNA from a *M. fortuitum* clinical isolate, also amplified above the cut-off threshold (PC, red curve). The NTC did not amplify.

3.5 Comparison of *M. fortuitum* LPA and qPCR results

The initial speciation of the twenty-four *M. fortuitum* samples was carried out by our working group at KCCR using the GenoType CM Mycobacteria LPA kit (Hain Lifescience, Nehren, Germany) as reference method. Subsequently, the samples were tested and mostly confirmed using the *M. fortuitum* qPCR assay. This was described under point 3.4 above. However, several different band patterns were observed on the LPA strips.

According to the manufacturer's instructions, LPA strips positive for *M. fortuitum* may exhibit two band patterns. Pattern 1: shows staining of bands 7 and 14, with optional weak staining of band 10 or facultative staining of band 14 (as noted in the updated version of the instructions). Pattern 2: shows staining of band 14, with optional weak staining of bands 7 or 8 (as noted in the updated version of the instructions) [87, 88]. Thus, LPA strips exhibiting the 7 band or the 7 and 14 band patterns are both interpreted as positive for *M. fortuitum* (for reference see Fig. 30 below). The manufacturer's instructions further explain that a heterogenous band pattern may appear for *M. fortuitum* samples, because sequence variations exist within this species.

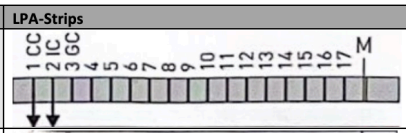
An overview of both the LPA test strips and their interpretation, as well as the results of the *M. fortuitum* qPCR assay can be seen in Table 8 below. The results are tabulated according to the sample ID used in previous experiments (MF1-24). The analysis of the LPA strips was carried out both manually and by use of the GenoScan® automated system (Hain Lifescience, Nehren, Germany) according to the manufacturer's instructions.

Out of the twenty-four *M. fortuitum* speciated samples, 7/24 LPA strips (29 %) displayed only the 7 band, whilst 12/24 LPA strips (50 %) exhibited the banding pattern with both the 7 and 14 band. Four samples (MF16, 19, 23, 24) were initially tested negative on the *M. fortuitum* qPCR and 3/4 had displayed the single 7 band pattern on the LPA, whereas only 1/4 negative samples had the 7 and 14 band pattern. Samples showing other forms of multiple band patterns (such as 7 and 10) made up just 5/24 and these were all correctly identified by the qPCR assay. Thus, 11/12 samples (91 %) with the 7 and 14 band pattern could be confirmed by subsequent *M. fortuitum* qPCR, whilst only 4/7 samples (57 %) with the single 7 band pattern were confirmed initially (Table 8).

In summary, only three suspected *M. fortuitum* samples exhibiting the 7 band pattern could not be confirmed by the initial *M. fortuitum* qPCR, while only one sample exhibiting both the 7 and 14 LPA band pattern could not be confirmed. Accordingly, this may indicate a problem with samples speciated by LPA as *M. fortuitum* exhibiting only the 7 band pattern.

Table 8: Overview of M. fortuitum LPA strips and concomitant qPCR results.

The GenoType CM LPA strips below were tabulated according to the sample ID used in previous experiments (MF1-24). Analysis of the LPA strips was carried out both manually and by use of the GenoScan® automated system according to the manufacturer's instructions. A reference scale for the manual interpretation of LPA band patterns can be seen at the top of the table. CC refers to the conjugate control, UC to the universal control detecting all mycobacteria as well as gram-positive bacteria with high G+C content and GC refers to the genus control detecting the genus *Mycobacterium.* The band pattern and interpretation for each LPA test strip is listed below. Note that the bands have faded in intensity over time. The samples were subsequently tested with the *M. fortuitum* qPCR assay and CT-values for each sample, as well as the interpretation of the qPCR results are also listed. Four samples (MF16, 19, 23, 24) were tested negative initially. They were re-extracted and re-tested by qPCR as described under 3.4 above, along with two other samples that had previously tested positive. The LPA testing of samples was carried out by Rejoice Arthur of KCCR, a scan of the LPA results was kindly provided by her.

Sample ID	LPA-Strips	Bands	Interpretation	1. qPCR	CT	2. qPCR	CT
							
MF1		7	*M. fortuitum*	*M. fort. pos.*	43.13		
MF2		7, 10, 16	*M. fortuitum*	*M. fort. pos.*	33.41		
MF3		7, 14	*M. fortuitum*	*M. fort. pos.*	32.49		
MF4		7, 14	*M. fortuitum*	*M. fort. pos.*	34.41	*M. fort. pos.*	33.42
MF5		7, 14	*M. fortuitum*	*M. fort. pos.*	26.40		
MF6		7, 14	*M. fortuitum*	*M. fort. pos.*	42.42		
MF7		7, 14	*M. fortuitum*	*M. fort. pos.*	32.04		
MF8		7, 9, 14	*M. fortuitum*	*M. fort. pos.*	38.03		
MF9		7, 14	*M. fortuitum*	*M. fort. pos.*	34.13		
MF10		7	*M. fortuitum*	*M. fort. pos.*	47.52		
MF11		7	*M. fortuitum*	*M. fort. pos.*	34.47	*M. fort. pos.*	30.02
MF12		7	*M. fortuitum*	*M. fort. pos.*	36.18		
MF13		7, 10	*M. fortuitum*	*M. fort. pos.*	34.11		
MF14		7, 10, 14	*M. fortuitum*	*M. fort. pos.*	34.20		
MF15		7, 14	*M. fortuitum*	*M. fort. pos.*	35.96		
MF16		7	*M. fortuitum*	***M. fort. neg.***	N/A	*M. fort. pos.*	39.90
MF17		7, 10, 14	*M. fortuitum*	*M. fort. pos.*	45.13		
MF18		7, 14	*M. fortuitum*	*M. fort. pos.*	44.41		
MF19		7	*M. fortuitum*	***M. fort. neg.***	N/A	***M. fort. neg.***	N/A
MF20		7, 14	*M. fortuitum*	*M. fort. pos.*	44.79		
MF21		7, 14	*M. fortuitum*	*M. fort. pos.*	42.45		
MF22		7, 14	*M. fortuitum*	*M. fort. pos.*	34.51		
MF23		7, 14	*M. fortuitum*	***M. fort. neg.***	N/A	***M. fort. neg.***	N/A
MF24		7	*M. fortuitum*	***M. fort. neg.***	N/A	*M. fort. pos.*	32.63

4.0 Discussion

The use of molecular biological methods for the identification of mycobacteria from sputum and culture samples is well documented, especially for *M. tuberculosis*. A study by our research group previously reported on the use of a kit-based DNA extraction method and the direct, *ex-vivo* identification of the NTM *M. abscessus* by means of qPCR [11]. In the renewed context of the differential diagnosis of mycobacterial infections in presumptive TB patients, the findings of this study extend the understanding of the molecular biological methods available for the identification of *M. fortuitum.* We were not able to replicate all methods that had been previously described by other authors for the diagnosis of mycobacteria [39]. However, here we report on new custom molecular biological methods for the identification and differentiation of *M. fortuitum* and *M. tuberculosis* from patient samples.

