



COURSEWORK



Mycobacterium smegmatis: Evaluation of isoniazid, ethionamide and a novel chemical entity using a modified Ziehl-Neelsen staining

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Introduction

Mycobacteria have a characteristic highly impermeable cell wall composed of around 80 mycolic acids forming a first carbon layer, which is covalently linked to the inner peptidoglycan layer. The linker consists of an arabinogalactan polysaccharide middle layer of three arabinan chains attached to the linear galactan polymer at position 5 (Figure 1.). Mycolic acids are synthesized in an anabolic bacterial fatty acid pathway (Figure 2.), that elongates fatty acyl chains in an enzyme-catalyzed post-translational process. It involves the production of short-chain primers by the FAS I (Rv2524c) protein which are attached to the mycolic acid-specific carrier protein AcpM by the β -ketoacyl ACP synthase III and FanH enzymes. These primers are then transported to the repetitive FAS II enzyme system which then extends the carbons. Extension is completed by the 2-trans enoyl ACP reductase InhA (Rv1484) ?. This rigid structure and the high amount of long fatty acid chains in the cell wall cause a low cell membrane permeability. High resistance to antibiotic molecules is therefore a consequence of a rigid cell wall, an active efflux of antibiotic molecules and chemical modification i.e., hydrolysis in β -lactams ?.

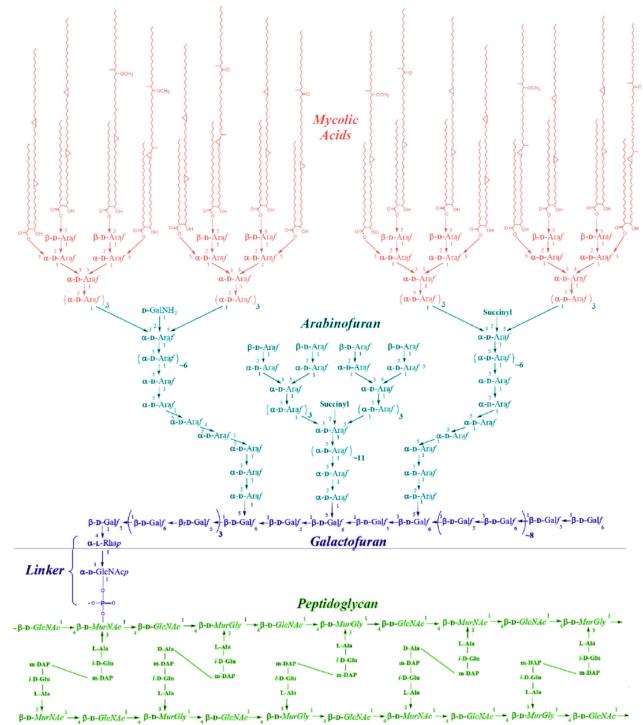


Figure 1. Cell wall structure of *M. smegmatis* mc²155. Long chain mycolic acids (red), 3 chains of arabinofuran (blue), a galactofuran layer (purple) covalently linked to the peptidoglycan layer (green). Figure was adopted from ?

