

KADRI KLAOS

The thin-layer agar MDR/XDR-TB
Colour Test: filling the gap in
diagnosing tuberculosis from
a laboratory perspective



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*To my husband Heigo,
who brings music to my heart.
To my children Evert, Herman and Elias,
who have given me plenty of colourful life lessons
and many more are still to come.*

*Never lose the ability to recognise true colours
of the world.*

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LIST OF PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals (I–IV):

- I. Kadri Toit, Sarah Mitchell, Yanina Balabanova, Carlton A. Evans, Tiina Kummik, Vladislav Nikolayevskyy and Francis Drobniewski. The Colour Test for drug susceptibility testing of *Mycobacterium tuberculosis* strains. *Int J Tuberc Lung Dis* 2012;16(8):1113–8.
- II. Kadri Klaos, Anna Agejeva, Tiina Kummik, Sirje Laks, Olesja Remets, Sirje Sasi, Anneli Tann, Piret Viiklepp and Alan Altraja. A successful introduction to a non-expert setting of the thin-layer agar Colour Test as an indirect phenotypic drug susceptibility test for *Mycobacterium tuberculosis*. *Int J Infect Dis* 2021;104:19–26.
- III. Kadri Klaos, Anna Agejeva, Kaja Hurt, Tiina Kummik, Anu Kurve, Julia Nirk, Lea Pehme, Olesja Remets, Sirje Sasi, Anneli Tann and Alan Altraja. Prospective evaluation of thin-layer agar colour test in routine diagnosis of multidrug-resistant TB. *Int J Tuberc Lung Dis* 2024;28(8):387–394.
- IV. Kadri Toit, Alan Altraja, Caroline D. Acosta, Piret Viiklepp, K. Kremer, Tiina Kummik, Manfred Danilovitš, Rafael Van den Bergh, Anthony D. Harries and Philip Supply. A four-year nationwide molecular epidemiological study in Estonia: risk factors for tuberculosis transmission. *Public Health Action* 2014;4(3):S34–40.

Author's contribution:

Studies I–IV: Participation in the planning of the study, teaching the study methods and/or performing the studies, gathering and analysing clinical and experimental data and drafting of the manuscript. All copyrights of the article II belong to the authors and permission to reproduce the published material is not needed. All copyrights of the article IV belong to the World Health Organization through a Creative Commons Attribution IGO licence (<http://creativecommons.org/licenses/by/3.0/igo/legalcode>), which permits reproduction in any medium provided the original work is cited. For the articles I and III, the reproductions are provided with permissions from the publisher.

ABBREVIATIONS

AFB	Acid-fast bacilli
BAL	Bronchoalveolar lavage
BCG	<i>Mycobacterium bovis</i> bacillus Calmette-Guerin
BDQ	Bedaquiline
BSC	Biological safety cabinet
CFX	Ciprofloxacin
CI	Confidence interval
CRI	Colorimetric redox indicator
CT	Thin-layer agar MDR/XDR-TB Colour Test
DNA	Deoxyribonucleic acid
DS	Drug susceptible
DST	Drug susceptibility test
FIND	Foundation of Innovative New Diagnostics
FLQ	Fluoroquinolone
GLM	Generalised linear modelling
HIV	Human immunodeficiency virus
Hr	Isoniazid-monoresistant
INH	Isoniazid
IQR	Inter-quartile range
IS	Insertion sequence
Lab	Laboratory
LAM	Latin American-Mediterranean
LJ	Löwenstein-Jensen
LVX	Levofloxacin
LZD	Linezolid
MDR	Multidrug-resistant
MGIT	Mycobacteria growth indicator tube
MIC	Minimal inhibitory concentration
MODS	Microscopic observation drug-susceptibility
MTB	<i>Mycobacterium tuberculosis</i>
MTBC	<i>Mycobacterium tuberculosis</i> complex
MXF	Moxifloxacin
NAAT	Nucleic acid amplification test
NALC	N-acetyl-L-cysteine
NaOH	Sodium hydroxide
NEMC	North Estonia Medical Centre
NRA	Nitrate reductase assay
OADC	Oleic-acid albumin dextrose catalase
Pa	Pretomanid
PAS	Para-aminosalicylic acid
PI	Principal investigator
pre-XDR	Pre-extensively drug-resistant

RFLP	Restriction fragment length polymorphism
RIF	Rifampicin
RR	Rifampicin-resistant
SD	Standard deviation
STC	2, -3-diphenyl-5-(2-thienyl) tetrazolium chloride
TB	Tuberculosis
TUH	Tartu University Hospital
UN	United Nations
UPGMA	Unweighted pair group method with arithmetic mean
VNTR	Variable numbers of tandem repeat
WHO	World Health Organization
WRD	WHO-recommended rapid diagnostic test
XDR	Extensively drug-resistant
ZN	Ziehl-Neelsen

1. INTRODUCTION

Tuberculosis (TB) is an air-borne infectious disease, caused by the bacteria of the *Mycobacterium tuberculosis* complex (MTBC), that has accompanied humanity for as long as it exists. The World Health Organization (WHO) has estimated that each year, TB causes the death of 1.25 million people, which equals the population of an entire country like Estonia (World Health Organization, 2024a). This huge mortality is against any common sense, because TB is a curable disease. These bacteria can successfully be inhibited by numerous antibacterial agents or even by the human immune response itself. Nevertheless, both have struggled to do so in the history of TB.

Now, as the United Nations (UN) general assembly has committed, is the time to finally stop TB transmission and TB associated deaths (United Nations General Assembly, 2018, 2023b). The fight against TB has reached the next level, where altogether persisting infections, active infections and infectiousness is tackled. Interventions prioritise the wellbeing of patients and the sustainability of households affected by TB. According to the latest treatment guideline, it is not necessary to hospitalise patients for the initiation of TB treatment and in addition, owing to the possibility of remotely observed treatment and community engagement, the patients do not have to leave their homes at all (World Health Organization, 2025b). Active case finding is performed and TB preventive treatment is offered to risk groups and close contacts of TB patients (World Health Organization, 2024c).

Notwithstanding, each year, the WHO releases awakening statistics which show limited improvement globally. There is a huge gap between the TB cases estimated by the WHO and the TB cases detected and treated. In addition, the treatment success rate for drug-resistant TB forms is far from optimal and if the treatment consists of antibiotics with severe side-effects, the treatment adherence becomes low too. This again gives rise to new infections and more severe forms of the disease.

The number of new TB infections can be reduced. Rapid diagnosis of contagious patients, rapid start of contact investigations and proper treatment together help to prevent the occurrence of further active cases. Important means to identify risk groups affected by TB transmission are molecular epidemiological tools. These methods tie a TB patient at a specific timepoint to a specific type or lineage of MTBC and in some settings molecular epidemiological tools can be used to confirm or disprove direct transmission (Teeter et al., 2017). Molecular epidemiological tools combined with patient data on comorbidities and other demographic indices can pinpoint the groups needed to be screened for TB infections in order to find persons at risk of developing TB (World Health Organization, 2021b).

The rapid diagnosis of TB disease remains a struggle, however. TB is very difficult to detect in a routine clinical microbiology laboratory, where most of the aerobic cultures have final assessments already in a few days. MTBC grows exceptionally slowly and therefore, phenotypic TB diagnosis needs a separate

workflow and, in many cases, a separate laboratory or centre due to the heightened risk of airborne infections (National TB elimination programme, 2024). Lately, TB laboratory diagnosis has evolved towards a “no culture” principle with the aim to move the diagnosis closer to the patients with safer methodologies for healthcare personnel and no amplification of infectious bacilli (World Health Organization, 2024d). Although the WHO-recommended rapid diagnostic tests (WRD) for the diagnosis of pulmonary TB are highly sensitive and safe at the same time they need specific operational prerequisites and infrastructure to function as a high throughput tool in a high-incidence setting (Brown et al., 2021). However, when these tests are used at a central level rather than peripheral, the effects of the aforementioned benefits are reduced (Haraka et al., 2021; Pooran et al., 2019).

It is thus time to search for a TB diagnostic strategy that is rapid, robust and closer to the point of care, i.e. can be conducted in a microscopy centre. The thin-layer agar MDR/XDR-TB Colour Test (CT) has proven to be highly sensitive in detecting TB directly from sputum samples and providing concurrent drug susceptibility results (Mekonnen et al., 2019). The CT was specifically designed for the use in high incidence resource-constrained settings (Herrera et al., 2007). Moreover, a positive CT for MTBC gives enough information to adequately start treatment for several forms of TB.

Here, we present a set of studies addressing the implementation, objectivity and reproducibility of the CT to be used in routine TB laboratory settings that test TB isolates, as well as routine respiratory specimens like sputum, bronchoalveolar lavage (BAL) and pleural fluid. In addition, this thesis also studies recent transmission of TB in Estonia during the years 2009–2014 with the emphasis on defining certain risk groups that are more affected by transmission than the others.

2. REVIEW OF LITERATURE

2.1. Origin of tuberculosis

TB has accompanied human society for as long as it exists. Already half a million years ago, TB-meningitis was present in the ancestor of the modern human, *Homo erectus*, discovered by investigating fossils (Kappelman et al., 2008). The first evidence of human TB infection by *Mycobacterium tuberculosis* (MTB) goes back nine thousand years from the Eastern Mediterranean (Hershkovitz et al., 2008). About 5,000 years ago, TB was quite common in the entire human community. Signs of TB from that time have been found both in Egyptian and Peruvian mummies, and in writing from India, China and ancient Hebrew territories (Barberis et al., 2017). With the wide spread of the human populations across Earth and the rise in population density, TB has caused several pandemics throughout history (Bynum, 2012).

Already in 1882, the future Nobel Prize winner, Robert Koch, discovered the tubercle bacillus (Heron, 1883). He described 140 years ago that the bacillus causing tubercle is of rod shape, grows very slowly compared to other bacteria, forms cords and is different physiologically from other bacteria, including other mycobacteria and even from the bacillus causing leprosy. All these facts are true today. For centuries, philosophers, physicians and researchers could not agree whether TB is an inheritable disease or a contagious one. At the end of the 19th century, Jean Antoine Villemin proved that TB is indeed contagious (Villemin, 1868). Today, we know that TB is caused by a group of bacteria forming the MTB complex (MTBC), which include the following species: MTB, *M. bovis*, *M. africanum*, *M. pinnipedii*, *M. microti*, *M. caprae* and *M. canetti*.

2.2. Epidemiology of tuberculosis

For more than ten years (2010–2023), the WHO has estimated that each year over ten million people fall ill with TB, a preventable and curable infectious disease (World Health Organization, 2019, 2024a). The estimated incidence declined very slowly for ten years and started to rise again during the COVID-19 pandemic between 2020 and 2021. Still, the estimated TB incidence has never reached under ten million cases despite the recent ultra-successful developments made in the diagnosis and treatment of TB.

Our modern globalised world has already seen two United Nations high-level meetings on the fight against TB (2023a), where government leaders of United Nations countries have committed to end the TB epidemic by 2030 (United Nations General Assembly, 2018, 2023b). Amongst others, the commitments include universal health care, increase of rapid testing for TB diagnosis and treatment coverage, reduction in TB-affected households facing catastrophic costs, as well as the reduction of TB-related deaths. Still, in 2023, a total of 8.2 million cases of newly diagnosed TB were reported. Five countries accounted for

56% of the people, who developed TB and India alone accounts for 26% of all TB cases in 2023 (World Health Organization, 2024a). The highest burden of TB is carried by working-aged men over 15 years old, which has catastrophic effects for families in societies, where the male is traditionally the sole income provider. These groups of patients would greatly benefit from an early-stage TB diagnosis and subsequent treatment closer to home. In addition, there are several reports of migrant miners, who pose a much higher risk of developing tuberculosis and dying of it than does the general population (Howlett et al., 2025; Morgan, 2014).

In 2023, TB caused an estimated 1.25 million deaths worldwide (World Health Organization, 2024a). The number of deaths caused by TB has been in a very small steady decline from 2010, except for the COVID-19 years in 2020–2021, when late diagnosis and problems with access to healthcare were common (Chanda-Kapata et al., 2022; McQuaid et al., 2021; Nikolayevskyy et al., 2021). Today, the countries most affected by TB in the African and South-East Asian regions account for 80% of the TB related deaths. India alone stands for 29% of TB related mortality (World Health Organization, 2024a). Research has proposed that 70% of the smear-positive TB patients would die in ten years when left untreated (Tiemersma et al., 2011).

Being untreated or being treated with a noneffective agent leads basically to a similar outcome. There are several forms of drug-resistant TB. The most prevalent form of drug resistance is isoniazid (INH) monoresistance (Hr), which is shown to be the probable progenitor of multidrug resistance (MDR), i.e., resistance to both INH and rifampicin (RIF) (Manson et al., 2017). Polyresistant-TB carries resistance to at least two of the first-line anti-TB drugs but is susceptible to RIF. Today, RIF-resistant (RR)-TB is treated exactly like MDR-TB, since RIF is the most important antibiotic in the treatment of drug susceptible (DS)-TB. In 2023, WHO estimated that 400,000 people developed RR-TB, of which, according to WHO, only up to 200,000 received a diagnosis (World Health Organization, 2024a). India accounts for more than a quarter of estimated yearly RR-TB cases. It is at least reassuring that 93% of the diagnosed RR-TB cases start adequate treatment. Unfortunately, even more resistant forms of TB have evolved, i.e. pre-extensively drug-resistant (pre-XDR) TB that is resistant to fluoroquinolones (FLQ) in addition to being MDR and extensively drug-resistant (XDR)-TB that is additionally resistant to linezolid (LZD) and/or bedaquiline (BDQ). Almost thirty thousand cases of pre-XDR and XDR-TB were diagnosed worldwide in 2023 and since the gap between MDR-TB and treatment is small, probably most of these patients received some kind of medication. Treatment of pre-XDR and XDR-TB has more adverse reactions, requires much more funds and is a lot longer than treatment of DS-TB, which is difficult to endure both by the patients and the health systems. However, in a recent systematic review, it was estimated that even under treatment conditions only close to 40% of extensively drug-resistant TB forms could be successfully cured (O. S. Pedersen et al., 2023). Therefore, it is of upmost importance to prevent and hinder further spread of drug-resistant TB.

The initial bacteriological diagnosis of TB is the basis of correct treatment initiation. However, there is still a diagnostic gap, where roughly 50% of the notified cases are tested for rifampicin resistance and/or with a WHO-recommended rapid diagnostic tool. On the other hand, the Global Fund has allocated billions of dollars over the last decades to invoke policy change, treatment and rapid diagnosis of TB, but TB still remains the leading cause of death of one infectious agent worldwide.

2.2.1. Epidemiology of tuberculosis in Estonia

The situation of TB in Estonia is quite different, when comparing that with the rest of the world. The TB incidence rate has declined for over 20 years and has reached 8.2 cases per 100,000 inhabitants in 2023 (European Centre for Disease Prevention and Control/WHO Regional Office for Europe, 2025). Estonia was able to detect 90% of the WHO estimated cases in 2023, e.g. 99 of the estimated 110 cases. Most of the cases in Estonia are laboratory-confirmed (83.8%; n=83) and almost all these cases have DST results available (European Centre for Disease Prevention and Control/WHO Regional Office for Europe, 2025). The estimated percentage of RR-TB among new TB cases for 2023 was 22%, however, the reported data show that the percentage was 24.4% among new pulmonary TB cases with DST results. This is one of the highest proportions of RR-TB cases in the world (World Health Organization, 2024a).

Diagnosis and treatment of TB is funded by the government and free of charge for everyone in Estonia like in numerous other countries. Molecular methods have been used for primary diagnosis from their release and all WHO-recommended antibiotics are tested phenotypically for susceptibility. Treatment decisions are supported by a TB expert committee and in most cases, the treatment is initiated in an inpatient ward. The drugs are procured and distributed centrally and administration of TB drugs by patients is monitored routinely. The newest TB regimens have been available in Estonia throughout recent years. All these points account for the success in the decline of TB cases in Estonia in the past two decades and keep the country on track in terms of meeting the targets agreed for the End TB strategy (STOP TB Partnership, 2022).

Although Estonia is advanced in the introduction of cutting-edge TB treatment and the support provided to the TB patients, still approximately 15% of patients die of TB, according to the Estonian Health Statistics and Health Research Database, mainly because of late diagnosis (Tuberculosis Registry in the National Institute for Health Development, 2001–2025a, 2001–2025b).

2.3. Treatment of tuberculosis

When compared to the overall long timeframe of TB infections in humans, the medical treatment of TB has rapidly evolved during the last century. In the beginning of the 20th century, TB was treated with having rest and surgical interventions. This era was the time of sanatoria. Besides rigorous rest and healthy nutrition, the medical procedures performed in the sanatoria included artificial pneumothorax and even lung resection (Gaensler, 1982). The era of sanatoria bloomed all over the world until the advent of antibiotics.

The discovery of the first “magic bullet” in the beginning of the 20th century started a massive search for compounds effective against the Koch’s bacillus. Some thirty years later, in 1944, streptomycin and para-aminosalicylic acid (PAS) were proven to be successful in the treatment of TB (Lehmann, 1946; Youmans & Mc, 1945). Streptomycin was the first drug to be used against TB. Very soon, it became clear that monotherapy with streptomycin initiated the rise of resistance. This was again demonstrated after the glorious discovery of INH in 1951 (Robitzek et al., 1952). Because INH was highly effective against MTB, it was first thought that monotherapy would be enough. However, after prolonged therapy, patients relapsed with developed fixed resistance. Therefore, the 1960s were the very end of anti-tuberculous drug monotherapy. After that, INH was used together with streptomycin or PAS for 18 to 24 months for the treatment of all TB cases (Medical Research Council, 1955).

In 1970s, ethambutol and RIF were released for TB treatment drastically shortening the therapy of TB to 9 months (British Thoracic and Tuberculosis Association, 1976). Already by the end of that decade, pyrazinamide was added to the regimen and treatment was even further shortened to six months (Singapore Tuberculosis Service/British Medical Research Council, 1979). The aforementioned drugs were used together to treat DS-TB in one regimen with several combinations throughout history (World Health Organization, 2010b, 2017). In the end of the millennium a more potent rifamycin, rifapentine, was discovered. Unfortunately, rifapentine was not widely available for decades. Until, after forty years, in 2022, a new recommendation was released by the WHO for the treatment of DS-TB that utilizes rifapentine and moxifloxacin (MXF), a highly potent FLQ, to shorten the all-oral treatment period from six to four months (World Health Organization, 2025b).

The overall economic situation in the 90’s and the collapse of the Soviet Union rapidly increased the number of cases infected with drug-resistant MTBC (Merker et al., 2015). This again activated the search for new or old compounds effective against drug-resistant MTBC. For instance, the years with the highest numbers of RR-TB cases in Estonia were around the beginning of the new millennium (Estonian Tuberculosis Registry, 2005). For several countries in the world, like Ukraine, Tajikistan, Viet Nam, Philippines, Indonesia and others, the rise of the cases resistant to first-line TB drugs has never stopped (World Health Organization, 2024a).

RR-TB, MDR-TB or even more resistant forms of TB have been historically treated with at least four effective drugs in an individualised regimen for at least 18 months after culture conversion (World Health Organization, 2010b). This included a fluoroquinolone, a few bacteriostatic drugs like cycloserine, ethionamide and PAS and 6 months of an injectable aminoglycoside, either kanamycin, capreomycin or amikacin, with daily dosing. It is basically impossible to carry this regimen out in an outpatient ward in a high incidence setting. Moreover, there were only a few anti-TB drugs left to use when the infecting MTBC strain harboured resistance to fluoroquinolones and aminoglycosides.

