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REDOX DYES IN THE DETECTION OF MYCOBACTERIAL VIABILITY

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ABSTRACT

Jenni Niemi: Redox dyes in the detection of mycobacterial viability
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In this master's thesis, I optimize the conditions of use of redox dyes to detect the viability of mycobacteria. *Mycobacterium tuberculosis* causes tuberculosis that infects millions of people every year. In 2019, 1.4 million people died of tuberculosis infection. *M. tuberculosis* is tolerant to several antibiotics, and therefore its investigation is important.

M. tuberculosis research can utilize *Mycobacterium marinum*, which is genetically very close to *M. tuberculosis*. In *in vitro* cultures, both *M. tuberculosis* and *M. marinum*, form biofilms containing antibiotic-tolerant persisting bacteria. In addition to bacteria, biofilms consist of extracellular polymeric material.

Redox staining of mycobacteria was studied with two different redox dyes, resazurin and 2,3,5-triphenyltetrazolium chloride (TTC). Metabolism of living cells reduces blue resazurin to pink, fluorescent resorufin. The yellowish colour of TTC, on the other hand, turns red by the cell metabolism as it is reduced to insoluble triphenylformazane crystals. Different concentrations of dyes and different measurement time points were optimized. Initial optimizations were performed using wild-type *M. marinum*. In optimized conditions, resazurin staining was used in the minimum duration of killing assay (MDK) of *M. marinum* and the sensitivity of resazurin staining was compared to bioluminescence from luciferase over-expressing *M. marinum* strain (MM-lux). Finally, resazurin staining was used to study the development of antibiotic tolerance in biofilms of avirulent *M. tuberculosis*.

A suitable protocol was found for resazurin staining of mycobacteria. Resazurin staining was found to act reliably with *M. tuberculosis* as well as with longer antibiotic exposures with *M. marinum*. In contrast, TTC staining of mycobacteria did not give reliable results under the conditions studied.

Based on resazurin staining, biofilm cultures of *M. tuberculosis* were found to be more tolerant to the antibiotic exposure than planktonic cultures. In biofilm cultures, the level of tolerance remained similar between five- and 12-day old biofilms.

Based on the results, resazurin staining is a rapid and promising way to study the viability of mycobacteria in samples, but it is less sensitive than for example luminescence-based detection methods. If it is not possible to measure the fluorescence or luminescence from the samples, the viability of the bacteria can also be detected visually by colour change.

Keywords: mycobacteria, resazurin, TTC, *M. tuberculosis*, *M. marinum*, tuberculosis
The originality of this thesis has been checked using the Turnitin OriginalityCheck service.

TIIVISTELMÄ

Jenni Niemi: Redox-väriaineet mykobakteerien elinkyvyn havaitsemisessa
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Tässä pro gradu -tutkielmassa optimoin redox-väriaineiden käyttöolosuhteita mykobakteerien elinkyvyn havaitsemiseksi. *Mycobacterium tuberculosis* infektoi miljoonia ihmisiä vuosittain ja aiheuttaa tuberkuloosia. Vuonna 2019 1,4 miljoonaa ihmistä kuoli tuberkuloosi-infektioon. *M. tuberculosis* on tolerantti useille antibiooteille, ja tämän vuoksi sen tutkiminen onkin tärkeää.

M. tuberculosis-bakteerin tutkimuksissa voidaan hyödyntää *Mycobacterium marinum*-bakteeria, joka on geneettisesti hyvin lähellä *M. tuberculosis*-bakteeria. *In vitro*-kasvatuksissa molemmat, sekä *M. tuberculosis* että *M. marinum*, muodostavat biofilmejä, jotka sisältävät antibiooteille toleranteja bakteereja. Bakteerien lisäksi biofilmit koostuvat solunulkoisesta polymeerisestä aineesta.

Mykobakteerien redox-värijäystä tutkittiin kahdella eri redox-väriaineella, resatsuriinilla sekä 2,3,5-trifenyylitetratsoliumkloridilla (TTC). Elävien solujen metabolia pelkistää sinisen resatsuriinin vaaleanpunaiseksi, fluoresoivaksi resorufiiniksi. TTC:n kellertävä väri sen sijaan muuttuu punaiseksi solujen metabolian vuoksi, kun se pelkistyy liukenemattomiksi trifenyyliformatsaanikiteiksi. Eri väriainepitoisuudet ja mittausajankohdat optimoitiin. Alustavat optimoinnit suoritettiin käyttämällä villityypin *M. marinum*-bakteeria. Optimoiduissa olosuhteissa resatsuriinivärijäystä käytettiin *M. marinum*-bakteerin toleranssin testauksessa (minimum duration of killing assay (MDK)), ja resatsuriinivärijäyksen herkkyyttä verrattiin lusiferaasia ilmentävän *M. marinum*-kannan (MM-lux) bioluminesenssiin. Lopuksi resatsuriinivärijäystä käytettiin tutkimaan antibioottitoleranssin kehittymistä avirulentin *M. tuberculosis*-bakteerin biofilmeissä.

Mykobakteerien resatsuriinivärijäykselle löydettiin sopiva protokolla. Resatsuriinivärijäyksen havaittiin toimivan luotettavasti *M. tuberculosis* -bakteerin kanssa sekä pidemmällä antibioottialtistuksilla *M. marinum*-bakteerien kanssa. Sen sijaan mykobakteerien TTC-värijäys ei antanut luotettavia tuloksia tutkituissa olosuhteissa.

Resatsuriinivärijäyksen perusteella *M. tuberculosis*-bakteerin biofilmikasvatusten havaittiin olevan planktonista kasvatusta tolerantimpia antibiootille. Biofilmiviljelmissä toleranssi pysyi samanlaisena viiden ja 12 päivän ikäisten biofilmien välillä.

Tulosten perusteella resatsuriinivärijäys on nopea ja lupaava tapa tutkia mykobakteerien elinkelpoisuutta näytteissä, mutta se ei ole yhtä herkkä kuin esimerkiksi luminesenssiin perustuvat detektiomenetelmät. Jos näytteistä ei ole mahdollista mitata fluoresenssia tai luminesenssiä, bakteerien elinkyky voidaan havaita myös visuaalisesti värimuutoksella.

Avainsanat: mykobakteerit, resatsuriini, TTC, *M. tuberculosis*, *M. marinum*, tuberkuloosi
Tämän julkaisun alkuperäisyys on tarkastettu Turnitin OriginalityCheck –ohjelmalla.

PREFACE

This thesis study was conducted at Faculty of Medicine and Health Technology, Tampere University, with the Infection Biology research group. I am grateful to the leader of the group Matalena Parikka, that I got the opportunity to work in this magnificent research group and got very interesting topic to my master's thesis. Many thanks also to Saara Lehmusvaara, from whom I received a lot of support and guidance while working in the laboratory and writing a master's thesis. Thanks also to all the other members of the research group that I got to know and from whom I got help whenever needed.

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Nokia, December 3rd, 2020

Jenni Niemi

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LIST OF SYMBOLS AND ABBREVIATIONS

BCG	<i>Mycobacterium bovis</i> bacilli Calmette-Guérin
EPS	Extracellular polymeric substance
MDK	Minimum duration of killing
MIC	Minimum inhibitory concentration
<i>M. marinum</i>	<i>Mycobacterium marinum</i>
<i>M. tuberculosis</i>	<i>Mycobacterium tuberculosis</i>
NTM	Nontuberculous mycobacteria
OD	Optical density
PE	Proline-glutamine
PPE	Proline-proline-glutamine
TNF- α	Tumor necrosis factor alpha
TTC	Triphenyl tetrazolium chloride
wt	Wild type

1. INTRODUCTION

Tuberculosis is caused by *Mycobacterium tuberculosis*. In 2019 about 10 million people were infected with *M. tuberculosis* and 1.4 million (including 208 000 people with HIV) died from tuberculosis. One of the health targets of the Sustainable Development Goals is ending the TB pandemic by 2030. (World Health Organization, 2020.) However, highly drug-resistant forms of tuberculosis, such as extensively drug-resistant tuberculosis (XDR), which is resistant to rifampicin and isoniazid, among others, and multidrug-resistant tuberculosis (MDR), threaten to achieve this goal (Conradie *et al.* 2020).

Tuberculosis is treated with prolonged multi-antibiotic therapy. Treatment of MDR-TB is difficult and its treatment duration in 2015 was normally 1.5 to 2 years. (Seung, Keshavjee, and Rich, 2015.) The development of tuberculosis research and drug research is therefore important to make treatment shorter.

M. marinum, which is virulent in ectotherms, can be used as a model organism for *M. tuberculosis*. *M. marinum* and *M. tuberculosis* have 3,000 similar genes with each other and have an amino acid identity of 85 %. In addition to this, *M. marinum* is a biosafety level 2 (BSL2) organism, so it is also safer to use than BSL3 *M. tuberculosis*. (Tükenmez *et al.* 2019.) BSL2 contains human disease agents that pose a reasonable health risk, while BSL3 contains agents that can cause very serious or even fatal diseases (Vaselek *et al.*, 2020).

Both non-tuberculous mycobacteria, such as *M. marinum*, and tuberculous mycobacteria, such as *M. tuberculosis*, form biofilms. Understanding the biofilms formed by *M. tuberculosis* opens new possibilities and perspectives for the treatment of tuberculosis but further research is required. (Chakraborty and Kumar, 2019, and Esteban and Garcia-Coca, 2018.)

Redox dyes, like resazurin and 2,3,5-triphenyltetrazolium chloride (TTC), can be used to assess bacterial viability. Resazurin is an oxidation-reduction indicator, and its change in color from blue to pink indicates bacterial growth and reduction of resazurin. (Palomino *et al.* 2002.) Previous studies have shown 2,3,5-triphenyltetrazolium chloride (TTC) to be a good alternative for testing the susceptibility of *M. tuberculosis* (Shinu, Singh, and Nair, 2016 and Mohammadzadeh *et al.* 2006). TTC is technically easy to use, cost effective, and is not time consuming. The yellow dye of TTC is reduced in living cells and produces red insoluble TTC formazan crystals. (Mohammadzadeh *et al.* 2006.)

2. LITERATURE REVIEW

2.1 Mycobacteria

Mycobacteria are bacteria that make up the genus *Mycobacterium*. Pathogens of this genus can cause diseases in humans and animals. *Mycobacterium tuberculosis*, which causes tuberculosis, is the best-known mycobacterium. The genus *Mycobacterium* includes 188 currently identified species. (O'Neill, Mortimer, and Pepperell, 2015; Gupta, Lo, and Son, 2018, and Smith, 2003.)

Mycobacteria can be divided into: *M. tuberculosis* complex, *M. leprae* and non-tuberculous mycobacteria (NTM), and they can be also divided based on their growth rate into fast-growing and slow growing (van Ingen *et al.*, 2012, Pullan *et al.*, 2016 and Porvaznik, Solovic, and Mokry, 2016). Fast-growing mycobacteria grow a visible culture from dilute inoculum in less than seven days and include for example *M. abscessus*, *M. smegmatis*, and *M. fortuitum*. Slow-growing mycobacteria, on the other hand, take more than seven days to grow the visible culture and include for example *M. celatum*, *M. tuberculosis*, and *M. kansasii*. (Stahl and Urbance, 1990; Helguera-Repetto *et al.*, 2014 and Porvaznik, Solovic, and Mokry, 2016.) *M. marinum* is often classified in the literature as slow growing, although it grows relatively rapidly compared to *M. tuberculosis* (Porvaznik, Solovic, and Mokry, 2016, and Stinear *et al.*, 2008). The distribution time of *M. marinum* is about four to six hours, while that of *M. tuberculosis* is more than 20 hours (Stinear *et al.*, 2008). In addition to slow-growing *M. tuberculosis*, the *M. tuberculosis* complex includes *M. microti*, *M. mungi*, *M. canettii*, *M. pinnipedii*, *M. bovis*, *M. africanum*, and *M. caprae* (van Ingen *et al.*, 2012). Examples of NTM are *M. chelonae*, *M. fortuitum*, *M. marinum*, *M. abscessus* and *M. avium* (Helguera-Repetto *et al.*, 2014).

Mycobacteria belonging to the *M. tuberculosis* complex cause tuberculosis in humans, but NTM also causes diseases. NTM can cause, for example, pulmonary disease as well as skin and soft tissue infections. (Kotilainen *et al.*, 2014; Simeone *et al.*, 2012 and Helguera-Repetto *et al.*, 2014.)

Recently, Gupta, Lo, and Son (2018) have made their own proposal for the division of species of the genus *Mycobacterium* based on genomic analyzes of 150 genomes as well as comprehensive phylogenomics. Based on the results, they would divide the species of the genus *Mycobacterium* into five groups, which are: "*Fortuitum-Vaccae*", "*Abscessus-Chelonae*", "*Terrae*", "*Triviale*", and "*Tuberculosis-Simiae*" clades. The "*Fortuitum-Vaccae*" and "*Abscessus-Chelonae*" clades contain fast-growing

species, and the "*Terrae*", "*Triviale*", and "*Tuberculosis-Simiae*" clades contain slow-growing mycobacterial species.

Mycobacteria have an outer membrane that is likely to contain the machinery required to produce biofilm (Niederweis *et al.*, 2010). For example, *M. abscessus* produces a glycopeptidic biofilm that can cover its surface lipids and make it more difficult to phagocytose delaying their uptake into macrophages (Helguera-Repetto *et al.*, 2014). In addition, the mycobacteria are acid-fast, which means that they can create a hydrophobic barrier that resists the decolorization process (Gupta, Lo, and Son, 2018 and Hettick *et al.*, 2006).

2.2 *Mycobacterium tuberculosis*

Mycobacterium tuberculosis causes tuberculosis in humans and is the deadliest human pathogen in the World. Features of *M. tuberculosis* include the ability to cause a latent infection, the ability to regenerate its metabolism during chronic infection, and a thick and waxy cell wall. (Gordon and Parish, 2018; Namouchi *et al.*, 2012, and Sharma and Bisht, 2017.) *M. tuberculosis* can be transmitted as an aerosol infection that can lead to chronic infection if *M. tuberculosis* persists in the host for a long time. However, not all aspects of chronic tuberculosis infection are yet fully understood. (Abomoelak *et al.*, 2011, and Seiler *et al.*, 2003.) To this end, several *in vitro* and *in vivo* models have been developed to elucidate the mechanisms of *M. tuberculosis* in the host (Gordon and Parish, 2018 and Abomoelak *et al.*, 2011).