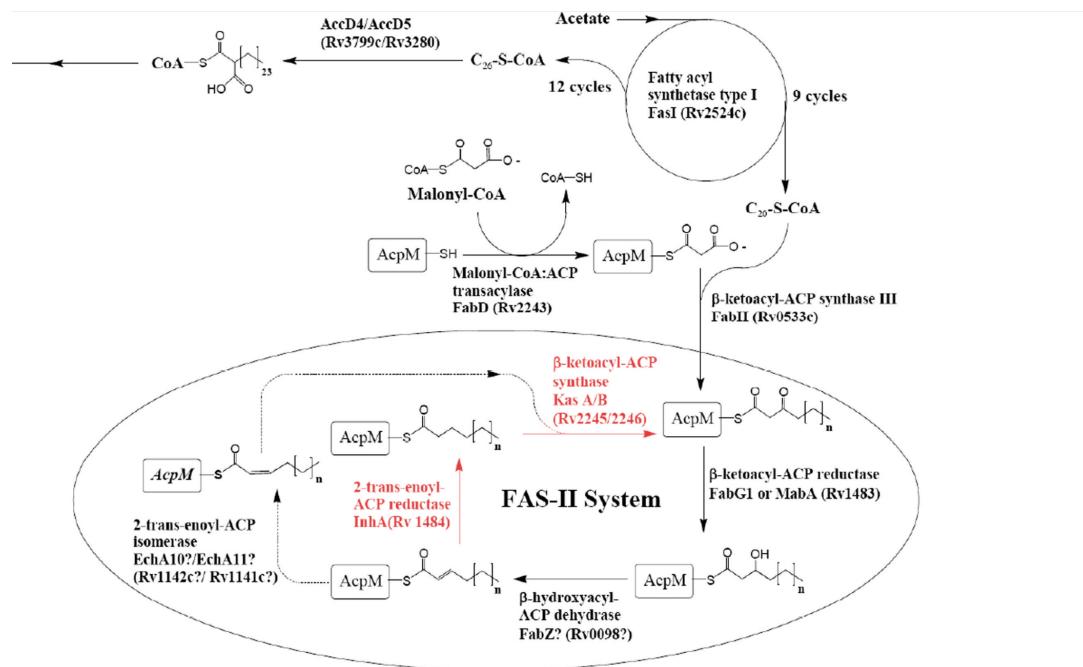


Figure 2. Bacterial fatty acid synthesis type II (FAS II). Isoniazid and Ethionamide target the InhA(Rv1484) and KatA(Rv2245) enzymes (red). Inhibiting the completion of the Fas II cycle. Figure was adopted from ?

M. smegmatis mutant mc²155, is a non-pathogenic, fast growing model for the study of mycobacteria. It is a saprophytic mycobacterium (does not enter the epithelial cells or remains inside phagocytes) ?. It therefore does not present the pathogenicity of *M. tuberculosis*. However, it is a good model to study antibacterial agents targeting the cell wall of *M. tuberculosis*, since both have the same architecture and physiology. This property also makes it a good model for genetic modification and cellular studies in mycobacteria in general ?. Ultimately, it is considered evolutionary too distant from the TB causing *M. tuberculosis* and is generally not a good model for virulence or pathogenicity studies on TB ?. Isoniazid (INH) and the related Ethionamide (ETH) target the same enzymes in the FAS II system of the mycolic acid synthesis: InhA (**Figure 2.**Rv1484) and β -ketoacyl-ACP synthase KasA (**Figure 2.**Rv2245/46). Both are prodrugs and require intra-cellular activation by katG (INH) or ethA (ETH) in the presence of NAD. Activation of ETH can additionally be regulated by ethR. Both exhibit a narrow spectrum bactericidal effect on fast-growing mycobacteria by disrupting the type II mycolic acid synthesis ???. *M. tuberculosis* and *M. smegmatis* are acid-fast bacilli (AFB). Their thick, hydrophobic cell wall confers the ability to resist decolorisation by acids during staining. A common technique is the carbol-fuchsin method also referred as the Ziehl-Neelsen staining (ZN-staining), to form a mycolate-fuchsin complex in the cell wall

resulting in AFB appearing red under the microscope, while the rest of the stain loses the fuchsin component due to the missing protective mycolic acid cell wall and can be counter stained for an effective visual contrast ?. We investigate the effect of INH, ETH and a novel chemical entity (NCE) on the the growth and morphology of *M. smegmatis* by verifying acid fastness using a cold ZN-staining method followed by bright-field microscopic examination.

Risk assessment and waste disposal

Although *M. smegmatis*. is a saprophytic microorganism, safety measures are nonetheless required since some opportunistic infections in human have been reported. It must therefore be handled according to Biosafety Level 1. This includes no drinking, eating, or smoking in the laboratory. Wearing a lab coat, nitrile gloves and eye/face protection all the time. All belongings must be placed in the metal cages. When leaving the laboratory, hands should be washed thoroughly. These measures are intended to avoid contamination and potential hazards and must be acknowledged with a signature prior to commencing the work. When working with the immersion oil, paying attention not to contaminate the other lenses and clean the 100x lens only with the Lense Cleansing Tissue ??. All liquid cultures and laboratory consumables, agar plates must be disinfected with a 5% (v/v) Trionic and disposed by autoclave using the make safe cycle. Sharp consumables must be disposed via the clinical waste stream

for incineration ?.