In short, the key findings of our study are the following: 1) *M. fortuitum* is clinically relevant in Ghana, 2.) we developed and evaluated new molecular biological diagnostic tools for *M. fortuitum* and 3.) these tools may outperform the existing reference methods.

4.1 Increasing Prevalence and Clinical Relevance of *M. fortuitum* in Ghana

The findings of our study reinforce the growing recognition of *M. fortuitum* as a clinically relevant NTM in Ghana. Consistent with available literature and unpublished work by our group, our methods confirmed *M. fortuitum* as the most commonly isolated NTM in samples of patients presenting with presumptive TB in Ghana [18-22]. This supports the hypothesis that *M. fortuitum* may be a significant cause of misdiagnosed pulmonary infections in high-TB-burden settings. This is of especial relevance and poses a critical challenge for public health in Ghana, a high-TB-burden setting, where diagnostic infrastructure is often focused solely on TB and at times limited. As previously mentioned, misdiagnosis of NTM can result in inappropriate treatment regimens leading to increased morbidity.

Although *M. fortuitum* is thought to rarely cause pulmonary disease in healthy, immunocompetent individuals [5, 6, 8, 12] studies showing high prevalence of

M. fortuitum in Ghana and elsewhere cast doubt on this [18-22]. The role of *M. fortuitum* as a pathogen of the immunocompetent is not yet entirely clear, but several risk factors and exposures are known. Studies have suggested a high prevalence of *M. fortuitum* infections in the cohort of presumptive TB patients in Ghana [20-22] and prior infections with TB are a known predisposing risk factor for NTM-PD, as they can lead to structural lung changes [5-7, 9]. An association with climate-related dust exposure may be another risk factor. A study from Nigeria found a connection between NTM-PD cases and dust exposure from the so-called "Harmattan". The "Harmattan" is a local weather pattern during the West African dry season in the months of January and February, that is also commonly present in Ghana [55].

As this study was primarily focused on methodological approaches for the diagnosis of *M. fortuitum,* drawing concrete conclusions on pathogenic relevance remains difficult. Larger scale sampling of suspected patients with concomitant WGS could offer insights into specific etiology and epidemiology of *M. fortuitum* infections in Ghana. Clinicians in high-prevalence TB settings need to be made aware of the possibility of NTM-PD as a differential diagnosis. Importantly, methods for the rapid identification of NTM in low resource settings are urgently needed.

4.2 New molecular biological methods for the identification and differentiation of *M. fortuitum* and *M. tuberculosis* from patient samples

Although progress has been made with new methods for the diagnosis of mycobacteria, most progress has been focused solely on the diagnosis of *M. tuberculosis*. In general, however, the diagnosis of mycobacteria and especially the diagnosis of NTM remains challenging. The culturing of mycobacteria has historically been the gold standard procedure for diagnosis and is still part of routine laboratory diagnostics. This is evident from the ATS criteria for diagnosis of NTM, which still require culture confirmation [5, 7, 9]. Recently, partial genetic sequencing of mycobacterial genes such as *erm, hsp65, rpoB* and *16S rRNA* has emerged as a sensitive and specific method for species diagnosis [5, 36]. However, both culturing of NTM and sequencing are work intensive and have a prolonged turnaround time. Culturing of mycobacteria may take several weeks and as

such brings with it the risk of contamination [5, 7]. While mycobacterial culture is affordable, sequencing can come at a prohibitive cost, which is especially relevant for diagnostics in resource-limited settings [5, 54, 89, 90].

Thus, our goal was the development of a rapid, specific and cost-effective molecular biological method for the direct, *ex-vivo* identification of *M. fortuitum* and *M. tuberculosis* from patient samples, possibly enabling a bypassing of mycobacterial culture as a means for diagnosis. Two critical steps would have to be accomplished to reach this goal: 1) the effective extraction of mycobacterial DNA from patient samples and 2) the detection of *M. fortuitum* and *M. tuberculosis* by means of nucleic acid amplification.

4.2.1 Evaluation of DNA Extraction Protocols for Mycobacterial Detection

Regarding our first goal, the effective extraction of mycobacterial DNA, several authors previously reported on the use of kit-based or in-house chemical lysis DNA extraction for the rapid identification of mycobacteria from patient and culture samples [39, 60, 61, 66, 67]. In most publications, these methods were used for the identification of *M. tuberculosis.* We reasoned that we could thus adapt these protocols for the use on another bacterium from the same genus, namely *M. fortuitum.*

Although we tested several extraction protocols, only two proved feasible. We were not able to consistently replicate the reported results for most protocols when working with *M. fortuitum*. Protocols such as the QIAGEN Blood DNA mini kit or the Norgen Biotek Sputum DNA isolation kit, as well as the chemical lysis protocols we tested proved insufficient. We reasoned that for some protocols this may be due to insufficient lysis of the thick mycobacterial cell wall in addition to the possibility of only paucibacillary *M. fortuitum* presence in samples [7, 39, 91]. This shows how challenging the work with mycobacteria can be.

One of the protocols we were able to successfully adapt was previously used for extraction of mycobacteria from tissue samples in our clinical pathology department. In contrast to some of the other protocols we tested, this protocol combined several different methods for cell lysis, for example repeated cycles of thermal degradation at

very high and very low temperatures in addition to chemical lysis with enzymes such as Lysozyme and Proteinase K with long periods of incubation. Possibly, this combination of techniques may have led to a better lysis of the thick mycobacterial cell wall. However, this combination of techniques made the extraction protocol time and work intensive, taking 2 days to complete.

Recent studies also reported on the use of Chelex® resin and ultrasonication based DNA extraction protocols for the identification of (paucibacillary) *M. tuberculosis* samples [39, 62, 64]. Consistently, this simpler protocol proved successful in initial experiments and we were able to utilize it to detect *M. fortuitum* up to a limit of 10^2-10^3 CFU/ml together with our qPCR assay. A combination of thermal lysis and ultrasonication may thus be especially effective at breaking up the mycobacterial cell wall. At a duration of approx. 1 ½ hours, the Chelex® extraction protocol has a short turnaround time, making it a potential time-saving alternative when working on patient samples. We also successfully used this protocol to extract *M. tuberculosis* DNA directly from sputum samples and initial experiments with the *IS6110* qPCR suggested high sensitivity. Nevertheless, we were not yet able to achieve similar results with *M. fortuitum* sputum samples, ultimately making the continued culturing of these samples necessary during our experiments. Bypassing of the mycobacterial culture through direct, *ex-vivo* identification from non-cultured patient material could thus so far not be achieved for *M. fortuitum*.

In short, the effective extraction of mycobacterial DNA remains challenging. We tested several DNA extraction approaches and identified two protocols that yielded DNA suitable for reliable amplification: 1) the Chelex®-based protocol involving thermal degradation and ultrasonication, this method proved relatively simple and fast, 2) the enzymatic lysis protocol adapted from our pathology department combining Tween lysis buffer, Proteinase K, Lysozyme digestion, and repeated heating. Both methods demonstrated compatibility with downstream qPCR assays and could be adapted to routine diagnostic workflows with appropriate training and validation. A caveat was that we could not directly identify *M. fortuitum* from sputum samples using these protocols. A more comprehensive laboratory validation of these protocols would be an important next step to achieve this goal.