M. tuberculosis was discovered by Robert Koch in 1882. It has been a human pathogen for a long time, according to coalescing analyzes, up to 70,000 years. (Gordon and Parish, 2018; Smith, 2003; Cambau and Drancourt, 2014, and Comas *et al.*, 2013.) The oldest evidence that *M. tuberculosis* has been a human pathogen for a long time is the 9000-year-old human remain discovery from the Eastern Mediterranean (HersHKovitz *et al.*, 2008, and Cambau and Drancourt, 2014). Most bacteria of *M. tuberculosis* infection are metabolically inactive or replicate slowly, leaving the infection latent in most cases. It is estimated that about a third of the world's population has a latent infection. (Gordon and Parish, 2018, and Helguera-Repetto *et al.*, 2014.) Infection usually results in the formation of granulomas (Gordon and Parish, 2018; Seiler *et al.*, 2003, and Helguera-Repetto *et al.*, 2014).

Vaccine and antibiotic treatments have been developed against *M. tuberculosis*, but it is still one of the most life-threatening pathogens of all time (Cambau and Drancourt, 2014; Simeone *et al.*, 2012, and Smith, 2003). Immunocompromised individuals, such as people with HIV, are highly susceptible to tuberculosis especially in

developing countries (Harries *et al.*, 2010; Simeone *et al.*, 2012, and Helguera-Repetto *et al.*, 2014).

2.2.1 Infection mechanism of *M. tuberculosis*

The infection with *M. tuberculosis* is special in that most of infected have latent state and it can be so for decades (Gordon and Parish, 2018; Houben and Dodd, 2016, and Lindenstrøm *et al.*, 2013). About 10% of latently infected individuals develop tuberculosis later (Lindenstrøm *et al.*, 2013).

M. tuberculosis infection is transmitted in most cases by inhalation (Concepcion *et al.*, 2017). Infection begins when inhaled aerosols of *M. tuberculosis* are deposited in the alveoli (Concepcion *et al.*, 2017; Scordo *et al.*, 2019, and Smith, 2003). In many cases of tuberculosis, the progression of tuberculosis can be divided into four different stages (Smith, 2003). In the first stage, which is about three to eight weeks after infection, the bacterium spreads to the lymph nodes and forms a so-called Ghon complex (Lin *et al.*, 2006, Concepcion *et al.*, 2017 and Smith, 2003). In the first stage, there is also a conversion to tuberculin reactivity, and during this stage, a probable tuberculosis infection can be tested with a tuberculin skin test (Concepcion *et al.*, 2017, and Smith, 2003). Calcification caused by the Ghon complex can also be seen on radiographs (Concepcion *et al.*, 2017, and Tomà *et al.*, 2017).

The second stage of tuberculosis infection lasts about three months. During this time, the bacteria pass to other parts of the lungs. The third stage of infection lasts three to seven months, but there can be a gap of up to two years between this and the second stage. In the third stage, pleurisy, or inflammation of the surfaces of the pleura often occurs, causing chest pain. (Smith, 2003.)

In the fourth or final stage, the resolution of the Ghon complex occurs. This stage can last for three years, during which time some people develop slowly developing extrapulmonary lesions, which can present as chronic back pain, for example. (Smith, 2003.) However, in most people with tuberculosis, the disease does not progress to active state, but remains in an asymptomatic, latent state (Wiggington and Kirschner, 2001, and Houben and Dodd, 2016). Tuberculosis persists in most individuals in latent state due to the effective immune response of the individual (Wiggington and Kirschner, 2001; Smith, 2003, and Lin *et al.*, 2006). The infection can remain latent for years or decades, but it can also become activated later (Lin *et al.*, 2006, and Lin *et al.*, 2014). However, in most individuals, the infection remains latent and does not reactivate later. (Wiggington and Kirschner, 2001).

M. tuberculosis is an intra- and extracellular pathogen capable of avoiding lysosomal fusion by manipulating host signaling pathways (Kotilainen *et al.*, 2014; Smith, 2003, and Simeone *et al.*, 2012). When *M. tuberculosis* is present in alveolar passages, the bacteria are phagocytosed by dendritic cells as well as alveolar macrophages (Simeone *et al.*, 2012, and Saini *et al.*, 2018). Dendritic cells are important in the early stages of infection and are better antigen presenters than macrophages. They are also likely to play an important role in the spread of *M. tuberculosis*. (Smith, 2003.) During tuberculosis infection, *M. tuberculosis* induces CCL2 production and thereby infects more CCR2-dependent permissive macrophages (Arias *et al.*, 2007, and Tang, Yam, and Chen, 2016). Granuloma then forms in the lungs, which is considered one of the hallmarks of tuberculosis. There are several different animal models for modeling granulomas, such as zebrafish and guinea pig models. (Tang, Yam, and Chen, 2016, and Singh and Gupta, 2018.)

The granuloma is thought to be a physical barrier that protects the host. The outer layer of granuloma consists of collagen and other components of the extracellular matrix. It is thought to prevent neighboring tissues from becoming infected. (Tang, Yam, and Chen, 2016.) Later during infection, the granuloma may shrink and calcify, indicating a successful response from the immune system (Tang, Yam, and Chen, 2016, and Flynn *et al.*, 2011). However, there is relatively little information on how the bacterium survives and grows in the later stages of infection. It is known that granulomas can heal later but leave small, calcified lesions. *M. tuberculosis* can replicate uncontrollably if the infected person is unable to control the initial infection in the lungs or if the immune system of the person carrying latent tuberculosis is weakened for any reason. In this case, *M. tuberculosis* can spread from the granuloma to the lungs and other tissues, making the person carrying the infection infectious and the disease must be treated with antibiotics. (Smith, 2003.)

The immune responses elicited by the host of *M. tuberculosis* infection can stop the progression of the disease, but they rarely kill the bacteria completely (Lindenstrøm *et al.*, 2013, and Saini *et al.*, 2018). Therefore, the vaccines to be developed must be effective and able to operate in complex conditions caused by, for example, regulatory signals for tuberculosis infection (Lindenstrøm *et al.*, 2013, and Singh and Gupta, 2018). By understanding how the host immune system is involved in preventing inflammation and granuloma formation from *M. tuberculosis*, more effective treatments for tuberculosis can be devised (Nagalingam *et al.*, 2017).

2.2.2 Virulence of *M. tuberculosis*

To better understand the formation of chronic infection with *M. tuberculosis*, it is also necessary to understand the regulation of its virulence at the molecular level (Abomoelak *et al.*, 2011, and Namouchi *et al.*, 2012). Different strains of *M. tuberculosis* have different virulence, some strains are more virulent than others (Smith, 2003, and Reiling *et al.*, 2013). The virulence of *M. tuberculosis* can be described by "mortality", "morbidity", and bacterial load. Mortality refers to the proportion of infected people who die and at what time after infection death occurs. Bacterial load refers to the number of bacteria in the host after infection. *M. tuberculosis* does not have classical virulence factors such as toxins formed by *Escherichia coli*. (Smith, 2003.) The cell wall structure of *M. tuberculosis* provides a particularly strong impermeable barrier to drugs, for example, and plays an essential role in virulence (Niederweis *et al.*, 2010, and Smith, 2003).

Protein secretion systems are important virulence factors for *M. tuberculosis*, and it has five ESX type VII secretion systems. These secretion systems are involved in the secretion of proline-glutamine (PE) and proline-proline-glutamine (PPE) proteins, among other things. (Gordon and Parish, 2018, and Ates and Brosch, 2017.) PE and PPE proteins are involved in, among other things, lipid metabolism and cell wall architecture (Gordon and Parish, 2018). ESX-1 is required to allow virulence of *M. tuberculosis* and it secretes important virulence factors such as ESAT-6 as well as CFP-10 (Niederweis *et al.*, 2010, and Ates and Brosch, 2017).

The virulence and various virulence factors of *M. tuberculosis* can be studied, for example, in animal models and by using macrophages and dendritic cells (Smith, 2003, and Reiling *et al.*, 2013). Protein virulence factors for *M. tuberculosis* include HspX, ESAT-6/CFP-10, 19 kD protein, and glutamine synthesis (Smith, 2003; Abomoelak *et al.*, 2011, and Niederweis *et al.*, 2010). Overexpression of HspX inhibits the growth of *M. tuberculosis*, and therefore HspX is an important regulator of latency and persistence (Smith, 2003, and Spratt, Britton, and Triccas, 2010). ESX-1-secreted proteins ESAT-6 and CF-10 are found in culture filtrates of *M. tuberculosis* and are immunodominant antigens (Smith, 2003; Harries *et al.*, 2010, and Niederweis *et al.*, 2010). The 19-kDa lipoprotein is an immunodominant antigen recognized, for example, by T cells in patients with *M. tuberculosis* infection (Smith, 2003, Liu *et al.*, 2015, and Abomoelak *et al.*, 2011). The presence of glutamine synthesis in *M. tuberculosis* culture filtrates is likely due to cell lysis (Smith, 2003).

M. tuberculosis' cell surface components such as erp and MmpL7 are also virulence factors (Smith, 2003, and Forrellad *et al.*, 2013). Of these, the function of erp is still unknown, but it is not found in non-pathogenic mycobacteria (Smith, 2003). MmpL7

is an important virulence factor because it is required, inter alia, in the transport of phthiocerol dimycocerosates (PDIM) (Smith, 2003, Forrellad *et al.*, 2013, and Niederweis *et al.*, 2010).

One of the cytokines necessary to control *M. tuberculosis* infection is tumor necrosis factor alpha (TNF- α) (Smith, 2003, and Arias *et al.*, 2007). However, it has been observed in mice that when TNF- α is present in large amounts, severe dermatitis and death occur. In contrast, if the mouse is unable to produce or react with TNF- α , granulomas to prevent the spread of bacteria cannot form. It has also been studied that apoptosis in *M. tuberculosis* macrophage infection is dependent on TNF- α . (Smith, 2003.)

2.2.3 Treatment and prevention of *M. tuberculosis*

Mycobacterium bovis bacille Calmette-Guérin vaccine (BCG), the only approved vaccine against *M. tuberculosis* infection was developed about a hundred years ago (Spratt, Britton, and Triccas, 2010, and Tang, Yam, and Chen, 2016). However, this vaccine does not provide protection against *M. tuberculosis* infection and provides protection from about 0 to 80 % for adults against pulmonary tuberculosis disease, depending on the continent and environmental mycobacteria (Moliva *et al.*, 2018; Roy *et al.*, 2014, and Tang, Yam, and Chen, 2016). The BCG vaccine protects children against severe tuberculosis disease in about 60 to 80% of cases, but has little effect on adult pulmonary disease, and the protection it provides lasts only about 10 to 15 years in humans (Moliva *et al.*, 2018; Fletcher, 2016; Roy *et al.*, 2014, and Abubakar *et al.*, 2013). Therefore, the development of more advanced tuberculosis vaccines, such as an improved whole organism vaccine or a vaccine to enhance the efficacy of the current vaccine, is needed (Moliva *et al.*, 2018, and Roy *et al.*, 2014). The effectiveness of BCG varies geographically and depends whether the vaccine is given before or after infection, and whether the person to be vaccinated has exposed to environmental mycobacteria (Roy *et al.*, 2014; Schaible *et al.*, 2017, and Tang, Yam, and Chen, 2016).

Tuberculosis has been treated with combination therapies since the 1950s, when antibiotics were developed and the first effective treatment for tuberculosis was found (Murray, Schraufnagel, and Hopewell, 2015). Tuberculosis is still treated with combination therapy and the effective treatment of tuberculosis lasts for at least six months and uses isoniazid, rifampicin, ethambutol, and pyrazinamide (Murray, Schraufnagel, and Hopewell, 2015; Suthar *et al.*, 2012, and Horsburgh, Barry, and Lange, 2015). However, this treatment can last up to 1.5 years (Horsburgh, Barry, and Lange, 2015). Recently, however, a promising form of treatment for tuberculosis has

been discovered that shortens the treatment period for tuberculosis to four months (Dorman and Nahid, 2020). The treatment uses a combination of four medicines: high dose rifapentine, isoniazid, pyrazinamide, and moxifloxacin. This study is called Study 31/A5349 phase 3. (Dorman *et al.* 2020, and Dorman and Nahid, 2020.)

Up to 60% of tuberculosis patients die without treatment (Smith, 2003). For these reasons, it is important to study *M. tuberculosis* infection to find a less toxic and shorter treatment for it in the future (Murray, Schraufnagel, and Hopewell, 2015; Horsburgh, Barry, and Lange, 2015, and Smith, 2003).

2.3 *Mycobacterium marinum*

Mycobacterium marinum is a pathogen in fish, amphibians, and humans which grows relatively rapidly compared to *M. tuberculosis* (Das *et al.*, 2018; Bouley *et al.*, 2001, and Stinear *et al.*, 2008). It causes tuberculosis-like mycobacteriosis in fish and skin damage in humans. Joseph Aronson isolated *M. marinum* in 1926 from infected fish with mycobacteriosis. (Das *et al.*, 2018, and Stinear *et al.*, 2008.) *M. marinum* infection can be treated with anti-tuberculosis agents such as rifampicin and ethambutol (Sridevi *et al.*, 2014, and Stinear *et al.*, 2008).

The genomes of *M. marinum* are highly diverse, and it has been suggested that they should be divided into two clusters: The M-type and the Aronson-type (Das *et al.*, 2018). The complete genome of M-type *M. marinum* was published in 2008 (Stinear *et al.*, 2008, and Das *et al.*, 2018).

2.3.1 Infection mechanism of *M. marinum*

M. marinum infection usually occurs in humans if the cut or scratch on the skin is in direct contact with infected fish or contaminated water, and *M. marinum* passes through the skin (Stinear *et al.*, 2008). This infection is called fish tank or aquarium granuloma (Sette *et al.*, 2015, and Stinear *et al.*, 2008).