Luckily today, after the successful search of new and reallocated antibiotics for the treatment of TB and numerous clinical trials, we have a guidance that gradually introduces all-oral regimens for the treatment of DS-TB, RR/MDR-TB and even pre-XDR-TB (World Health Organization, 2025a). The re-evaluation of clofazimine, LZD and cycloserine as effective anti-TB drugs and the concurrent development of new anti-TB drugs BDQ, delamanid and pretomanid (Pa) in the 2020's gave rise to new regimens. Currently, the latest recommendations include a 4-months regimen for the treatment of DS-TB incorporating rifapentine, MXF, INH and pyrazinamide (World Health Organization, 2025b). For the treatment of RR/MDR-TB, the new guidelines introduce two all-oral six-months regimens including BDQ, Pa, LZD with or without MXF and two 9-month regimens incorporating one FLQ, clofazimine, ethionamide or LZD, ethambutol, high-dose INH and pyrazinamide (World Health Organization, 2025a). The recommendations emphasize that FLQ susceptibility must be known to correctly prescribe these regimens. Therefore, one could conclude that the susceptibility testing against INH, RIF and FLQ is the basis of correct treatment initiation.

2.3.1. Vaccination against tuberculosis

Soon after the discovery of the TB bacillus, the *M. bovis* bacillus Calmette-Guerin (BCG) vaccine was developed and first administered to humans in 1921 (Calmette et al., 1927). BCG is the first and only licenced TB vaccine so far. It consists of live attenuated bacteria and is widely used in high TB risk regions with the aim to prevent childhood TB, disseminated TB and other difficult forms of the disease (Lange et al., 2022).

2.4. Laboratory diagnosis of tuberculosis

The gold standard of laboratory diagnosis of tuberculosis is culture, because it proves the presence of proliferating bacteria in the patient specimen. Although nucleic acid amplification-based methods can enormously speed up the initial diagnosis of new TB cases, culture will remain the gold standard until there is another method that can reliably prove the viability of bacteria in a specimen.

2.4.1. Traditional diagnosis of tuberculosis

Traditionally, the fastest, most cost-effective and high-throughput way for diagnosing TB has been sputum smear microscopy. This is the preparation of air-dried sputum stained with carbol-fuchsin using the Ziehl-Neelsen (ZN) method and examined with a 1,000 $\times$ magnification under a light microscope (Angra et al., 2007). This has historically been done and is still performed today in microscopy centres or TB wards with no other special equipment than an ambient air laboratory gas burner and a light microscope. Although the preparation of smears is fast, the evaluation of these slides takes time and requires experience. Therefore, fluorescence microscopy was endorsed by WHO, i.e. the smear slides are stained with a fluorescent stain like auramine or acridine orange (World Health Organization, 2010a). Although this does not enormously enhance the sensitivity of the method, such staining allows for faster examination of the stained slides under a fluorescence microscope. Smear microscopy is fast in detecting the most infectious patients; however, it is less sensitive than any culture method and its detection limit is close to 5,000 bacilli/mL.

Alternative pulmonary specimens like induced sputum, bronchoalveolar lavage (BAL) fluid and pleural fluid can be used for diagnosing TB in cases, where the patient exhibits high clinical suspicion of pulmonary TB, but is unable to produce sputum. It has been shown that specifically the use of BAL enhances the diagnosis of otherwise smear-negative pulmonary TB (Musso et al., 2025).

Solid media have been used to culture mycobacteria for more than half a century. In principle, there are two types of solid media for the cultivation of mycobacteria: egg-based media like the Löwenstein-Jensen (LJ) and the Ogawa and agar-based Middlebrook 7H medium supplemented with oleic-acid albumin dextrose catalase (OADC) that allows mycobacteria to populate on the medium. The egg-based media are more widely used, because they are cheaper and withstand the long duration of incubation, whereas Middlebrook agar medium is prone to drying during long incubation periods. Still, culture on solid media has a detection limit of 10 bacilli/mL and therefore, it can detect up to 50% more TB cases than microscopy (European Centre for Disease Prevention and Control, 2023). Slow growing mycobacteria like the MTBC have a generation time of over 20 hours on solid media and require at least two to four weeks to become detectable. This slows down the diagnosis of TB and meanwhile gives rise to new infections. Moreover, egg-based media require heating or cooking to solidify during preparations, which makes the addition of several antibiotics to the medium impossible due to possible drug inactivation and therefore, the use of this type of medium for susceptibility testing is limited.

Of the liquid media, Middlebrook 7H9 broth supplemented with OADC is widely used for faster cultures of mycobacteria. Liquid media withstand long incubation times and give faster results than solid media, because of a bigger amount of concentrated specimen added to the medium and a shorter generation time. The automated system BACTEC MGIT from BD (Becton, Dickinson and Company, NJ, USA) with the use of Mycobacteria growth indicator tube (MGIT)

that together enable automated detection of growth in the tubes revolutionised the TB diagnostics in the late 2000s (Chien et al., 2000; Hanna et al., 1999; Tortoli et al., 1999). The MGIT tube incorporates fluorescent dye at the bottom, which indicates the consumption of oxygen by microorganisms. Although high-throughput and convenient, the BACTEC MGIT system requires a permanent source of electricity and a biological safety cabinet (BSC) level II to confirm grown bacilli in the tubes and to identify mycobacteria on microscopy slides. In addition, drug susceptibility testing (DST) from a MTBC positive MGIT can easily be performed in the same system with consumables provided by BD. Still, the whole algorithm is solely dependent on one company and leaves consumers in difficult situations when manufacturing errors or unsuccessful quality controls become evident during production. Today, this method is recommended by WHO for culture and phenotypic DST of MTBC (World Health Organization, 2024b).

Mycobacteria have a specific colony morphology, when grown on solid media (Fregnan & Smith, 1962; Runyon, 1970). Their colonies have an off-white colour, assemble cauliflowers and are dry and bumpy. When grown in liquid medium, mycobacteria mostly appear as white clumps and the acid-fastness of the specific growth must be confirmed by ZN staining and microscopy. Further from this point, mycobacteria are identified on species level to perform DST and to assign correct treatment.

In clinical microbiology in general, the susceptibility of a microorganism is evaluated through measuring the minimal inhibitory concentration (MIC) of a certain microorganism to certain drugs. However, cultivation of MTBC requires a lot of time and interpretation of the MIC for all anti-TB drugs from solid medium would be very time consuming and laborious, although it is still done in many laboratories worldwide. Therefore, in DST of MTBC, only one critical concentration is tested, which represents the cut-off between the susceptible and the resistant population(s). It is evaluated whether the tested concentration can inhibit 99% of the growth. These critical concentrations differ between the media used and are therefore not directly comparable.

With the advancements in molecular microbiology during the first decade of the 21st century, the identification and in some cases, also the DST of MTBC transitioned to a molecular-based form. Nucleic acid amplification tests (NAATs) allow for a very small limit of detection compared to traditional culture-based methods. NAATs are faster and highly specific however require experienced personnel and a BSC level two to handle pure cultures. Today, the TB diagnostics are moving towards safer molecular methods that do not rely on cultivating hazardous bacteria.

2.4.2. Modern routine of the diagnosis of tuberculosis

The current WHO guidelines recommend collecting one specimen from a TB suspect. This one specimen should then be tested with a WHO-recommended rapid diagnostic test (WRD) rather than smear microscopy or culture (World Health Organization, 2024d). In 2025, the WRDs for the initial diagnosis of TB

included a manual molecular assay that does not detect drug resistance, a urine assay that detects lipoarabinomannan and several low- and moderate-complexity automated NAATs (World Health Organization, 2024d). Follow-on tests after the initial diagnosis include molecular tests that reliably detect several additional resistances, for instance resistance to INH, pyrazinamide, FLQ, aminoglycosides, ethionamide and ethambutol. The newest guideline even includes targeted next-generation sequencing assays that can detect resistance to up to ten TB medicines (World Health Organization, 2024d). Exploiting these methods allows proper diagnosis and rapid treatment initiation for DS-TB, Hr-TB and RR/MDR-TB in high-incidence settings, based on one specimen without the need for culture. However, in any case of suspected or confirmed resistance to any drug, a culture is needed for phenotypic DST.

The indirect phenotypic DST recommended for most of the antibiotics used for treatment of TB is the proportion method with the BACTEC MGIT liquid media system. This method needs a pure log-phased culture grown in liquid or on solid medium as an input. It employs one MGIT tube for every drug concentration tested and in addition growth control tubes without any drug that are injected with the 100-fold dilution of the original bacterial suspension. The system keeps track of growth in all tubes until the control tube is flagged positive with 400 growth units, after which the DST is interpreted and the results obtained. The BACTEC MGIT system is the only MTBC DST method that has a standardised commercially available kit. The method has been rigorously evaluated for most of the drugs recommended for TB treatment with very good results (Bemer et al., 2002). WHO takes the BACTEC MGIT method as the gold standard for DST. However, the BACTEC MGIT method is expensive and requires specialised equipment, personnel and a laboratory with sufficient bio-safety precautions to handle pure liquid cultures of MTBC.

At the moment, most of the WHO recommended techniques for the laboratory diagnosis of TB are used in Estonia. Here, we use low-complexity and moderate complexity NAAT for initial diagnosis of TB with concurrent fluorescence staining for smear microscopy and LJ and MGIT media for solid and liquid culture, respectively. Positive cultures are identified using molecular methods and finally, all TB strains isolated in Estonia are sequenced for relatedness analysis and further analysis of variants related to drug resistance.

The reality in the global TB diagnostic landscape differs a lot from the recommended version, however. According to WHO, in 2023, only 48% (3.9 million cases) of the new TB diagnoses were based on a WRD as an initial test (World Health Organization, 2024a). The numbers might be biased because of countries or parts of countries not collecting and reporting this variable. Even in the European Union, there are several larger countries that report 0% of cases detected with a WRD (European Centre for Disease Prevention and Control/WHO Regional Office for Europe, 2025).

Yet not all sites in the world can afford these high-complexity techniques. TB is most spread in high-density populations that need to take budgetary considerations in the design of their national TB programme. India, one of the leaders in

the TB incidence table that accounts for a quarter of yearly TB cases, struggles under a huge number of patients and a limited number of healthcare workers (Teibo et al., 2024). Today, India must cope with diagnosing roughly 10,000 new TB cases a day. The cost of diagnosing all these patients with a WRD is overwhelming, even when the costs for these tests are negotiated through the Global Fund, because of the massive need of machinery and employees. In many low-resource settings, as well as in India, which support TB diagnosis and treatment from government funds, most of the cost for TB diagnosis and treatment must come from patients' pockets because of the inevitable use of private sector healthcare (Sinha et al., 2020). Therefore, the patients, as well as the healthcare workers choose low-cost solutions like microscopy, which is quite fast and has been used for ages although it lacks sensitivity, or another traditional or in-house method.

2.5. Non-commercial laboratory techniques for the diagnosis of tuberculosis

Non-commercial laboratory diagnostics are techniques that do not have a manufacturer. These solutions are usually based on traditional microbiology, they can be very low-cost, prepared in-house and of course, they must be quality-controlled in-house as well.

Many non-commercial techniques for the detection and DST of TB have been developed in the recent half century because of the cost and limitations that commercial culture methods present like the need for infrastructure and experienced personnel.

One of the most extensively studied non-commercial assays is the microscopic observation drug-susceptibility (MODS) assay and its developments like the MODS-Wayne test. This method was developed in Peru already over 20 years ago (Caviedes et al., 2000). It utilises a 24- or 12-well culture plate with Middlebrook 7H9 broth supplemented with OADC, a general antibiotic mixture to prevent contamination and respective anti-tuberculosis drugs in separate wells. The principle of the method is growth in liquid media that can be detected very fast with an inverted microscope. In addition, the specific growth morphology of mycobacterial colonies in liquid media viewed at 50× magnification allows correct identification of the MTBC. The method has been extensively evaluated on sputum specimens as a direct detection and DST method, as well as an indirect DST method (Brady et al., 2008; Caviedes & Moore, 2007; Elinav et al., 2012; Ha et al., 2009; Limaye et al., 2010) with very good results. The time to positivity for the direct MODS assay ranges from 10 to 13 days, which is much faster than the three to four weeks until a MGIT culture and indirect DST result (Agarwal et al., 2019). Several cost-effectiveness analyses have been conducted showing the advances in implementing MODS to routine laboratory diagnosis of TB (Groessl et al., 2018; Walusimbi et al., 2016; Wikman-Jorgensen et al., 2017) and even studies that tie MODS to better treatment outcomes (Obregon et al., 2018).

Other direct or indirect non-commercial DST methods include colorimetric redox indicator (CRI) assays that use Middlebrook 7H9 broth combined with a mycobacterial growth indicator like alamar blue, rezasurin, tetrazolium, crystal violet or malachite green. The principle of the assays is the colour change of the broth that indicate growth and can be easily detected. These assays are usually performed using liquid medium in a 96-well microtiter plate or in tubes and therefore, it is quite difficult to perform a direct DST using these methods. Still, a few studies have attempted a direct detection of mycobacteria using CRI with promising results (Shinu et al., 2016; Wan Nor Amilah et al., 2016).

One of the non-commercial assays that can be performed both in liquid and with solid media is the nitrate reductase assay. The principle of the assay lies in the ability of mycobacteria to reduce nitrate to nitrite. The latter can be detected by adding Griess reagent to the medium (Golyshevskaya et al., 1996). The method has been assessed as a direct or indirect DST method, with both solid and liquid media, on acid-fast bacilli (AFB) smear-positive sputum with the time to detection being 10–19 days and promising accuracy (Affolabi et al., 2007; Halwai et al., 2018; Huang et al., 2018).

The WHO assessed the suitability of these non-commercial methods in 2009 and recommended the MODS assay, the CRI assay and the NRA to be used at the reference laboratory level. However, the WHO failed to recommend decentralization of these assays, because of the need for a biosafety level 3 environment and lack of scientific evidence from these settings (World Health Organization, 2011). Of the WHO-recommended techniques, only NRA is performed on solid media and could therefore potentially be implemented using direct patient specimen in a biosafety level 2 laboratory setting. On the other hand, this method requires opening of the culture tubes to add nitrate reductase, which poses high risk of aerosol formation and has to be carried out in a level 2 biosafety cabinet.

One of the promising non-commercial methods that WHO aimed at assessing, but failed because of lack of evidence, was the thin-layer agar method. This method is one of the techniques that utilise a safer solid culture method and therefore, it was taken into consideration in 2019, when Kontsevaya *et al.* re-evaluated the non-commercial methods in a systematic review (Kontsevaya et al., 2020). Yet, no pooled diagnostic performance calculations could be made because of the low number of studies incorporating thin-layer agar methods (Kontsevaya et al., 2019).

2.5.1. The thin-layer agar MDR/XDR-TB Colour Test

The CT is not a newly developed method. However, it utilizes many of the historical discoveries made in early mycobacterial laboratories. The basis of the CT method, the Middlebrook 7H11 agar media, is a descendant of the Middlebrook and Cohn 7H-10 oleic-acid albumin agar medium that was already in documented laboratory use over 70 years ago (Middlebrook & Cohn, 1953). In 1958, Middlebrook and Cohn evaluated several media for diagnosing TB and showed that mycobacterial growth on 7H-10 is faster than on LJ slants, it might stand for less

contamination and provide reliable direct DST results (Middlebrook & Cohn, 1958). Direct DST results are obtained by plating decontaminated patient specimen directly onto the antibiotic containing and growth control media. Direct DST is in principle faster than traditional indirect DST because of no need for previous growth of pure culture.

The other principle of CT is microscopic colony detection of tubercle bacilli on a thin layer of agar. Microcolony culture techniques for diagnosing tuberculosis have also been in development and use for a very long time (Welch et al., 1993). They utilise a thin layer of agar that allows for the microscopic evaluation of growth with a 40–60× magnification of a stereo or inverted microscope. Research has shown that the microcolony detection technique is a lot faster than conventional cultivation on solid egg-based medium (Esteban et al., 1996; Idigoras et al., 1995; Mejia et al., 1999). The time to MTBC detection on Middlebrook 7H10 thin layer agar has shown to be on average 11 days compared to other conventional solid media that take 17 days on average for a positive culture (Welch et al., 1993).

Compared to solid media prepared into slants, the microcolony plate method has another advantage. Because it utilizes microscopic observation of bacterial colonies, the identification of mycobacterial species might be readily made on initial detection. After the introduction of clear solid media to mycobacterial diagnostics in the 1960s, there were several studies, which searched for possibilities of identifying mycobacteria according to the morphology of the colony (Fregnan & Smith, 1962; Fregnan et al., 1961; Navalkar et al., 1964; Runyon, 1970; Vestal & Kubica, 1966). Welch *et al.* demonstrated that the microcolony techniques give a remarkable reduction in detection times for many mycobacterial species that can be differentiated early on using a light microscope (Welch et al., 1993).

Even after the introduction of the fully automated BACTEC MGIT that revolutionised mycobacterial diagnostics (Chien et al., 2000; Hanna et al., 1999; Tortoli et al., 1999), some settings searched for a low-cost, fast and safe alternative to the commercial techniques. The addition of an oxidation-reduction indicator to the thin-layer agar or other growth medium simplified the detection of early colonies by colouring them red (Kanai & Yanagisawa, 1954a, 1954b; S. Lee et al., 2006; S. M. Lee et al., 2007; Yamane et al., 1996). One of these indicators, the 2, -3-diphenyl-5-(2-thienyl) tetrazolium chloride (STC) was particularly suitable, because it did not inhibit any mycobacterial growth and could be added to the medium before autoclaving (Kakimoto & Yamamoto, 1973; Shin et al., 2004; Yamane et al., 1996).

With the support of the Foundation of Innovative New Diagnostics (FIND), CT was born combining all the previously mentioned advantages of the thin-layer transparent media: the detection of microcolonies and the addition of a redox reagent to the medium. In addition to these advantages, the development of the CT utilised a four-quadrant Petri dish, which provided the possibility of performing direct susceptibility tests for INH, RIF and ciprofloxacin (CFX) (Herrera et al., 2007). The CT quadrants containing antimicrobials were supplemented with food colouring to allow for easier interpretation of the plates.

The primary reason behind developing CT was to use it in densely populated high incidence settings, for example in Latin America. A TB suspect might give one sputum specimen, which is then transported to the laboratory and plated onto the CT. Former studies have shown that one culture on a CT is able to detect over 80% of cases of sputum smear-negative TB and almost all cases of sputum smear-positive TB (Shibabaw et al., 2019; Zhang et al., 2018). CT as a robust and low-cost in-house method has the potential of giving an enormous advantage in TB detection, when used in the right settings. CT can be performed in a biosafety level II laboratory because the plate with the primary specimen is sealed and contained in a zip-lock bag. There is no need for biosafety level III infrastructure until the plate with a positive culture remains unopened.

The CT has been extensively evaluated for the rapid diagnosis of MTBC and direct DST from sputum in low RR-TB settings with promising results (Ardizzoni et al., 2021; Herrera et al., 2007; S. Lee et al., 2006; Mekonnen et al., 2019; Zhang et al., 2018). It has also been evaluated as an indirect DST test on MDR isolates (Martin et al., 2009). Many of the studies evaluating the CT prepared the test plates in a remote laboratory and shipped the prepared plates to the testing laboratories (Ardizzoni et al., 2015; Mekonnen et al., 2019). CT has not been evaluated with other pulmonary specimens apart from sputum in a non-expert setting conducting local media preparations.