4.2.2 Evaluation of qPCR for Mycobacterial Detection

Regarding our second major objective, the detection of *M. fortuitum* and *M. tuberculosis* by means of nucleic acid amplification, previous studies reported on the use of the *IS6110* and *rpoB gene* regions for the qPCR-based identification of *M. tuberculosis* and *M. fortuitum* respectively [11, 31, 39, 40, 75-80]. When designing an assay, selecting an appropriate genetic target is crucial for the sensitivity, specificity and overall reliability. In this study, we targeted the *rpoB gene* for the detection of *M. fortuitum* and the *IS6110* insertion sequence for *M. tuberculosis*. Both targets are well-established in literature [11, 31, 39, 40, 75-80]. Consistently, our custom qPCR assays successfully utilized these regions for the detection of *M. fortuitum* and *M. tuberculosis*. Novel *rpoB*-specific primers and probes for the identification of *M. fortuitum* were designed, which demonstrated high reliability in our assay system. However, our chosen targets differ significantly in their genomic characteristics, which can affect assay performance. Thus, when evaluating assay performance, these characteristics warrant further discussion.

The *IS6110* is a commonly used target for molecular biological detection of *M. tuberculosis*. Typically, the *IS6110* is present in multiple copies within the *M. tuberculosis* genome, in some cases it may even be present up to 200 times. Thus, using *IS6110* as a target may offer a sensitivity advantage, especially when testing paucibacillary samples. However, the copy number variability can also be a limitation of this target, as some *M. tuberculosis* strains lack the element altogether and cases of NTM harboring *IS6110* homologues have been described, potentially making this a less specific genetic target [81-86, 92-94].

In contrast, the *rpoB gene*, which encodes the beta-subunit of the bacterial RNA polymerase, is a single-copy gene found in all mycobacterial species. It has also been commonly used in molecular diagnostics of mycobacteria [11, 31]. Due to its sequence specificity, *rpoB* may be advantageous for designing more selective primers and probes. While our novel *rpoB*-specific primers and probes successfully detected *M. fortuitum* in our study, demonstrating the robustness of this target, the single-copy nature of *rpoB* may limit sensitivity. Unlike *IS6110*, *rpoB* provides no advantage from the presence of multiple copies per genome, potentially making it less reliable when trying to detect paucibacillary NTM infections [71-74].

Overall, both *IS6110* and *rpoB* are robust qPCR targets**.** The *IS6110* may offer a sensitivity advantage due to its multicopy genomic presence whereas the *rpoB gene* is more specific but may provide limited sensitivity in comparison due to its single-copy nature. Together these targets illustrate the trade-offs in qPCR assay design: weighing sensitivity versus specificity. Accordingly future NTM diagnostics could benefit from the identification of novel multicopy or highly expressed gene targets specific to *M. fortuitum*, possibly enhancing detection sensitivity without compromising specificity. Employing techniques such as WGS of our local isolates could aid in identifying such targets and enable the development of molecular assays tailored to regional genetic profiles. The WGS of our *M. fortuitum* isolates in further studies would thus be a critical next step.

4.2.3 Decreased cost of reagents for *M. fortuitum* qPCR vs. NTM LPA

Although not a major objective of this study, cost and procurement of reagents can be a limiting factor for laboratory diagnostics, especially in resource-limited settings [54, 89, 90] and as such must be considered critically. The costs for two of the diagnostic methods used in this study are discussed below. The cost for required machinery itself (e.g. centrifuges, qPCR) is not listed, as these machines and other consumables (such as pipette tips and microdilution tubes) were readily available at KCCR.

The GenoType CM Kit (Hain Lifescience, Nehren, Germany) for LPA-based identification of NTM was procured from a distributor in Kenya for 1128 € per kit. As each kit contains reagents for 96 reactions, the cost per test would be 11,75 €. However, in practice the actual cost per test is higher, as one needs to account for more generous use of reagents, for example due to pipetting error.

The total cost of our eight *M. fortuitum* primers and probe was 113,82 €. The primers were delivered and transported in lyophilized form and had to be reconstituted. The primers had a volume between 280 µl at the lowest (reverse primer 6) and 359 µl at the highest (reverse primer 1). The probe (86 €) had a volume of 149 µl. In our assay, the primers and probe were used in a 1:10 dilution. Thus, the 10-fold volume of each was available. For example, when running our *M. fortuitum* assay with a 1:10 dilution of the

probe, 1490 µl are available for use instead of just 149 µl. Using a 1:10 dilution of the primers allowed for 3460 µl and 3490 µl of forward primer 3 (2,80 €) and reverse primer 4 (2,80 €) to be available, respectively. As 1 µl of 1:10 diluted forward and reverse primer was used per reaction, the cost per reaction would be approximately 0,0008 € per primer used. For each reaction, 0.5 µl of 1:10 diluted probe was used and thus the cost per reaction would be approximately 0,028 € for the probe. The qPCR kit we used was the QuantiTect Multiplex PCR NoROX Kit (QIAGEN N.V., Venlo, Netherlands) at a cost of 905 € per 200 tests (approximately equal to 4,52 € per test). One should note that the cost per test using our qPCR assay was also higher in practice, as additional reagents were used to account for pipetting error.

All in all, the total cost of reagents for our *M. fortuitum* assay is approximately 4,55 € per qPCR reaction, less than half of the cost of LPA analysis. Thus, the use of the *M. fortuitum* qPCR assay constitutes a cost-effective alternative to the GenoType Mycobacteria CM LPA kit.

4.3 Improved accuracy and discrepant results compared to reference methods

This study demonstrates the potential of molecular biological techniques, specifically qPCR, for the differential diagnosis of *M. fortuitum* and *M. tuberculosis*. Several commercial methods for the identification of both *M. tuberculosis* and *M. fortuitum* already exist. The GeneXpert® (Cepheid®, Sunnyvale, CA, USA) system can detect *M. tuberculosis* directly from patient samples and the GenoType Mycobacterium CM LPA kit (Hain Lifescience, Nehren, Germany) can differentiate several relevant NTM species from cultured samples. These two methods were used as reference methods in our experiments.

Our qPCR assay targeting the *rpoB gene* of *M. fortuitum* achieved a LOD between 10^2 and 10^3 CFU/ml and showed high concordance with the GenoType CM LPA reference method when testing DNA extracted from sputum culture samples. Similarly, the *M. tuberculosis IS6110* qPCR assay showed strong initial performance when used together with the Chelex® DNA extraction protocol on sputum samples, indicating

potential for accurate and timely discrimination between TB and NTM infections in clinical specimens.

Our methods offer a promising alternative to the available reference methods regarding diagnostic performance and clinical utility. Initial experiments suggested a higher accuracy when compared to the reference methods. Interestingly, we observed discordant results for several samples that had been speciated as *M. fortuitum* by LPA when they were tested using our *M. fortuitum* qPCR assay. The key observation being that samples with only the 7 band LPA pattern showed notably reduced confirmation rate by qPCR (57%) when compared to samples exhibiting the 7 and 14 banding pattern (91%). This ambiguity led us to question whether we could elucidate a cause or explanation for the discordant results.

One possible explanation for these discordant results between the LPA and qPCR may be the misclassification of another closely related NTM present in the samples.