M. marinum infection in its natural hosts has been modeled with various models, such as zebrafish and frog models (Chen *et al.*, 2017, Bouley *et al.*, 2001 and Davis *et al.*, 2002). Both adult zebrafish and their embryos/larvae can be utilized in infection modeling. Fetuses and early-swimming larvae have benefits such as optical transparency. However, they do not have adaptive immune cells, whereas adult zebrafish do. (Davis *et al.*, 2002, and Oehlers, Hortle, and Cook, 2020.) Zebrafish embryos do not have lymphocytes, but they develop pathological granuloma hallmarks in *M. marinum* infection and mycobacterial granuloma-specific genes are activated. In

adult zebrafish, on the other hand, *M. marinum* infection produces granulomas such as tuberculosis produces them in humans. (Davis *et al.*, 2002.)

In one study, *M. marinum* infection was monitored in zebrafish embryos by injecting *M. marinum* into caudal vein. In zebrafish, phagocytosis of blood macrophages occurred directly after injection and only macrophages phagocytosed *M. marinum*. In the early stages of infection, some of the bacteria migrated between the two macrophages, and the infected macrophages were phagocytosed into uninfected macrophages, which may give rise to new mechanisms of bacterial spread. Three days after infection, macrophages in the tissues began to form tight aggregates of the homogeneous cell type. The aggregates formed resembled giant cells with cell membranes or cell boundaries found in established granulomas. As in adult tuberculous granulomas, both infected and uninfected cells were found in these structures and the bacteria were intracellular. (Davis *et al.*, 2002.) The formation of granulomas from macrophage aggregates also occurs rapidly in the frog model. The frog *M. marinum* granuloma also has many similarities to human tuberculous granulomas. (Bouley *et al.*, 2001, and Cosma *et al.*, 2008.)

The amount of *M. marinum* injected also affected zebrafish embryo survival. Individuals injected with more than 500 bacteria died after six to nine days, while most of those injected with less than 20 bacteria survived the entire nine-day follow-up. (Davis *et al.*, 2002.)

2.3.2 Virulence of *M. marinum*

Like *M. tuberculosis*, *M. marinum* has a type VII secretory system, and the ESX-1 genes are major virulence factors (Cosma *et al.*, 2008; Das *et al.*, 2018, and Stinear *et al.*, 2008). The major substrates for ESX-1 are ESAT-6/esxA and CFP-10/esxB, which are transported through the cytoplasmic membrane into the cell. The association of esxA and esxB has been found to correlate with virulence in *M. marinum*. (Stinear *et al.*, 2008; Tan *et al.*, 2006, and Mba Medie *et al.*, 2014.) All *M. marinum* strains also have partial replication of ESX-1, resulting in multiple copies of several genes such as ESAT-6 and CFP-10 (Das *et al.*, 2018, and Stinear *et al.*, 2008). In addition, there is an inverse relationship between ESX-1-mediated virulence and esxA acetylation in *M. marinum* (Mba Medie *et al.*, 2014).

ESX-5 has also been suggested to affect the virulence of *M. marinum* and it has been shown to be mandatory in the export of PPE41 protein (Abdallah *et al.*, 2008; Das *et al.*, 2018, and Stinear *et al.*, 2008). In addition to PE/PPE- and ESX-proteins, *M.*

marinum lipoproteins are also important for virulence. Lipoproteins interact with host immune responses, for example, signaling through receptors. (Stinear *et al.*, 2008.)

2.4 Similarities between *M. tuberculosis* and *M. marinum*

M. marinum is used to model and study the molecular pathogenesis of tuberculosis (Bouley *et al.*, 2001; Chen *et al.*, 2017, and Davis *et al.*, 2002). *M. marinum* and *M. tuberculosis* have many common factors, such as major virulence factors and more than 85 % genomic similarity (Abdallah *et al.*, 2008; Das *et al.*, 2018, and Stinear *et al.*, 2008). The genome of *M. tuberculosis* is 2.1 Mb shorter than the genome of *M. marinum*, which is 6.5 Mb in size (Das *et al.*, 2018, and Tobin and Ramakrishnan, 2008). Based on genomic similarity, *M. marinum* and *M. tuberculosis* are diverged from common generalist environmental *mycobacteria* (Stinear *et al.*, 2008).

M. marinum forms a chronic infection of macrophages that results in the formation of granulomas, as in tuberculosis (Bouley *et al.*, 2001; Davis *et al.*, 2002, and Stinear *et al.*, 2008). In addition, infections caused by both *M. tuberculosis* and *M. marinum* can be treated with anti-tuberculosis agents such as rifampicin and ethambutol (Stinear *et al.*, 2008).

M. marinum and *M. tuberculosis* also have the same virulence factors and it has been found that, for example, the ESX-3 system is found in both bacteria in addition to the ESX-1 system. Both bacteria also secrete PE- and PPE-proteins, and lipoproteins are important for the virulence of both. (Stinear *et al.*, 2008.) The *esxA* proteins of *M. marinum* and *M. tuberculosis* are very similar, having 97.9% amino acid similarity and 91.5% identity (Mba Medie *et al.*, 2014).

Manipulation of *M. marinum* is also relatively easy and partly resembles *M. tuberculosis* in terms of growth conditions and physiological properties. Both can be grown in Middlebrook 7H9 medium, but optimal growth temperatures differ. The difference in optimal growth temperature is likely because both bacteria have different natural hosts with different normal body temperatures. (Stinear *et al.*, 2008.)

However, there are also differences between *M. tuberculosis* and *M. marinum*. For example, upon light exposure, *M. marinum* is unable to reduce the amount of nitrate, as *M. tuberculosis* can, and bright yellow pigments are formed. In addition, *M. marinum* has been found in drinking water and swimming pools, for example. However, *M. tuberculosis* have not been found in such aquatic environments. (Stinear *et al.*, 2008.)

M. marinum is used in tuberculosis research not only for its similarity to *M. tuberculosis* but also for cost and safety reasons. Non-human primates are often quite expensive, and treatment of *M. tuberculosis* requires a biosafety laboratory level 3.

(Chen *et al.*, 2017.) *M. marinum* is also relatively fast growing compared to *M. tuberculosis*, so time is saved by utilizing it (Bouley *et al.*, 2001, and Stinear *et al.*, 2008).

2.5 Biofilms

Microorganisms such as bacteria can adhere to surfaces and form biofilms (Karatan and Watnick, 2009; Donlan, 2002, and López, Vlamakis, and Kolter, 2010). Attachment to the surfaces of microorganisms involves many factors, such as the properties of the medium and the cell surface, and this process is complex (Donlan, 2002). The biofilm structure allows the exchange of genetic material between cells and consists of microbial cells and an extracellular polymeric substance (EPS) matrix (Donlan, 2002, and Aggarwal, Stewart, and Hozalski, 2015). The EPS matrix contains mainly polysaccharides, but also proteins, lipids, and DNA (Aggarwal, Stewart, and Hozalski, 2015; Donlan, 2002, and López, Vlamakis, and Kolter, 2010). Biofilms as well as the microorganisms in them are very persistent for antimicrobials such as antibiotics as well as physical removal such as brushing and coughing (López, Vlamakis, and Kolter, 2010; Aggarwal, Stewart, and Hozalski, 2015, and Karatan and Watnick, 2009).

Biofilms consist of microbial cells enclosed in an EPS matrix and they were first discovered by van Leeuwenhoek on the surface of teeth (Aggarwal, Stewart, and Hozalski, 2015, and Donlan, 2002). Prior to this, microorganisms were generally considered planktonic (Donlan, 2002). The organisms in the biofilm differ from the planktonic ones in the transcribed genes and in biofilms conjugation rates between cells are accelerated compared to conjugation rates between planktonic cells (Watnick and Kolter, 2000, and Donlan, 2002). Biofilms differ a lot from each other, and it can be said that there are as many different biofilms as there are different bacteria (López, Vlamakis, and Kolter, 2010, and Karatan and Watnick, 2009).

Biofilms can be formed in a wide variety of locations, such as in living tissues, minerals and drinking water system piping (Karatan and Watnick, 2009; Donlan, 2002, and Aggarwal, Stewart, and Hozalski, 2015). Particles of the material to which it is attached can also be found in the structure of the biofilm, such as clay particles and mineral crystals. It has been observed that as the roughness of the attachment surface increases, the extent of microbial colonization increases with it and biofilms do not come off the attachment surfaces by gentle rinsing alone. (Donlan, 2002.)

Cell attachment and biofilm formation are significantly affected by the properties of the attachment surface, bulk fluid, and cells (Donlan, 2002). Factors influencing the formation of the biofilm of the adhesion surface include surface roughness and hydrophobicity (Donlan, 2002, and Aggarwal, Stewart, and Hozalski, 2015). Among the

properties of the bulk fluid, the flow rate, and the presence of antimicrobial agents, among others, affect the formation of biofilms (Karatan and Watnick, 2009; Donlan, 2002, and López, Vlamakis, and Kolter, 2010). In addition to the bulk fluid and surface properties mentioned above, cellular flagella and fimbriae affect biofilm formation (Karatan and Watnick, 2009; Donlan, 2002, and Watnick and Kolter, 2000).

To form a biofilm, the bacteria can adhere to the surface either individually to form a single-layer biofilm or as clusters, whereby the resulting biofilm is multilayered. A single-layer biofilm is usually formed when cell-adhesion surface interactions prevail rather than cell-cell interactions. Multilayer biofilms, on the other hand, are formed when bacteria can adhere to each other in addition to the adhesion surface. (Karatan and Watnick, 2009.)

The EPS of biofilms can contain even large amounts of water through hydrogen bonds and its solubility can vary. EPS has two important properties that affect the biofilm: the composition and structure of polysaccharides determine its conformation, and EPS can change spatially and over time. (Donlan, 2002.) EPS also has biofilm protective properties. It can prevent the biofilm from drying out due to the large amount of water and it can also affect antimicrobial resistance by preventing the transport of antibiotics through the biofilm. (Aggarwal, Stewart, and Hozalski, 2015; Donlan, 2002, and López, Vlamakis, and Kolter, 2010.)

Biofilms are very heterogeneous and different from each other, but all biofilms usually have a base film, which, however, can vary much in thickness (Karatan and Watnick, 2009; Donlan, 2002, and López, Vlamakis, and Kolter, 2010). Biofilms are different between different species and even between different strains of the same species (Donlan, 2002, and López, Vlamakis, and Kolter, 2010).

Cell-to-cell signaling (quorum-sensing) is important for attachment and detachment of cells from the biofilm and is one of the most important biological determinants of biofilm architecture (Karatan and Watnick, 2009, and Donlan, 2002). Quorum-sensing molecules are called acyl-homoserine lactones and are produced not only in cultured biofilms but also in natural biofilms (López, Vlamakis, and Kolter, 2010, and Watnick and Kolter, 2000).

2.5.1 Mycobacterial biofilms

Both NTM and tuberculous mycobacteria form biofilms. Biofilm-forming mycobacteria include, for example, *Mycobacterium ulcerans*, *M. tuberculosis*, and *M. marinum*. (Chakraborty and Kumar, 2019, and Esteban and Garcia-Coca, 2018.) Several molecules are involved in the formation of mycobacterial biofilms, such as shorter-chain

mycolic acids and GroEL1-chaperone, and the formation of the biofilm is a pathogenic factor (Esteban and Garcia-Coca, 2018; Chakraborty and Kumar, 2019, and Zambrano and Kolter, 2005). In biofilm, mycobacteria have been found to be more tolerant to antibiotics and disinfectants than the planktonic form (Chakraborty and Kumar, 2019, and Esteban and Garcia-Coca, 2018). Treatment of mycobacterial disease may fail due to biofilm-induced tolerance, and therefore an understanding of mycobacterial biofilms is essential in the treatment of mycobacterial diseases (Esteban and Garcia-Coca, 2018).

The first articles on mycobacterial biofilms were published in the late 1980s, although similar structures were described as early as the 1920s and 1930s (Chakraborty and Kumar, 2019, and Esteban and Garcia-Coca, 2018). However, the current concept of biofilms did not emerge until the 1990s (Esteban and Garcia-Coca, 2018).

Like other biofilms, mycobacterial biofilms can form on surfaces, but also on the liquid-air interface. The fact that the biofilm can also form at the liquid-air interface is probably due to the special properties of the mycobacterial cell wall, such as the high lipid content, as well as the different composition of the extracellular matrix of the biofilm. (Chakraborty and Kumar, 2019, and Esteban and Garcia-Coca, 2018.) Components that cause hydrophobicity on the surface of a mycobacterial cell, such as glycopeptidolipids and short-chain mycolic acids, facilitate intercellular interaction. However, they are not necessarily part of the extracellular matrix of mycobacterial biofilms. (Chakraborty and Kumar, 2019.)

There are many differences between biofilm living mycobacteria and planktonic form, including tolerance to antibiotics as well as environmental aggression (Chakraborty and Kumar, 2019, and Esteban and Garcia-Coca, 2018). The tolerance of biofilms to disinfectants and antibiotics is mainly due to the permeability-inhibiting mycolic acids as well as the physical barrier caused by the biofilm, its homogeneity, and its metabolic states (Esteban and Garcia-Coca, 2018; Chakraborty and Kumar, 2019, and Zambrano and Kolter, 2005). Another possible factor for disinfectant tolerance as well as human infections may be NTM biofilms formed together by eukaryotes and prokaryotes, like for example relationship between NTM and amoeba in biofilm (Esteban and Garcia-Coca, 2018).

2.5.2 Biofilms in tuberculosis

The role of biofilm in the pathogenesis of *M. tuberculosis* and whether *M. tuberculosis* forms biofilms *in vivo* is still unclear, but it has been suggested that the role of biofilm in tuberculosis is to participate for example in the cavitation formation in lung tissue.

Because *M. tuberculosis* also develops biofilm *in vitro*, it can be used to study the pathogenesis of tuberculosis as well as possible new therapies against tuberculosis. (Chakraborty and Kumar, 2019, and Esteban and Garcia-Coca, 2018.)

Bacteria are studied in planktonic cultures for the development of a homogeneous population and other benefits. However, bacteria growing in biofilms have different phenotypic characteristics to bacteria growing in planktonic cultures, such as higher minimum inhibitory concentration. (Chakraborty and Kumar, 2019.) Biofilm study of pathogenic mycobacterial species is important because biofilms can protect pathogenic mycobacteria against the host immune system (Chakraborty and Kumar, 2019, and Esteban and Garcia-Coca, 2018).