2.6. Molecular epidemiology of tuberculosis

One of the ways to fight TB is the early detection and successful treatment of new cases, another is the prevention of further spread of the disease by active case finding and community interventions.

Molecular epidemiology is a discipline that utilises methods from molecular microbiology to study the spread, certain characteristics and risk factors of a disease (Riley & Blanton, 2018). Studies using molecular epidemiological methods have for instance helped to determine what proportion of active TB is due to reinfection or reactivation and to identify risk factors for TB transmission within certain communities (Herranz et al., 2017; van der Zanden et al., 2007). For such studies, the most commonly used methods for MTBC deoxyribonucleic acid (DNA) fingerprinting have been insertion sequence (IS) 6110 restriction fragment length polymorphism (RFLP) (van Embden et al., 1993) and 24-locus variable numbers of tandem repeat (VNTR) typing (Supply et al., 2001), which were internationally standardised in 1993 and in 2006, respectively (Supply et al., 2006). VNTR typing has various advantages over RFLP typing, including its ease of use, short turnaround time and digital output format (M. K. Pedersen et al., 2018). In addition, several studies have shown that the predictive value of VNTR typing to study TB transmission is similar to that of RFLP typing in different European settings (Allix-Beguec, Fauville-Dufaux, et al., 2008; J. L. de Beer et al., 2013). Moreover, the VNTR loci are predicted to mutate extremely

slowly, which make them a good marker for studying epidemiologically linked MTBC strains (Grant et al., 2008).

The previous gold standard after IS 6110 RFLP typing in molecular epidemiology of TB was VNTR typing, which produces a numeric pattern enabling the identification of MTBC strain lineages (J. de Beer et al., 2014). It is assumed that patients infected with MTBC strains accompanying identical DNA fingerprints are 1) infected with the same MTBC strain and 2) have infected each other, have a common source of infection or were independently infected by a highly prevalent strain circulating in the community. Conversely, it is assumed that patients, who are infected with a strain with a unique DNA fingerprint in a population, are either suffering from a reactivation of a previous infection or from infection with a strain that was newly introduced into the population by the patient. Investigators have thus used molecular clustering, defined as matching of identical DNA fingerprints as an index of TB transmission.

Today the gold standard for studying TB transmission is whole genome sequencing of the infecting TB strain and the subsequent analysis of single nucleotide variations between strains. This method has a much higher resolution than the previous gold standards, IS 6110 RFLP typing and VNTR typing, providing a perfect solution for in-depth transmission analysis (Walker et al., 2018).

The first molecular epidemiological study to use RFLP DNA fingerprinting results conducted in Estonia showed high clustering rates among MDR-TB isolates and suggested an association between the Beijing genotype and drug resistance (Krüüner et al., 2001). A later study that compared DNA fingerprints from MDR-TB cases reported in Europe during the years 2003–2007 showed that these Beijing strains persisted in Estonia and were part of a large cluster of MDR-TB strains (designated as cluster E0051) detected in 12 different countries (Devaux et al., 2009). In 2009, Estonia started using VNTR typing within the framework of the TB PAN-NET project with the aim of studying TB transmission in European countries and Estonia (Cirillo, 2009–2013).

2.7. Rationale of the studies

In recent years, the grim numbers of TB incidence have showed no mercy despite the enormous advancements made in the laboratory diagnosis of TB and the development of new treatment regimens. On the contrary, today, the modern laboratory diagnostics of TB relies on a few very successful companies. Since the few privileged companies have been advocating and merchandising their innovative products with the outcome of being recommended by the WHO, these can now afford to do further product development and become even more persistent in their positions. Still, these advancements have not contributed, as expected, to the extinction of TB.

Non-commercial methods for the diagnosis of TB are widely used without any recognition by international authorities, scientific publications or merchandise, mainly because of cost implications, lack of experienced personnel and historical

reasons. Today, however, when we live in the times of crisis and full of unexpectedness, the use of in-house assays for TB diagnosis might be more secure than the dependence on a few commercial companies and courier services. Therefore, even in high-income settings, a back-up method should be in place in cases, when routine diagnostics become unavailable for any reason. On the other hand, there are several huge regions in the world, where the wide use of advanced laboratory diagnostic methods is inhibited by the lack of infrastructure and dedicated personnel. These are the hotspots of TB, where the gap between patients diagnosed and people on treatment arises. These places would reap the benefits of using a rapid and safe laboratory diagnostic method for identifying MTBC and performing direct DST from any kind of specimens. The present thesis investigates the possibility of an in-house MTBC diagnostic method, the CT, of being introduced into previously naïve settings with local media preparations, procurement of supplies and small introductory training. In addition, this thesis evaluates the diagnostic performance characteristics and the objectivity of the CT readouts with different pulmonary specimens than sputum.

One possibility to hinder the epidemic of TB is the rapid diagnosis of new cases with the concurrent initiation of correct treatment. Another contributory form to tackle the epidemic is active pursuit for new TB infections. In Estonia, this has been modestly done by screening close contacts of patients with active TB and, where applicable, screening for TB infection and providing preventive treatment. However, in Estonia, such an active case finding with addition of molecular epidemiological tools to confirm or to refute recent transmission has not been done and therefore, the risk factors for recent transmission are unknown. Because MDR-TB is still actively spreading in Estonia, the deep knowledge about MDR clusters, which the current thesis aims to provide, might give insight into the routes of TB transmission.

3. AIMS OF THE STUDIES

The general aim of the studies was to determine the performance characteristics, reproducibility and objectivity of the CT as a direct MTBC detection and DST method, as well as an indirect method for DST determination of MTB cultures.

The specific aims of the studies were:

- Study I** To evaluate the performance characteristics and feasibility of the CT in the identification of TB from cultures, concurrently with the detection of MDR-TB strains and resistance to CFX.
- Study II** To assess the reproducibility, objectivity and ease of implementing the CT as an indirect DST method of MTB isolates in a non-expert setting.
- Study III** To evaluate the performance and objectivity of the CT as a direct DST and MTBC identification method on a routine clinical patient cohort with different routine pulmonary specimen.
- Study IV** To detect molecular clusters, based on the VNTR method and to identify the risk factors associated with recent transmission of TB in Estonia.

4. MATERIALS AND METHODS

4.1. Ethics

Ethics approval was not sought for Study I due to no sensitive information being used. The protocol for Studies II and III was approved by the Research Ethics Committee of the University of Tartu, Tartu, Estonia (protocol number 269/T-12). During Study III, a written informed consent was obtained from every participating individual. For Study IV, an ethics approval was sought from both the Research Ethics committee of the University of Tartu (protocol number 219/T-18) and the International Union Against Tuberculosis and Lung Disease Ethics Advisory Group, Paris, France. Permission for collecting, handling and analysing patient data was obtained from the Estonian Data Protection Inspectorate and the Ministry of Social Affairs, Tallinn, Estonia.

4.2. Study design

Studies I and II were blinded retrospective diagnostic evaluation studies designed to compare indirect DST results obtained from the CT and BACTEC MGIT. In addition, the results from Study II were used to assess the agreement between technicians evaluating the results. All technicians involved in studies I and II were previously unfamiliar with the CT method and media preparations. However, all technicians participated in a hands-on training prior to the respective study initiation to be introduced with CT plate preparations and evaluation of the results. Study III was a prospective study, carried out at the same two sites as Study II, where patients' pulmonary specimens were tested in parallel with the direct CT and the routine diagnostic methods. The patients were invited to participate in Study III from January 1st 2018 for two years at the Tartu University Hospital (TUH) and for 13 months at the North Estonia Medical Centre (NEMC) starting in March 2020. Study IV was a retrospective cross-sectional analysis of the nation-wide data.

4.3. Study population

Studies I and II tested randomly selected MTB strains isolated from nationwide TB patients during 2004–2010 (n=201 strains in Study I) and 2009–2014 (n=150 strains in Study II; involving two sites, 6 technicians) archived at -80°C in the National TB Reference Laboratory, Tartu University Hospital, Tartu, Estonia.

The patients invited to participate in Study III during a combined period of three years were all adults, who 1) were referred to a pulmonologist due to developing symptoms indicative of tuberculosis or mycobacteriosis, 2) had already been diagnosed with active pulmonary TB or pulmonary mycobacteriosis, but having been treated for less than one month, 3) had been diagnosed and treated

for active pulmonary TB or pulmonary mycobacteriosis, but had developed signs of relapse and therefore needed to be re-screened for the respective disease. Patients' pulmonary specimens (sputum, BAL and pleural fluid) were collected only for routine diagnostic purposes and never solely for the study. BAL and pleural fluid specimens were divided in the laboratory as routine and research samples. The sputum specimens were collected separately as routine and study specimens on the same day or on the closest possible date to each other.

Study IV included all patients with a positive culture and both DST and MIRU-VNTR typing results available for that culture during a four-year period from January 2009 through December 2012.

4.3.1. Strain panel preparation (I-II)

During Study I, the strain panel of 201 isolates was prepared by plating 0.25 mL of the thawed isolate from archive on to a LJ slope. The cryovial was checked for contamination by plating a drop of thawed isolate onto a blood agar and incubating at 37°C for 48 h. The growth on LJ media was confirmed as detecting acid-fast bacilli by ZN staining and light microscopy (Ellis & Zabrowarny, 1993). All the strains were pseudonymised by an independent staff member not involved in the experiments.

The panels for Study II were prepared in triplicate by inoculating 0.5 mL of thawed isolate from archive into an OADC-supplemented MGIT tube and incubating the tube in BACTEC MGIT system until flagged positive. The culture was evaluated by eye for MTBC specific growth and for contamination by plating on to a blood agar and incubating at 37°C for 48 h. The MGIT tube was further incubated at 37°C for one week and divided into three cryovials each containing 1 mL of culture. The three batches were renumbered according to random numbers generated in Microsoft Excel (Microsoft, Redwoods, WA, USA) and frozen as panel A, B, and C until study initiation. The algorithm of panel preparation, study process and number of obtained results is shown in Figure 1.

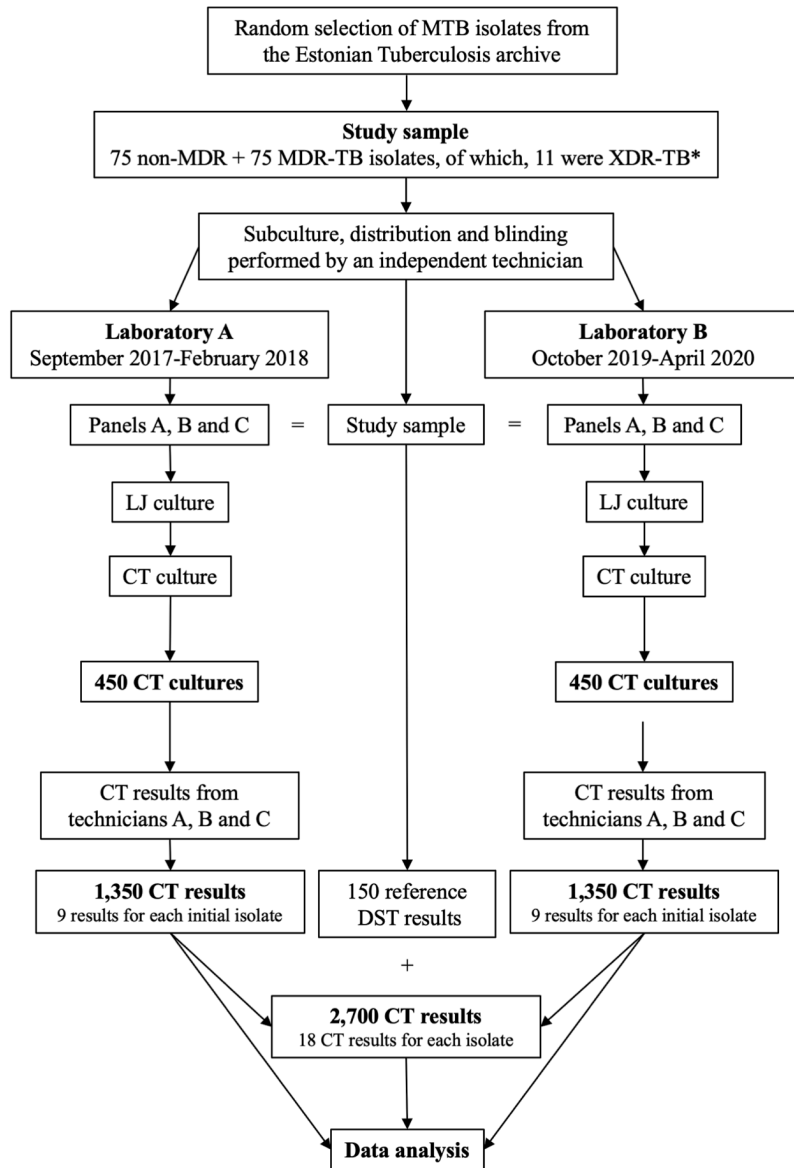


Figure 1. Flow chart of Study II. Schematic view of the study algorithm indicating the number of initial isolates, number of CT cultures in each laboratory and final CT results. CT – thin-layer agar MDR/XDR-TB Colour Test; DST – drug susceptibility test; LJ – Löwenstein-Jensen; MDR – multidrug-resistant; TB – *Mycobacterium tuberculosis*; XDR – extensively drug-resistant. *XDR according to the World Health Organization definition valid until 2021 (World Health Organization, 2021a).

4.4. MDR/XDR-TB thin-layer agar Colour Test method

4.4.1. Introduction to the Colour Test

Prior to Study I, the principal investigator (PI) participated in a 3-day hands-on training at the UK Health Protection Agency National Mycobacterium Reference Laboratory in London, followed by a practice with online support testing 10 local isolates on CT plates. All technicians participating in Study II attended a two-day practical training of the CT method at their facilities in Estonia lead by the PI prior to study initiation. The same technicians as in Study II participated in Study III that evaluated direct DST results from the CT plates. The PI was not involved in CT plate preparations, quality control, plate inoculation or any evaluations during Studies II and III.

4.4.2. Preparation of the Colour Test plates

All CT plates were prepared on site and all reagents were ordered locally in Estonia. Quadrant Petri dishes were used to prepare the plates. Base medium for the CT was prepared as Middlebrook 7H11 agar supplemented with 10% oleic acid albumin dextrose complex (OADC) supplement, Mycobacteria Selectatab (Kirchner, Mast Laboratories Ltd, Merseyside, UK) and 50 mg/L of STC (TCI Europe, Zwijndrecht, Belgium). One of the quadrants in the CT plate acted as a growth detection and contained solely the base medium without colouring and additional anti-TB drugs. Each of the remaining three quadrants acted as a direct DST field and therefore contained base medium additionally supplemented with 0.2 mg/L INH and green food colouring, with 1 mg/L RIF and yellow food colouring, with 2 mg/L CFX (I) or 1 mg/L levofloxacin (LVX) (II–III) and blue food colouring, respectively. All food colouring was sterilised prior usage with a 0.22 µm filter (Millipore, Billerica, MA, USA). The CT plates were poured as approximately 4 mL of media in each respective quadrant with a Pipetboy (INTEGRA Biosciences AG, Zizers, Switzerland) and a 10 mL sterile serologic pipette or with a MediaJet machine (INTEGRA Biosciences AG).

The prepared CT plates were kept at 4°C and used within one month (I–II) or three months (III). Every batch of the CT media was quality-controlled by incubating one plate from the beginning, middle and end of pouring at 37°C, room air for 48 h and by growing the reference strain MTB H37Ra and a resistant strain (I).

4.4.3. Strain inoculum preparation (I–II)

During Study I, the inoculum for plating 201 MTB strains on CT plates was prepared adding several colonies from LJ medium growing each of the strains into two drops of water with glass beads in a 50 mL centrifuge tube. The tube was vortexed for 30 s, allowed to settle for 20 min and diluted to 1/10 and 1/100 dilutions of McFarland 1.0.

The MTB strain inoculum in Study II was prepared by adding a 1 μ L loop full of fresh MTB culture grown on LJ media into 0.5 mL of sterile water and glass beads in a 5 mL-tube. The tube was vortexed for 30 s, allowed to settle for 20 min before 1 mL of additional sterile water was added to the tube. A 1/5 dilution of the resulted inoculum was plated to the CT plates.

4.4.4. Colour Test plate inoculation

The CT plates were inoculated with the respective strain (I–II) or the decontaminated concentrated respiratory specimen (III) inoculum by placing one drop (II) or two drops (I–III) into each quadrant of the plate with a 3-mL Pasteur pipette and spreading with a 10 μ L loop, changing the loops for each quadrant. Every plate was sealed with parafilm (American National Can Company, Norwalk, CT, USA) and placed into a zip-lock bag.

4.4.5. Interpretation of the Colour Test results

Inoculated CT plates were incubated at 37°C in room air. The plates were checked for growth three times per week on Mondays, Wednesdays, and Fridays. First, the control quadrant was evaluated for growth and if growth was detected by eye as small red colonies, the colony morphology specific for mycobacteria was verified microscopically at a 50 $\times$ magnification (Herrera et al., 2007).

The indirect DST results from the CT plates were interpreted, when at least 50 colonies had appeared in the control quadrant (I–II). Resistance was classified comparing the number of colonies in the control quadrant to the respective drug-containing quadrants. A strain was evaluated as resistant to a certain drug, when the number of colonies in a drug-containing quadrant was >1% compared to the control quadrant. After a maximum of 6 weeks or when the result was evaluated, the plate was photographed and discarded.

The direct CT was deemed positive, when at least one red colony with a confirmed morphology specific to mycobacteria appeared in the control quadrant (III). However, the direct DST result could be evaluated if at least ten such colonies had grown in the control quadrant. Susceptibility to a certain drug was confirmed, when the control quadrant contained at least ten colonies and no colonies grew in the respective drug-containing quadrants. On the other hand, resistance to INH, RIF or LVX was determined, if at least ten colonies grew in the control quadrant and at least one colony grew in any of the respective drug containing quadrants. If by the time of the initial growth detection, less than ten colonies appeared in the control quadrant, the resistance to INH, RIF or LVX could only be evaluated, if colonies had appeared in the respective control quadrants. If at the time of the initial growth detection, less than ten colonies were detected in the growth control quadrant and the drug-containing quadrants did not contain any colonies, the plate was incubated further and re-evaluated a week later. If after further incubation the control quadrant still showed less than ten colonies and no colonies had appeared in the drug-containing quadrants, the

direct DST could not be evaluated and the strain could not be deemed as susceptible to the respective drug.

4.5. Routine diagnostic methods

Either the in-house N-acetyl-L-cysteine (NALC)-sodium hydroxide (NaOH) method (European Centre for Disease Prevention and Control, 2023) with a 2% NaOH end-concentration or the Myco-TB kit (Copan, Brescia, Italy) was used for decontamination of all pulmonary specimens in Study III. The decontaminated specimens were concentrated by centrifugation at 3000×g for 15 min.

