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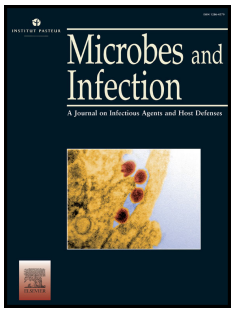

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Nanomotion technology in combination with machine learning: a new approach for a rapid antibiotic susceptibility test for *Mycobacterium tuberculosis*

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Abstract

Nanomotion technology is a growth-independent approach that can be used to detect and record the vibrations of bacteria attached to microcantilevers. We have developed a nanomotion-based antibiotic susceptibility test (AST) protocol for *Mycobacterium*

tuberculosis (MTB). The protocol was used to predict strain phenotype towards isoniazid (INH) and rifampicin (RIF) using a leave-one-out cross-validation (LOOCV) and machine learning techniques.. This MTB-nanomotion protocol takes 21 hours, including cell suspension preparation, optimized bacterial attachment to functionalized cantilever, and nanomotion recording before and after antibiotic exposure. We applied this protocol to MTB isolates (n=40) and were able to discriminate between susceptible and resistant strains for INH and RIF with a maximum sensitivity of 97.4% and 100%, respectively, and a maximum specificity of 100% for both antibiotics when considering each nanomotion recording to be a distinct experiment. Grouping recordings as triplicates based on source isolate improved sensitivity and specificity to 100% for both antibiotics. Nanomotion technology can potentially reduce time-to-result significantly compared to the days and weeks currently needed for current phenotypic ASTs for MTB. It can further be extended to other anti-TB drugs to help guide more effective TB treatment.

Keywords:
Antibiotic-susceptibility test
Atomic force microscopy
Nanomotion
Machine learning
Multi-drug resistant tuberculosis.
Mycobacterium tuberculosis

1. Introduction

Tuberculosis (TB) represents a major health concern. According to the World Health Organization, there were more than 10 million new infections and 1.5 million deaths caused by the bacterium in 2021. Furthermore, TB infection has become more difficult to treat in recent decades due to the emergence of drug-resistant TB (DR-TB), multidrug-resistant TB (MDR-TB), pre-extensively drug-resistant TB (pre-XDR-TB), and extensively drug-resistant TB (XDR-TB), all of which are resistant to the core currently employed antibiotics [1]. Effective TB treatment therefore requires (i) the use of a combination of antibiotics to minimise the risk of resistance development and emergence of MDR-TB, and (ii) a sufficient treatment duration to ensure cure effectiveness and avoid relapse. Antibiotic susceptibility tests (ASTs) are used to determine effective antibiotic combinations at start of treatment [2-5].

Currently, a combination of phenotypic and molecular methods are used to assess *Mycobacterium tuberculosis* (MTB) antibiotic susceptibility. Culture-based phenotypic assays are impaired by MTB's slow growth, meaning that data typically only becomes available after patient treatment regimen decision-making and initiation has already taken place [6, 7]. Molecular techniques for assessing drug susceptibility and resistance such as polymerase chain reaction (PCR), probe hybridization assays, or whole genome sequencing (WGS) offer a more rapid time-to-result [8-10]. However, determining what targets to probe with genotypic AST assays depends on the presence of previously characterised resistance mechanisms, which are not always clearly defined for newer anti-TB drugs. These limitations call for new approaches for the determination of antibiotic sensitivity for MTB.

Nanomotion-based AST is a novel approach using atomic force microscopy (AFM) and nanomotion technology to detect and record nanoscale movements in

biological samples [11-14]. In AFM, nanomechanical sensors (micro-cantilevers) oscillate when triggered by the organisms attached to their surface [15-17]. These oscillations are detected by an optical readout system with a sensitivity of less than 0.1 Angstrom (0.01 nm) (Fig. 1a) [15, 11, 12, 14]. As such, nanomotion technology can be adapted for bacterial phenotypic AST. Bacteria on the surface of a microcantilever will generate cantilever deflections through their metabolic activity. Differences in cantilever oscillations upon drug exposure depends on antibiotic susceptibility, hence permitting the delineation of susceptible and resistant bacteria (Fig. 1) [15, 18]. Nanomotion-based AST requires small sample sizes (fewer than 1,000 bacteria), is replication-independent, and can determine the presence of a resistance phenotype within a few hours [15, 18]. It has already been successfully applied for various bacterial strains, including *Staphylococcus aureus, Escherichia coli*, and *Bordetella pertussis* [15, 18, 19].