Materials and methods

Cryo-preservation of Mycobacterial cultures

A culture of actively replicating *M. Smegmatis* was preserved in 25% (v/v) glycerol and cryo-preserved in the freezer at -80°C, for about 6-12 months.

Starter culture

1% (v/v) of the cryo-preserved *M. Smegmatis* mc2155 culture was added to the Middlebrook liquid medium (7H9) and enriched with Albumine Dextrose Catalyst (ADC). It was then grown until an Optical Density (OD) reading of OD₆₀₀ = 1. One (1) mL was then transferred to a fresh 7H9 Middlebrook broth containing the ADC supplement and the process was repeated at least three times. Followed by inoculating a 1% (v/v) of the culture into 100 mL 7H9 Middlebrook broth containing 10% (v/v) of the ADC (Difco) supplement and 0.05% (v/v) Tween 80 (Sigma). The starting culture was then left to incubate in a 50 mL falcon tube at 37°C and constantly shaken at 180 rpm in a shaker to avoid floating biofilm formation.

Treatment of the Culture with INH, ETH and NCE

Optical density (OD) readings were taken using an Eppendorf Bio Photometer at 600 nm. At an OD₆₀₀ of 0.4, the starting culture was allocated into 4 separate falcon tubes and treated with 250 µg/mL INH, 500µg/mL ETH and 500µg/mL NCE and then returned to the shaker for 6 hours.

Determination of Growth

A four (4) – fold dilution of the culture was performed prior to the readings. After zeroing against the liquid broth media only, individual readings for the three treated samples with INH, ETH and NCE and the untreated wildtype (WT) were taken for λ = 595 nm. Thereby loading a 1 mL sample from every culture into the Eppendorf Bio Photometer using 12.5 mm² cuvettes and paying attention to mix the culture thoroughly using a pipette to avoid false readings due to the bacillus tending to sediment towards the bottom of the culture.

Preparation for ZN-staining

Four (4) slides were labeled U (untreated), X (unknown), H (isoniazid) and E (ethionamide) and 50 µL of the respective bacterial culture was pipetted

onto the slides to create a thin smear and allowed to fully air dry. Each smear was then inserted into the Genlab oven and heat-fixed at 80°C for 20 minutes.

Ziel-Neelsen cold Staining from Culture

The heat-fixed smears were then individually stained by covering every smear in carbol-fuchsin for 5 minutes using the TB-COLOUR Cold Staining Kit from the Bund Deutscher Hebammen Laboratory (BDH). Tap water was then used to carefully rinse the slides for about 5 seconds until all excess dye is removed. The slides were then treated with a few drops of TB-COLOUR de-staining solution to completely remove all excess carbol-fuchsin dye. This step is then followed by another careful washing step using tap water. The stained slides are then counterstained with malachite green and left to stain for about 5 seconds before carefully rinsing again with tap water for another 5 seconds. The washed counterstained slides are then left to fully air dry.

Mounting slides for microscopic observations

To prevent contamination with the immersion oil of the 100x lens, one drop of Eukitt medium containing 45% acrylic resin and 55% xylenes is placed in the middle of the slide. The specimen is then covered with a cover slip using a needle to avoid bubble formation. The prepared slides are then left to solidify for 5 minutes.

Examination under the microscope

The slides are individually placed under the bright-field microscope and observations made for x20, x40 and x100 (immersion oil) lenses, x10 ocular lens and the magnification caused by the refractive index of glass and Eukitt medium (1.510) resulting in a maximal magnification of x1000. All slides were observed using the immersion oil lens (x100) and recorded by a photograph.

Results

Data Analysis

All four treatment groups follow a normal distribution ([Figure 3](#)). Homoscedasticity criteria (variance is approximately equal) is only fulfilled between INH, NCE and ETH. There is no overlap between the untreated sample and the antibiotic treatment group, suggesting different population means ([Figure 4](#)).