Concentrated routine clinical specimens were cultivated in MGIT tubes (0.5 mL) with the BACTEC MGIT (BD) incubator and on LJ slants (0.2 mL). The LJ cultures were incubated at 37°C and checked weekly for positivity. The presence of acid-fast bacilli was confirmed by ZN staining and light microscopy. The identification of cultures during Study III was performed with BD MAX MDR-TB (Becton Dickinson), Xpert MTB/Rif Ultra, TBc Identification test (TBc ID) (Becton Dickinson), GenoType MTBC, MTBDRplus or MTBDRsl (Hain Lifescience GmbH, Nehren, Germany). In addition to the identification of MTBC, the Xpert MTB/Rif Ultra allows for detection of resistance to RIF, the BD MAX MDR-TB kit and the HAIN MTBDRplus kit provide detection if concomitant INH and RIF resistances and the HAIN MTBDRsl kit enables the detection of resistance to FLQ and second-line injectable anti-TB drugs.

Routine indirect DST results for each respective strain were obtained at the time of initial isolation from the BACTEC MGIT system using WHO-recommended drug concentrations valid at that specific moment, i.e. INH 0.1 mg/L, RIF 1.0 mg/L and LVX 1.5 mg/L until 18.09.2019 and since then, 1.0 mg/L and 2.0 mg/L for OFX (Siddiqi et al., 2012; World Health Organization, 2008, 2018). Isolates susceptible to INH and RIF only had first-line DST results from BACTEC MGIT.

4.6. MIRU-VNTR typing and cluster analysis (IV)

Genotyping of the MTBC isolate DNA was performed by the Institute Pasteur de Lille (Lille, France) within the TB PAN-NET project (FP7/2007–2013; FP7–223681) using the standard VNTR typing method and mycobacterial interspersed repetitive units (MIRU) VNTR typing kits from Genoscreen (Lille, France) (Supply et al., 2006). The typing was quality-controlled by the inclusion of positive (BCG DNA) and negative controls and internal procedures. Cluster analysis of VNTR patterns and lineage assignment was performed using the MIRU-VNTRplus database (Weniger et al., 2010).

Cluster analysis was conducted using the unweighted pair group method with arithmetic mean (UPGMA) and the categorical coefficient (Sokal & Michener, 1958). A cluster was defined as at least two isolates with 100% identical VNTR

typing patterns. An index case was defined as chronologically the first case in a cluster. Clustering of VNTR patterns was used as a measure of TB transmission. Lineages were assigned using the similarity search provided in the MIRU-VNTRplus database using a maximal genotypic distance of 0.17, which allows for a tolerance of, at most, four locus differences when 24-locus-based MIRU-VNTR typing and tree-based identification using the neighbour-joining algorithm (Allix-Beguec, Harmsen, et al., 2008; Saitou & Nei, 1987; Takezaki & Nei, 2008). The number and sizes of clusters and the genetic background of these clusters were tabulated.

4.7. Data collection and formation of the study databases

All results obtained during Studies I and II were entered into MS Excel spreadsheets, unblinded and linked with original BACTEC MGIT DST results. During Study III, the following data was collected for every tested sample: the date of sampling, type of sample, sample number, dates of culture on CT, MGIT and LJ, DST results, date of DST result, date of culture results, number of colonies on LJ or CT media, routine molecular DST results and dates of the routine molecular DST results.

In Study IV, all patient data was obtained from the Estonian Tuberculosis Registry. VNTR patterns were collected from the Department of Mycobacteriology, United Laboratories, Tartu University Hospital acting as the Estonian TB reference laboratory and DST results were also obtained from the Estonian Tuberculosis Registry. Data from different sources were matched using unique patient identifiers, names and birth dates. Study data was collected into an EpiData database (EpiData Association, Odense, Denmark).

4.8. Statistical analyses

Sample size and proportion of different DST patterns in the study population in Study I was calculated assuming an at least 85% sensitivity and specificity for each drug tested, based on data published at the time of study protocol preparation (Cohen, 1988). Sample size in Study II was calculated using a predetermined sensitivity of 0.95, an α of 0.05 and a marginal distribution of 0.05 (Hajian-Tilaki, 2014). Half of the 150 samples used in Study II were MDR according to the results of the routinely performed indirect DST. All patients willing to participate in Study III were invited and a maximum sample size and distribution of resistances was not predetermined.

The diagnostic performance characteristics of the CT were calculated using STATA (version 11.2, StataCorp LP, College Station, TX, USA), IBM SPSS Statistics software (version 20.0.0, Armonk, NY, USA) and MS Excel. The intra- and interobserver agreements in Study II were assessed by the Fleiss' kappa

(Fleiss, 1971). The level of agreement between observers was deemed almost perfect, when Fleiss' kappa was >0.8 (Landis & Koch, 1977).

In Study III, Mann-Whitney U-test was used to compare between the methods for detection of the MTBC with susceptibility testing results for the time spent from material collection. To assess the time from material collection until the detection of MTBC or until drug susceptibility testing results by the detection method, the drug and the type of analyte collected, generalised linear modelling (GLM) with scale response was applied on rank-transformed data and used where appropriate (IBM SPSS).

EpiData version 2.2.2.182 (EpiData Association) was used during study IV to determine the associations between patient characteristics and isolate clustering, and risk ratios with 95% confidence intervals (CIs) were calculated. Differences at the 5% level ($p < 0.05$), calculated using Pearson's χ^2 test, were considered statistically significant.

R, a language and environment for statistical computing (version 4.0.0, R Foundation for Statistical Computing, Vienna, Austria) was used with the "meta" package to draw the forest plots in Study II and with the "ggplot2" package to draw the notched boxplots in Study III. The notches indicate the 95% confidence intervals and are calculated according to McGill *et al.* (McGill et al., 1978).

5. RESULTS

5.1. Evaluation of the Colour Test (I–III)

5.1.1. Implementation of the Colour Test method in non-expert settings

A total of 351 archived isolates were used in Studies I and II. According to the BD BACTEC MGIT indirect DST results, 75 (21.4%) of these were susceptible to the first-line anti-TB drugs; 6 (1.7%) were mono-resistant to RIF and 24 (6.8%) were susceptible to RIF, but resistant to at least INH. Approximately 70% (n=246) of the isolates used in Studies I and II were MDR and 51 of these were resistant to FLQ and an injectable drug (amikacin, kanamycin or capreomycin), i.e. being XDR according to the WHO definition valid until 2021 (World Health Organization, 2021a). During the combined study period of 37 months, in Study III, a total of 156 patients, 106 (67.9%) in the TUH and 50 (32.1%) in the NEMC, were enrolled providing 177 pulmonary specimens cultured on the CT plates (Table 1).

Table 1. Performance of the CT in Study III partitioned by the type of specimen. Reproduced with permission from the publisher (III).

Material	Count	TUH	NEMC	CT MTB positive	CT DST results INH+RIF+LVX	CT DST results INH+RIF
All	177	125 (70.6%)	52 (29.4%)	67	54 (80.6%)	4 (6.0%)
Sputum	147	97 (66.0%)	50 (34.0%)	61	49 (80.3%)	3 (4.9%)
BAL	27	25 (92.6%)	2 (7.4%)	6	5 (83.0%)	1 (17.0%)
Pleural fluid	3	3 (100.0%)	0	0	0	0

BAL – Bronchoalveolar lavage; CT – thin-layer agar MDR/XDR-TB Colour Test; DST – drug susceptibility testing; INH – isoniazid; LVX – levofloxacin; MTBC – *Mycobacterium tuberculosis* complex; NEMC – North Estonia Medical Centre; RIF – rifampicin; TUH – Tartu University Hospital.

During Studies I–III, a total of 1,525 CT plates (402, 943 and 180 in Studies I, II and III, respectively) were cultured for diagnostic purposes. The cost for locally purchased materials for one CT plate was 2.4€ in Study I and 1.21€ in Studies II and III. All positive control plates showed growth and none of the negative controls was contaminated during the whole study period (I–III).

Each participating technician prepared at least 3 batches of CT media during Study II and in total 20 batches of media were prepared in the two participating laboratories. The time taken to prepare and pour one batch of CT plates (median of 59 CT plates (inter-quartile range (IQR) 55–60 plates)) without the time for

autoclaving was 112.5 min in both laboratories with an IQR of 91.25–123.75 minutes and 106.25–130 minutes for laboratory (Lab) A and Lab B, respectively.

Standardisation of the plate inoculum in Study I was necessary to determine the best dilutions for plating to avoid too dense or too lean dilutions. No uncertain results in interpreting DST from the CT plates were therefore obtained during Study I. In Studies II or III, no issues were discovered in the plate inoculation or plate reading steps. Every cultured CT plate was read 3 times per week until positive, or to a maximum of 6 weeks after which the plate was deemed to be negative and discarded. Reading and documenting results for one CT plate in Study II took a median of 153 s (IQR 143–181 s) in Lab A and 154 s (IQR 143–157 s) in Lab B for one technician. Four MTB strains in Study I and 5 strains in Study II showed insufficient growth and were excluded from the subsequent analysis.

5.1.2. Reproducibility and objectivity of the Colour Test (II)

The reproducibility of the CT was thoroughly addressed in Study II. The intra-isolate agreement of DST results for the 150 isolates used was 0.95, 0.96 and 0.92 for INH, RIF and LVX, respectively (II), when performing 18 readings of 6 CT plates for each isolate (Figure 1). The agreement of DST results of triplicate testing of 150 isolates from one observer was the same for all the technicians in Lab A ranging from 0.96 for INH to 0.97 for RIF and LVX (Table 2). The intra-observer agreement rates of the DST results for triplicate testing of 150 isolates in Lab B ranged from 0.85 for LVX to 0.97 for RIF (Table 2).

The interobserver agreement between the three technicians in Lab A was 100%. The Fleiss' Kappa values for interobserver agreement in Lab B were 0.99 for the DST results for INH and RIF and 0.97 for LVX (Table 2).

Table 2. Fleiss' kappa values of inter- and intraobserver agreements of the CT results.

Interobserver agreement (450 CT plates; 3 technicians)				
		Fleiss' kappa	SD	p-value
Lab A	INH	1.0	0.027	$<10^{-3}$
	RIF	1.0	0.027	$<10^{-3}$
	LVX	1.0	0.026	$<10^{-3}$
Lab B	INH	0.99	0.027	$<10^{-3}$
	RIF	0.99	0.027	$<10^{-3}$
	LVX	0.97	0.027	$<10^{-3}$
Intraobserver agreement (150 isolates; 3 CT plates; 1 reader)				
Lab A A, B, C	INH	0.96	0.046	$<10^{-3}$
	RIF	0.97	0.046	$<10^{-3}$
	LVX	0.97	0.045	$<10^{-3}$
Lab B A	INH	0.95	0.047	$<10^{-3}$
	RIF	0.97	0.047	$<10^{-3}$
	LVX	0.85	0.046	$<10^{-3}$
Lab B B	INH	0.95	0.047	$<10^{-3}$
	RIF	0.96	0.047	$<10^{-3}$
	LVX	0.89	0.047	$<10^{-3}$
Lab B C	INH	0.96	0.047	$<10^{-3}$
	RIF	0.96	0.047	$<10^{-3}$
	LVX	0.91	0.047	$<10^{-3}$
Intra-isolate agreement (150 isolates; 6 CT plates; 18 readings)				
	INH	0.95	0.006	$<10^{-3}$
	RIF	0.96	0.006	$<10^{-3}$
	LVX	0.92	0.006	$<10^{-3}$

Interobserver agreement was calculated in each laboratory for 3 technicians reading 450 CT plates. Intraobserver agreement was calculated for each technician reading triplicate CT plates of 150 isolates. Intra-isolate agreement was calculated for 18 readings of 6 duplications of CT for 150 isolates. CT – thin-layer agar MDR/XDR-TB Colour Test; INH – isoniazid; Lab A – Laboratory A; Lab B – Laboratory B; LVX – levofloxacin; RIF – rifampicin; SD – standard deviation.

5.1.3. Evaluation of Colour Test indirect DST results (I-II)

The median time until a DST result and the reliability of the CT results was assessed in Studies I and II. A combined total of 2,882 CT indirect DST result assessments were compared to respective BACTEC MGIT indirect DST results during Studies I (197 results) and II (2,685 results). The median time from the start of the test until a BACTEC MGIT first- and second-line indirect DST result during the years 2010–2014 was 12 (IQR 9–15) days and 13 (IQR 10–15) days, respectively. The median time to a CT DST result in Study I was 14 days (IQR 12–14). However, in Study II, the median time until a DST result from the CT for two sites together was 13 days (IQR 11–15) and specifically 12 days (IQR 10–15) for Lab A and 13 days (IQR 11–15) for Lab B. The time to result ranged from 5 to 45 days (Figure 2). However, the CT plate result pictures from Study II showed that 5 out of 6 plates in Lab A (15 readings in total) and 5 plates in Lab B (9 readings in total) that grew >35 days were overgrown and could have been assessed earlier (Figure 3).

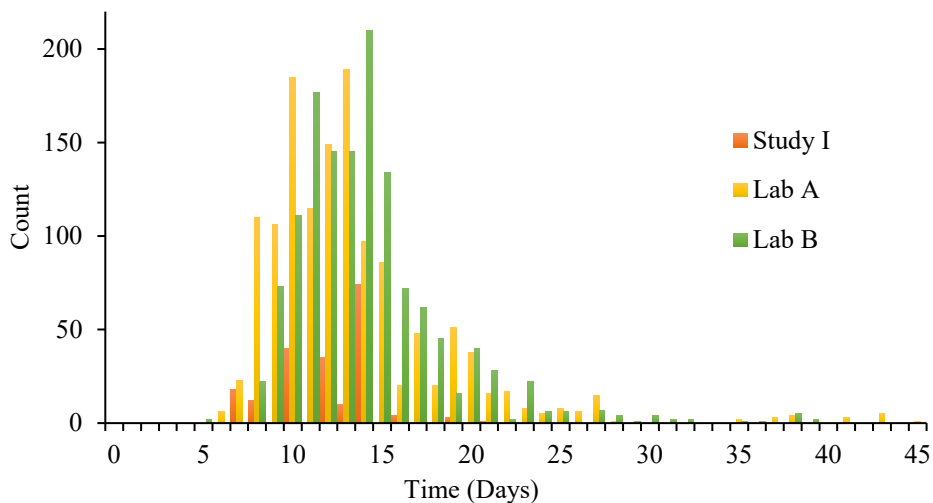


Figure 2. Time in days until a CT result during Study I and Study II (Laboratory A and Laboratory B). A histogram was drawn from the times until a positive *Mycobacterium tuberculosis* thin-layer agar MDR/XDR-TB Colour Test (CT) result in Study I (dark blue, 197 CT readings), laboratory A (Lab A) (yellow, 1,337 CT readings) and the laboratory B (Lab B) (green, 1,347 CT readings).

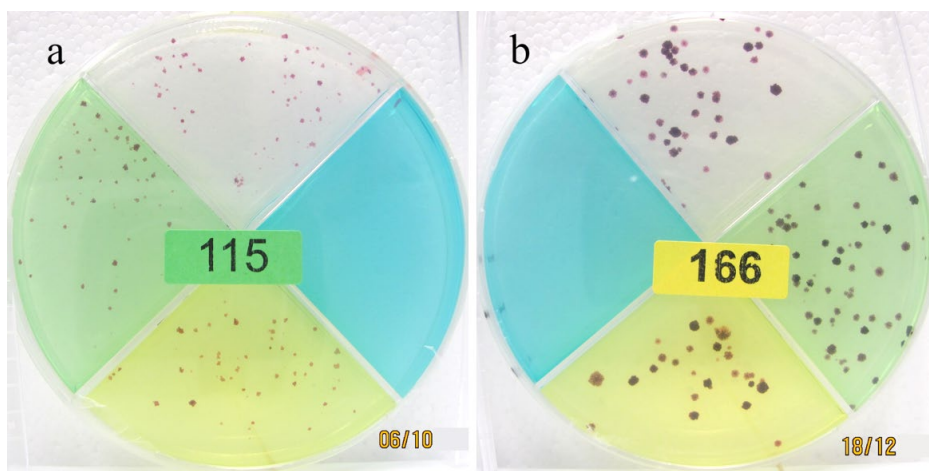


Figure 3. Photographs of positive CT plates. Examples of *Mycobacterium tuberculosis* thin-layer agar MDR/XDR-TB Colour Test results on day 14 (a) and 43 (b) after inoculation.

The performance of the CT indirect DST was assessed by combining results from Studies I–II to a sample of 2,882 results that were compared to BACTEC MGIT indirect DST results (Table 3 and Figure 4). The overall agreement between the CT and BACTEC MGIT indirect DST was 96.8%, 97.2% and 97.5% for INH, RIF and LVX (CFX), respectively (Table 3). The combined sensitivities for detecting resistance to tested drugs varied between 95.2% for LVX (CFX) to 96% and 97.2% for INH and RIF, respectively. All of the overall specificities exceeded 97% being 98.1% for INH, 97.0% for RIF and 97.9% for LVX (CFX).

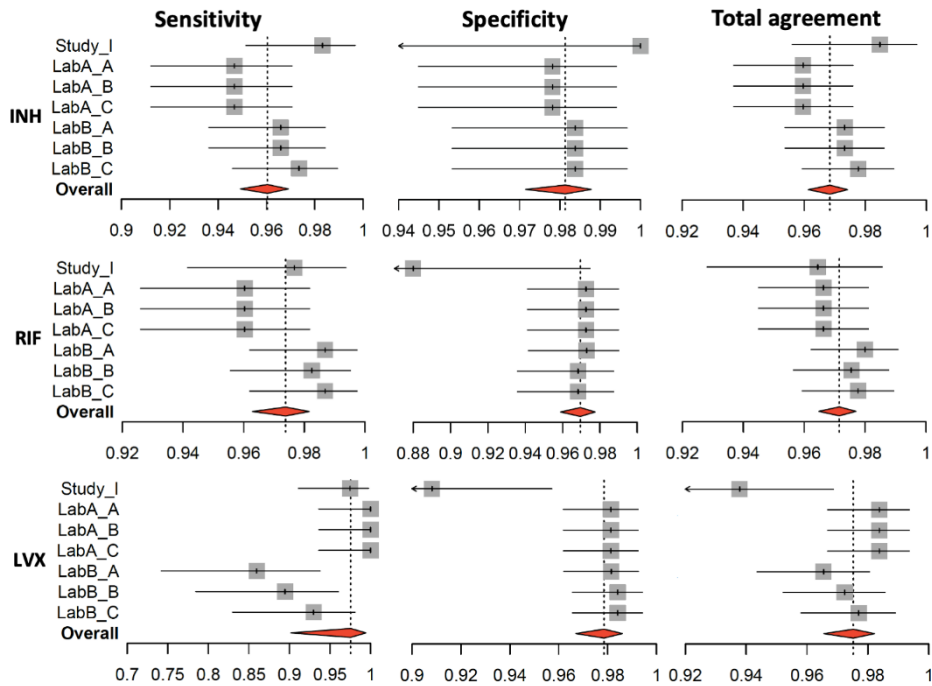


Figure 4. Individual and pooled sensitivities, specificities and total agreements of the thin-layer agar MDR/XDR-TB Colour Test indirect DST of *Mycobacterium tuberculosis* isolates. Grey squares indicate the value for the respective technician and red diamonds indicate pooled estimates. INH – isoniazid; Lab A – Laboratory A; Lab B – Laboratory B; LVX – levofloxacin; RIF – rifampicin; Study I – estimates from Study I.

Table 3. Combined CT indirect drug susceptibility testing performance of the *Mycobacterium tuberculosis* compared to the BACTEC MGIT (I–II).