MTB's cell wall and surface charge properties differ from previously studied bacteria strains, thereby requiring a unique protocol for cantilever attachment. We therefore aimed to develop and validate a rapid nanomotion-based AST method for MTB that could overcome the limitations imposed by the strain's slow replication rate. We herein present how nanomotion technology can drastically accelerate time-to-result compared to existing phenotypic methods (Fig. 1b), representing a paradigm shift in MTB phenotypic AST.

2. Materials and methods

2.1. Bacterial strains, culture conditions, and antibiotic susceptibility tests

Mycobacterium bovis BCG (ATCC® 35737), *M. smegmatis* mc²155 (ATCC® 700084), and *M. tuberculosis* H37Rv (ATCC® 27294) strains were used to study mycobacteria

attachment to the cantilever. Clinical strains susceptible or resistant to isoniazid (INH) and rifampicin (RIF) were obtained from the collection of the Institute of Microbiology of Lausanne University Hospital. Bacteria were cultured in mycobacteria growth indicator tubes (MGIT) incubated using an automated mycobacterial detection system (BD BACTEC™ MGIT™). The MGIT method was also used to perform phenotypic AST for first-line drugs. Molecular and phenotypic susceptibility testing for all strains was conducted as previously described to determine susceptibility to INH and RIF [20, 21]. Bacterial minimal inhibitory concentrations (MICs) were determined using resazurin microtiter plate assays (REMA). Molecular resistance to INH was determined by analysing *inhA*, *katG*, and *oxyR* gene expression, as well as via WGS [21]. Molecular resistance to RIF was determined by analysing *rpoB* gene expression [20, 22].

2.2. Reagents and antibiotics

Chitosan, dimethyl sulfoxide (DMSO), glutaraldehyde, phosphate buffered saline (PBS), poly-diallyl-dimethylammonium chloride (pDADMAC), poly-D-lysine (PDL), resazurin sodium salt, TWEEN 80, INH, and RIF were purchased from Sigma-Aldrich (St. Louis, MO). MGITs and MGIT 960 supplement kits were purchased from Becton Dickinson (Franklin Lakes, NJ). INH and other chemical agents were dissolved in sterile water, while RIF was dissolved in DMSO.

2.3. Nanomotion-AST

To perform nanomotion-AST for MTB, we prepared MTB cell suspensions, attached them to a functionalized cantilever, and recorded and analysed nanomotions in the absence and presence of antibiotic agents. The overall protocol takes 21 hours, and the full, detailed protocol can be found in the supplementary materials. We confirmed

efficient bacterial adhesion via two approaches: monitoring resonance frequency and visual examination of bacterial presence on the cantilever before and after each recording.

2.4. Biosafety

Initial validation was performed using *M. smegmatis*, *M. bovis* strain BCG, and *E. coli* strains in BSL-1 and -2 laboratories. Subsequent validation using ATCC® and clinical MTB strains were all performed in our BSL-3 laboratory. The nanomotion instrument was placed under the laminar flow hood located in a BSL-3 environment, and was connected to a computer using a cable (Fig. 2). All experiments were performed by biologists or biomedical technicians with BSL-3 training and authorization to handle MTB strains.

3. Results

3.1. Microcantilever functionalization for M. tuberculosis *attachment.*

Mycobacteria have waxy cell surfaces due to an abundance of mycolic acids and various other lipids. Therefore, we tested a panel of functionalizing agents with different chemical properties when examining MTB attachment to the cantilever. The cantilevers were functionalized with either 20% (*v/v*) pDADMAC, 0.1 mg/mL PDL, 0.1 mg/mL chitosan, or 0.5% (*v/v*) glutaraldehyde, after which *M. smegmatis*, *M. bovis* BCG, and *M. tuberculosis* H37RV were sequentially applied. Immediately post-application, bacteria were observed on the microcantilever for all four functionalizing agents. However, after several washes with MGIT media, 20% (*v/v*) pDADMAC yielded the best results. Although attachment with glutaraldehyde worked to some extent for *M. smegmatis* and *M. bovis* BCG, it failed to maintain MTB attachment at the micro-

cantilever for which a superior attachment was obtained with pDADMAC 20% (Fig. S2). We therefore decided to continue subsequent nanomotion experiments with 20% *(v/v)* pDADMAC-functionalized micro-cantilevers.