The effects of antibiotic treatment at different stages of biofilm development have been studied, and it has been found that as biofilms mature, they become more difficult to treat and antibiotics are less effective but the use of this information in the treatment of tuberculosis is still unclear. The development of biofilm is therefore an important factor in the tolerance to antimicrobials. (Esteban and Garcia-Coca, 2018.)

Higher treatment tolerance of biofilm structure can lead to treatment failure, and in some cases, it is necessary to physically destroy the biofilm to eliminate infection (Chakraborty and Kumar, 2019, and Esteban and Garcia-Coca, 2018). It has also been found that biofilm bacteria die better with a combination of antibiotics and antibiofilm agents than in the case of antibiotics alone. Such antibiofilm agents include N-acetylcysteine as well as Tween® 80, of which Tween® 80 has been found to thin the thickness of the biofilm and to reduce the area covered by the biofilm more. N-acetylcysteine degrades the disulfide bridges of the biofilm that connect the polysaccharide fibers. Tween® 80, on the other hand, can alter membrane, lipids, and proteins, and this property explains why it is a more active antibiofilm agent in high-lipid mycobacteria. (Muñoz-Egea *et al.*, 2016, and Esteban and Garcia-Coca, 2018.)

Understanding the biofilms of many NTM-induced diseases is very important. Understanding the biofilms formed by *M. tuberculosis* also provides new perspectives and possibilities for the treatment of tuberculosis. (Chakraborty and Kumar, 2019, and Esteban and Garcia-Coca, 2018.) The treatment time for tuberculosis can potentially be shortened by better understanding the EPS of *M. tuberculosis* biofilms as well as the bacteria in the biofilm (Chakraborty and Kumar, 2019).

2.6 Resazurin

Resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide), also known as Alamar Blue, is a dye commonly used to measure cell viability (Pace and Burg, 2015; Csepregi *et al.*, 2018,

and Martin *et al.*, 2003). Resazurin, which is blue in color, itself does not fluoresce, but bacterial and mammalian cells cause it to be reduced to the highly fluorescent pink resorufin (Figure 1) (Pace and Burg, 2015, and Csepregi *et al.*, 2018).

Resazurin assay can be used to study, among other things, antibiotic resistance in mycobacteria and the formation of bacterial biofilms (Csepregi *et al.*, 2018; Van den Driessche *et al.*, 2014, and Martin *et al.*, 2003). The number of viable cells in biofilms can be measured by resazurin staining, where the amount of fluorescent resorufin is proportional to the number of metabolically active cells and the resulting fluorescence can be measured with plate readers (Van den Driessche *et al.*, 2014). Traditionally, resazurin is added to samples in a volume of about 10 % of the sample volume, and reduction to resorufin can be measured by colorimetry or fluorescence (Pace and Burg, 2015, and Csepregi *et al.*, 2018). The fluorescence of resazurin-stained samples is generally measured using an excitation wavelength of 530 to 580 nm and an emission wavelength of 570 to 620 nm (Csepregi *et al.*, 2018). The change in color of the samples from blue to pink describes the growth and metabolism of bacteria (Castilho *et al.*, 2015; Martin *et al.*, 2003; Van den Driessche *et al.*, 2014, and Palomino *et al.*, 2002).

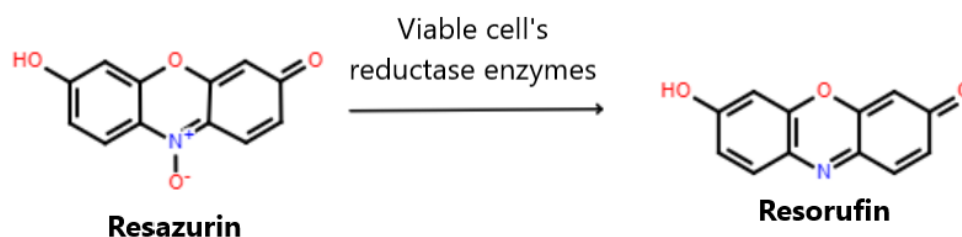


Figure 1. Chemical structure of resazurin and resorufin. Reduction reaction of resazurin to resorufin by chemical structures. Drawn with ChemSpider.

The susceptibility of *M. tuberculosis* to anti-tuberculosis drugs can be assessed visually by a resazurin microtiter assay. This assay is based on the oxidation and reduction of resazurin and is rapid and inexpensive compared to traditional methods. (Castilho *et al.*, 2015; Martin *et al.*, 2003, and Palomino *et al.*, 2002.) In addition, the facilities and biosafety of this assay are improved because microplates do not need to be further treated to add resazurin and no contamination occurs (Castilho *et al.*, 2015). The cytotoxicity of resazurin is also relatively low (Csepregi *et al.*, 2018).

2.7 Triphenyl tetrazolium chloride

Living cells reduce the yellow dye 2,3,5-triphenyltetrazolium chloride, also known as triphenyl tetrazolium chloride, (TTC) to insoluble red TTC formazan crystals (TPF) (Figure 2). The TTC assay can be used, among other things, to determine the drug

susceptibility of *M. tuberculosis*, and the assay is technically easy, fast, and cost-effective. (Mohammadzadeh *et al.*, 2006, and Shinu, Singh, and Nair, 2016.)

The specificity and sensitivity of TTC have been shown to be quite high at critical antibiotic concentrations. These features, as well as speed, cost-effectiveness, and simplicity, make the TTC colorimetric method a promising alternative for testing *M. tuberculosis*, especially when monitoring drug resistance in tuberculosis requires a rapid and inexpensive method. (Mohammadzadeh *et al.*, 2006.) Relatively few studies have yet been published on direct TTC assay (Shinu, Singh, and Nair, 2016).

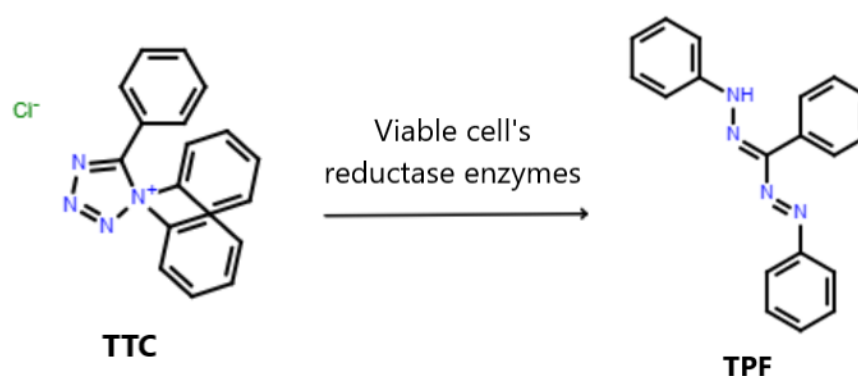


Figure 2. Chemical structure of TTC and TPF. Reduction reaction of TTC to TPF by chemical structures. Drawn with ChemSpider.

2.8 MDK-assay

Resistance and tolerance are properties of the entire bacterial population, whereas persistence is a property of a subpopulation. Resistance reflects an increase in drug concentration at which bacteria can grow indefinitely. Tolerance, on the other hand, is an extension of the period during which bacteria can survive at lethal antibiotic levels before dying. Persistence is the ability of a subset of the bacterial population to survive at high levels of antibiotics. (Brauner *et al.*, 2016, and Brauner *et al.*, 2017.)

Resistance is generally measured using the minimum inhibitory concentration (MIC), but instead there has not previously been a suitable indicator of tolerance. This can lead to resistant strains being considered tolerant, and vice versa, in which case the treatments used may be ineffective. It has therefore been proposed that the minimum duration of killing (MDK) can be used as a quantitative indicator of tolerance. (Brauner *et al.*, 2016, and Brauner *et al.*, 2017.)

Based on the results of the MDK-assay and MIC, it can be determined whether the bacterial strain studied under specific conditions is tolerant, resistant, or persistent (Brauner *et al.*, 2016, and Brauner *et al.*, 2017). In MDK-assay, bacterial populations are

exposed to different antibiotic concentrations for different lengths of time. The MDK is determined by the presence or absence of surviving bacteria, for example, by bacterial regrowth. For this reason, it is important that MDK-assay also has growth control to which no antibiotic has been added. (Brauner *et al.*, 2017.)

The sub-index of the MDK parameter can be marked as a percentage. For example, MDK₉₉ means a minimum duration of killing of 99 % of the population and so on. A high MDK means that it takes a long time to kill the bacteria. It also indicates that the bacteria have a high tolerance. (Brauner *et al.*, 2016, and Brauner *et al.*, 2017.)

3. AIMS OF THE STUDY

The aim of this master's thesis was to optimize redox staining so that they can be utilized in tuberculosis research as well as in the research of other mycobacteria. The goal was that either resazurin or TTC, or both, could be a suitable dye for these purposes. These optimized methods are needed for MDK-assay of mycobacterial biofilms to test the efficacy of drugs. The efficacy of drugs on biofilm bacteria cannot be tested by measuring optical density (OD), as would be done with planktonic bacteria.

Among other things, the appropriate bacterial concentration, dye concentration and dye exposure/incubation times were optimized. *M. marinum* staining was optimized with both redox dyes, resazurin and TTC, and *M. tuberculosis* staining was optimized with resazurin.

4. MATERIALS AND METHODS

In this experiment, the conditions of use of two different redox dyes, resazurin and TTC, for mycobacteria were optimized. Resazurin was tested with both *Mycobacterium marinum* and avirulent strain of *Mycobacterium tuberculosis*, while TTC was tested with *M. marinum*.

Both redox dyes were also tested in antibiotic exposure to see if redox dyes can be used to detect the number of dead bacteria in both *M. marinum* biofilm culture and *M. tuberculosis* biofilm and planktonic cultures. The correctness of the antibiotic exposure results of *M. marinum* biofilm culture was checked using the bioluminescent *M. marinum* (MM-lux) strain.

4.1 Culturing of mycobacteria

For all resazurin and TTC assay optimizations and antibiotic exposure experiments, wild-type *M. marinum* (ATCC 927wt) was first grown for one week on 7H10 agar plates at 28 °C in an incubator (Thermo Scientific). For bioluminescent *M. marinum* experiments, MM-lux (*M. marinum* ATCC-927 with luminescent plasmid insertion) was first grown for one week on 7H10 agar plates including 50 µg/ml Kanamycin at 28 °C in an incubator (Thermo Scientific). After one week of incubation, the bacterial mass was collected from the agar plate into Middlebrook 7H9 biofilm medium including ADC enrichment, the OD₆₀₀-value was adjusted to 0.1.

For all *Mycobacterium tuberculosis* assay optimizations and antibiotic exposure experiments, avirulent *M. tuberculosis* (ATCC, 25177, *Mycobacterium tuberculosis*, Strain H37Ra, Lot: 70017057) was first grown for three weeks on 7H10 agar plates at 37 °C in an incubator, 5 % CO₂ (Thermo Scientific, Forma Steri-cycle i160, CO₂ incubator). After three weeks of incubation, the bacterial mass was collected from the agar plate in 7H9-medium. The medium always has a 7H9-base and (O)ADC. The planktonic culture medium also contains 0.2 % Tween® and glycerol. Before experiments, the OD₆₀₀-value was adjusted either to 0.1 or to 0.5.

4.2 Measurement conditions for resazurin and TTC

All fluorescence measurements of resazurin-stained samples were made using either PerkinElmer EnVision 2104 Multilabel Plate Reader or PerkinElmer Wallac Victor² 1420 Multilabel Counter. Used excitation wavelength was 550 nm and emission wavelength

was 590 nm by EnVision. The excitation wavelength used by Victor was 544 nm and emission wavelength was 590 nm instead.

All absorbance measurements of TTC-stained samples were made using EnVision. The wavelength used was 490 nm. Wavelength was optimized to determine which wavelength, according to EnVision, gives the strongest signal in the calibration wells. In the first TTC optimizations, the absorbance values of the samples were measured at some time points with Victor, also using 490 nm as the wavelength.

Prior to measuring the sample plates, the EnVision was calibrated with the calibration plate and the instrument chamber was heated to the same temperature as the incubator (28 °C). Victor was not calibrated prior measurements, and the chamber temperature was the same as room temperature (25 °C).

4.3 Resazurin assay optimizations

4.3.1 Preparation of resazurin stock solution

A 0.5 % stock solution of resazurin (Resazurin sodium salt, Lot: MKBZ4934V, Sigma) was prepared to sterile water. The solution was stored in - 20 °C.

Dilutions of resazurin (0.02% and 0.06%) used in the experiments were prepared from this stock solution. All dilutions were made to sterile water.

4.3.2 Sensitivity of resazurin for biofilm culture staining

The ability of resazurin to distinguish different bacterial counts in biofilm was tested by preparing a 96-well plate of *M. marinum* biofilm culture. After four days of incubation of bacterial suspension in biofilm medium, suspension was centrifuged with 2000 g for 5 minutes (Sigma Laboratory Centrifuges, 6K15), and the pellet was resuspended to 1 ml of old medium. The rest of the medium was used for further bacteria dilutions. The use of old medium prevents the induction of bacterial growth as fresh medium would do.

A 1:10 dilution series of *M. marinum* biofilm culture was then performed up to 1:10⁸ to a volume of 180 µl per well. Three parallel wells were prepared for each strength of the dilution series and for two different concentrations of resazurin (0.02 % and 0.06 %). Sterile water was added to the surrounding wells to prevent evaporation of the contents of the sample wells.

For resazurin staining, 10 µl of 0.06 % resazurin and 30 µl 0.02 % resazurin per well was used. After the addition of resazurin, both well plates were incubated in 28 °C

for 16, 24 and 48 hours. At each time point, the fluorescence of the samples was measured using EnVision.

4.3.3 Differences in the number of dead bacteria with resazurin

The ability of resazurin to distinguish differences in the number of dead bacteria in biofilm was tested by preparing a 96-well plates of *M. marinum* biofilm culture. The bacterial suspension was added into sample wells at 190 μ l per well. Biofilm medium was used as a control. Sterile water was added into the wells flanking the samples. Two such well plates were prepared: one for two days of antibiotic exposure and one for seven days of exposure. Prepared bacterial suspension plates were incubated in 28 °C for four days.