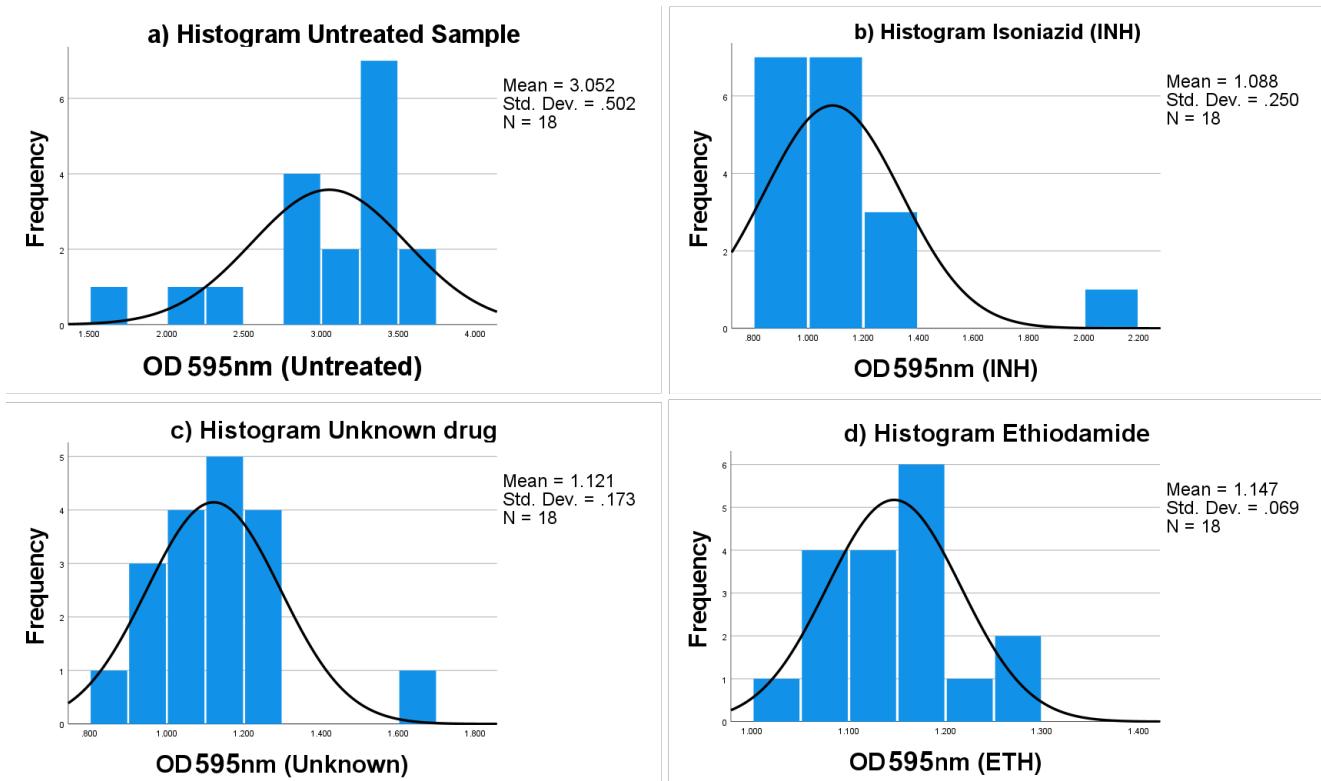


Figure 3. Histogram showing normal distribution for a) Untreated sample (UT), b) Isoniazid (INH), c) Unknown sample (NCE) and d) Ethionamide (ETH). Analysis was performed in SPSS.