3.2. Development of a nanomotion-AST assay for M. tuberculosis.

We developed a five-step workflow for nanomotion-based MTB AST (Fig. 1c). Nanomotion technology is highly sensitive to external sources of vibrations, but working with viable MTB strains requires a biosafety cabinet in a BSL-3 laboratory that provides laminar air flow. We therefore had to use a noise isolation device to overcome the vibrations emitted by the biosafety cabinet (Fig. 2), as well as test the reliability of the instrument under laminar air flow. These tests were performed first using *E. coli* in a BSL-1 laboratory before proceeding to MTB in a BSL-3 setting.

Each nanomotion recording began by reading the deflections generated by an unloaded (bacteria-free) pDADMAC-functionalized cantilever for five minutes. This was referred to as the blank portion of the recording, and is necessary to ensure optimal sensor functionality and detect potential bacterial contamination within the supplemented MGIT media in the measurement chamber. After this, the sensor would be removed, loaded with bacteria, and returned to the measurement chamber. Nanomotion recordings of the attached bacteria in supplemented MGIT were first carried out for one hour in the absence of any antibiotic agent (the “bac” phase) in order to capture baseline/endogenous oscillations. After this, INH or RIF was added and bacterial oscillations were recorded for 20 hours (“drug” phase). The protocol duration therefore totalled 21 hours.

Cantilever deflections were sampled at a frequency of 60 kHz. To visualize the data, deflection variance for every ten seconds of recording was calculated during data

acquisition using a custom LabVIEW code (Fig. 3). However, raw data was used later for classification model development. This workflow summarizes the first protocol for a nanomotion-based AST for MTB under BSL-3 conditions.

3.3. Prediction of isoniazid resistance using nanomotion-AST

In order to use nanomotion technology for AST, there must be a set of parameters within the nanomotion signal that can be used to discriminate between resistant and susceptible strains. We therefore examined the clinical applicability of our AST protocol by testing a set of forty sensitive and resistant clinical isolates from our MTB collection.

To examine whether our protocol could predict INH resistance, we examined 27 clinical isolates sensitive or resistant to INH. Each isolate was independently recorded in triplicate (Table S1 and Fig. 3). Each recording within a triplicate was considered a distinct experiment, which allowed us to compare separate recordings from the same isolate and determine if experimental repetition would be needed for acceptable performance. The same set-up was used for each isolate, while the raw data of variance evolution throughout the experiments were visualised using a custom LabVIEW code. In total, our INH dataset contained 39 recordings for INH-susceptible strains and 38 recordings for INH-resistant strains, upon which we applied leave-one-out cross validation (LOOCV).

Sensitivity assesses how well our method can predict a susceptible isolate, while specificity assesses performance for prediction of resistance. A 100% performance indicates no recordings falsely classified as a resistant strain. The correct identification of a resistant strain is the most critical factor for avoiding the administration of an ineffective antibiotic. Using seven free parameters, the algorithm was able to classify the data with an accuracy of 96.1%, a sensitivity of 92.3% and a specificity of 100%.

With eight parameters, performance reached approximately greater-or-equal to 95% for all three metrics (97.4% accuracy, 94.9% sensitivity and 100% specificity) (Fig. 3).

Similar performances were achieved when applying LOOCV at the sample level — that is, each individual test set comprised all three triplicate recordings generated from a given individual sample. Here, recording level performance was slightly lower (seven parameters: 94.7% accuracy, 89.5% sensitivity, and 100% specificity; eight parameters: 96.1% accuracy, 92.1% sensitivity, and 100% specificity) (Fig. 4). However, we achieved the best performance by using LOOCV on single replicates and subsequently combining the predictions for each triplicate using majority voting. Here, a five parameter algorithm was sufficient to attain 100% accuracy, sensitivity, and specificity (Fig. 4).