After four days of incubation, biofilm was formed, and *M. marinum* well plates were exposed to rifampicin. Solutions producing different final concentrations (5, 25 and 125 μ g/ml) of rifampicin were diluted from rifampicin stock solution (TOKU-E, Cas 13292-02-US, Batch R041-02US, 10 mg/ml) to sterile water.

Prepared rifampicin solutions, or sterile water for controls were added to 96-well plates in a volume of 10 μ l per well. Six parallel sample wells and three parallel blank wells were made for each rifampicin concentration. The sample well plates were then returned to the 28 °C incubator.

After seven days of rifampicin exposure, the seven-day samples and blank wells were stained with 0.06 % resazurin. Resazurin solution was added to the wells in a volume of 10 μ l.

After the addition of resazurin, 7-day well plate was incubated in 28 °C for 16, 24 and 48 hours. At each time point, the fluorescence of the samples was measured using EnVision.

Resazurin staining and fluorescence measurements of rifampicin-exposed *M. marinum* well plates were repeated twice more. In both times, similar two- and seven-day exposure well plates were prepared as before. At these times, staining was done by adding 10 μ l of 0.06 % resazurin to the sample.

Unlike previous measurement time points, the fluorescence values of the plates were now measured 19, 24 and 48 hours after staining. Measurements were done with both Victor and EnVision.

4.3.4 Comparison between resazurin staining and bioluminescence detection

To confirm the accuracy of fluorescence measurements of resazurin-stained rifampicin-exposed *M. marinum* samples, a corresponding well plate was also prepared from MM-lux. The bacterial suspension was added into 96-well plate at 190 μ l per well. Biofilm medium was used as a control and sterile water per well was added into the wells flanking the samples. Prepared 96-well plate was incubated in 28 °C for four days.

After four days of incubation, MM-lux samples were exposed to rifampicin and for each rifampicin concentration (5, 25 and 125 μ g/ml), three parallel sample wells and three parallel blank wells were prepared. Solutions of different concentrations of rifampicin were prepared to sterile water and were added to the wells in a volume of 10 μ l. To wells without rifampicin, 10 μ l of sterile water was added.

In addition to the normal time points, the bioluminescence of MM-lux samples was also measured immediately after the addition of rifampicin. The bioluminescence of the samples was measured with PerkinElmer EnVision 2104 Multilabel Plate Reader's luminescence measurement program, by five-point measurement. Prior to measurement, the instrument was calibrated using calibration wells.

4.4 TTC assay optimizations

4.4.1 Preparation of TTC stock solution

A 10 mg/ml stock solution of TTC (2,3,5-Triphenyl-2H-tetrazolium chloride, ab145043, Lot: GR237488-11, Abcam) was prepared to sterile water. The solution was stored at -80 °C.

All dilutions of TTC (5, 50 and 500 μ g/ml) used in the experiments were prepared from this stock solution. All dilutions were made in sterile water.

4.4.2 Sensitivity of TTC for biofilm culture staining

The ability of TTC to distinguish between different amounts of bacteria in a biofilm was tested by preparing a 96-well plate from a culture of *M. marinum* biofilm like that of resazurin. Exceptions to the resazurin assay are summarized in this section.

This time the bacterial suspension was centrifuged using 3000 g. A 1:10 dilution series was made up to 1: 10⁵ to a volume of 190 µl per well. Three parallel wells were prepared for each TTC concentration (5, 50 and 500 µg/ml).

For TTC, dye incubation times were 4, 19, 27, 51, and 75 hours. At 4-, 19-, and 75-hour time points, samples were measured with EnVision and at other time points with Victor.

4.4.3 Differences in the number of dead bacteria with TTC

The ability of TTC to distinguish between differences in the number of dead bacteria in the biofilm was also tested, as with resazurin, by preparing 96-well plates of two and seven days of antibiotic exposure in *M. marinum* biofilm culture. This section summarizes the differences between the resazurin assay and the TTC assay.

Three parallel wells and one blank were made for each rifampicin and TTC concentration. The final concentrations of TTC used in the wells were 5, 50 and 500 µg/ml, which were added to the wells in a volume of 10 µl.

After the addition of TTC, the incubation times were 3, 19, 24, and 48 hours. The absorbance values of the samples were measured at all time points with EnVision.

TTC assay plates were prepared in the same manner for the second time. The only difference was that the incubation times were now 2, 24, and 48 hours.

4.5 MDK-assay

4.5.1 MDK-assay with resazurin

To determine whether resazurin staining is a suitable assay to detect the kinetics of persistent cell formation and to validate the results of bioluminescence measurements, *M. marinum* 96-well plates were prepared. The bacterial suspension was added into sample wells at 192 µl per well. Six parallel biofilm wells and three parallel blank wells were prepared for each rifampicin concentration (0 and 400 µg/ml). Biofilm medium was used as a control and sterile water was added into the wells flanking the samples. To let biofilm form, plates were incubated in 28 °C for four days.

After four days of incubation, *M. marinum* well plates were exposed to rifampicin (400 µg/ml). Sterile water was added to the untreated wells.

Samples of the well plates prepared for each measurement time point (24 hours, 2 days, 4 days, and 7 days after the rifampicin exposure) were stained with 0.06 %

resazurin solution (10 µl per well) 24 hours before measurement. After resazurin staining, fluorescence values were measured with Victor.

4.5.2 MDK-assay with MM-lux

To confirm the results and for comparison, MDK-well plates were also prepared using MM-lux. Two MM-lux well plates were prepared in the same manner as in the *M. marinum* wt well plates. After four days of incubation, the bioluminescence values of the MM-lux well plates were measured with EnVision, after which they were exposed to rifampicin.

Rifampicin exposure was performed in the same manner as with *M. marinum* wt. The bioluminescence values of the plates were measured at the same time points as the *M. marinum* wt plates and at a 6-hour time point with EnVision.

4.6 Monitoring the development of antibiotic tolerance in *M. tuberculosis*

4.6.1 Determination of the growth rate of planktonic culture

Before testing the development of antibiotic tolerance of *M. tuberculosis* with resazurin, the growth rate of planktonic culture was determined. After three weeks of incubation, the bacterial mass was collected from the agar plates into Middlebrook 7H9 medium including 0.2 % Tween®, (O)ADC and glycerol. The OD₆₀₀-value was adjusted to 0.1.

4.6.2 Optimization of the time of fluorescent measurement

The optimal time for fluorescence measurement of *M. tuberculosis* samples after resazurin staining was determined by preparing two 96-well plates. The bacterial suspension, which had an OD₆₀₀-value set to 0.1 or 0.5, was added into sample wells at 200 µl per well. Six parallel wells were prepared. 7H9-medium was used as a control. Sterile water was added into the wells flanking the samples. Two such well plates were prepared: one was stained with resazurin one day after preparation and the other five days after preparation. Both prepared 96-well plates were incubated in 37 °C and sealed with Parafilm.

After incubation, the test wells were stained with 10 µl of 0.06 % resazurin. The sample well plates were then returned to the 37 °C incubator. Fluorescence values of resazurin stained one- and five-day samples were measured with Victor 24 and 48 hours after the addition of resazurin.

4.6.3 Antibiotic tolerance determination

Antibiotic tolerance of *M. tuberculosis* samples was determined by preparing three 96-well plates: for a zero-day, a two-day, and a six-day time point from rifampicin exposure. For all rifampicin exposure time points, biofilm of different age (five or 12 days), or planktonic culture (five days old) were prepared (in a volume of 192 μ l of the bacterial suspension per well). Three parallel wells for each culture and rifampicin concentration (0 μ g/ml, 5 μ g/ml, 25 μ g/ml, 125 μ g/ml, 250 μ g/ml, and 400 μ g/ml) were prepared for two-day- and six-day-well plates. Nine parallel wells were prepared for zero-day-well plates.

7H9-medium without bacteria +/- rifampicin was used as a control. Sterile water was added into the wells flanking the samples. All prepared 96-well plates were incubated in 37 °C and sealed with Parafilm.

At proper time point (zero, two, and six days) samples were stained with 10 μ l of 0.06 % resazurin. Fluorescence values were measured 24 hours after the addition of resazurin with Victor.

4.7 Analysing the results

Fluorescence and absorbance values measured from the samples were analyzed using Microsoft® Excel®. The data obtained from the measuring devices were entered into an Excel spreadsheet, and the mean values given by the blank wells were removed from the values of the sample wells.

In all experiments, blank wells were prepared in the same volume as the sample wells, but without bacteria. They thus contained the same amount of used medium, rifampicin and redox dye as the sample wells.

Based on the results obtained, graphs were prepared. In the graphs, the standard deviations of the determined values, calculated with Excel, were performed as error bars.

5. RESULTS

5.1 Resazurin assay optimizations

5.1.1 Sensitivity of resazurin staining in biofilm culture

The sensitivity of resazurin for *M. marinum* biofilm culture staining was examined at two different stock concentrations of resazurin: 0.02 % and 0.06 % (Figure 3a and b). The aim was to study the sensitivity of resazurin staining with decreasing bacterial counts. As the Figure 3 shows, the fluorescence value is lower in lower *M. marinum* dilution. As the resazurin incubation time increased from 16 to 48 hours, the fluorescence value also appeared to increase (especially at higher *M. marinum* concentration).

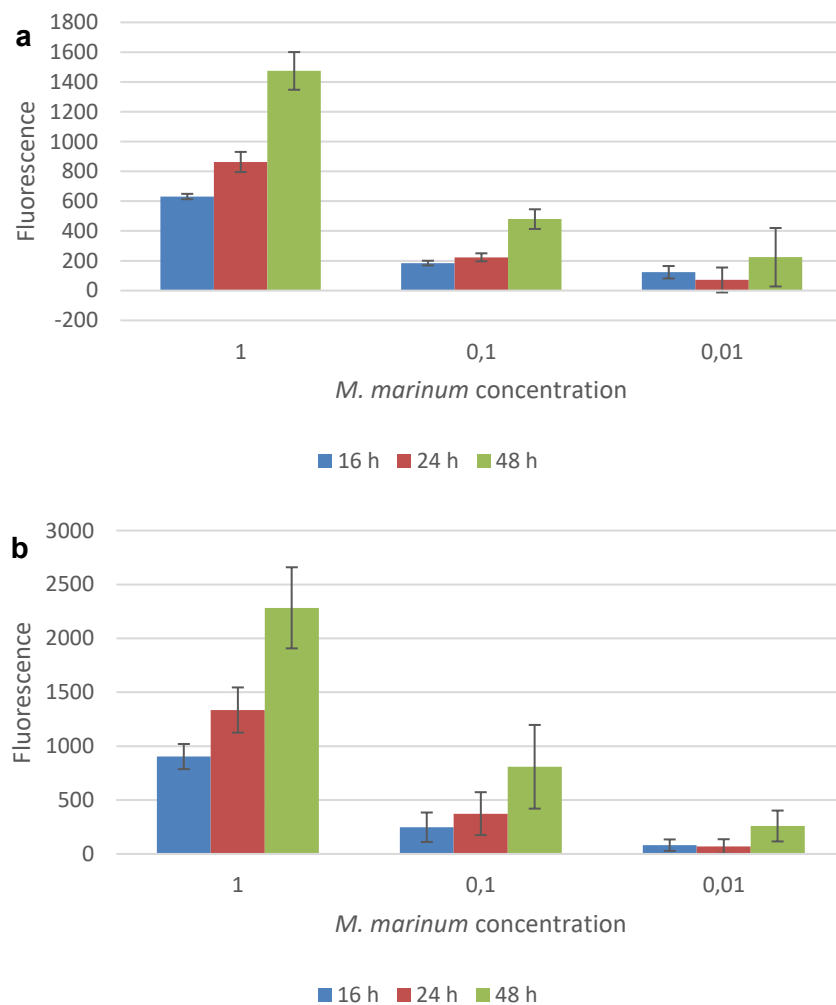


Figure 3. Fluorescence values of the resazurin sensitivity experiment. a 0.02 % resazurin, **b** 0.06 % resazurin. N = 3. The bacterial concentrations in the graphs are artificial because the lower concentrations are diluted 1:10 from the previous one. Values measured with EnVision.

Based on these results, it appeared that resazurin staining is not very sensitive at lower bacterial concentrations. It was decided to use a stock concentration of 0.06% resazurin in the future.

5.1.2 Differences in the number of dead bacteria measured with resazurin

Next, the ability of resazurin to differentiate the number of dead bacteria after rifampicin exposure was studied. First, samples of *M. marinum* biofilm cultures were exposed to rifampicin for seven days. After rifampicin exposure, samples were stained with 10 μ l of 0.06% resazurin and incubated for 16, 24, and 48 hours (Figure 4). The fluorescence values of the untreated samples were the highest, and the fluorescence values decreased with increasing rifampicin concentration, indicating reduced bacterial viability.

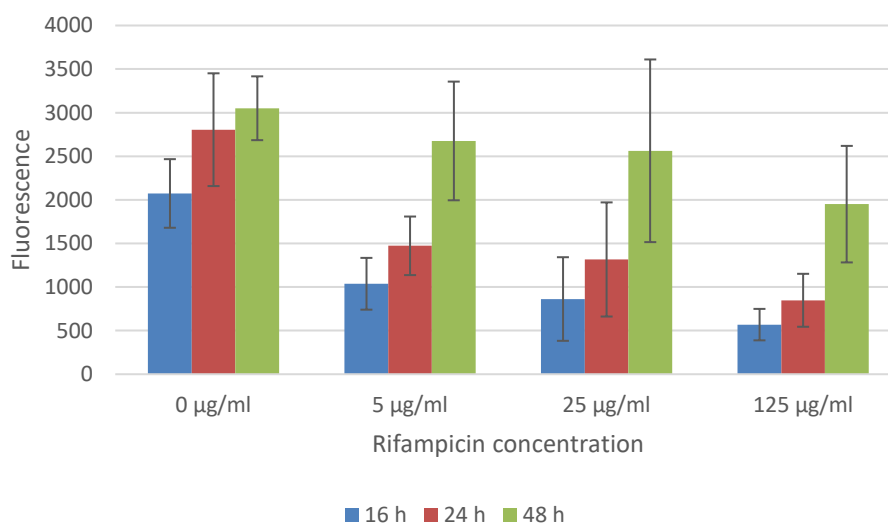


Figure 4. Fluorescence values of the first rifampicin exposure experiment. Exposure time seven days and staining with 0.06 % resazurin. N= 6. Values measured with Victor.