			BACTEC MGIT		Total agreement (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	LR+ (95% CI)	LR- (95% CI)
			S	R					
CT INH	Pooled	S	1102	70	96.8% (96.1–97.5)	96.0% (95.0–96.9)	98.1% (97.2–98.8)	51.3 (33.6–78.5)	0.04 (0.03–0.05)
		R	21	1689					
	Study I	S	19	3	98.5% (95.6–99.7)	98.3% (95.2–99.7)	100% (82.4–100)	NA	0.02 (0.01–0.05)
		R	0	175					
	Lab A Study II	S	537	42	96.0% (94.8–97.0)	94.7% (92.9–96.1)	97.8% (96.2–98.9)	43.3 (24.8–75.8)	0.05 (0.04–0.07)
		R	12	747					
	Lab B Study II	S	546	25	97.5% (96.5–98.3)	96.8% (95.4–98.0)	98.4% (96.9–99.3)	59.7 (31.2–114.2)	0.03 (0.02–0.05)
		R	9	767					
CT RIF	Pooled	S	1304	41	97.2% (96.5–97.7)	97.3% (96.4–98.1)	97.0% (95.9–97.8)	31.9 (23.6–43.2)	0.03 (0.02–0.04)
		R	41	1496					
	Study I	S	22	4	96.5% (92.8–98.6)	97.7% (94.2–99.4)	88.0% (68.8–97.5)	8.1 (2.8–23.5)	0.03 (0.01–0.07)
		R	3	168					
	Lab A Study II	S	639	27	96.6% (95.5–97.5)	96.0% (94.3–97.4)	97.3% (95.7–98.4)	35.1 (22.2–55.3)	0.04 (0.03–0.06)
		R	18	654					
	Lab B Study II	S	643	10	97.8% (96.8–98.5)	98.5% (97.2–99.3)	97.1% (95.6–98.2)	34.2 (22.2–52.6)	0.02 (0.01–0.03)
		R	20	674					

			BACTEC MGIT		Total agreement (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	LR+ (95% CI)	LR- (95% CI)
			S	R					
CT LVX	Pooled	S	2305	20	97.5%	95.2%	97.9%	45.7	0.05
		R	49	398	(96.9–98.1)	(92.7–97.1)	(97.3–98.5)	(34.6–60.4)	(0.03–0.07)
	Study I (CFX)	S	89	2	93.8%	97.5%	90.8%	10.61	0.03
		R	9	77	(89.2–96.9)	(91.2–99.7)	(83.3–95.7)	(5.7–19.8)	(0.01–0.11)
	Lab A Study II	S	1104	0	98.4%	100%	98.1%	53.6	0.00
		R	21	168	(97.5–99.0)	(97.8–100)	(97.2–98.8)	(35.1–81.8)	
	Lab B Study II	S	1112	18	97.2%	89.5%	98.3%	53.3	0.11
		R	19	153	(96.1–98.0)	(83.9–93.6)	(97.4–99.0)	(34.0–83.4)	(0.07–0.17)

CT – thin-layer agar MDR/XDR-TB Colour Test; CFX – ciprofloxacin; CI – confidence interval; DST – drug susceptibility test; INH – isoniazid; Lab A and Lab B – laboratories A and B; LR+ – positive likelihood ratio; LR- – negative likelihood ratio; LVX – levofloxacin; MGIT – Mycobacterium Growth Indicator Tube; S – susceptible; R – resistant; RIF – rifampicin.

Figure 5 illustrates the CT colony counts from each of the drug containing compartments in Study I and demonstrates that there were very few borderline results. The CT re-readings on day 21 in Study I did not influence INH and RIF results. However, the late growth in the CFX-containing plate quadrant caused significant decrease in the agreement between CT DST and BACTEC MGIT ($p = 0.03$, data not shown).

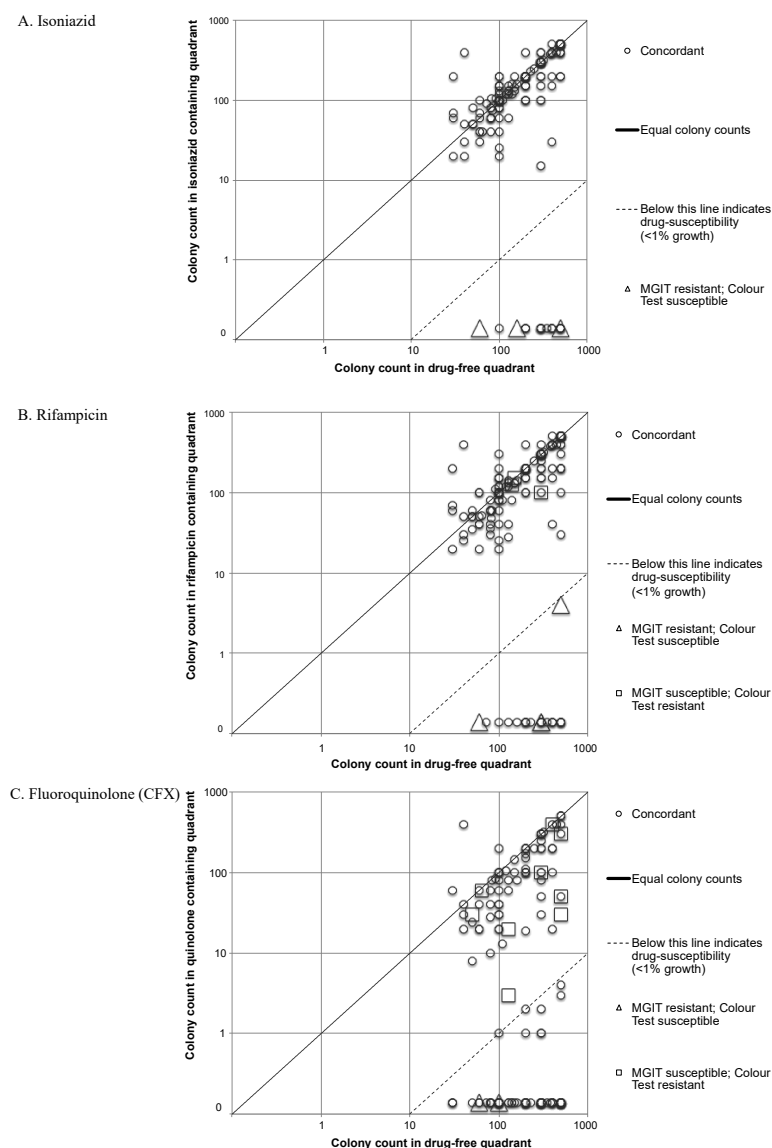


Figure 5. Thin-layer agar MDR/XDR-TB Colour Test colony count results from Study I ($n=197$). The graphs show the number of *Mycobacterium tuberculosis* complex colonies visible to the naked eye on the day that each test became culture positive. Drug resistance

results that were concordant and discordant with the BACTEC MGIT indirect DST are indicated by different symbols (see the legend). The strains were considered to be susceptible to a drug if it prevented more than 99% of TB growth (dotted line). Reproduced with permission from the publisher (1).

Out of the 18 CT result readings produced for every MTB isolate in Study II (n=150), at least one result was discordant with the BACTEC MGIT indirect DST for 19 isolates. LVX false negative results were recorded for 6 samples in Lab B for which the result photographs showed microcolonies in the LVX quadrant (Figure 6). For 5 isolates a discordant result was recorded on one of 6 CT culturing. Of these, there was one LVX false positive result in Lab B, where the photograph showed an empty LVX quadrant, one INH false sensitive result, where the photograph showed <1% of colonies in the INH quadrant and 3 results, where the reported CT DST was in concordance with the CT plate photographs.

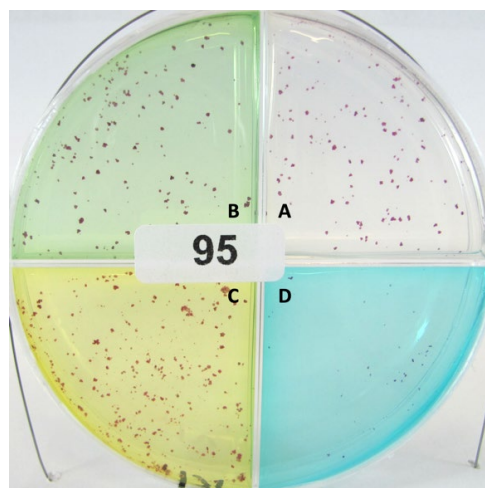


Figure 6. Photograph of a positive CT plate. An example of *Mycobacterium tuberculosis* thin-layer agar MDR/XDR-TB Colour Test result with microcolonies in the levofloxacin containing quadrant. A – growth control quadrant; B – INH containing quadrant; C – RIF containing quadrant; D – LVX containing quadrant.

Eight MTB isolates in Study II produced 77% of the discordant results between the CT and the BACTEC MGIT indirect DST that could not be resolved by re-evaluating results photographs. These 8 isolates were tested twice (once from the frozen study panel and once from the original archived culture in the Estonian TB archive) with the HAIN MTBDRplus and MTBDRsl line-probe assays (Table 4). Three of these isolates had consistently discordant results in 100% of the readings. The molecular DST results of these samples (30, 48 and 82) that cumulatively produced 41.1% of the discordant results in Study II were in concordance with the CT results.

Table 4. Molecular drug susceptibility test results obtained with HAIN MTBDRplus and MTBDRsl assays compared to the previous phenotypic results for 8 *Mycobacterium tuberculosis* isolates, which produced discordant indirect phenotypic drug susceptibility testing results between the CT and the BACTEC MGIT system.

Isolate No.	Estonian TB Registry data (BACTEC MGIT)			HAIN MTBDRplus/sl			CT			
	INH	RIF	FLQ	INH	RIF	FLQ	INH	RIF	LVX	Count
13	R	R	S	R	S	S	R	R	S	12
							S	S	S	6
30	R	S	S	R	R	R	R	R	R	18
48	S	S	S	R	R	R	R	R	R	18
67	R	R	S	S	S	S	S	S	S	4
							S	R	S	3
							R	R	S	11
68	R	R	S	S	S	S	S	S	S	15
							R	R	S	3
82	R	S	S	S	S	NA	S	S	S	18
117	R	S	S	S	S	NA	S	S	S	7
							R	S	S	11
138	R	R	S	S	S	S	S	S	S	12
							R	R	S	6

Count – the number of times a respective CT result was observed out of 18 readings; CT – thin-layer agar MDR/XDR-TB Colour Test; FLQ – fluoroquinolone; INH – isoniazid; MGIT – Mycobacterium Growth Indicator Tube; NA – not applicable; No. – isolate number (out of 150 samples in Study II); R – resistant; RIF – rifampicin; S – sensitive; TB – tuberculosis.

5.1.4. Evaluation of the ability of the Colour Test to detect MTBC (III)

The ability to detect and identify MTBC directly from the CT plates was assessed in Study III by culturing all types of pulmonary specimens in parallel on the CT and MGIT. Additionally, 164 (92.7%) of these were also cultured on LJ media (Table 5). Three of the 177 pulmonary specimens were re-decontaminated and re-plated because of contamination on CT plates.

The sensitivities for the detection of MTBC with different media were 81.3% (95% CI 71.0–89.1), 93.5% (95% CI 85.5–97.9) and 81.7% (95% CI 70.7–89.9), for CT, MGIT and LJ, respectively (Table 5). Non-tuberculous mycobacteria were not detected in any of the study samples and at least one of the cultures (CT, MGIT or LJ) grew positive with MTBC in 83 (46.9%) of the specimens. Two (2.4%) of the 83 MTBC positive cultures were INH mono-resistant and almost

one third (31.3%) of the positive MTBC cultures appeared to be MDR or RR strains according to the indirect DST results from the BACTEC MGIT system.

Table 5. Performance characteristics of the CT, LJ and MGIT media in *Mycobacterium tuberculosis* complex detection. Reproduced with permission from the publisher (III).

MTBC detection	TP	FP	TN	FN	Positivity (%)	Sensitivity (95% CI)	Specificity (95% CI)	Median TTD (days)
CT	65	2	95	15	37.9	81.3% (71.0–89.1)	97.9% (92.8–99.8)	20 (IQR 14.5–27)
LJ	58	1	92	13	36.0	81.7% (70.7–89.9)	98.9% (94.2–100)	24 (IQR 17–29)
MGIT	72	5	95	5	43.5	93.5% (85.5–97.9)	95.0% (88.7–98.4)	13 (IQR 7–18)

CT – thin-layer agar MDR/XDR-TB Colour Test; CI – confidence interval; FN – false negative; FP – false positive; IQR – interquartile range; LJ – Löwenstein-Jensen; MGIT – Mycobacterium growth indicator tube; MTBC – *Mycobacterium tuberculosis* complex; TN – true negative; TP – true positive; TTD – Time to MTBC detection.

Adjusted for the type of specimen (sputum, BAL and pleural fluid), the median time from collection to MTBC detection with the CT was 20 (IQR 14.5–27.0) days, which was longer than that with MGIT media [13 (IQR 7–18) days] ($p < 10^{-3}$), but not different from that on LJ media [24 (IQR 17–29) days] ($p = 0.29$) (Figure 7). Adjusted for the culture media, the median time from specimen collection to MTBC detection from sputum was 18 (IQR 12.0–23.3) days being significantly shorter than that from BAL [24 (IQR 17.0–42.0) days] ($p = 0.004$), but quite not significantly different from that from the pleural fluid specimens [26.5 (IQR 21.0–32.0) days] ($p = 0.071$) (data not shown).

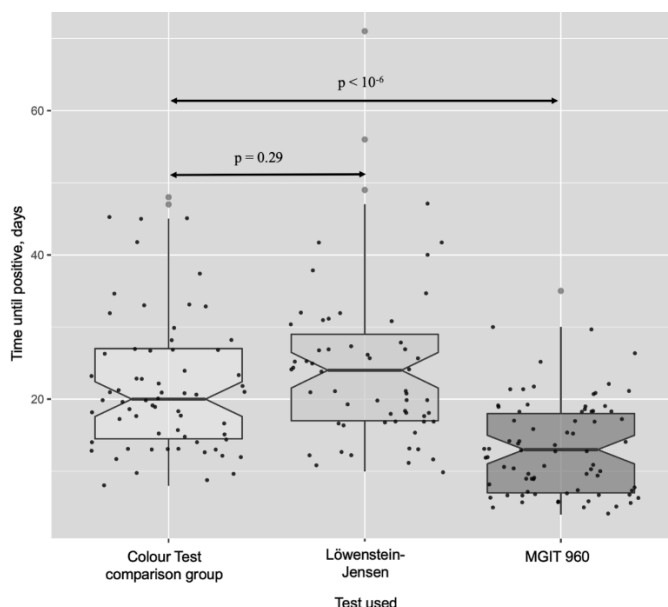


Figure 7. The time in days from material collection to *Mycobacterium tuberculosis* complex detection by method drawn as notched boxplots showing median as the bold horizontal lines and 95% confidence intervals of the medians as notches. Generalised linear modelling with scale response, adjusted for the type of sample (sputum, broncho-alveolar lavage and pleural fluid), was applied on rank-transformed data, and used to compare the time spent with other methods against that spent with the thin-layer agar MDR/XDR-TB Colour Test (CT). Reproduced with permission from the publisher (III). MGIT 960 – BACTEC Mycobacterium growth indicator tube system; • – individual data points; • – outliers.

5.1.5. Evaluation of the Colour Test direct DST results (III)

CT detected 67 MTBC cultures during Study III. These were also used for the evaluation of direct DST results obtained from the CT compared to indirect DST results found with the BACTEC MGIT system.

The direct DST results from the CT plates for all the drugs tested (INH, RIF and LVX) could be read for 54 (80.6%) out of 67 CT MTBC-positive specimens. DST could be evaluated for INH and RIF only for 4 (6.0%) specimens. The direct DST from the CT plates could not be evaluated for 9 (13.4%) of the 67 CT MTBC positive specimens due to insufficient growth (less than 10 colonies) in the control quadrant (Table 6).

The median time from specimen collection to a DST result for INH and RIF together was significantly shorter with the application of the CT than that of BACTEC MGIT: 20 (IQR 14–26.75) vs. 24.5 (IQR 18.25–32) days, $p = 0.029$ (Figure 8). The median time from specimen collection to a DST result for LVX was also significantly shorter with the CT than that with BACTEC MGIT: 19 (IQR 13–22) vs. 29.5 (23–40.3) days, $p < 10^{-3}$ (Figure 8).

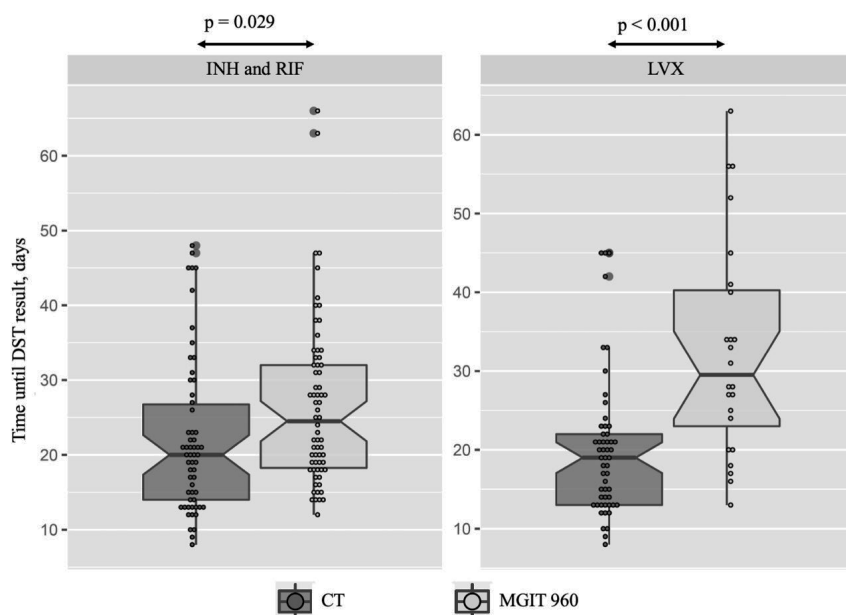


Figure 8. Time in days from material collection until drug susceptibility testing results for isoniazid (INH) and rifampicin (RIF) together or levofloxacin (LVX) by method drawn as notched boxplots showing medians as the bold horizontal lines and 95% confidence intervals of the medians as notches. Analyses between the methods were performed with use of Mann-Whitney U-test. Reproduced with permission from the publisher (III). CT – thin-layer agar MDR/XDR-TB Colour Test; DST – drug susceptibility testing; MGIT 960 – BACTEC Mycobacterium growth indicator tube system; ○ – individual data points; • – outlier.

The total agreement between the CT direct DST and the BACTEC MGIT indirect DST results was 98.2% (95% CI 90.6–100), 96.4% (95% CI 87.7–99.6) and 94.3% (95% CI 84.3–98.8), for INH, RIF and LVX, respectively (Table 6). The corresponding sensitivities for drug resistance detection with the CT were 100% for INH (95% CI 83.2–100) and LVX (95% CI 15.8–100) and 94.7% (95% CI 74.0–99.9) for RIF. The specificity for LVX DST results was slightly higher at 98.0% (95% CI 89.2–100) than that for INH and RIF DST results, which were both 97.3% (95% CI 85.8–99.9). The likelihood of an isolate with a CT resistant result of being also resistant to a given drug according to the BACTEC MGIT indirect DST was 37 (95% CI 5.4–255.8), 35 (95% CI 5.1–243) and 17 (95% CI 5.7–51) times higher for INH, RIF and LVX, respectively, than being sensitive on the BACTEC MGIT (Table 6). The likelihood of an isolate showing resistance to a given drug on BACTEC MGIT despite a susceptible result on the CT was 0 for INH and LVX and 0.05 (95% CI 0.01–0.36) for RIF (Table 6).