Although rare, according to the manufacturer's instructions, misidentification of closely related bacterial strains by the LPA is possible, because a similar banding pattern may form [87, 88]. For example, the instructions note that *M. mageritense* and *M. smegmatis* samples may also show the 7 banding pattern. An interpretation chart along with an explanation for the different band patterns can be found in Fig. 30 below. The updated instructions by the manufacturer give more insights on differentiation of the facultative banding patterns, stating that *M. mageritense* may show a banding pattern with just the 7 band staining, while rare *M. smegmatis* variants may also show a similar banding pattern with band 7 showing only a weak staining (see Fig. 30, second from right column for comparison). The instructions further note that sequence variations within the *M. fortuitum* species may contribute to the heterogeneous banding patterns observed in these samples [87, 88]. All four samples display a rather weak staining reaction of the 7 band when compared with the other test strips, described by the manufacturer`s instructions as a characteristic cross-reaction for *M. smegmatis* (see Table 8 above for reference).

Moreover, several studies have reported misidentification of mycobacterial isolates as *M. fortuitum* [95-98]. Notably, two reports describe *M. smegmatis* isolates misidentified

as *M. fortuitum* by line probe assays, albeit not using the GenoType LPA [95, 98]. A larger comparative study evaluating the two commercially available LPA (INNO LiPA and GenoType) on 317 mycobacterial strains found that both assays misidentified between 20 and 28 taxa, with the majority of cross-reactions affecting the *M. fortuitum*-specific probes [97].

It is therefore plausible that the four samples tested negative by qPCR may in fact represent *M. smegmatis* isolates. This suggests that the single-band pattern, with staining only at the 7 band, is more prone to misclassification. Further testing of the samples in question, for example using the "Pan-Mycobacteria" *16srRNA* primers and WGS could be an important next step and necessary in elucidating the suspected presence of other mycobacteria.

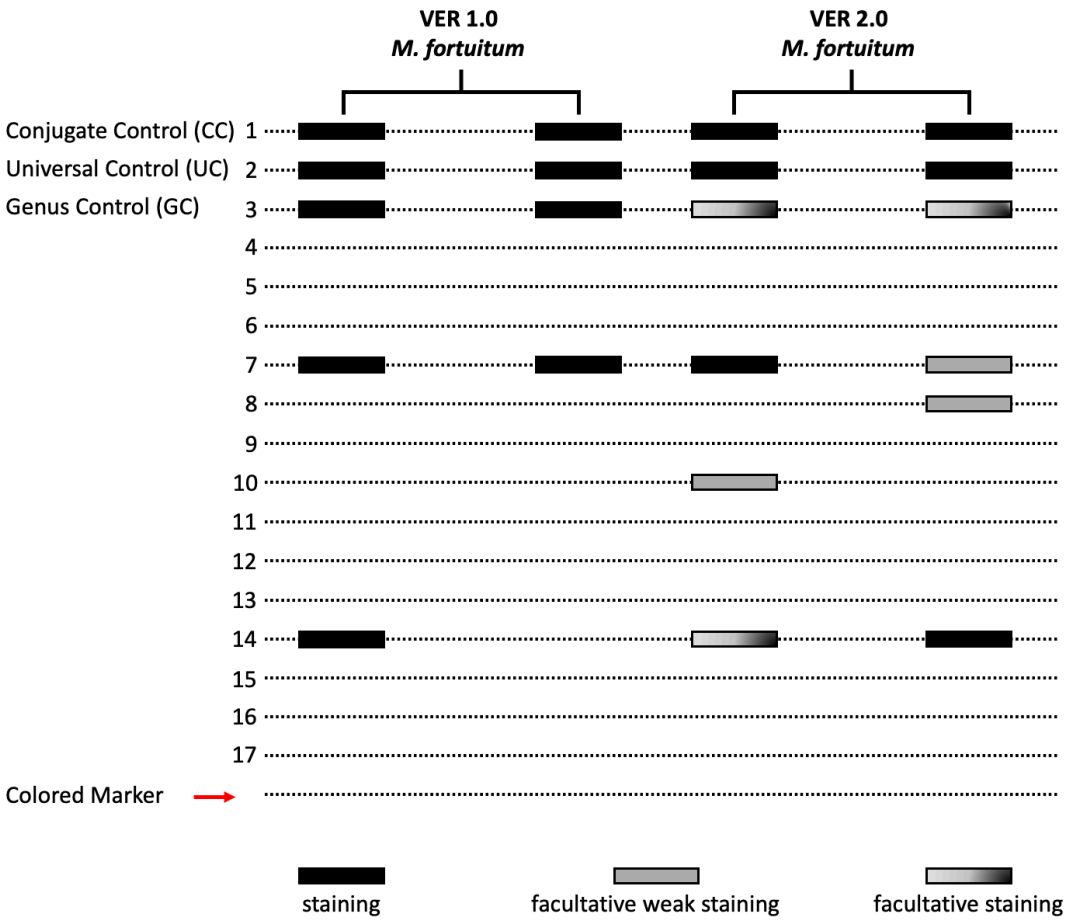


Fig. 30: Interpretation Chart for *M. fortuitum* on GenoType Mycobacterium CM LPA.
Side by side comparison of the different banding patterns for *M. fortuitum* when using the GenoType Mycobacterium CM LPA kit (Hain Lifescience, Nehren, Germany). The interpretation of the banding patterns was adopted from the manufacturer`s instruction manual [87, 88]. The columns on the left side show the two different banding patterns possible for *M. fortuitum* according to the VER 1.0 instructions. Here, staining occurring at the 7 and 14 bands (first from left) or just at 7 band (second from left) is interpreted as a positive result for *M. fortuitum*. The columns on the right side show the two different banding patterns possible for *M. fortuitum* according to the VER 2.0 instructions. Here staining occurring at the 7 band with facultative weak staining of the 10 band and facultative staining of the 14 band (second from right) is interpreted as positive for *M. fortuitum*, whilst staining of the 14 band together with facultative weak staining of the 7 or 8 bands (first from right) is also interpreted as positive for *M. fortuitum.* These different banding patterns occur due to sequence variations within the species. The instructions (for the VER 1.0) further state that *M. mageritense* may show the same banding pattern as depicted in second from left column. For VER 2.0 it is noted that *M. mageritense* may show the banding pattern as depicted in the second from right column without band 14. Additionally, *M. smegmatis* variants may show the banding pattern depicted in the second from right column without band 14 and with band 7 showing only a weak signal.

Another possible explanation for the discrepant results could be the test quality criteria of the LPA. Based on the specificity data of the GenoType Mycobacterium CM in literature, which is around 92 %, the false positive rate (false positive rate is defined as: $1 - specificity$) can be calculated at around 8 % [99, 100]. Accordingly, with a false positive rate of around 8 %, a certain number of false positive results is to be expected with the LPA. Since 8 % out of 24 samples would roughly amount to 2 samples, the discrepant results may simply lie within the normal limits of the LPAs test quality criteria.

In short, these observations led us to the conclusion that our initial testing indicated an improved accuracy of the *M. fortuitum* qPCR assay over the GenoType Mycobacterium CM LPA reference method, when testing presumptive *M. fortuitum* samples.