On the second repeat, samples were stained with 0.06% resazurin after two and seven days of rifampicin exposure (Figure 5). After two days of rifampicin exposure (Figure 5a), the fluorescence values did not counter-correlate with the amount of rifampicin, as they did after seven days of rifampicin exposure (Figures 4 and 5b).

From all the measurement results, it seems that the addition of 10 μ l of 0.06 % resazurin to a 200 μ l *M. marinum* sample gives the best results. Based on the results, the 24-hour resazurin staining seems to be the best, as it has the largest difference in the fluorescence values of the untreated and treated samples in the most experiments (Figures 4 and 5b). However, resazurin may not be sensitive enough to detect the change in bacterial viability caused by two days of rifampicin exposure at the antibiotic

concentrations used here (Figures 5a and 5b). Of the measuring devices, Victor gave results with smaller standard deviations, and the differences between the measurement reproductions were also smaller. Thus, the wavelengths of 544/590 nm used by Victor appear to be suitable for resazurin staining.

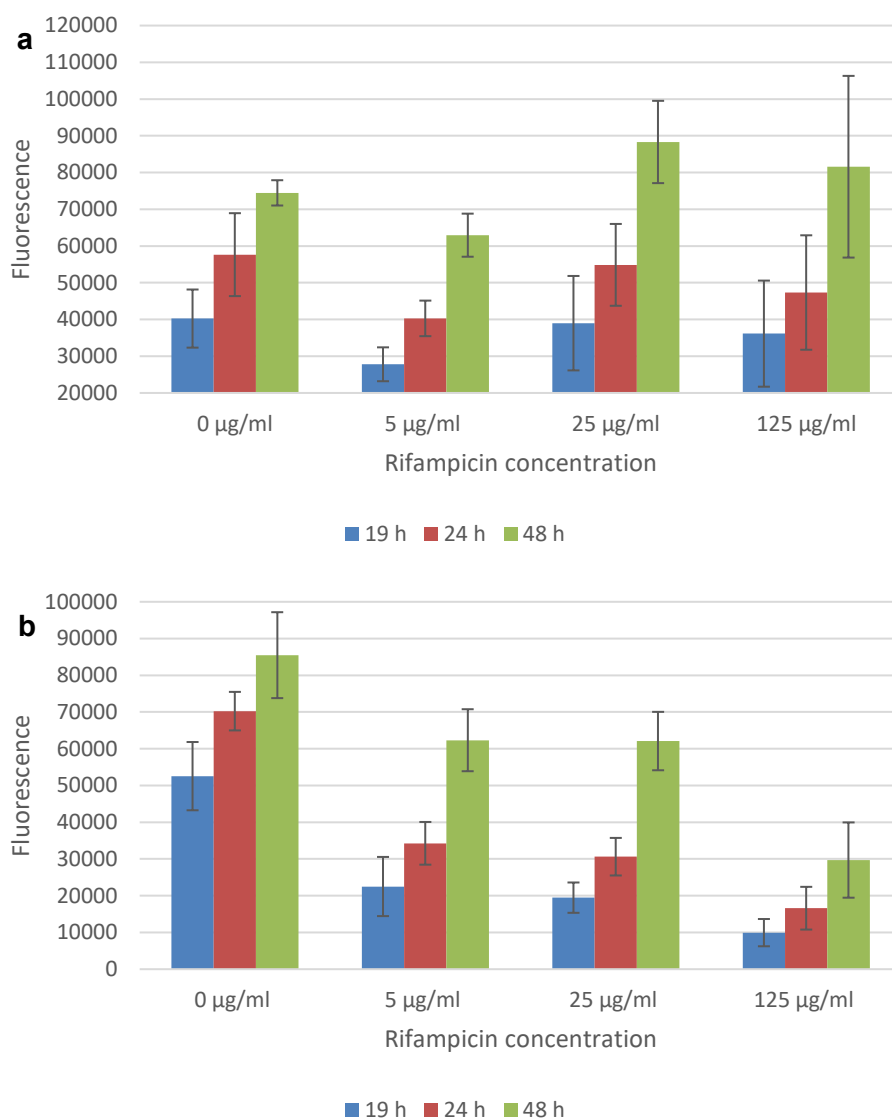


Figure 5. Fluorescence values of the second rifampicin exposure experiment. a Two-day rifampicin exposure, **b** seven-day rifampicin exposure. N = 6. Values measured with Victor; samples stained with 0.06 % resazurin.

5.1.3 Comparison of the results between resazurin staining and luminescent detection

The accuracy of resazurin staining to detect changes in bacterial viability after rifampicin exposure was confirmed using *M. marinum* strain which expresses bioluminescence as

control. Bioluminescence values after two and seven days of antibiotic exposure are presented in Tables 1 and 2. The measurement time points of MM-lux and resazurin stained *M. marinum* samples are the same in both tables, for example, the time point of 19 h MM-lux is measured at the same time point as the *M. marinum* samples are measured 19 hours after the addition of resazurin.

Table 1. Comparison of relative bacterial viability of two-day rifampicin exposed samples. The table shows the percentage of bacteria viability measured with bioluminescence (MM-lux) or fluorescence (resazurin) detection. Untreated sample set to 100 %. N = 3 for bioluminescence values and N = 6 for fluorescence values. The measurement time point shown in the top row. Values measured with EnVision.

RIF	19 h MM-lux	19 h resazurin	24 h MM-lux	24 h resazurin	48 h MM-lux	48 h resazurin
0 µg/ml	100 %	100 %	100 %	100 %	100 %	100 %
5 µg/ml	15.6 %	69.1 %	12.4 %	69.9 %	5.0 %	84.5 %
25 µg/ml	5.3 %	96.9 %	5.5 %	95.2 %	2.7 %	118.6 %
125 µg/ml	2.0 %	89.8 %	2.4 %	82.1 %	1.3 %	109.5 %

Table 2. Comparison of relative bacterial viability of seven-day rifampicin exposed samples. The table shows the percentage of bacteria viability measured with bioluminescence (MM-lux) or fluorescence (resazurin) detection. Untreated sample set to 100 %. N = 3 for bioluminescence values and N = 6 for fluorescence values. The measurement time point shown in the top row. Values measured with EnVision.

RIF	19 h MM-lux	19 h resazurin	24 h MM-lux	24 h resazurin	48 h MM-lux	48 h resazurin
0 µg/ml	100 %	100 %	100 %	100 %	100 %	100 %
5 µg/ml	1.9 %	42.8 %	1.8 %	48.8 %	4.5 %	72.9 %
25 µg/ml	1.1 %	37.1 %	1.1 %	43.6 %	2.5 %	72.6 %
125 µg/ml	0.6 %	18.9 %	0.5 %	23.6 %	3.1 %	34.8 %

As can be seen from the Tables 1 and 2, both the bioluminescence values and fluorescence values of the resazurin-stained samples decreased with increasing rifampicin concentration except for two days of exposure at a 48-hour measuring point for fluorescence measurement. Overall, based on the bioluminescence values, it can be said that the fluorescence values of the resazurin-stained samples are in the right direction, because the bioluminescence values also followed the same trends. However, the percentage differences between the signals of untreated and treated samples were much larger in the bioluminescent samples than in the resazurin-stained samples.

5.1.4 MDK-assay with resazurin staining and luminescent detection

To detect persister cell formation kinetics, biofilm samples were exposed to high concentration of rifampicin followed by the detection of bacterial viability with both luciferase signal and resazurin staining detection (Figure 6). As Figure 6a shows, the bioluminescence values of the untreated MM-lux samples increased over time. The values of antibiotic treated MM-lux samples instead decreased as antibiotic exposure continued. However, fluorescence signals from resazurin staining differs significantly only at seven days after antibiotic exposure both in untreated and treated *M. marinum* samples and the standard deviations are then quite large for the treated samples (Figure 6b).

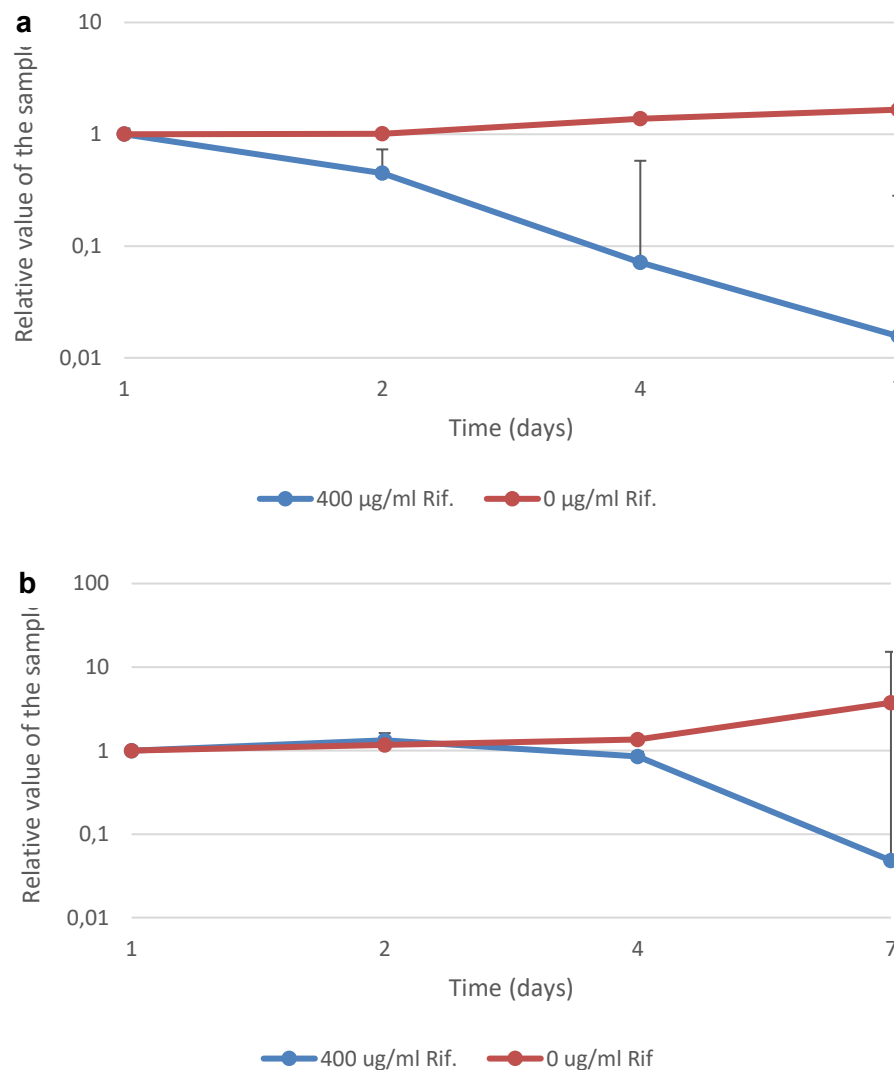


Figure 6. Bioluminescence and fluorescence values of MDK-assay. The values of the one-day samples are set to 1. Relative values are presented as logarithmic. Only positive error bars are shown in the graphs. **a** Bioluminescence values measured with EnVision, **b** Fluorescence values of 0.06 % resazurin stained samples measured with Victor. N = 6.

Table 3 shows the comparison between relative values between untreated and treated samples in the MDK-assay. MM-lux and 0.06 % resazurin stained *M. marinum* samples have been compared to the bioluminescence value of one-day untreated samples.

In summary, the results of resazurin-stained samples do not fully correspond to the results of bioluminescence measurements. Especially at shorter antibiotic exposure times (1 to 4 days), the differences in resazurin-stained samples between untreated and treated samples are very small compared to bioluminescence-measured samples. However, after seven days of exposure, the resazurin and bioluminescence results were closer to each other, although the percentage differences between untreated and treated samples were still not at the same level (0.2 % vs. 4.6 %).

Table 3. Comparison of relative values of untreated and treated MM-lux- and resazurin stained *M. marinum* samples. The table shows the measured bioluminescence/fluorescence values relative to the one-day untreated sample. One-day untreated sample value is set to 100 %. N = 3 for bioluminescence values and N = 6 for fluorescence values.

Rifampicin exposure time (days)	MM-lux	Resazurin stained <i>M. marinum</i>
1	11.9 %	94.7 %
2	5.3 %	125.7 %
4	0.8 %	80.3 %
7	0.2 %	4.6 %

5.2 TTC assay optimizations

5.2.1 Sensitivity of TTC staining in biofilm culture

The aim was to study the sensitivity of TTC staining in *M. marinum* biofilm culture with decreasing bacterial counts. This was examined at three different concentrations of TTC: 5 µg/ml, 50 µg/ml, and 500 µg/ml, and different incubation times. Figure 7 shows the absorbance values of the undiluted *M. marinum* samples at different concentrations of TTC and at five different incubation times (4 to 75 hours).

The TTC staining *per se* is able to distinguish the bacterial sample from background since the absorbance values are 0.3 – 0.6 units higher compared to background (which has been normalized to zero). However, the TTC concentration increasement seems to increase the absorbance value only after the 19-hour TTC incubation (Figure 7). Interestingly, the level of absorbance seems to peak at 19-hour incubation time.

At this stage, it was decided to exclude the 75-hour measurement time point from the further rifampicin exposure trials. Also, device switching between measurements did not appear to have much effect on absorbance values, so it was decided to measure samples using EnVision only.