Table 6. Performance of the drug susceptibility testing of the thin-layer agar MDR/XDR-TB Colour Test compared to BACTEC MGIT. Reproduced with permission from the publisher (III).

		CT direct DST					
		INH		RIF		LVX	
		R	S	R	S	R	S
BACTEC MGIT indirect DST	R	21	0	18	1	4	0
	S	1	36	1	36	1	49
	Total	58		56		54	
Agreement (95% CI)		0.98 (0.91–1.00)		0.96 (0.88–1.00)		0.98 (0.90–1.00)	
Sensitivity (95% CI)		1.00 (0.84–1.00)		0.95 (0.74–1.00)		1.00 (0.40–1.00)	
Specificity (95% CI)		0.97 (0.86–1.00)		0.97 (0.86–1.00)		0.98 (0.89–1.00)	
LR+ (95% CI)		37.0 (5.4–255.8)		35.05 (5.1–243)		17.0 (5.7–51)	
LR- (95% CI)		0		0.05 (0.01–0.36)		0	

CI – confidence interval; CT – thin-layer agar MDR/XDR-TB Colour Test; DST – drug susceptibility testing; INH – isoniazid; MGIT – Mycobacterium growth indicator tube; LR- – negative likelihood ratio; LR+ – positive likelihood ratio; LVX – levofloxacin; R – resistant; RIF – rifampicin; S – susceptible.

GLM with scale response revealed that the median time until a DST result was significantly shorter using the CT (20 days, IQR 13–23 days) compared to the BACTEC MGIT (27 days, IQR 19–34 days) regardless of the drugs tested or the type of specimen used ($p < 10^{-3}$) (Figure 9a). The median time until a DST result was not affected by the drugs tested regardless of the method or the type of specimen used, i.e. 21 (IQR 16.0–29.8) and 21 (IQR 15.0–30.3) days for INH and RIF pooled together and LVX, respectively, ($p = 0.82$) (Figure 9b). The median time until a DST result from BAL specimens was significantly longer compared to that from sputum specimens regardless of the method used or the drugs tested, i.e. 33.5 (IQR 24.0–41.8) and 20 (IQR 15.0–27.0) days, respectively, ($p < 10^{-3}$) (Figure 9c). Producing a DST result from pleural fluid specimens took insignificantly more time compared to sputum specimens regardless of the method used or the drugs tested [38.5 days (IQR not shown because of too few data points)] ($p = 0.15$) (not shown on Figure 9c).

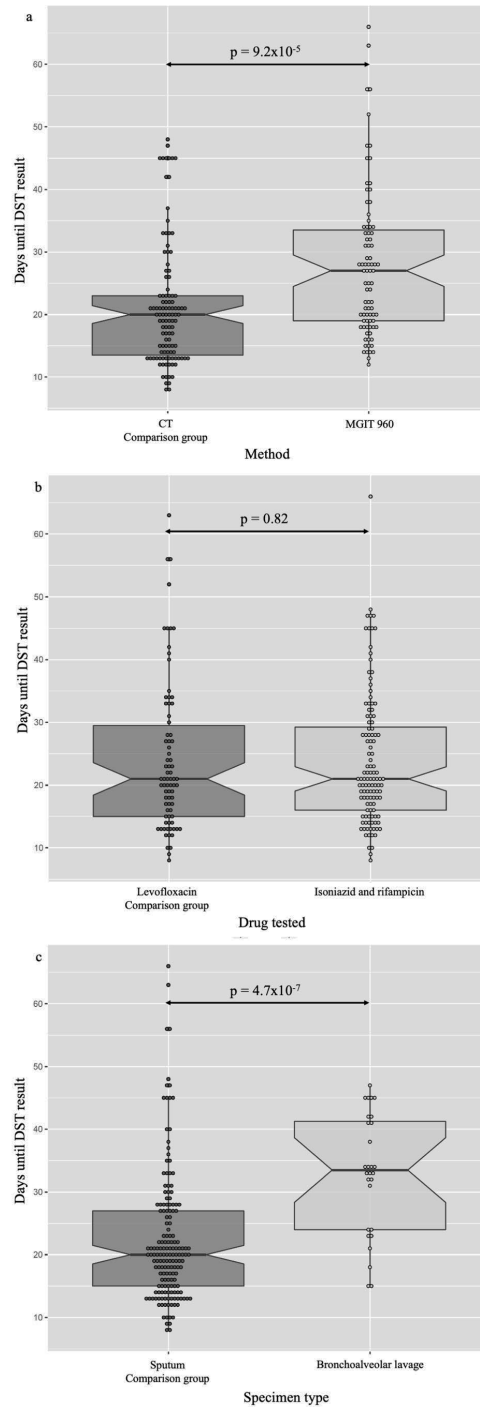


Figure 9. Time in days from material collection until drug susceptibility testing results by method (a), by drug group tested, i.e. rifampicin and isoniazid together or levofloxacin (b) or by the specimen type used (c) drawn as notched boxplots showing median as the

bold horizontal line and 95% confidence intervals as notches. The impact of the method used, the drug group tested and the specimen used was analysed on rank-transformed data with generalised linear modelling with scale response, adjusted for the drugs tested and types of specimens (a), for the culture methods and types of specimens (b) and for the culture methods and drugs tested (c). Reproduced with permission from the publisher (III). CT – thin-layer agar MDR/XDR-TB Colour Test; DST – drug susceptibility testing; MGIT 960 – BACTEC Mycobacterium growth indicator tube system; ○ – individual data points.

5.2. Phylogenetic distribution of MTBC strains and risk factors associated with recent TB transmission in Estonia (IV)

During the four-year period from 2009 to 2012 of Study IV, 1,371 TB cases were diagnosed in Estonia. Of these, 1,286 (93.8%) were diagnosed with pulmonary TB, 1,080 (78.8%) were new cases with no previous treatment record and 1,066 (77.8% of total) were culture-positive TB cases. Over three quarters (86.0%, n=917) of the culture positive cases had VNTR patterns available for their isolates in the National TB Reference Laboratory database. In 912 cases, a link between the patient and the strain could be established retrospectively, including 9 patients registered twice because of recurrent TB.

Lineages could be assigned for 86.2% of the 912 strains. The distribution of lineages according to DST pattern is shown in Table 7. The largest overall proportion of strains (n=357, 39.2%) was from the Beijing lineage. DS strains were evenly distributed across the four main lineages (Beijing, Haarlem, Latin American-Mediterranean [LAM] and Ural). In contrast, most MDR-TB and XDR-TB strains were from the Beijing lineage (94.0% and 96.3%, respectively). In addition, 5 out of 7 RIF-monoresistant strains (71.4%) were from the LAM lineage.

Table 7. Drug susceptibility testing result patterns and the distribution of lineages in culture-positive tuberculosis cases in Estonia, 2009 – 2012. Reproduced with permission from the publisher (IV).

Resistance pattern	Total	Proportion of <i>M. tuberculosis</i> lineages N (%)					
		Beijing	Haarlem	LAM	Ural	Un-known	Other
DS	602	101 (16.8)	168 (27.9)	131 (21.8)	72 (12.0)	122 (20.3)	8 (1.4)
INH-mono-resistant	15	2 (13.3)	4 (26.7)	6 (40.0)	-	3 (20.0)	-
RIF-mono-resistant	7	1 (14.3)	-	5 (71.4)	-	1 (14.3)	-
Polyresistant	47	30 (63.8)	2 (4.3)	7 (14.9)	8 (17.0)	-	-
MDR	134	126 (94.0)	2 (1.5)	3 (2.2)	3 (2.2)	-	-
MDR+AG/CP	54	45 (83.3)	-	9 (16.7)	-	-	-
MDR+FLQ	26	26 (100.0)	-	-	-	-	-
XDR	27	26 (96.3)	-	1 (3.7)	-	-	-
Total	912	357 (39.2)	176 (19.3)	162 (17.8)	83 (9.1)	126 (13.8)	8 (0.8)

Proportions are presented as n (%). DS – drug susceptible; INH – isoniazid; LAM – Latin American-Mediterranean; MDR – multidrug-resistant; MDR+AG/CP – MDR plus resistance to any of the injectable second-line drugs; MDR+FLQ – MDR plus resistance to any fluoroquinolone; Polyresistant – resistant to at least two of the first-line drugs and not MDR; RIF – rifampicin; XDR – extensively drug-resistant according to WHO definition valid until 2021 (World Health Organization, 2021a).

5.2.1. Clustering analysis (IV)

The clustering analysis detected 87 clusters comprising 630 (69.1%) MTBC strains (Figure 10) (IV). Overall, 10 clusters, each consisting of more than ten strains, were detected. Moreover, each of the 4 largest clusters contained over 20 strains. The largest cluster consisted of the Beijing lineage strain MtbC 15-9 type 100-32, according to the standard VNTR typing nomenclature (Allix-Beguec, Harmsen, et al., 2008) containing 178 strains of which 164 (92.1%) were MDR. In contrast, the second largest cluster comprised 32 isolates from the LAM lineage, of which 30 (94.0%) were susceptible. The third largest cluster comprised 30 isolates from another Beijing lineage strain (MtbC 15-9 type 94-32), of which 9 (30.0%) were MDR.

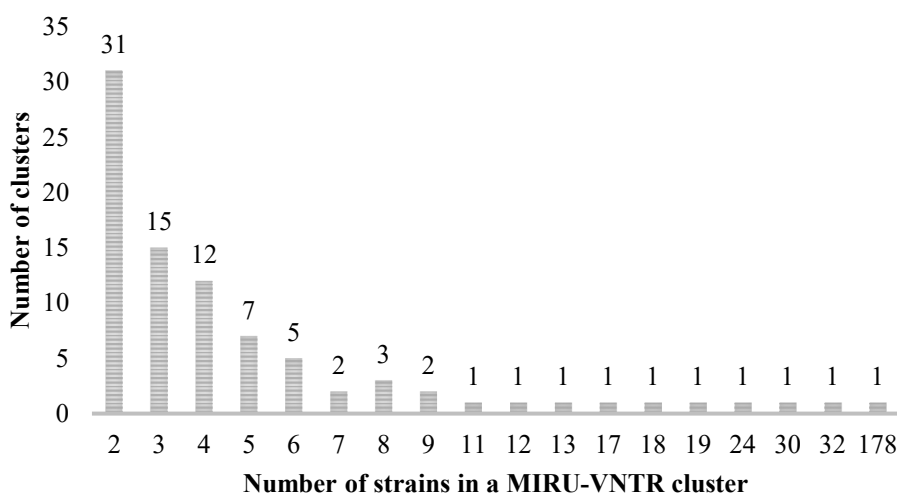


Figure 10. Number and sizes of *Mycobacterium tuberculosis* strain clusters, based on mycobacterial interspersed repetitive unit-variable number of tandem repeat (MIRU-VNTR) typing patterns, in Estonia, 2009–2012. Reproduced with permission from the publisher (IV).

Socio-demographic factors associated with an increased risk of clustering were Russian ethnicity, non-permanent living conditions, alcohol abuse and history of incarceration. Factors associated with a reduced risk of clustering were older age, being born in Ukraine, being of Belarusian ethnicity, living in the city of Tartu and having a lower education level (Table 8). The clinical factors associated with an increased risk of clustering were polyresistant, MDR- and XDR-TB, and being human immunodeficiency virus (HIV)-positive (Table 9). XDR-TB cases had the highest risk of clustering (RR 1.63, $p < 10^{-3}$). Extrapulmonary TB was associated with a reduced risk of clustering (Table 9) (IV).

Table 8. Socio-demographic characteristics of patients with culture-positive tuberculosis in relation to clusters, Estonia, 2009–2012. Reproduced with permission from the publisher (IV).

Risk factor	Total number	Clustered N (%)	Risk Ratio (95% CI)	p-value
Sex				
Male	638	449 (70.4)	1.07 (0.97–1.18)	0.20
Female	274	181 (66.0)	Reference group	
Age in years				
<20	16	12 (75.0)	1.01 (0.75–1.35)	1.0
20–29	110	91 (82.7)	1.11 (0.99–1.25)	0.10

Risk factor	Total number	Clustered N (%)	Risk Ratio (95% CI)	p-value
30–39	178	144 (80.9)	1.09 (0.97–1.21)	0.14
40–49	200	149 (74.5)	Reference group	
50–59	198	142 (71.7)	0.96 (0.85–1.08)	0.53
60–69	101	59 (58.4)	0.78 (0.65–0.94)	<10 ⁻²
≥70	109	33 (30.3)	0.41 (0.30–0.55)	<10 ⁻³
Country of birth				
Estonia	772	550 (71.2)	Reference group	
Russia	96	62 (64.6)	0.91 (0.78–1.06)	0.18
Ukraine	15	4 (26.7)	0.37 (0.16–0.87)	<10 ⁻³
Belarus	11	6 (54.5)	0.77 (0.45–1.32)	0.23
Ethnicity of the patient				
Estonian	515	348 (67.6)	Reference group	
Russian	320	242 (75.6)	1.12 (1.03–1.22)	0.01
Belarusian	19	8 (42.1)	0.62 (0.37–1.06)	0.02
Other	43	24 (55.8)	0.83 (0.63–1.08)	0.12
Living area				
Urban	428	307 (71.7)	1.09 (1.00–1.19)	0.05
Rural	452	297 (65.7)	Reference group	
City of residence				
Tallinn	269	198 (73.6)	Reference group	
Tartu	46	27 (58.7)	0.80 (0.62–1.03)	0.04
Kohtla-Järve	43	33 (76.7)	1.04 (0.87–1.25)	0.66
Narva	45	30 (66.7)	0.91 (0.73–1.13)	0.33
Pärnu	25	19 (76.0)	1.03 (0.82–1.30)	0.79
Living situation				
Permanent home	804	541 (67.3)	Reference group	
Non-permanent home	64	54 (84.4)	1.25 (1.12–1.41)	<10 ⁻²
Homeless	42	34 (81.0)	1.20 (1.03–1.40)	0.06
Level of education				
Primary	61	33 (54.1)	0.76 (0.60–0.97)	<10 ⁻²
Basic	303	215 (71.0)	1.00 (0.91–1.10)	0.99
Secondary	476	338 (71.0)	Reference group	
Higher	62	23 (37.1)	0.89 (0.73–1.08)	0.19
Alcohol abuse				
Yes	421	325 (77.2)	1.24 (1.13–1.35)	<10 ⁻³
No	464	290 (62.5)	Reference group	
Drug abuse				
Yes	65	47 (72.3)	1.05 (0.89–1.23)	0.57
No	526	362 (68.8)	Reference group	
History of detention				
Yes	171	137 (80.1)	1.19 (1.09–1.30)	10 ⁻³
No	688	463 (67.3)	Reference group	

CI – confidence interval.

Table 9. Clinical characteristics of patients with culture-positive tuberculosis in relation to clusters, Estonia: 2009–2012. Reproduced with permission from the publisher (IV).

Risk factor	Total number	Clustered N (%)	Risk Ratio (95% CI)	p-value
Type of TB				
Pulmonary	725	513 (70.8)	Reference group	
Extra-pulmonary	32	8 (25.0)	0.35 (0.19–0.65)	<10 ⁻³
Both	148	105 (70.9)	1.00 (0.90–1.12)	0.96
Category of TB				
New	752	507 (67.4)	Reference group	
Relapse	100	76 (76.0)	1.13 (1.00–1.27)	0.08
Treatment after failure	28	22 (78.6)	1.17 (0.95–1.42)	0.22
Treatment after LFU	32	25 (78.1)	1.16 (0.96–1.40)	0.20
Drug resistance pattern				
DS	602	355 (59.0)	Reference group	
INH-mono-resistant	15	11 (73.3)	1.24 (0.91–1.70)	0.30
RIF-mono-resistant	7	4 (57.1)	0.97 (0.51–1.85)	1.00
Poly-resistant	47	38 (80.9)	1.37 (1.18–1.60)	<10 ⁻²
MDR	144	122 (91.0)	1.54 (1.42–1.68)	<10 ⁻³
MDR+AG/CP	54	50 (92.6)	1.57 (1.42–1.74)	<10 ⁻³
MDR+FLQ	26	24 (92.3)	1.57 (1.38–1.78)	<10 ⁻³
XDR	27	26 (96.3)	1.63 (1.48–1.80)	<10 ⁻³
HIV testing done				
Positive	107	90 (84.1)	1.23 (1.12–1.36)	<10 ⁻³
Negative	757	517 (68.3)	Reference group	

CI – confidence interval; DS – drug susceptible; INH – isoniazid; HIV – human immunodeficiency virus; LFU – loss to follow up; MDR – multidrug-resistant; MDR+AG/CP – MDR plus resistance to any of the injectable second-line drugs; MDR+FLQ – MDR plus resistance to any fluoroquinolone; Poly-resistant – resistant to at least two of the first-line drugs and not MDR; RIF – rifampicin; TB – tuberculosis; XDR – extensively drug-resistant according to the WHO definition valid until 2021 (World Health Organization, 2021a).

The distribution of transmission between index cases and secondary cases in relation to different age groups is shown in Figure 11. Transmission occurred most frequently from the 30–39-year age group. The largest proportion of secondary cases were among patients aged between 30–59 years.

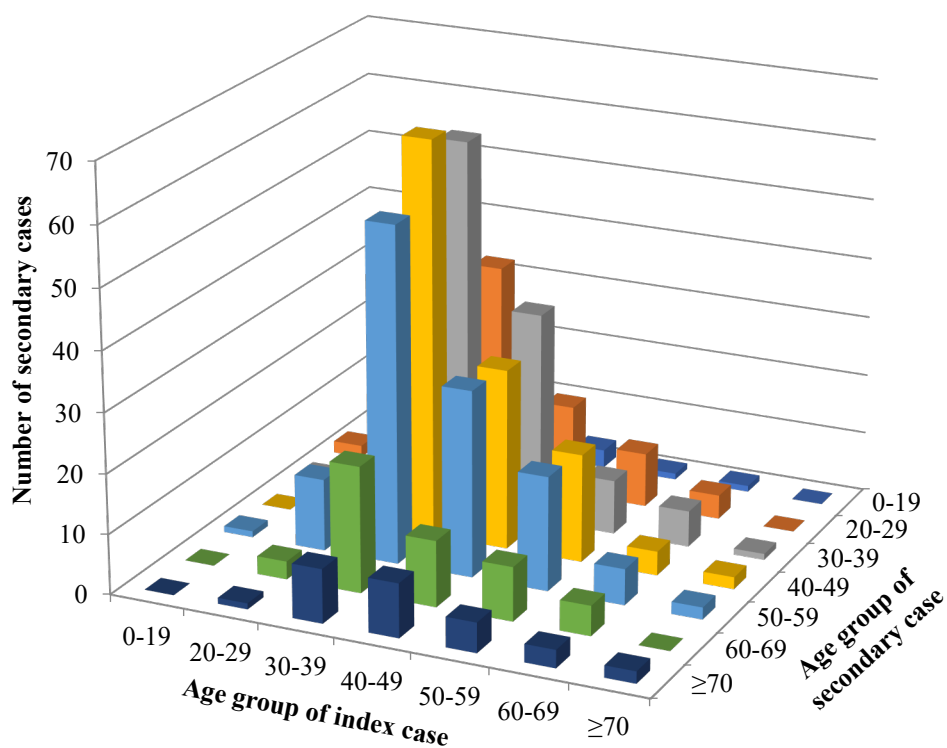


Figure 11. Distribution of tuberculosis transmission between age groups of index cases and secondary cases in Estonia, 2009–2012. Reproduced with permission from the publisher (IV).