When comparing the results of our *M. tuberculosis* assay to the GeneXpert® (Cepheid®, Sunnyvale, CA, USA) reference method, we also found discordant results in part. Six sputa from presumptive TB patients were tested on GeneXpert® and using our *M. tuberculosis IS6110* qPCR assay. On the GeneXpert® 2/6 samples were positive for TB and all samples that were tested positive by the GeneXpert® could concomitantly be confirmed by our qPCR. Thus, there was 100 % agreement/concordance between the two methods. However, one sample was apparently not correctly identified by the GeneXpert®. Literature indicates a sensitivity of around 88 % when the GeneXpert® system is used as the initial test for samples from presumptive TB patients. The false negative rate (false negative rate defined as: $1 - sensitivity$) can be calculated at 12 % [37]. This is very roughly equal to 1/6 samples. Accordingly, the discordant false negative result lies roughly within the normal limits of the test quality criteria found in literature when using the GeneXpert® system as an initial testing tool for presumptive TB samples. Arguably, this points to better accuracy of the *M. tuberculosis IS6110* qPCR assay for testing presumptive TB samples.

Additionally, our *M. tuberculosis* qPCR assay together with the Chelex® DNA extraction method was able to identify *M. tuberculosis* directly from patients' sputum without prior decontamination of samples. No clear negative effect of decontamination on the qPCR assays performance could be observed in the initial experiment, even though available literature suggests negative ramifications of sample decontamination for diagnosis of

mycobacteria. Apart from decreasing workload and turn-around-time, foregoing decontamination may increase the diagnostic yield of a mycobacterial sample [7].

While our initial comparison of the *M. fortuitum* and *M. tuberculosis* qPCR assays with their respective reference methods led us to the conclusion that our assays had improved test accuracy, we were not yet able to perform comprehensive laboratory validation of both assays. Final conclusions regarding the sensitivity and specificity of the assays are thus limited. More testing would be a sensible next step, for example: including ATCC reference strains of other mycobacteria to evaluate assay specificity and cross-reactivity; testing additional replicates of serially diluted mycobacterial DNA to evaluate assay performance and calculate inter-/intra-run variability; and testing larger numbers of patient specimens using the custom assays and reference methods for comparison. Attempts at multiplexing the assay would also be of interest for a more streamlined workflow when handling samples from presumptive TB/NTM patients. Regrettably, the Pan-Mycobacteria primers arrived too late in Ghana to be used in experiments, further work by the team at KCCR to implement these primers as part of the assay in the future is necessary.

4.4 Limitations of the Study

Despite the promising results of the novel methods we developed, this study has several limitations that are important to note.

Firstly, although recruitment of participants and sample collection was carried out across the entire Ashanti Region, the amount of available presumptive NTM samples we tested (n = 24) remained small and represents only limited geographic coverage within Ghana. As such, the samples we tested may not fully represent the national NTM burden in Ghana. Due to at times long sample transport and storage within Ghana, the quality of our samples may have also been impeded.

Secondly, despite our goal of direct, *ex-vivo* diagnosis from sputum samples, most of the testing of our assays was performed on DNA extracted from cultures. This in turn may distort diagnostic sensitivity of our methods.

Thirdly and most importantly, while early results are encouraging, they represent only preliminary clinical validation. As mentioned previously, prior to implementation in routine diagnostics, a larger scale laboratory validation is necessary and further studies would be needed to achieve this.

4.5 Conclusion and outlook

Knowledge on NTM and especially on *M. fortuitum* as well as its associated infections remains limited. Our research offers important insights into the relevance of *M. fortuitum* and the methods developed in this study showcase the possibility for the molecular biological differentiation of mycobacterial infections, namely *M. tuberculosis* and *M. fortuitum* in a high-prevalence setting.

We present novel molecular tools for the detection of *M. fortuitum*, demonstrating their potential application in diagnostic laboratories in Ghana. As testing for both infections is carried out by the same qPCR method and not on two different systems (i.e. GeneXpert® and GenoType CM LPA), a streamlined laboratory workflow utilizing this custom assay could simplify the differential diagnosis in the future, in turn improving clinical decision making. Optimally, the direct, *ex-vivo* identification from sputum samples would be possible for both *M. tuberculosis* and *M. fortuitum* with this assay. However, comprehensive laboratory assay validation is still pending. Expanding the qPCR platform to detect other clinically relevant NTM species, either by incorporating Pan-Mycobacteria primers or by developing additional primers for individual clinically relevant NTM species could facilitate the development of a multiplex diagnostic tool suited for routine use.

Continuing work is necessary to identify an effective method for the direct extraction of *M. fortuitum* DNA from sputum samples and for the extraction of gDNA from cultured samples that meets the high-quality standards for WGS. A possible alternative extraction method that is currently being evaluated and will be tested by our team in the future is the cetyltrimethylammonium bromide or CTAB method [56].

Several of our samples have been submitted to the Genomics and Transcriptomics Laboratory (GTL) at Heinrich-Heine-University Düsseldorf for quality control evaluation

prior to WGS. Unfortunately, only few have met the quality criteria for WGS so far. Once enough gDNA samples of sufficient quality have been generated from the 22 presumptive *M. fortuitum* samples at KCCR, WGS will commence. This is an important next step in the project, necessary not only for identifying novel genetic regions to be utilized for diagnostic applications in the future, but also for elucidating the pathogenetic relevance of *M. fortuitum* and its antimicrobial resistance profiles, genetic strain diversity and epidemiological patterns in Ghana.

In conclusion, our study highlights the potential of novel custom molecular biological methods for the identification of *M. fortuitum* and the differentiation of NTM-PD from TB. Furthermore, our findings reinforce the urgent need for timely, accurate and accessible diagnostic tools for NTM infections, especially in low-resource countries with a high TB burden. By avoiding misdiagnosis and enabling appropriate treatment, these tools could improve patient outcomes and help to better understand the impact of NTM infections. As infections with NTM continue to rise globally, integrating methods for the diagnosis of NTM infections into national TB programs and local laboratory infrastructures would be an essential next step for effective disease management and surveillance in sub-Saharan Africa and beyond.