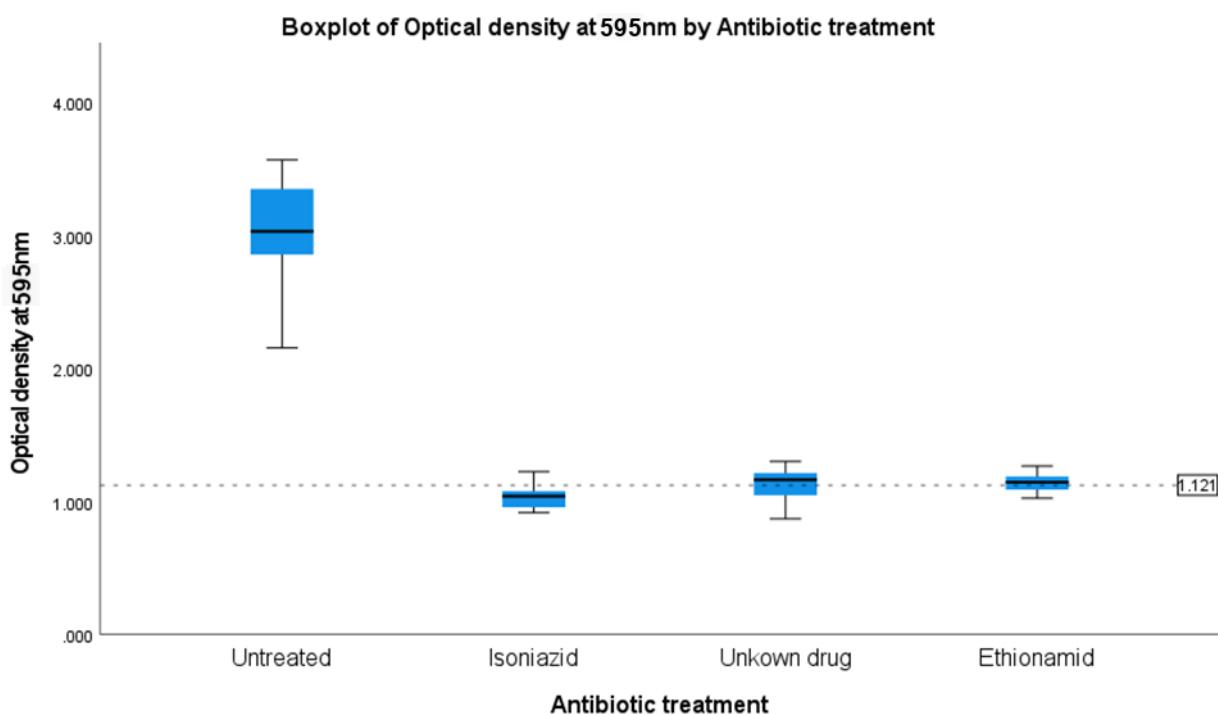


Figure 4. Boxplot of antibiotic agents, showing difference in variance between the means of samples treated with antibiotics and untreated samples. The dotted line shows the average OD595 between the antibiotics. Analysis was performed in SPSS.

Our null hypothesis (H_0) is therefore $\mu_1 = \mu_2 = \mu_3$ and our **alternative hypothesis (H_1)** can be stated as:

*One or more of the four treatments leads to unequal mean OD600 values and therefore influences the growth of *M. smegmatis*.*

Table 1 shows, that there is a high variance between the untreated and the treated groups and low variance within the treated groups, which confirms our observation of the non-homoscedasticity. Our P value is given with $P < 0.001$. With $P << \alpha$ (0.05). We therefore have a significant result and reject H_0 . We accept H_1 .

Table 1 Analysis of Variance (ANOVA) for optical density at 595 nm values. Analysis for INH, ETH, NCE and WT showing high variance between treated and untreated culture and low variance within the treated cultures. Analysis was performed in SPSS.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	37.609	3	12.536	45.385	<.001
Within Groups	18.783	68	276		
Total	56.392	71			

To investigate further which pairs of means contributed to the significant F-value, we perform the Kruskal-Wallis test to identify overall significance and examine each pair for significance in the post hoc study **Table 2**. Looking at Table 2, the Null hypothesis is rejected and the pairwise comparison **Figure 5** shows, that all antibiotic agents, including the unknown drug had a significant result ($p < 0.001$) when compared to the untreated sample. Figure 5 also shows, that all antibiotics when compared between each other had an insignificant result. Their mean OD595 value therefore has the same population mean.

Table 2 Hypothesis Test Summary. Kruskal-Wallis test for nonparametric data, testing H_0 with a significance of 0.050. Analysis was performed in SPSS.