6. DISCUSSION

We aimed at evaluating the CT for MTBC detection and simultaneous DST compared to routine commercial diagnostic tests (I–III). The current combined investigation is the most comprehensive evaluation so far of the CT method for routine TB diagnostic application. Studies I and II had the highest prevalence of MDR among isolates used for retrospective evaluation (85.1% and 50.0%, respectively), whereas in Study III, the highest proportion of MDR strains (31.3%) was detected during prospective phases. These studies together represent one of the few evaluations of the CT, where all study methods are performed in one single laboratory (I–III) (Ardizzoni et al., 2021; Mekonnen et al., 2019; Shibabaw et al., 2019). Moreover, Study II is the first and only one to systematically evaluate the reproducibility and objectiveness of the CT.

6.1. Implementation of the Colour Test in non-expert settings (I–III)

In a short period of time, it was possible to incorporate CT into two TB diagnostic laboratories and introduce the method to six technicians (II). We did not identify any bottlenecks in the incorporation of CT into a routine laboratory workflow.

None of the CT plate batches prepared by seven different technicians was contaminated, which indicates that the medium is robust enough for the use in resource-constrained setting (I–III). The addition of Selectatab to the CT medium did not inhibit the growth of MTBC and all positive controls grew throughout the whole study period. The incorporation of STC to the CT medium made the growing MTBC colonies red and visible to the naked eye in early stages and the addition of food colouring to the detection quadrants facilitated easier reading of the plates. The prior standardisation of the dilutions used in Study I allowed for fast and easy interpretation of the DST results.

Although one of the laboratories in Studies II and III used a MediaJet machine to pour the 4-quadrant Petri dishes with the CT media, whereas the other did the same by hand, the median time to prepare and pour one batch of CT plates was the same (112.5 min). The most time-consuming part of the media preparations lies in the one-by-one addition of antimycobacterial drug working solutions and food colourings to the autoclaved and still warm Middlebrook 7H11 agar.

During Study II, we introduced CT into two previously inexperienced laboratories. It took equally 2.5 min in both laboratories to continuously read a CT plate until a result was documented. One could predict that this time could be slightly different and perhaps even shorter in routine settings, where most cultured plates remain negative for 6 weeks and can quickly be examined three times weekly.

6.2. Cost of the Colour Test reagents (I–III)

Throughout the study period of almost ten years and the gap of over five years in purchasing reagents for the CT media, the test proved inexpensive with the cost for one CT plate being 2.4€ and 1.21€ in 2012 and 2017, respectively. This cost appears to be similar to that previously published by Martin *et al.* for TLA (Martin *et al.*, 2009). The largest reduction in cost was made by changing the FLQ from CFX to LVX. For comparison, the local cost for one MGIT tube during our study period was 6.05€, which is 5 times higher than for a CT plate. Still, our study only gives an indication of the plate retail cost in our setting and appropriately designed cost-effectiveness studies are needed to formally assess this in specific settings.

6.3. Performance of the Colour Test in detecting drug resistance in clinical isolates (I–II)

Studies I and II investigated the overall ability of seven previously inexperienced technicians to define drug susceptibility using the CT. The median time of investigating 2,882 CT plates until a DST result was 13 days, which is consistent with previous results by others using the plate either as an indirect or direct DST method (Hussain *et al.*, 2019). The median time for a BACTEC MGIT indirect DST result during the years 2010–2014 was 12 days. Although it might be that the CT DST performance time was slightly slower than the BACTEC MGIT indirect DST at 13 days, it was significantly faster than other solid-media-based DST (Bemer *et al.*, 2002). A significant proportion of the diagnostic laboratories worldwide still use LJ medium for initial mycobacterial isolation and send MTBC cultures thousands of kilometres into reference laboratories for DST. Our study shows that as a cost-effective alternative indirect or direct CT DST might be implemented in these laboratories.

The high total agreement between BACTEC MGIT DST and excellent LR– and LR+ values suggest that this technique can serve as an accurate and promising test (I–II). The total agreement between the CT and the BACTEC MGIT system was excellent being over 93% for all the anti-TB drugs tested (Figure 4). Furthermore, the specificities found in Study II were >97% for all technicians, which is in good concordance with previous studies using the method either as a direct or indirect DST (Ardizzoni *et al.*, 2015; Iftikhar *et al.*, 2017; Shibabaw *et al.*, 2020). The sensitivities varied between the study laboratories being slightly higher in Study I and Lab B for the detection of INH and RIF resistance and higher in Lab A with 100% for the detection of LVX resistance (I–II). In the Lab B, microcolonies present in LVX quadrants caused false susceptible results as the technicians did not take these as evidence of growth, because the respective microcolonies did not have the cauliflower-like cording, when viewed under a light microscope and were smaller than the colonies in other quadrants (Figure 6) (II). In Study I, slightly lower specificity was observed for CFX compared to OFL in BACTEC MGIT than that in Study II for Lab A and Lab B (98.1 and 98.3%,

respectively). This might be due to CFX being a less potent drug than OFL or LVX and therefore, not a good marker indicating susceptibility of FLQ.

During Study II, each of the participating six technicians evaluated 450 CT plates that originated from triplicate culturing of the study sample. This structure resulted 18 CT readings for each sample. Inconsistent results between at least one of the individual CT readings and the BACTEC MGIT DST were recorded for 19 samples. For three samples, a discordant result was produced in 100% of the readings, i.e. on all 18 readings of the 6 CT plates. However, the HAIN MTBDRplus and/or MTBDRsl results were in concordance with the CT DST results for these isolates (Table 4, isolates no. 30, 48 and 82) (II). These 3 samples accounted for over 40% of all discordant results (II).

Our study protocol for indirect DST using the CT plates required having at least 50 colonies in the control quadrant before reporting DST results and if <50 colonies were present at the first MTBC detection, the incubation period needed to be prolonged (I). We found that a few of the CT plates were read very late, even 45 days after culturing (Figures 2 and 3) (II). Our results indicate that the initial protocol should be modified stating for how long one should prolong the incubation after the first detection of mycobacterial colonies in the control quadrant (I–II). The prolonged plate incubation during indirect DST can be avoided with a more concentrated inoculum when culturing on CT plates. However, increasing the volume of the inoculum is not recommended because of potential carryover between plate quadrants. On the other hand, one could say that diluted inoculum was one of our study strengths, because it better reflects the diversity between patient specimens.

We also showed that the CT gives consistent results on repetitive culturing of 1 isolate and is therefore highly reproducible and not dependent on the experience of laboratory personnel, moreover, the readings of one CT plate by different inexperienced readers are highly consistent (II). The interobserver agreement rates were close to 1.0 except for LVX in Lab B (Table 2 and Figure 4) (II). The difference for the DST results for LVX in Lab B might be due to the fact that one of the technicians in Lab B read CT plates up to 1 week later and had better concordance with the reference regarding the DST for LVX (Figure 4). In general, the intra-observer agreement and the intra-isolate agreement exceeded 0.9, except for that in case of LVX in Lab B, where microcolonies were not considered as growth in some of the CT plates.

6.4. Feasibility of the Colour Test to identify MTBC and to detect drug resistance from routine pulmonary specimen (III)

In Study III, we found that one culture on a CT plate gave enough information to detect DS, Hr and MDR-TB cases. Our data showed that one culture on a CT plate was able to detect 81.3% of the MTBC-positive specimens, comparable to

a LJ culture, but still inferior to a MGIT culture (Table 5). One explanation for this difference could be the amount of sediment cultured with each of the studied methods, i.e. only two drops of sediment being added to the control quadrant of the CT plate compared to 500 µL into MGIT medium.

The analysis of detection times in our sample suggested that despite the BACTEC MGIT system being seven days faster than the CT plate in detecting MTBC growth, it was significantly slower in producing an indirect DST result for INH, RIF and LVX than the direct DST on the CT (III). GLM with scale response revealed that this difference in DST times was not affected by the drug tested or the specimen used; however, sputum specimens produced the fastest DST results regardless of the method used (III) indicating that the additional use of the CT gives the most added value in a peripheral setting where sputum smears are normally examined.

Compared to the BACTEC MGIT DST results, RIF resistance was misclassified by the CT in two specimens (Table 6). LVX resistance was missed by BACTEC MGIT DST on one occasion, where the CT and the routine molecular DST result (data not shown) showed genetic FLQ resistance (III). The latter indicates that the critical concentration used in BACTEC MGIT indirect DST for determining susceptibility to LVX might still be too high to accurately differentiate between the wild-type and the resistant populations (Köser & Maurer, 2023).

Study III showed that the CT could reliably be used with different pulmonary specimens as a primary diagnostic test. The sensitivity and the speed of the CT compared to MGIT in the detection of MTBC could be enhanced, when two CT cultures instead of one were used for each specimen. However this is just a logical thought and needs to be proven in future research. Nevertheless, the capacity of the CT to provide faster DST results than MGIT 960 without the necessity for a biosafety level III laboratory leaves the test appropriate to every high-burden setting. Because of its benefit of providing simultaneous diagnosis of TB diagnosis and accurate DST results, the CT is also suitable for peripheral settings where, together with Xpert Ultra, it could provide enough information for faster treatment initiation for RR/MDR-TB patients.

6.5. Molecular epidemiology of tuberculosis in Estonia (IV)

Study IV is the first molecular epidemiologic study in Estonia using the VNTR method to examine associations between patients, their characteristics, strains of *M. tuberculosis* and clustering to determine where recent TB transmission has occurred.

Our study pointed out that over 90% of patients with MDR-TB, MDR-TB with additional resistance to second line drugs and XDR-TB in Estonia were infected with isolates from the Beijing lineage. The association between severe drug resistance and the Beijing lineage is well established worldwide, (European Concerted Action on New Generation Genetic Markers Techniques for the Epidemiology Control of Tuberculosis, 2006; Merker et al., 2015; Mokrousov et al.,

2006) and in Estonia this association was already demonstrated in 2001 with isolates that originated 20 years ago (Krüüner et al., 2001). Our data thus demonstrate a continuous, longitudinal persistence of this association in Estonia. Another interesting but more unexpected finding was that the small number of isolates with RIF monoresistance largely came from the LAM lineage (5/7). Nevertheless, patients with RIF monoresistance require MDR-TB treatment rather than first-line anti-TB treatment, indicating that this pattern of drug resistance needs to be carefully monitored in the future.

Almost 70% of the isolates were clustered (IV). This is significantly higher than that shown previously for Estonia being at 49% from cases examined 20 years ago (Krüüner et al., 2001). Importantly, there were several clusters suggesting transmission chains involving large groups of ten people or more (IV). The largest cluster of 178 isolates consisted of the Beijing lineage strain (IV), which was recently reported to be the major source of MDR/XDR-TB in many European Union countries (J. de Beer et al., 2014). In this largest cluster, the prevalence of MDR/XDR-TB exceeded 90% (IV). This evidence for active and extensive spread of MDR/XDR clones of MTB Beijing isolates in Estonia is of particular concern and calls for the need for new measures to better prevent transmission in the populations involved.

Certain socio-demographic and clinical factors were significantly associated with clustering indicating that the detected clustering reflects recent transmission to a good extent (IV). These factors included an absence of a permanent home, alcohol abuse, previous imprisonment and having polyresistant TB, MDR/XDR-TB or HIV-infection. While the associations of clustering with detention in prison, alcohol abuse, MDR/XDR-TB and HIV are well established (Alland et al., 1994; Mokrousov et al., 2009), the other socio-demographic associations are novel. However, it makes an intuitive sense that those without a permanent home are at increased risk of TB (Lonnroth et al., 2009) and are likely to congregate and spread the infection. We are unaware of previous reports that polyresistant TB is significantly associated with recent transmission. Most of the polyresistant isolates were clustered and were also from the Beijing lineage (IV). This is important because polyresistant Beijing strains are associated with a risk of developing amplified resistance leading to MDR-TB (Cox et al., 2007). Transmission patterns of polyresistant isolates, therefore, also need to be carefully monitored.

Some factors were associated with reduced transmission of TB. Patients with extra-pulmonary TB had a 65% reduced risk of recent transmission compared with those, who had pulmonary TB, and this reflects the predominantly non-infectious nature of extra-pulmonary disease (IV). In patients, who were born in Ukraine, there was a significantly lower risk of clustering, possibly because these patients acquired latent infection in their home country, which reactivated after coming to Estonia. Old age was not associated with recent transmission (IV), which fact may reflect the increasing isolation of elderly people from today's modern societal communication.

6.6. Limitations of the studies

All studies, which form the basis of this dissertation, are carried out with MTBC isolates spreading in Estonia. The methods presented here might perform slightly differently in another population of strains. Although both, the CT and MIRU-VNTR have been used elsewhere with similar results.

6.6.1. Limitations in association with evaluation of the Colour Test results (I–III)

There were a few more limitations to our studies. First, the laboratories participating in our studies had previous experience in medical microbiology and TB diagnostics (II). However, the use of solid medium as a simultaneous MTBC detection and DST method and the evaluation of DST results by naked eye from the CT was unknown and new for all participating technicians.

Secondly, we aimed at having some nontuberculous mycobacteria in our sample for Study III. However, we did not detect any and did not include any NTM in the sample sets of Studies I and II. However, just for educational purposes, during the introduction phase in Study II we cultured *M. avium* on to the CT plate to illustrate the difference in colony morphology between these species (data not shown).

Ideally, in Study III, we aimed at collecting two parallel sputum specimens. However, on several occasions, the specimens were collected on different days and even in different hospitals. Nonetheless, all specimens were collected prior initiation of treatment and therefore, the samples still represented the original infecting organisms.

Lastly, our statistical analysis indicated that times until a result on a CT plate differed in-between Studies I–III, specifically taking longer in Study III, 13 days vs. 20 days, respectively. This might reflect the diversity of bacillary load in patient specimen or perhaps the insecurity of the participating technicians in reporting direct DST results based in a new method.

6.6.2. Limitations around the molecular epidemiological study in Estonia (IV)

There were two limitations in our molecular epidemiological study with MTBC strains circulating in Estonia. It remains unclear how person-to-person transmission occurred within each cluster, because a detailed epidemiological contact investigation was not performed. Second, we used 24-locus VNTR typing data for clustering analysis and lineage assignment. The additional use of VNTR hypervariable loci on Beijing isolates would have allowed a degree of independent confirmation of lineages and more precise discrimination of cases within clusters (Allix-Beguec et al., 2014; Oelemann et al., 2007). Thus, there might be some overestimation in our assessment of recent transmission. Still, our analysis

of risk factors for clustering allowed a good degree of confidence on the epidemiological relevance of the clusters identified.

6.7. Implications for practice and future research

CT is a robust MTBC detection and direct DST method best suitable for resource-constrained settings and might be one of the methods reducing the gap between the people diagnosed with having TB and the people, who started TB treatment. It is feasible to use this method in Biosafety level 2 or smear microscopy laboratory conditions, when coupled with an in-tube on-transport decontamination procedure. The format of the CT evaluated during Studies I–III perfectly conforms with the most recent WHO recommendations to test for RIF, INH and FLQ susceptibility prior to the initiation of treatment. One positive culture on a CT plate potentially allows for the correct initiation of treatment for DS, Hr and in some cases, even MDR TB.

Further research is needed using CT accompanied with in-transport decontamination to assess the actual ability of the CT to fill the TB diagnostic gap in high-density high TB-burden populations. In addition, appropriately designed cost-effectiveness studies are necessary to promote the uptake of CT.

It is necessary to search for a possibility to use positive CT cultures in follow-on tests like targeted next-generation sequencing or other molecular or phenotypic assays testing susceptibility to new and repurposed antibiotics. In addition, a second-line CT DST plate could be easily developed and used in the aforementioned purpose.

The most important implication of Study IV is to show the value of molecular epidemiology in understanding the transmission of the TB epidemic in Estonia. It will be important to continue to support this field, invest in new molecular epidemiology technology and improve the already good linkages with the National Tuberculosis Programme. Starting from 2021, all the MTBC strains isolated in Estonia are routinely whole genome sequenced. Still, future epidemiological studies are needed with a higher resolution using single-nucleotide variation-based clustering or core-genome multi-locus sequence typing for the detection of recent transmission.

7. CONCLUSIONS

The conclusions drawn from the studies incorporated into this thesis (I–IV) are the following:

- The CT provides indirect DST results as rapidly as the commercial DST methods in a cost-effective manner. Moreover, CT proves to be highly accurate in the detection of resistance from TB isolates. For an inexperienced technician, it was feasible to learn the method in a few days.
- The CT evaluations were proven to be reproducible both between the observers and several retesting occasions. There were no bottlenecks identified in introducing the CT into non-expert routine TB diagnostic laboratories.
- There are no significant hurdles in the use CT with different pulmonary specimens from patients. Moreover, the use of CT as a direct culturing method provides a DST result earlier than the routinely performed phenotypic DST method.
- The most prominent MTBC lineage circulating in Estonia is the Beijing lineage. According to VNTR-based clustering of MTBC strains, Estonia has a high clustering rate, especially between MDR strains. The main socio-demographic risk factors associated with recent transmission of TB are absence of a permanent home, alcohol abuse, previous imprisonment and having poly-resistant TB, MDR/XDR-TB or HIV-infection.

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SUMMARY IN ESTONIAN

Multiresistentse ja eriti resistentse tuberkuloosi diagnostika õhukesel agaril põhineva värvustestiga korvamaks seniseid puudujääke labori poolel

Tuberkuloos (TB) on ravitav haigus, mis on inimühiskonnaga kaasas käinud selle algusest saadik. Esimesed dokumenteeritud viited TB-le pärinevad uuringust, mis tuvastas *Homo erectuse* fossiililt pool miljonit aastat vanad TB-meningiidi tunnused (Kappelman et al., 2008). Alles 1882. aastal avastas Robert Koch, et TB tekitajaks on bakter ja 19. sajandi lõpuks tõestas Jean Antoine Villemin, et TB on nakkav (Heron, 1883; Villemin, 1868). Tänapäeval teame, et TB on nakkushaigus, mida põhjustavad *Mycobacterium tuberculosis* kompleksi (MTBC) kuuluvad bakterid: *Mycobacterium tuberculosis* (MTB), *M. bovis*, *M. africanum*, *M. pinnipedii*, *M. microti*, *M. caprae* ja *M. canetti*.