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References

1. *Global tuberculosis report 2025*. 2025, WHO: Geneva.
2. Meier-Kolthoff, J.P., et al., *TYGS and LPSN: a database tandem for fast and reliable genome-based classification and nomenclature of prokaryotes.* Nucleic Acids Research, 2021. **50**(D1): p. D801–D807.
3. Parte, A.C., et al., *List of Prokaryotic names with Standing in Nomenclature (LPSN) moves to the DSMZ.* International Journal of Systematic and Evolutionary Microbiology, 2020. **70**(11): p. 5607–5612.
4. Bös, L., N. Schönfeld, and T. Schaberg, *Nichttuberkulöse Mykobakterien – eine kurze Übersicht zur Epidemiologie, Diagnostik und Therapie.* Pneumologie, 2015. **69**(05): p. 287–294.
5. Griffith, D.E., et al., *Nontuberculous Mycobacterial Disease, A Comprehensive Approach to Diagnosis and Management*. Respiratory Medicine. 2019: Springer Nature Switzerland AG.
6. Daley, C.L., et al., *Treatment of Nontuberculous Mycobacterial Pulmonary Disease: An Official ATS/ERS/ESCMID/IDSA Clinical Practice Guideline.* Clin Infect Dis, 2020. **71**(4): p. e1–e36.
7. Griffith, D.E., et al., *An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases.* Am J Respir Crit Care Med, 2007. **175**(4): p. 367–416.
8. Schönfeld, N., et al., *Empfehlungen zur Diagnostik und Therapie nichttuberkulöser Mykobakteriosen des Deutschen Zentralkomitees zur Bekämpfung der Tuberkulose (DZK) und der Deutschen Gesellschaft für Pneumologie und Beatmungsmedizin (DGP) / Recommendations for Diagnosis and Treatment of Nontuberculous Mycobacterioses of the German Central Committee against Tuberculosis and the German Respiratory Society.* Pneumologie, 2013. **67**: p. 605–633.
9. McShane, P.J. and J. Glassroth, *Pulmonary Disease Due to Nontuberculous Mycobacteria: Current State and New Insights.* Chest, 2015. **148**(6): p. 1517–1527.
10. Prevots, D.R. and T.K. Marras, *Epidemiology of human pulmonary infection with nontuberculous mycobacteria: a review.* Clin Chest Med, 2015. **36**(1): p. 13–34.
11. Steindor, M., et al., *Rapid detection and immune characterization of Mycobacterium abscessus infection in cystic fibrosis patients.* PLoS One, 2015. **10**(3): p. e0119737.
12. Lange, C., et al., *Consensus management recommendations for less common non-tuberculous mycobacterial pulmonary diseases.* Lancet Infect Dis, 2022. **22**(7): p. e178–e190.
13. Cassidy, P.M., et al., *Nontuberculous Mycobacterial Disease Prevalence and Risk Factors: A Changing Epidemiology.* Clinical Infectious Diseases, 2009. **49**(12): p. e124–e129.
14. Winthrop, K.L., et al., *Pulmonary Nontuberculous Mycobacterial Disease Prevalence and Clinical Features.* American Journal of Respiratory and Critical Care Medicine, 2010. **182**(7): p. 977–982.
15. Bryant, J.M., et al., *Whole-genome sequencing to identify transmission of Mycobacterium abscessus between patients with cystic fibrosis: a retrospective cohort study.* Lancet, 2013. **381**(9877): p. 1551–60.
16. Bryant, J.M., et al., *Emergence and spread of a human-transmissible multidrug-resistant nontuberculous mycobacterium.* Science, 2016. **354**(6313): p. 751–757.
17. Aitken, M.L., et al., *Respiratory Outbreak of Mycobacterium abscessus Subspecies massiliense in a Lung Transplant and Cystic Fibrosis Center.* American Journal of Respiratory and Critical Care Medicine, 2012. **185**(2): p. 231–232.

18. Okoi, C., et al., *Non-tuberculous Mycobacteria isolated from Pulmonary samples in sub-Saharan Africa - A Systematic Review and Meta Analyses.* Sci Rep, 2017. **7**(1): p. 12002.
19. Mawak, J.D., et al., *Human pulmoary infections with bovine and environment (atypical) Mycobacteria in Jos, Nigeria.* Ghana Medical Journal, 2006. **40**(4): p. 132–136.
20. Addo, K.K., et al., *Genotyping and drug susceptibility testing of mycobacterial isolates from population-based tuberculosis prevalence survey in Ghana.* BMC Infect Dis, 2017. **17**(1): p. 743.
21. Bjerrum, S., et al., *Tuberculosis and non-tuberculous mycobacteria among HIV-infected individuals in Ghana.* Trop Med Int Health, 2016. **21**(9): p. 1181–90.
22. Arthur, R.A., et al., *The burden of non-tuberculous mycobacterium species in presumptive tuberculosis patients in Ghana.* [Unpublished manuscript], 2022.
23. van Ingen, J. and D. Wagner, *Epidemiologie der nichttuberkulösen mykobakteriellen Erkrankungen in Deutschland und weltweit.* Der Pneumologe, 2011. **8**(6): p. 396–403.
24. Pennington, K.M., et al., *Approach to the diagnosis and treatment of non-tuberculous mycobacterial disease.* J Clin Tuberc Other Mycobact Dis, 2021. **24**: p. 100244.
25. The American Thoracic Society Official Statement, *Diagnosis and Treatment of Disease Caused by Nontuberculous Mycobacteria.* American Journal of Respiratory and Critical Care Medicine, 1997. **156**(2): p. S1–S25.
26. Richard J. Wallace, J., et al., *Diagnosis and Treatment of Disease Caused by Nontuberculous Mycobacteria.* American Review of Respiratory Disease, 1990. **142**(4): p. 940–953.
27. Larsen, M.H., K. Biermann, and W.R. Jacobs, Jr., *Laboratory maintenance of Mycobacterium tuberculosis.* Curr Protoc Microbiol, 2007. **Chapter 10**: p. Unit 10A.1.
28. Siddiqi, S.H. and S. Rüsch-Gerdes, *MGIT Procedure Manual*. 2006, Foundation for Innovative New Diagnostics.
29. Nathavitharana, R., et al., *Multi-center Study to Assess the Non-inferiority of Nipro NTM + MDRTB and Hain Genotype Mtbdrplus V2 Line Probe Assays Compared to Hain Genotype Mtbdrplus V1.* Open Forum Infectious Diseases, 2015. **2**(suppl_1).
30. Richardson, E.T., D. Samson, and N. Banaei, *Rapid Identification of Mycobacterium tuberculosis and nontuberculous mycobacteria by multiplex, real-time PCR.* J Clin Microbiol, 2009. **47**(5): p. 1497–502.
31. Rocchetti, T.T., et al., *Detection of Mycobacterium chelonae, Mycobacterium abscessus Group, and Mycobacterium fortuitum Complex by a Multiplex Real-Time PCR Directly from Clinical Samples Using the BD MAX System.* The Journal of Molecular Diagnostics, 2017. **19**(2): p. 295–302.
32. Rocchetti, T.T., et al., *Validation of a Multiplex Real-Time PCR Assay for Detection of Mycobacterium spp., Mycobacterium tuberculosis Complex, and Mycobacterium avium Complex Directly from Clinical Samples by Use of the BD Max Open System.* Journal of clinical microbiology, 2016. **54**(6): p. 1644–1647.
33. Sarro, Y.D.S., et al., *Development and clinical evaluation of a new multiplex PCR assay for a simultaneous diagnosis of tuberculous and nontuberculous mycobacteria.* EBioMedicine, 2021. **70**: p. 103527.
34. Sevilla, I.A., et al., *Detection of mycobacteria, Mycobacterium avium subspecies, and Mycobacterium tuberculosis complex by a novel tetraplex real-time PCR assay.* J Clin Microbiol, 2015. **53**(3): p. 930–40.
35. Wang, H.-y., et al., *Performance of a real-time PCR assay for the rapid identification of Mycobacterium species.* Journal of Microbiology, 2015. **53**(1): p. 38–46.
36. Buckwalter, S.P., et al., *Mycobacterium abscessus subspecies identification using the Deeplex Myc-TB targeted NGS assay.* J Clin Microbiol, 2023. **61**(10): p. e0048923.
37. Steingart, K.R., et al., *Xpert® MTB/RIF assay for pulmonary tuberculosis and rifampicin resistance in adults.* Cochrane Database Syst Rev, 2013(1): p. Cd009593.