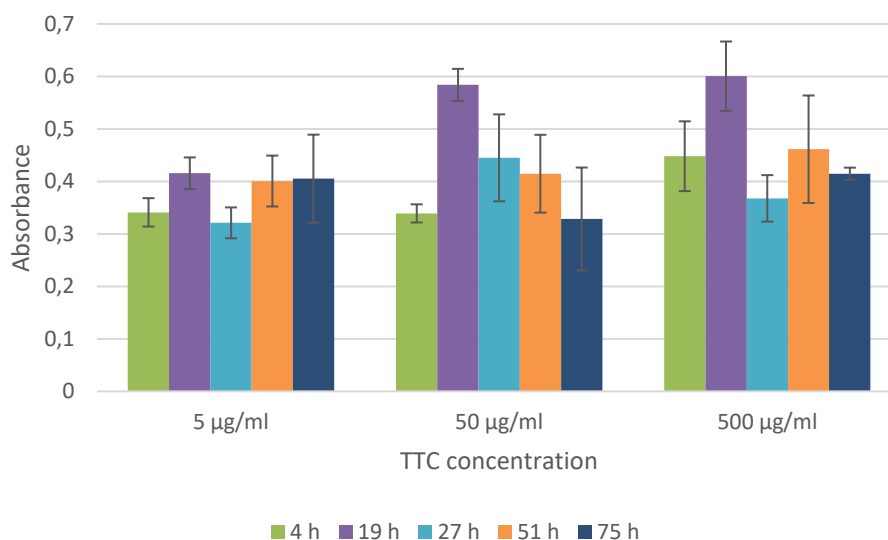


Figure 7. Fluorescence values of the TTC sensitivity experiment. N = 3. Values measured at 4-, 19-, and 75-hour time points with EnVision. At 27- and 51-hour time points, values were measured with Victor.

5.2.2 Differences in the number of dead bacteria measured with TTC

The ability of TTC to differentiate the number of dead bacteria at two and seven days after rifampicin exposure was studied in the same way as resazurin. In each measurement, samples were stained using three different TTC concentrations: 5, 50, and 500 µg/ml. At the first time, samples were measured at 3, 19, 24, and 48 hours after TTC addition (Figure 8).

Figures 8a and b show a well plate exposed to antibiotics for seven days and stained with different concentrations of TTC at three- (Figure 8a) and 24-hour (Figure 8b) time points. Absorbance values measured from those samples from different timepoints are represented in Figure 8c. As the Figures 8a and b show, the different rifampicin concentrations differ well from each other, as the orange color of the sample increases as the rifampicin concentration increases. In contrast, the color change of TTC is hardly visible, especially at lower TTC concentrations (black and red boxes), and in samples exposed to rifampicin.

The graphs in Figure 8c show that at each time point, the absorbance value of the highest rifampicin concentration sample was higher than that of the untreated sample. Otherwise, the absorbance values did not decrease with increasing rifampicin

concentration but rather correlated with the amount of rifampicin so that as the concentration of rifampicin increased, the measured absorbance value also increased.

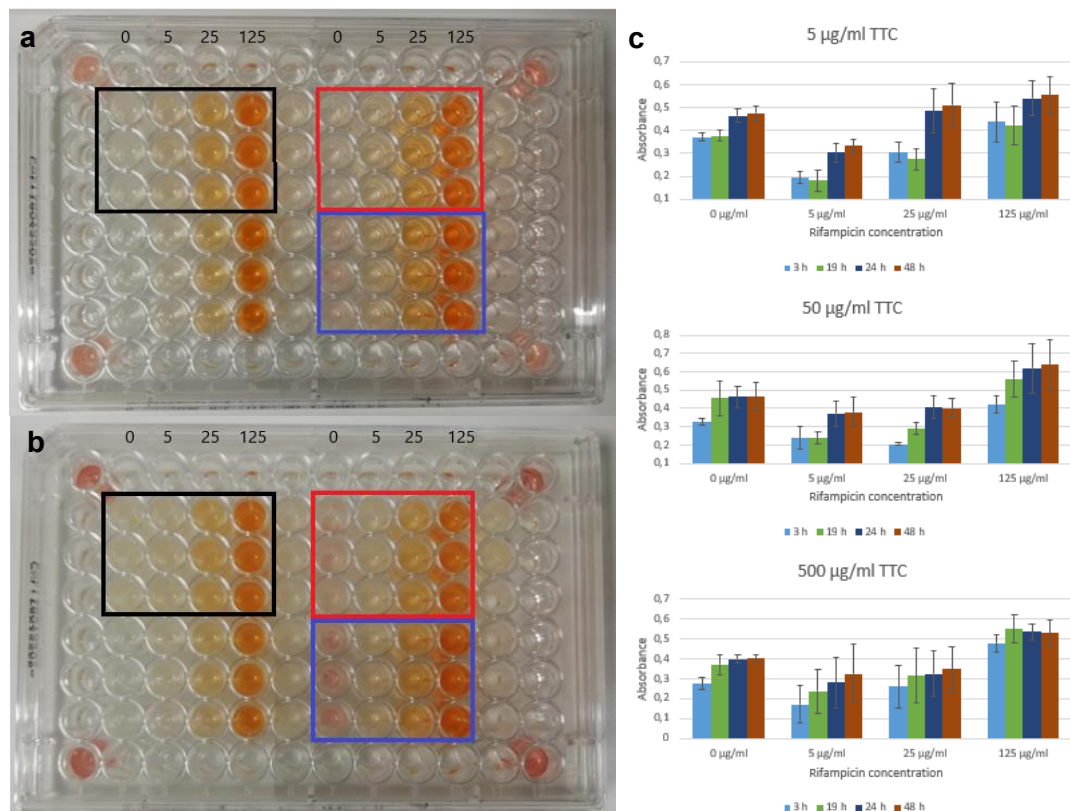


Figure 8. Seven-day rifampicin exposure well plate stained with TTC and absorbance values measured from those samples. a & b, the concentration of TTC is marked in the figures as follows black: 5 µg/ml, red: 50 µg/ml and blue: 500 µg/ml. The numbers at the top of the well plates represent the rifampicin concentration (µg/ml). The wells under the black box contain only medium and rifampicin, **a** three-hour TTC incubation, **b** 24-hour TTC incubation, **c** absorbance values of samples at different time points stained with TTC. N = 3, values were measured with EnVision.

In the repeated experiment, the absorbance values of the samples were measured at 2, 24, and 48 hours after TTC addition. After two days of rifampicin exposure, the absorbance values decreased logically with increasing rifampicin concentration at the 24- and 48-hour TTC staining (5 µg/ml) (Figure 9a). At a two-hour TTC staining (5 µg/ml) TTC time point and at all time points with other TTC concentrations, the absorbance values of treated samples were close to that of the untreated sample in lower antibiotic concentrations. The standard deviations were again quite large similarly as the results in the Figure 8c.

Figure 9b shows the absorbance values of the seven-day rifampicin exposure stained with different TTC concentrations. As can be seen from the graphs, the absorbance values of the highest antibiotic concentration were higher than those of the untreated sample.

The shapes of the graphs correspond quite well to the shapes of the graphs in Figure 8c. Higher concentrations of rifampicin appear to increase the absorbance of the

solution, although the background of rifampicin and medium has already been subtracted from the sample values. Therefore, the method based on absorbance measurement cannot be used in this case. The absorbance values of the wells containing rifampicin and medium alone also increased with increasing rifampicin concentration (data not shown). It is therefore concluded that TTC staining is not suitable for the reliable detection of *M. marinum* bacterial counts under the conditions studied.

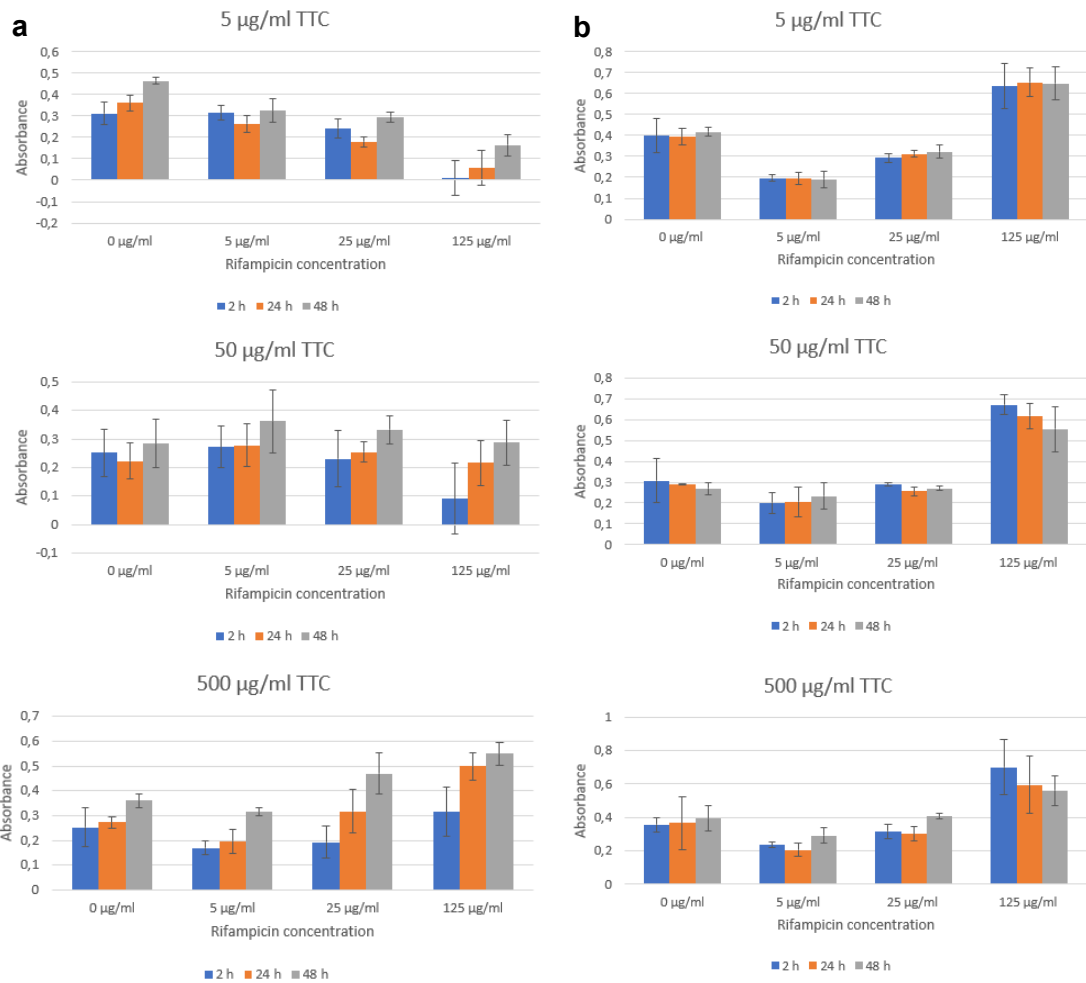


Figure 9. Absorbance values of the two- and seven-day rifampicin exposure experiment. a Two-day rifampicin exposure, **b** seven-day rifampicin exposure. N = 3. Values measured with EnVision.

5.3 Monitoring the development of antibiotic tolerance in *M. tuberculosis*

5.3.1 Optimization of the time of resazurin staining

The optimal time for resazurin staining of avirulent *M. tuberculosis* samples was determined by staining one- and five-days old planktonic culture (OD₆₀₀-values were adjusted to 0.1 and 0.5) with resazurin. At the one-day-old planktonic culture, the 48-hour resazurin staining gave a higher fluorescence value than at the 24-hour staining (Figure 10, blue bar). The standard deviations were also smaller at 48-hour time point.

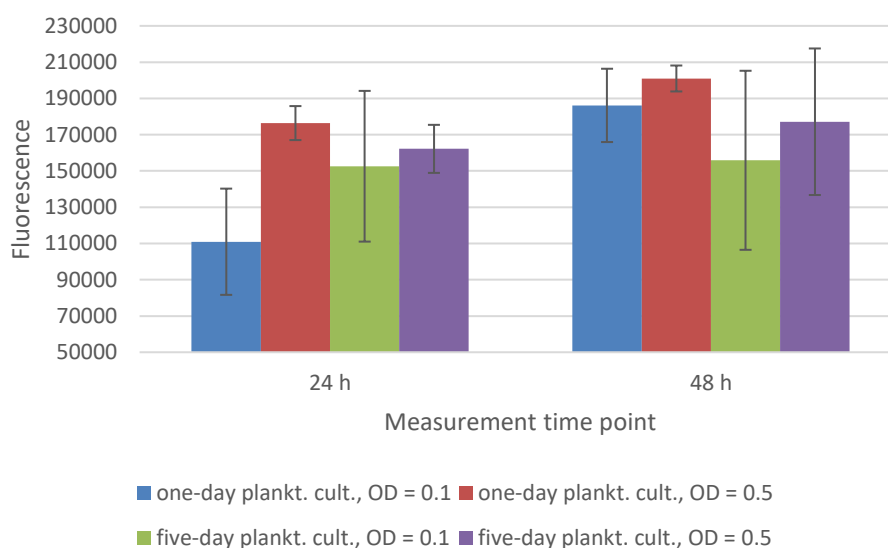


Figure 10. Fluorescence values of the measurement time optimization experiments. Planktonic culture of *M. tuberculosis* with different ages and OD-values, stained with 0.06 % resazurin stock solution. N = 6. Values measured with Victor.

In the case of a five-day-old *M. tuberculosis* planktonic culture, however, the fluorescence value did not change much between the 24- and 48-hour measurement points. At the 48-hour measurement time point, the standard deviations were also higher than at the 24-hour time point. Since five-day-old planktonic culture and five-day and 12-day-old biofilm cultures were used to determine antibiotic tolerance of *M. tuberculosis*, it was decided to use a 24-hour resazurin staining for these experiments.

5.3.2 Antibiotic tolerance determination

Next, we determined the differences of antibiotic tolerance at different *M. tuberculosis* cultures (at planktonic, 5 days old, and 12 days old biofilm). Cultures were exposed to rifampicin for two and six days before resazurin staining detection.