TB kiire diagnostika on keeruline, kuna MTBC bakterid kasvavad väga aeglaselt ja on seetõttu rutiinsetes kliinilise mikrobioloogia laborites tuvastamatud. Lisaks on fenotüübilise MTBC ravim tundlikkuse eelduseks bakteri puhaskultuur, mille ohutuks paljundamiseks on vajalik eraldi kolmanda ohuastme nõuetele vastavat laborit (World Health Organization, 2020). Seetõttu püüab kaasaegne laboratoorne TB diagnostika minna üle turvalisematele meetoditele, mis ei vaja bakterite paljundamist ja mida saab teostada patsiendile lähemal. Maailma Terviseorganisatsioon (MTO) soovib TB laboratoorseks diagnostikaks kasutada pigem molekulaarseid kiirteste kui külvi ja mikroskoopiat. Molekulaarsed kiirtestid on muutnud TB laboratoorset diagnostikat kiiremaks, olles väga tundlikud ja MTBC suhtes spetsiifilised. Samal ajal on need aga kulukad ja vajavad usaldusväärseks toimimiseks korralikku taristut ja kogenud personali. Neid tingimusi on keeruline täita kõrge haigestumusega piirkondades, mis võitlevad maailma suurima arvu TB juhtudega. Taolistesse piirkondadesse tuleb leida TB laboratoorse diagnostika strateegia, mis on kiire, lihtne ja vastupidav ning mida on võimalik teostada patsientidele lähemal, ehk ka mikroskoopiakeskustes.

MTO avaldab juba üle kümne aasta sarnaseid statistilisi numbreid TB ülemaailmse leviku ja suremuse kohta (World Health Organization, 2019, 2024a). 2023. aastal diagnoositi 8,2 miljonit uut TB juhtu (World Health Organization, 2024a). Nendest üle poole (56%) tuvastati viies kõrge TB levikuga riigis: Indias 26%, Indoneesias 10%, Hiinas 6,8%, Filipiinidel 6,8% ja Pakistanis 6,3% (World Health Organization, 2024a). Samal ajal põhjustab TB aastas 1,25 miljonit surma. Arvatakse, et ravi puudumisel sureb 70% nakkusohtlikest TB patsientidest 10 aasta jooksul (Tiemersma et al., 2011).

Lisaks suurele hulga ravim tundlikku TB (DS-TB) haigestumistele on suurenenud ka nakatumine ravimresistentsetesse TB vormidesse. Kõige levinum ravimresistentne TB vorm on monoresistentsus isoniasiidile (INH), mida loetakse multiravimresistentsuse (MDR) (resistentsus nii isoniasiidile kui ka rifampitsiinile (RIF-le)) eellaseks. MTO hinnangul haigestub iga-aastaselt RIF-resistentsesse-

TB-i (RR-TB) või MDR-TB vormi 400 000 inimest. Nendest vaid 200 000 saavad diagnoositud, kuigi tehnoloogiafirmad on välja töötanud mitmeid patsiendi-lähedasi molekulaarseid kiirteste. Eestis on TB haigestumiste arv vähenenud juba 20 aastat, jõudes 2023 aastal 8,2 haigusjuhu 100 000 elaniku kohta (European Centre for Disease Prevention and Control/WHO Regional Office for Europe, 2025). Eesti ja meie lähiriikide probleem on ravimresistentsete TB juhtude suur osakaal kõikidest TB juhtudest, mis ulatub mõnel aastal isegi 24%-ni (European Centre for Disease Prevention and Control/WHO Regional Office for Europe, 2025). See on üks kõige kõrgemaid MDR-TB osakaale maailmas. Eesti on TB ravis ja juhtude arvu vähendamises väga edukas olnud – ravi on tasuta, kasutusel on kõige uuemad TB ravimid ja raviskeemid, ravi toimub patsiendile kõige paremal viisil ja kodus keskkonnas, kui võimalik. Siiski sureb keskel läbi 15% Eesti TB patsientidest, peamiselt hilinevad diagnooside tõttu (Tuberculosis Registry in the National Institute for Health Development, 2001–2025a, 2001–2025b).

Kiire ja soodne TB diagnostika koos INH, RIF ja fluorokinolonide tundlikkuse hinnanguga on tänapäeval korrektse raviskeemi baasiks ning aitaks vähendada lünka MTO poolt ennustatud TB juhtude arvu ja diagnoositud juhtude arvu ning ravitud juhtude arvu vahel. Õhukesel agaril põhinev MDR/XDR-TB värvustest (Colour Test, CT) arendati välja just nendele põhimõtetele tuginevalt. See võimaldab külvata kopsumaterjali õhukesele Middlebrook'i agarile, mis on rikastatud redoksindikaatoriga. Viimase tõttu värvuvad kasvavad MTBC kolooniad punasteks. Lisaks kiirele diagnostikale võimaldab CT ka otsest ravimitundlikkuse määramist INH, RIF ja levofloksatsiini (LVX) suhtes. Nende omaduste tõttu annab CT MTBC positiivne tulemus piisavalt informatsiooni, et alustada kiiret ja spetsiifilist TB ravi.

Lisaks kiirele laboratoorsele diagnostikale on uusi TB haigestumisi võimalik ära hoida ka aktiivse juhtude leidmisega. See tähendab riskigruppide leidmist, nende skriinimist TB suhtes ja mõnel juhul ka TB profülaktilist ravi. Molekulaarse epidemioloogia meetodid, mis ühendavad kindlal ajahetkel patsiendi kindla TB tüve liiniga aitavad leida TB leviku riskigruppe, aitavad kinnitada või ümber lükata TB ülekannet isikute vahel.

Antud väitekiril põhineb uuringutel, mis hindavad CT kasutuselevõttu, kordustäpsust ja objektiivsust rutiinses TB laboris testides kopsumaterjale nagu röga, bronhiloputusvedelik ja pleuravedelik. Lisaks käsitletakse TB levikut Eestis 2009–2014 aastate jooksul ja tuvastatakse TB leviku riskigrupid.

UURINGUTE EESMÄRGID

Uuringu üldeesmärgiks oli hinnata CT diagnostilisi näitajaid, korratavust ja objektiivsust tuberkuloosi diagnoosimisel ja ravimitundlikkuse otsesel määramisel kui ka ravimitundlikkuse hindamisel MTB kultuuridest.

Töö alameesmärkideks olid:

- Osatöö I** Hinnata CT diagnostilist võimekust MTBC samastamisel puhas-kultuuridest samaaegse multiresistentsete vormide tuvastamise ja tsiprofloksatsiini (CFX) resistentsuse määramisega.
- Osatöö II** Hinnata CT korratavust ja vastuste objektiivsust testi juurutamisel uutesse oludesse määrates ravimite tundlikkust MTB puhaskultuuridest.
- Osatöö III** Määrata CT tulemuslikkus, diagnostiline võimekus ja objektiivsus otsese ravimitundlikkuse ja MTB samastamismeetodina, kasutades Eesti patsientide kopsumaterjale nagu röga, bronhiloputusvedelik ja pleuravedelik.
- Osatöö IV** Leida tandemkorduste muutuva mustri (ingl. k. variable numbers of tandem repeat, VNTR) meetodi põhjal MTB tüvede klastrid ja hinnata tuberkuloosi leviku riskifaktoreid Eestis.

UURINGUTE METOODIKA JA VALIM

CT diagnostilise täpsuse hindamine ehitati üles pimestatud mitmeetapilise uurin-guna. Osatöodes I ja II võrreldi retrospektiivselt Eestis aastatel 2004–2014 isolee-ritud MTBC isolaatide CT tassidega saadud ravimitundlikkuse tulemusi rutiinselt BACTEC MGIT süsteemiga saadud ravimitundlikkuse tulemustega. Osatöö II hindas lisaks veel CT kordustäpsust ja hindajatevahelist erinevust. Selleks viidi töö osa II läbi kuuekordse uuringuvalimiga, mis koosnes 150 MTBC isolaadist, mille ravimitundlikkusi hindasid CT tassidel kahes laboris (SA Tartu Ülikooli Kliinikumi Ühendlabori Mükobakterioloogia laboris ja Põhja-Eesti Regionaal-haigla Laboratooriumi Mükobakterioloogia laboris) kuus varasema kogemusega bioanalüütikut. Osatöö III oli prospektiivne uuring, mis hindas CT diagnostilist täpsust, aega tulemuseni ja võimet tuvastada MTBC otse kopsumaterjalidest võrreldes paralleelselt saadud rutiinsete TB laboratoorse diagnostika meetodite tulemustega. Isikud, kes osalesid osatöös III ja andsid selleks informeeritud nõus-oleku, olid TB või mükobakterioosi kahtluse, sümptomite või diagnoosiga täis-kasvanud, kes pöördusid pulmonoloogi vastuvõtule, kuid ei olnud veel ravi alus-tanud või oli ravi algusest möödas vähem kui üks kuu. Osatöö IV oli retrospek-tiivne läbilõikeuuring, mis kasutas üleriigiliselt aastatel 2009–2012 kogutud TB patsientide andmeid, kelle proovimaterjalist oli varasemalt isoleeritud ravimi-tundlikkuse ja VNTR tüpiseerimise tulemustega MTBC kultuur. Klastriks loeti gruppi, mis koosnes vähemalt kahest identse VNTR mustriga MTBC tüvest.

Uuringute andmebaasid ja statistiline analüüs

Osatööde I–III andmebaasidesse kogutud andmestik koosnes rutiinsetest retro-spektiivsetest või prospektiivselt omandatud TB laboratoorse diagnostika tulemus-test, mis lingiti CT tassidelt saadud tulemustega. Osatöö IV andmestik, mis koos-nes Eesti Tuberkuloosiregistrilt küsitud patsiendiandmetest, vastavatest ravimi-tundlikkuse andmetest ja nende MTBC tüvede VNTR mustritest, koguti EpiData andmebaasi.

CT tundlikkus ja spetsiifilisus ravimitundlikkuse hindamisel ja MTBC tuvastamisel arvutati kasutades STATA, IBM SPSS ja MS Excel-i tarkvararakendusi. CT kordustäpsust ja lugejatevahelist erinevust hinnati Fleiss'i kapp statistikuga, mille puhul loetakse väärtused üle 0,8 suurepäraseks. Selleks, et osatöös III võrrelda erinevate meetodite MTBC tuvastamise võimet ja aega ravimitundlikkuse tulemuseni materjali kogumisest, kasutati Mann-Whitney U-testi. Erinevate meetodite, ravimite ja proovimaterjali tüüpide mõju MTBC tuvastamise ja/või ravimitundlikkuse tulemuste saamise kiirusele uuriti üldistatud lineaarse mudeliga.

TB nakkuse leviku ja patsientide sotsiaalmajanduslike ning kliiniliste näitajate vaheliste seoste leidmiseks osatöös IV kasutati EpiData andebaasi. Statistilise analüüsi programmi R "meta" ja "ggplot2" lisadega kasutati osatööde II ja III jooniste tegemiseks.

UURINGUTE PEAMISED TULEMUSED

Üle 70% (n=246) osatöodes I ja II käsitletud 351 MTBC isolaadist olid multiravimresistentsed. Nendest omakorda üle viiendiku ehk 51 MTBC isolaati olid lisaks veel resistentsed ka ühele fluorokinoloonile ja ühele süstitavatest ravimitest (amikatsiinile, kanamütsiinile või kapreomütsiinile) kuuludes kuni 2021. aastani kehtinud MTO definitsiooni järgi eriti ravimresistentsete MTBC tüvede hulka. Uuringu prospektiivses faasis osales 156 isikut, kellelt koguti uuringuks ja rutiinseteks analüüsideks kokku 177 proovimaterjali (147 röga, 27 bronhiloputusedelikkku ja 3 pleuravedelikkku).

Kolme uuringu jooksul (I–III) valmistasid, külvasid ja hindasid seitse erinevat bioanalüütikut kahes mükobakterioloogia laboris kokku 1525 CT tassi (vastavalt 402, 943 ja 180 osatöodes I, II ja III). Kogu uuringuperioodi jooksul ei tuvastatud probleeme kontrollide tegemisel, söötme reostumisega, ega ka CT tassidele külvamisel ja tulemuste interpreteerimisel.

Osatöodes I ja II võrreldi kokku 2882 CT tassilt saadud ravimitundlikkuse tulemust BACTEC MGIT ravimitundlikkuse tulemustega (vastavalt 197 tulemust osatöös I ja 2685 tulemust osatöös II). CT koondtundlikkus ravimitundlikkuse määramisel LVX (CFX) suhtes oli 95,2% ja 96% ning 97,2% vastavalt INH ja RIF suhtes. Mediaanaeg aastatel 2010–2014 teostatud BACTEC MGIT ravimitundlikkuse tulemuseni oli 12 päeva (IQR 9–15 päeva) esimese rea ravimite (isoniasiid ja rifampitsiin) kohta ning 13 päeva (kvartiilidevahemik [IQR] 10–15 päeva) reservrea ravimite (fluorokinoloonid, aminoglükosiidid jt.) kohta. Mediaanaeg ravimitundlikkuse tulemuseni kasutades CT oli 14 päeva (IQR 12–14) (I) või 13 päeva (IQR 11–15) (II).

Kõik CT ravimitundlikkuse tulemused Töö osas II laboris A kattusid (Fleiss'i kapp 1,0). Hindajatevaheline ravimitundlikkuse tulemuste kokkulangevus ehk Fleiss'i kapp laboris B oli 0,99 INH ja RIF suhtes ning 0,97 LVX suhtes. CT kordustäpsust määrati 150 MTBC isolaadi ravimitundlikkuse tulemuste 18 kordse hindamisega. Isolaadipõhiseks kokkulangevuseks Fleiss'i kapp statistikuga saadi 0,95, 0,96 ja 0,92 vastavalt INH, RIF ja LVX jaoks.

Töö prospektiivse faasi käigus (III) võrreldi CT, LJ ja MGIT kiirust ja täpsust MTBC detekteerimisel erinevatest kopsumaterjalidest. Kokku tuvastati 83 MTBC-positiivset materjali. Erinevate söötmete tundlikkuseks MTBC identifitseerimisel saadi 81,3% (95% usaldusintervall [CI] 71,0–89,1), 93,5% (95% CI 85,5–97,9) ja 81,7% (95% CI 70,7–89,9), vastavalt CT, MGIT ja LJ jaoks. Aeg MTBC tuvastamiseni sõltumata algmaterjalist oli statistiliselt oluliselt aeglasem CT meetodit kasutades kui BACTEC MGIT süsteemiga, vastavalt 20 (IQR 14,5–27) päeva ja 13 (IQR 7–18) päeva ($p < 0,001$). Samas ei erinenud oluliselt aeg MTBC tuvastamiseni CT ja LJ meetodite vahel olles LJ meetodi jaoks 18 (IQR 12,0–23,3) päeva ($p = 0,29$).

CT meetodiga isoleeriti uuringu prospektiivses faasis 67 MTBC kultuuri, millest üheksa puhul (13,4%) ei olnud võimalik ravimitundlikkusi hinnata, kuna kontrollsektoris kasvas alla 10 MTBC koloonia. Ülejäänud isoleeritud kultuuride ravimitundlikkuse tulemuste kokkulangevus CT ja BACTEC MGIT vahel oli INH, RIF ja LVX jaoks vastavalt 98,2% (95% CI 90,6–100,0), 96,4% (95% CI 87,7–99,6) ja 94,3% (95% CI 84,3–98,8). CT tundlikkus ravimitundlikkuse testimisel otse algmaterjalist võrreldes BACTEC MGIT meetodiga oli 100% INH (95% CI 83,2–100,0) ja LVX (95% CI 15,8–100,0) korral ning 94,7% (95% CI 74,0–99,9) RIF puhul. Aeg materjali kogumisest ravimitundlikkuse tulemuseni oli CT otsest meetodit kasutades oluliselt kiirem kui BACTEC MGIT meetodiga, kus ravimitundlikkuse teostamiseks vajatakse eelnevalt puhaskultuuri. Vastavalt võttis ravimitundlikkus INH ja RIF suhtes CT otsest meetodit kasutades 20 päeva (IQR 14–26,75) võrreldes BACTEC MGIT 24,5 (IQR 18,25–32) päevaga, $p = 0,029$. Ravimitundlikkuse hinnang LVX suhtes võttis CT otsest meetodit kasutades samuti vähem aega kui BACTEC MGIT süsteem, vastavalt 19 (IQR 13–22) ja 29,5 (23–40,25) päeva, $p < 0,001$.

Eestis aastatel 2009–2012 isoleeritud MTBC tüvede liine ja levikumustreid uuriti Töö osas IV. Selleks kasutati retrospektiivselt 912 MTBC tüve VNTR mustreid. Kõige suurem osa MTBC tüvedest kuulus Beijing liini ($n=357$; 39,2%). Samasse fülogeneetilisse gruppi (Beijing) kuulusid ka enamus Eestis isoleeritud MDR ja XDR-TB tüved, vastavalt (94,0% ja 96,3%). Ravimitundlikud tüved jagunesid nelja enamlevinud fülogeneetilise liini vahel ühtlaselt (Beijing [16,8%], Haarlem [27,9%], Latin American-Mediterranean [21,8%] ja Ural [12,0%]).

Klastritesse kuulus 630 (69,1%) osatöö IV valimis olnud MTBC tüvedest. Neli suuremat klastrit koosnesid üle 20 MTBC tüvest ja suurim klaster sisaldas 178 identse VNTR mustriga MTBC tüve. Suurim klaster pärines Beijing liinist ja 164 (92,1%) sinna kuuluvatest tüvedest olid vähemalt MDR.

Osatöös IV leiti, et MTBC leviku riski tõstvateks faktoriteks on vene rahvus, kodutus, alkoholi kuritarvitamine, vangistus, ravimresistentsus ja HIV-nakkus. Sealjuures oli XDR-TB tüvedel kõrgeim risk klasterduda (riskide suhe 1,63; $p < 0,001$). MTBC leviku riski vähendavateks faktoriteks osutus kõrge vanus, Ukrainas sündimine, valgevene rahvus, Tartus elamine, madal haridustase, kopsuväline TB.

JÄRELDUSED

Käesoleva väitekirja järeldused on järgmised:

- Varasema kogemusega bioanalüütik on võimeline edukalt CT metoodika õppima paari päeva jooksul. Lisaks on MTB puhaskultuuridest CT meetodiga saadud ravimitundlikkuste tulemused automaatse referentsmeetodiga võrreldes soodsamad, sama kiired ja niisama täpsed.
- CT juurutamisel mükobakterioloogia laboritesse ei leitud ühtegi kitsaskohta. CT hinnangud on korratavad nii erinevate hindajate kui ka korduskülvide vahel.
- Eri sorti kopsumaterjalide külvil CT tassile ei tuvastatud ühtegi takistust. CT kasutamine otsese ravimitundlikkuse meetodina on kiirem kui rutiinselt kasutusel olev fenotüübiline ravimitundlikkuse referentsmeetod.
- Eestis enamlevinud MTB tüvi pärineb Beijing liinist. Eestis levinud MTB tüved, aga eriti just MDR tüved, klasterduvad VNTR meetodi põhjal olulisel määral. Tuberkuloosi leviku riskifaktoriteks on antud uuringu järgi alkoholi kuritarvitamine, kinnipidamisasutuses viibimine, kodutus, polüresistentne, MDR või XDR-TB ja HIV nakkus.

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PUBLICATIONS

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List of publications:

- Klaos, K;** Agejeva, A; Hurt, K; Kummik, T; Kurve, A; Nirk, J; Pehme, L; Remets, O; Sasi, S; Tann, A; Altraja, A. (2024). Prospective evaluation of thin-layer agar colour test in routine diagnosis of multidrug-resistant TB. The International Journal of Tuberculosis and Lung Disease, 28 (8), 387–394. DOI: 10.5588/ijtld.23.0536.
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Teadustöö:

Teadustöö põhisuunaks on mükobakterite laboratoorse diagnostika arengud nii geneetilise kui ka traditsioonilise mikrobioloogia suunal.

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DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS

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