38. Zifodya, J.S., et al., *Xpert Ultra versus Xpert MTB/RIF for pulmonary tuberculosis and rifampicin resistance in adults with presumptive pulmonary tuberculosis.* Cochrane Database of Systematic Reviews, 2021(2).
39. Kolia-Diafouka, P., et al., *Optimized Lysis-Extraction Method Combined With IS6110-Amplification for Detection of Mycobacterium tuberculosis in Paucibacillary Sputum Specimens.* Front Microbiol, 2018. **9**: p. 2224.
40. Reed, J.L., et al., *Highly sensitive sequence specific qPCR detection of Mycobacterium tuberculosis complex in respiratory specimens.* Tuberculosis (Edinb), 2016. **101**: p. 114–124.
41. Savelkoul, P.H., et al., *Detection of Mycobacterium tuberculosis complex with Real Time PCR: comparison of different primer-probe sets based on the IS6110 element.* J Microbiol Methods, 2006. **66**(1): p. 177–80.
42. Sanjuan-Jimenez, R., et al., *Comparative clinical study of different multiplex real time PCR strategies for the simultaneous differential diagnosis between extrapulmonary tuberculosis and focal complications of brucellosis.* PLoS Negl Trop Dis, 2013. **7**(12): p. e2593.
43. Philley, J.V. and D.E. Griffith, *Management of nontuberculous mycobacterial (NTM) lung disease.* Semin Respir Crit Care Med, 2013. **34**(1): p. 135–42.
44. Schön, T. and E. Chryssanthou, *Minimum inhibitory concentration distributions for Mycobacterium avium complex-towards evidence-based susceptibility breakpoints.* Int J Infect Dis, 2017. **55**: p. 122–124.
45. Lange, C., et al., *Consensus management recommendations for less common non-tuberculous mycobacterial pulmonary diseases.* The Lancet Infectious Diseases, 2022. **22**(7): p. e178–e190.
46. Maiga, M., et al., *Failure to recognize nontuberculous mycobacteria leads to misdiagnosis of chronic pulmonary tuberculosis.* PLoS One, 2012. **7**(5): p. e36902.
47. López-Varela, E., et al., *High Rates of Non-Tuberculous Mycobacteria Isolation in Mozambican Children with Presumptive Tuberculosis.* PLoS One, 2017. **12**(1): p. e0169757.
48. Pokam, B.T. and A.E. Asuquo, *Acid-fast bacilli other than mycobacteria in tuberculosis patients receiving directly observed therapy short course in cross river state, Nigeria.* Tuberc Res Treat, 2012. **2012**: p. 301056.
49. Badoum, G., et al., *Failing a re-treatment regimen does not predict MDR/XDR tuberculosis: is "blind" treatment dangerous?* European Respiratory Journal, 2011. **37**(5): p. 1283–1285.
50. Awokola, B.I., et al., *Chronic obstructive pulmonary disease in sub-Saharan Africa.* Int J Tuberc Lung Dis, 2022. **26**(3): p. 232–242.
51. Rademacher, J., *Nichttuberkulöse Mykobakterien im Sputum.* Der Internist, 2017. **58**(11): p. 1163–1170.
52. Schaberg, T., et al., *Tuberkulose im Erwachsenenalter.* Eine S2k-Leitlinie zur Diagnostik und Therapie, Chemoprävention und Chemoprophylaxe der Tuberkulose im Erwachsenenalter des Deutschen Zentralkomitees zur Bekämpfung der Tuberkulose e. V. (DZK) und der Deutschen Gesellschaft für Pneumologie und Beatmungsmedizin e. V. (DGP), 2022. **76**(11): p. 727–819.
53. Buijtels, P.C., et al., *Nontuberculous mycobacteria, zambia.* Emerg Infect Dis, 2009. **15**(2): p. 242–9.
54. Petti, C.A., et al., *Laboratory Medicine in Africa: A Barrier to Effective Health Care.* Clinical Infectious Diseases, 2006. **42**(3): p. 377–382.
55. Aliyu, G., et al., *Prevalence of non-tuberculous mycobacterial infections among tuberculosis suspects in Nigeria.* PLoS One, 2013. **8**(5): p. e63170.

56. Larsen, M.H., et al., *Genetic Manipulation of Mycobacterium tuberculosis.* Curr Protoc Microbiol, 2007. **Chapter 10**: p. Unit 10A.2.
57. Epola Dibamba Ndanga, M., et al., *Non-tuberculous mycobacteria isolation from presumptive tuberculosis patients in Lambaréné, Gabon.* Tropical Medicine & International Health, 2022. **27**(4): p. 438–444.
58. Hoza, A.S., et al., *Increased isolation of nontuberculous mycobacteria among TB suspects in Northeastern, Tanzania: public health and diagnostic implications for control programmes.* BMC Res Notes, 2016. **9**: p. 109.
59. Thangavelu, K., et al., *Prevalence and speciation of non-tuberculous mycobacteria among pulmonary and extrapulmonary tuberculosis suspects in South India.* J Infect Public Health, 2021. **14**(3): p. 320–323.
60. Thakur, R., S. Sarma, and R. Goyal, *Comparison of DNA Extraction Protocols for Mycobacterium Tuberculosis in Diagnosis of Tuberculous Meningitis by Real-time Polymerase Chain Reaction.* J Glob Infect Dis, 2011. **3**(4): p. 353–6.
61. Aldous, W.K., et al., *Comparison of six methods of extracting Mycobacterium tuberculosis DNA from processed sputum for testing by quantitative real-time PCR.* Journal of clinical microbiology, 2005. **43**(5): p. 2471–2473.
62. Bello, G.L., et al., *Improvement of Mycobacterium tuberculosis detection in sputum using DNA extracted by sonication.* Braz J Infect Dis, 2020. **24**(5): p. 398–404.
63. de Almeida, I.N., et al., *Evaluation of six different DNA extraction methods for detection of Mycobacterium tuberculosis by means of PCR-IS6110: preliminary study.* BMC Research Notes, 2013. **6**(1): p. 561.
64. Rabodoarivelo, M.S., et al., *Optimizing of a protein extraction method for Mycobacterium tuberculosis proteome analysis using mass spectrometry.* Journal of Microbiological Methods, 2016. **131**: p. 144–147.
65. Tarhan, G. and İ. Ceyhan, *Evaluation of three DNA extraction methods of Mycobacterium tuberculosis DNA from Processed Sputum for testing by Three Real Systems.* Advances in Biotechnology & Microbiology, 2017. **2**(2): p. 0029–0034.
66. Pholwat, S., et al., *Digital PCR to detect and quantify heteroresistance in drug resistant Mycobacterium tuberculosis.* PLoS One, 2013. **8**(2): p. e57238.
67. Mwesige, S., et al., *Prevalence of pulmonary tuberculosis and the associated clinical symptoms in Western Uganda.* International Journal of Life Science Research Archive, 2022. **03**(01): p. 155–162.
68. Beissner, M., K.-H. Herbinger, and G. Bretzel, *Laboratory diagnosis of Buruli ulcer disease.* Future Microbiology, 2010. **5**(3): p. 363–370.
69. Siegmund, V., et al., *Dry-reagent-based PCR as a novel tool for laboratory confirmation of clinically diagnosed Mycobacterium ulcerans-associated disease in areas in the tropics where M. ulcerans is endemic.* J Clin Microbiol, 2005. **43**(1): p. 271–6.
70. Peñuelas-Urquides, K., et al., *Measuring of Mycobacterium tuberculosis growth. A correlation of the optical measurements with colony forming units.* Braz J Microbiol, 2013. **44**(1): p. 287–9.
71. Adékambi, T., P. Colson, and M. Drancourt, *rpoB-Based Identification of Nonpigmented and Late-Pigmenting Rapidly Growing Mycobacteria.* Journal of Clinical Microbiology, 2003. **41**: p. 5699 – 5708.
72. Ben Salah, I., et al., *rpoB sequence-based identification of Mycobacterium avium complex species.* Microbiology, 2008. **154**(12): p. 3715–3723.
73. de Zwaan, R., J. van Ingen, and D. van Soolingen, *Utility of rpoB gene sequencing for identification of nontuberculous mycobacteria in the Netherlands.* J Clin Microbiol, 2014. **52**(7): p. 2544–51.
74. Kim, B.J., et al., *Identification of mycobacterial species by comparative sequence analysis of the RNA polymerase gene (rpoB).* J Clin Microbiol, 1999. **37**(6): p. 1714–20.