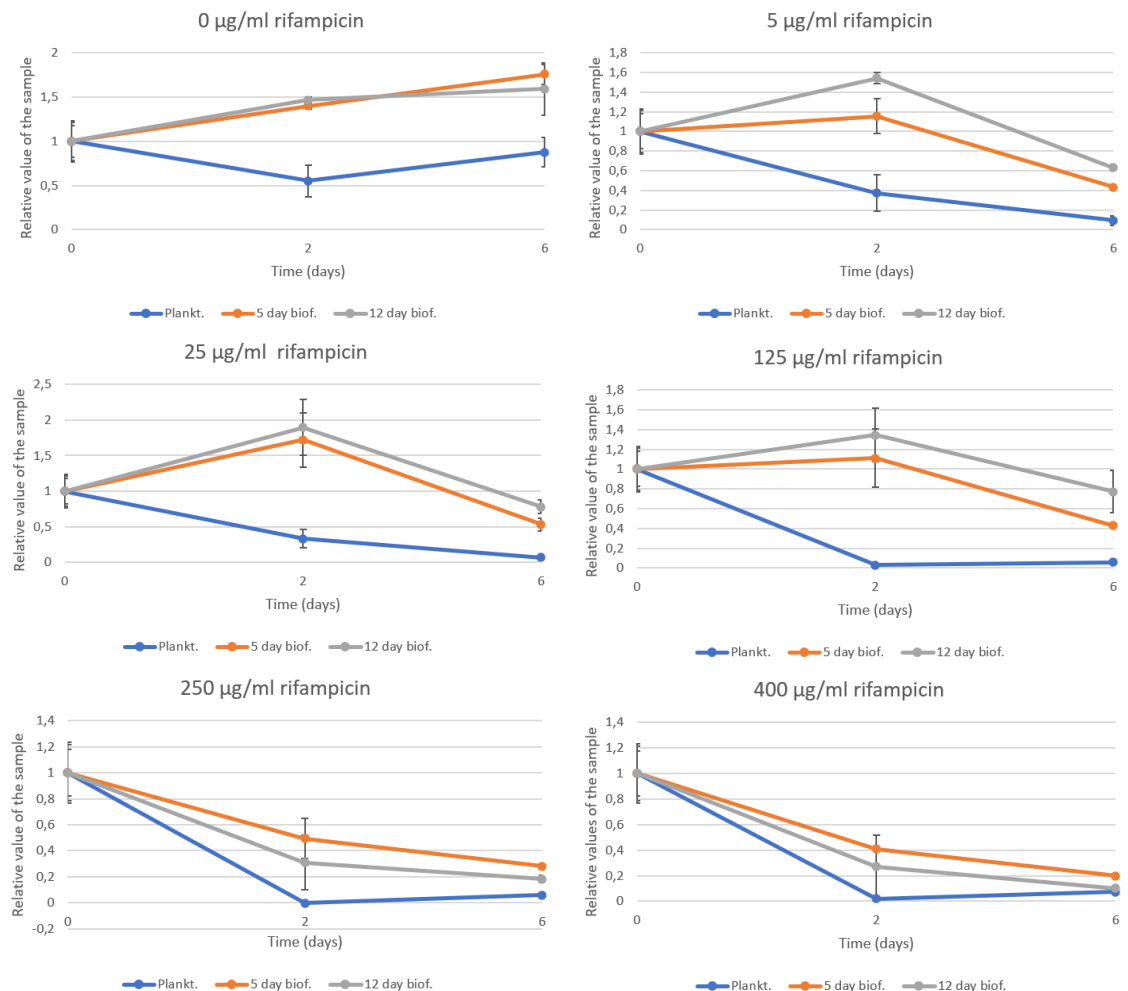


Figure 11. Fluorescence values of the antibiotic tolerance of *M. tuberculosis* experiment. N = 3 for two and six days of exposure, N = 9 for zero-day samples. Values measured with Victor. The values for the zero-day samples are set to one.

As Figure 11 shows, planktonic bacteria (blue line) are more sensitive to rifampicin than biofilm cultures. In planktonic culture bacteria begin to die as early as two days after exposure to all rifampicin concentrations. Of note, in the untreated planktonic sample, the number of metabolically active bacteria was lower at two-day timepoint than that in the zero-day sample, so, bacterial growth was no longer in the logarithmic growth phase. In contrast to planktonic culture, bacteria in biofilm cultures do not begin to die at lower antibiotic concentrations (0 to 125 µg/ml) until six days of antibiotic exposure. The

antibiotic concentration needs to increase to 250 and 400 µg/ml until the bacteria of the more tolerant biofilm cultures start to die earlier.

Thus, based on the results, it appears that planktonic culture is the most sensitive to rifampicin exposure because more than 90 % of bacteria from planktonic culture can be killed at a concentration of 5 µg/ml of rifampicin in six days. In contrast, biofilm cultures require a rifampicin concentration of 400 µg/ml to kill more than 80% of bacteria in six days. Overall, however, there was little difference in antibiotic tolerance between biofilm cultures of different ages.

In addition, based on the results, it appears that resazurin is more suitable for *M. tuberculosis* than for *M. marinum* viability detection. In the case of *M. tuberculosis*, the differences between the untreated and the treated sample were already distinguished after two days of antibiotic exposure and at lower antibiotic concentrations, whereas in *M. marinum* the differences were better distinguished only with longer antibiotic exposures and higher antibiotic levels.

6. DISCUSSION

The purpose of this work was to find a suitable protocol and optimize its use for redox staining of mycobacteria. The results of this work may be used in future mycobacterial studies, such as the determination of antibiotic tolerance in mycobacterial biofilms.

Redox dyes appear to be a potential candidate for mycobacterial staining because they allow studies to be done relatively quickly and inexpensively. Resazurin staining is relatively commonly used detection method for mycobacteria, but far fewer publications can be found on TTC staining.

6.1 Research on mycobacterial biofilms is important

Research on mycobacterial biofilms may play a very important role in the more advanced treatment of mycobacterial infections. Because mycobacterial biofilms are more tolerant to antibiotics than planktonic culture, and presumably presents *in vivo* conditions better than planktonic culture, they are potentially better and more reliable culturing form at exploring new therapies.

Further research is needed into whether *M. tuberculosis* also forms biofilms *in vivo*. If so, biofilms may be one possible cause of antibiotic tolerance in *M. tuberculosis*, or they may, for example, protect *M. tuberculosis* against host immune defence.

Although biofilms of *M. tuberculosis* and other infectious mycobacteria may be structurally different *in vitro* and *in vivo*, they may still be treated by the same means. However, it is also worth noting that NTM also forms biofilms, so biofilms are not necessarily part of the virulence mechanism of *M. tuberculosis* (Chakraborty and Kumar, 2019).

Mycobacterial biofilms have unique properties, such as the ability to form not only on a surface but also on a liquid-air interface (Esteban and Garcia-Coca, 2018). By studying biofilms, more unique features and potential weaknesses can be found. Therefore, the treatment of mycobacterial biofilms and the inhibition of biofilm formation are potentially important in the development of new treatment for *M. tuberculosis*.

6.2 Resazurin is suitable for use in mycobacterial research

In our experiments, it was found that adding 10 μl of a 0.06 % resazurin stock concentration to a *M. marinum* biofilm culture sample of about 200 μl gave a stronger fluorescence signal than adding 30 μl of 0.02 % resazurin stock concentration. In

previous studies, either 0.02 % resazurin stock concentration or 0.01 % resazurin stock concentration was often used for *M. tuberculosis* resazurin staining and added to a 100 μl sample in a volume of 30 μl (Palomino *et al.*, 2002, and Martin *et al.*, 2003). Resazurin staining of fast-growing mycobacteria has also been implicated in a 0.02 % stock concentration of resazurin by adding it in a volume of 30 μl (Castilho *et al.*, 2015). Thus, it may be that the addition of 30 μl of 0.02 % resazurin stock concentration to a larger sample volume was slightly too small amount of dye, although after addition of 10 μl of 0.06 % resazurin stock concentration, the final concentration of resazurin is almost the same, but still slightly higher, as different resazurin stock solutions were added to a 180 μl sample in different volumes, giving a final volume of 210 μl for 0.02 % resazurin stained samples and 190 μl for 0.06 % resazurin stained samples.

The wavelengths used, excitation 544 nm and emission 590 nm, are well suited for resazurin measurements. These wavelengths were within the wavelength ranges of resazurin measurements found in the literature, which were excitation 530 to 580 nm and emission 570 to 620 nm (Csepregi *et al.*, 2018).

The 24-hour fluorescence measurement time point was suitable for *M. marinum* as well as *M. tuberculosis*. In fact, resazurin staining appears to be even better in *M. tuberculosis* studies than in *M. marinum* studies, as even after two days of antibiotic exposure, *M. tuberculosis* samples showed a difference between untreated and treated sample, whereas *M. marinum* samples showed differences only after seven days of antibiotic exposure. The conversion of resazurin to resorufin was more clearly seen in *M. tuberculosis* by fluorescence measurement as well as by visual inspection alone, as the untreated samples of *M. tuberculosis* were pinker than those of *M. marinum* (data not shown). One possible reason that the differences between the treated and untreated sample were better seen in *M. tuberculosis* may be the difference in incubation temperature. *M. tuberculosis* samples were incubated at 37 °C after resazurin addition, while *M. marinum* samples were incubated at 28 °C. Other possible reason could be that *M. tuberculosis* may have more metabolism than *M. marinum*, which reduces resazurin to resorufin in a shorter time.

Resazurin staining was not as sensitive as bioluminescence measurement of MM-lux, especially at lower bacterial counts. Resazurin could possibly have shown better the differences between untreated and treated if the samples had been incubated at 37 °C instead of 28 °C. It may also be that rifampicin may affect the bioluminescence of MM-lux by decreasing the amount of bioluminescence with increasing rifampicin concentration. However, resazurin staining works reliably with *M. tuberculosis* as well as with longer antibiotic exposures with *M. marinum*.

A good aspect of mycobacterial resazurin staining, in addition to time saving and cheapness, is that the colour induced by rifampicin did not appear to affect fluorescence values of the samples. The background signal was successfully removed with blanks containing medium and rifampicin. It is also a great advantage that resazurin staining can be used to visually detect the viability of mycobacteria without fluorescence measurement by colour change. This can be exploited when fluorescence measurement is not available, such as in developing countries.

6.3 Planktonic culture of *M. tuberculosis* is most sensitive to antibiotics

Resazurin-stained samples of *M. tuberculosis* showed that biofilm cultures were more tolerant to rifampicin than planktonic cultures. This was assumed, as mycobacteria have been reported to be more tolerant in biofilms than in planktonic culture (Chakraborty and Kumar, 2019). This is probably due to the persister cells that the biofilms have been shown to contain (Ojha *et al.*, 2008).

Interestingly, the five- and 12-day-old biofilms of *M. tuberculosis* did not differ much in terms of antibiotic tolerance. This suggests that, at least *in vitro*, the tolerance of the *M. tuberculosis* biofilm develops as early as five days and is only slightly enhanced in the older biofilm. Probably a biofilm younger than five days would have been more sensitive to rifampicin. Due to the antibiotic tolerance of biofilms, it is particularly important that they are investigated further.

6.4 TTC is not suitable dye for mycobacteria under studied conditions

TTC staining of mycobacteria could not be performed as desired, although different TTC concentrations and measurement time points were experimented, and the absorbance measurement wavelength used (490 nm) was optimized (data not shown). The wavelength used was very close to the wavelengths used in the studies for TTC absorbance measurements, such as for maize (485 nm), fungal chitosan (480 nm), and *Campylobacter jejuni* (500 nm) (Zhao, Zhu, and Wang, 2010; Moussa *et al.*, 2013, and Brown *et al.*, 2013). One possible reason for this may be the incubation temperature of *M. marinum*, which was 28 °C, whereas in the other studies, samples were generally incubated at 37°C, as in the case of fungal chitosan and *M. tuberculosis* (Moussa *et al.*, 2013, and Mohammadzadeh *et al.*, 2006).

The colour induced by rifampicin also appeared to increase the absorbance value of the measured samples, although absorbance values of blank wells containing only rifampicin and medium were removed from the samples. The absorbance values of the blank wells alone also increased with increasing rifampicin concentration. Removal of rifampicin from exposed samples prior to the addition of TTC could have helped in this.

Also, by visual detection, rifampicin interfered with the detection of the red colour of TTC reduced TPF. In calibration wells using a TTC concentration of 500 µg/ml, the colour change was more pronounced than in the wells of untreated samples. This was probably due to the slight evaporation of the bacterial suspension from the calibration wells because the calibration wells were located in the corner wells of the 96-well plate and not in the centre of the plate where the samples were located, resulting in a slightly higher final TTC concentration.

6.5 Study limitations

Although a workable protocol for resazurin staining of mycobacteria was found in this study, some limitations must also be considered. The study optimized resazurin staining and TTC staining for *M. marinum*, which were not found in previous study publications. In contrast, resazurin staining of *M. tuberculosis* had already been used in previous studies.

Another limitation is the number of bacterial strains used in the study. Only one strain of both *M. tuberculosis* and *M. marinum* was used to optimize redox dyes. In addition, viability determinations with *M. tuberculosis* stained with resazurin were not repeated, so it may be necessary.

The limitation for MDK-assay was that the plates were measured only after the first day of antibiotic exposure and not before exposure. Therefore, the measured values could not be compared with the results before exposure.

The next step could be to test the functionality of resazurin staining also with a virulent strain of *M. tuberculosis*. Various antibiotics could also be tested in addition to rifampicin.

6.6 Future prospects

Resazurin staining of mycobacteria can be used to quickly and inexpensively determine whether there are living bacterial cells in a sample. Resazurin-stained samples can be examined by both fluorescence measurement and visual inspection. Visual inspection is

also a good tool, especially in places where fluorescence measurement may not be possible, such as in developing countries.

In the future, the optimized mycobacterial resazurin staining protocol can be utilized in tuberculosis research when determining, for example, the efficacy of new drugs. Resazurin staining works especially with *M. tuberculosis* and longer antibiotic exposure measurements of *M. marinum*.

Since *M. tuberculosis* forms biofilms that are tolerant to antibiotics, at least *in vitro*, it is good that the development of antibiotic tolerance and cell viability in biofilms can be monitored by resazurin staining. If it is found that *M. tuberculosis* also forms biofilms *in vivo*, it is great advantage that biofilm research is carried out and new ways are developed to treat them.

7. CONCLUSIONS

The main objective of this study was to optimize a redox dye protocol suitable for mycobacterial research. This was accomplished for resazurin staining and suitable conditions for resazurin staining were found. In contrast, the TTC staining protocol did not work under the tested conditions.

Based on resazurin staining, biofilm growth of *M. tuberculosis* was found to be more tolerant to the antibiotic than planktonic growth. There were no major differences between the tolerance of the five- and 12-day biofilm.

Resazurin staining was also found to work better with *M. tuberculosis* than with *M. marinum*. This was probably due to the higher incubation temperature of *M. tuberculosis*.

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