Null Hypothesis	Test	Sig. ^a	Decision
The distribution of the optical density at 595 nm is the same for INH, ETH, NCE and WT.	Independent-Samples Kruskal-Wallis Test	< .001	Reject null hypothesis

^a The Significance level is .050

^b Asymptotic significance is displayed

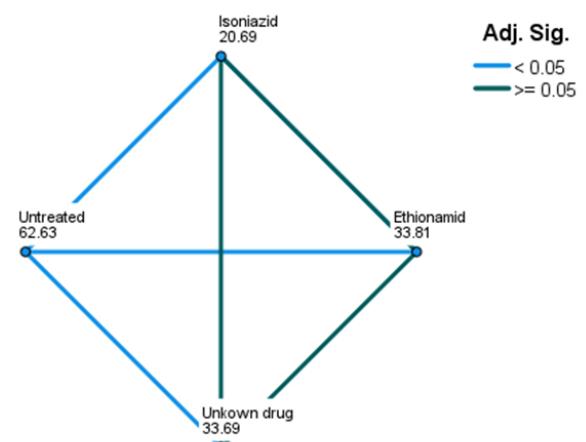


Figure 5. Pairwise comparison of antibiotic treatment. Graph showing pairwise test of null Hypothesis (H_0) at a significance level of .050. Each node shows the sample average rank of antibiotic treatment. Analysis was performed in SPSS.

Growth

Our statistical analysis showed that all treated cultures reduced the untreated OD of 3.052 by an average of 63.3%. INH reduced the OD by 64.4% (1.088), ETH reduced the OD by 62.4% (1.147) and the NCE reduced the OD by 63.3% (1.121). The reduction in OD compared to the untreated culture is significant for all three drugs. They also show a similar efficacy, since the differences in OD reduction between the individual drugs are not significant (**Figure 5**).

Morphology

Wild type culture (U). The untreated wildtype culture (U) containing *M. smegmatis* appeared as a clustered group of clumped bacilli under the microscope and has retained the pink carbol-fuchsin stain **Figure 6..** Compared to the treated groups (Figs ttreated), the wildtype culture also appears to have more bacilli per area.

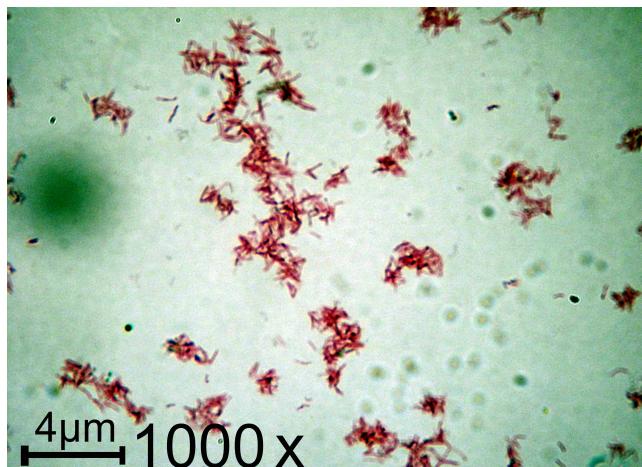


Figure 6. Wild type containing *M.smegmatis* under bright-field microscope. The Bacilli appear in red on a green media after the ZN-staining procedure. Observed at 1000x using 100x immersion oil lens and a 10x ocular. The figure was supplied by Dr Sanjib Bhakta.

Isoniazid (I)/ Ethionamide (E) culture. We analysed the INH and ETH (I, E) *M. smegmatis* culture and observed under the microscope a non-clustered aggregation of single bacilli cells [Figure 7.](#) It appeared to have less bacilli per area than the untreated culture (U) and a similar morphology and size compared to the unknown drug culture (X). It has obtained a green/blue color.