75. Shapiro, A.E., et al., *Complementary Nonsputum Diagnostic Testing for Tuberculosis in People with HIV Using Oral Swab PCR and Urine Lipoarabinomannan Detection.* J Clin Microbiol, 2022. **60**(8): p. e0043122.
76. Luabeya, A.K., et al., *Noninvasive Detection of Tuberculosis by Oral Swab Analysis.* J Clin Microbiol, 2019. **57**(3).
77. Wood, R.C., et al., *Characterization of oral swab samples for diagnosis of pulmonary tuberculosis.* PLoS One, 2021. **16**(5): p. e0251422.
78. LaCourse, S.M., et al., *Diagnostic performance of oral swabs for non-sputum based TB diagnosis in a TB/HIV endemic setting.* PLoS One, 2022. **17**(1): p. e0262123.
79. Fakhreddine, M., et al., *Rapid detection of Mycobacterium tuberculosis complex by real-time polymerase chain reaction (PCR) in pulmonary and extra-pulmonary samples in Casablanca, Morocco.* Pan Afr Med J, 2020. **36**: p. 134.
80. Zida, S., et al., *Combined testing for herpes simplex virus and Mycobacterium tuberculosis DNA in cerebrospinal fluid of patients with aseptic meningitis in Burkina Faso, West Africa.* J Clin Lab Anal, 2019. **33**(3): p. e22719.
81. Thierry, D., et al., *Characterization of a Mycobacterium tuberculosis insertion sequence, IS6110, and its application in diagnosis.* J Clin Microbiol, 1990. **28**(12): p. 2668–73.
82. Thierry, D., et al., *IS6110, an IS-like element of Mycobacterium tuberculosis complex.* Nucleic Acids Res, 1990. **18**(1): p. 188.
83. van Embden, J.D., et al., *Strain identification of Mycobacterium tuberculosis by DNA fingerprinting: recommendations for a standardized methodology.* J Clin Microbiol, 1993. **31**(2): p. 406–9.
84. Cave, M.D., et al., *IS6110: Conservation of sequence in the Mycobacterium tuberculosis complex and its utilization in DNA fingerprinting.* Molecular and Cellular Probes, 1991. **5**(1): p. 73–80.
85. Gordon, S.V., et al., *New insertion sequences and a novel repeated sequence in the genome of Mycobacterium tuberculosis H37Rv.* Microbiology (Reading), 1999. **145 (Pt 4)**: p. 881–892.
86. McEvoy, C.R.E., et al., *The role of IS6110 in the evolution of Mycobacterium tuberculosis.* Tuberculosis, 2007. **87**(5): p. 393–404.
87. Hain Lifescience GmbH. *GenoType Mycobacterium CM VER 1.0 Instructions for Use*. 2014 [cited 2025, Nov. 2]; Available from: https://www.hain-lifescience.de/include_datei/kundenmodule/packungsbeilage/download.php?id=776.
88. Hain Lifescience GmbH. *GenoType Mycobacterium CM VER 2.0 Instructions for Use*. 2016 [cited 2025, Nov. 5]; Available from: https://www.hain-lifescience.de/include_datei/kundenmodule/packungsbeilage/download.php?id=1333.
89. Bates, I. and K. Maitland, *Are Laboratory Services Coming of Age in Sub-Saharan Africa?* Clinical Infectious Diseases, 2006. **42**(3): p. 383–384.
90. Okeke, I.N., *Diagnostic Insufficiency in Africa.* Clinical Infectious Diseases, 2006. **42**(10): p. 1501–1503.
91. Wani, S.R., C. Wattal, and R. Raveendran, *Epidemiology and risk factors associated with NTM pulmonary and extrapulmonary infections in a high tuberculosis endemic Region.* Indian J Med Microbiol, 2020. **38**(2): p. 169–175.
92. Comín, J., I. Otal, and S. Samper, *In-depth Analysis of IS6110 Genomic Variability in the Mycobacterium tuberculosis Complex.* Front Microbiol, 2022. **13**: p. 767912.
93. Roychowdhury, T., S. Mandal, and A. Bhattacharya, *Analysis of IS6110 insertion sites provide a glimpse into genome evolution of Mycobacterium tuberculosis.* Scientific Reports, 2015. **5**(1): p. 12567.
94. Patel, D., et al., *Rapid Quantification of IS6110 Copy Number in M. tuberculosis Using Dual-Target qPCR Assay.* medRxiv, 2025: p. 2025.04.04.25325287.

95. Fida, M., E. Beam, and N.L. Wengenack, *Misidentification of Mycobacterium smegmatis as Mycobacterium fortuitum by DNA line probe assay.* Journal of Clinical Tuberculosis and Other Mycobacterial Diseases, 2021. **25**: p. 100268.
96. Richter, E., S. Rüsch-Gerdes, and D. Hillemann, *Evaluation of the GenoType Mycobacterium Assay for identification of mycobacterial species from cultures.* J Clin Microbiol, 2006. **44**(5): p. 1769–75.
97. Tortoli, E., et al., *Commercial DNA probes for mycobacteria incorrectly identify a number of less frequently encountered species.* J Clin Microbiol, 2010. **48**(1): p. 307–10.
98. van den Broek, T., et al., *INNO-LiPA DNA line probe assay misidentification of M. smegmatis as Mycobacterium fortuitum complex.* Diagn Microbiol Infect Dis, 2019. **95**(3): p. 114858.
99. Lee, A.S., et al., *Identification of non-tuberculous mycobacteria: utility of the GenoType Mycobacterium CM/AS assay compared with HPLC and 16S rRNA gene sequencing.* Journal of Medical Microbiology, 2009. **58**(7): p. 900–904.
100. Russo, C., E. Tortoli, and D. Menichella, *Evaluation of the new GenoType Mycobacterium assay for identification of mycobacterial species.* J Clin Microbiol, 2006. **44**(2): p. 334–9.