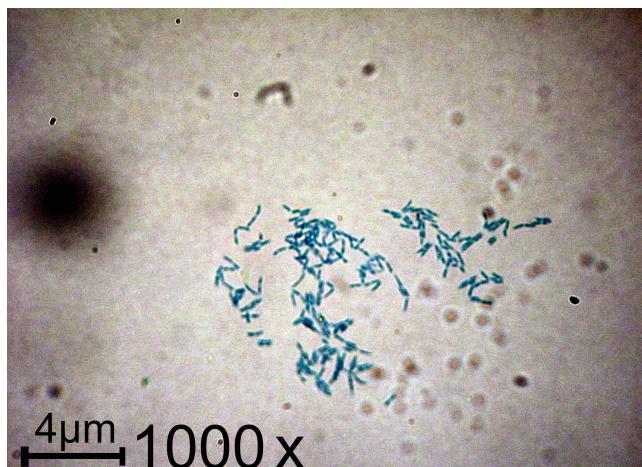


Figure 7. ZN-staining of the Isoniazid/Ethionamide treated culture of *M. smegmatis* showed the rod-shaped bacilli adopting a green/blue color. Observed at 1000x using 100x immersion oil lens and a 10x ocular. The figure was supplied by Dr Sanjib Bhakta.

Unknown drug culture (X). The bacilli appear aggregated, un-clustered with a green stain and similar morphology to the INH/ETH-stained culture (I,E). Compared to the wild type (U), it appears to have less bacilli per area.

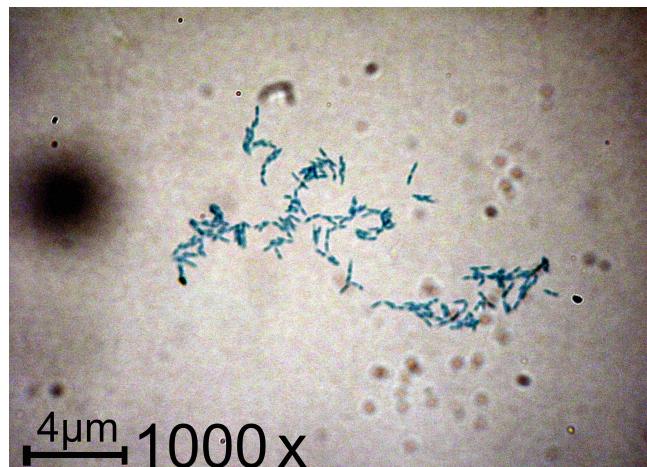


Figure 8. ZN-staining of the NCE treated culture of *M. smegmatis* showed the rod-shaped bacilli adopting a green/blue color. Observed at 1000x using 100x immersion oil lens and a 10x ocular. The figure was supplied by Dr Sanjib Bhakta.

Discussion

After the ZN-staining and decolorization procedure the untreated sample appeared pink ([Figure 6.](#)), while the treated samples appeared green ([Figure 7.](#), [Figure 8.](#)). This suggests the formation of a mycolate-fuchsin complex in the cell wall of the *M. smegmatis* wild type and a lack thereof in the groups treated with INH, ETH, and the NCE. Isoniazid and Ethionamide are known narrow spectrum, bactericidal, anti-TB drugs which affect the bacterial viability by inhibiting a correct mycolic acid synthesis in the cell wall [??](#). This leads to our hypothesis, that the treated samples could not retain the pink stain, because they lost the protective effect from the hydrophobic, tightly packed, and rigid mycolic acid outer layer leading to the cells being susceptible for treatment with acids or alcohols as contained in the decolorising solution. The morphology showed a reduction in clumping and number of bacilli, which could be verified by an OD reduction of 63.3% on average for INH, ETH and the NCE. This suggests a potential bactericidal mode of action of the NCE, because it showed similar morphology to INH and ETH and the difference in OD was not significant between the three treatment groups.

Conclusion

Cold ZN-Staining could successfully identify *M. smegmatis* as an acid-fast bacterium (ACB). The unknown drug (NCE) could significantly reduce the number of viable *M. smegmatis* bacilli and is therefore a promising new candidate for the treatment of TB caused by *M. tuberculosis*. We can conclude

this from our findings because *M. smegmatis* could be successfully decolorised after treatment with all three drugs. This result can be attributed to the inhibition of the cell wall formation by the treated group. The morphology showed a reduction in clumping for all drugs. Mycolic acids stitch together and form clumps during normal growth, as seen in the untreated sample. The reduction in the bacilli numbers, which was verified by an average significant OD reduction of 63.3%. The antibiotic treatment rank between the groups showed no significance, indicating that all three drugs have a similar mode of action. Considering INH and ETH being bacte-

ricidal, we assume a bactericidal mode of action of the NCE.

Acknowledgments

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