3.4. Prediction of rifampicin resistance using nanomotion-AST

We next applied the same approach to a dataset of 13 clinical isolates sensitive or resistant to RIF with corresponding susceptible (n=18) or resistant (n=19) recordings. A model based on a three-parameter algorithm was able to classify resistant and susceptible strains using LOOCV at the recording level with a performance of 94.6% accuracy, 94.4% sensitivity, and 94.7 % sensitivity. When the algorithm was extended to include additional parameters (from four to twelve parameters), the dataset could be perfectly delineated with 100% accuracy, 100% sensitivity, and 100 % specificity (Fig. 4). Applying LOOCV at the sample level (with triplicates grouped together) yielded a performance of 97.2% accuracy, 94.4% sensitivity, and 100% specificity for four-parameter algorithms and perfect delineation for five parameter algorithms (100% accuracy, 100% sensitivity, and 100% specificity) (Fig. S7). When all three replicates

were utilised through the application of majority voting, both four- and five-parameter algorithms exhibited 100 % accuracy, 100% sensitivity, and 100% specificity (Fig. S8).

4. Discussion

We have combined nanomotion technology and machine learning to develop a novel rapid AST approach for MTB, and validated it for INH and RIF using a set of clinical isolates with different minimum inhibitory concentrations (MICs). Though nanomotion technology has been previously applied to investigate the impact of beta-lactam antibiotics on various bacteria including *E. coli*, *B. pertussis, and S. aureus* [23, 24, 18, 19], this study is the first to establish a nanomotion-based AST that, in combination with machine learning, can classify MTB isolates that are resistant or susceptible to two major clinically-used antibiotics. A previous study on nanomotion using the virulence-attenuated strain *M. bovis* BCG showed that mycobacterial viability can be measured in response to antibiotic exposure [25]. However, this proof-of-concept study did not aim to generally distinguish between sets of resistant and susceptible strains [25].

By applying LOOCV, the models we built in the one-to-twelve-dimensional parameter space were able to classify susceptible and resistant strains with high confidence (over 95% accuracy, sensitivity, and specificity) for both INH and RIF. The RIF model showed slightly better performance (incorporating fewer parameters). We believe that this is because there is a greater gap between MICs of susceptible and resistant isolates, as determined by resazurin microplate assay (REMA).

Nevertheless, prediction performance for both antibiotics could be improved by applying majority voting to the three biological replicates and then combining sample-level individual prediction outcomes, as well as finding parameters which can perfectly

delineate (100% accuracy) the data at the sample level. Therefore, a combination of nanomotion recordings with advanced analyses by machine learning can be applied as a highly sensitive and specific rapid AST for INH and RIF.

Working with MTB is very challenging since it possesses a slow growth rate and work must be done under BSL-3 conditions. Nonetheless, we were able to include 40 clinical isolates in this study. Although limited, this number is still significant as the isolates were not genetically related strains, but rather clinical isolates with different genetic backgrounds. Since nanomotion-based AST performs well with antibiotics that differ in their modes of action, this method can presumably be applied to other, more recently-developed antibiotics such as bedaquiline, linezolid, and future drug candidates [10, 21, 26].

Effective treatment of TB relies on drug regimen selection, which in turn depends on reliable and timely access to AST data. As such, commonly used growth-dependent assays underperform because of MTB's slow growth. Molecular and genomic methods such as PCR and WGS are faster and show high performance for some agents such as INH and RIF [27-29, 20], but are limited by high costs, little applicability to novel drugs, and low flexibility. Nanomotion-based AST is a growth-independent method, and thus circumvents the limitations of conventional phenotypic ASTs, leading to a turnaround time of less than 24 hours.

There are several rapid antibiotic susceptibility tests available for more common bacteria with known mechanisms of resistance. Enzymatic tests, for instance, can be used to detect the degradation of agents such as β-lactamase or carbapenemase. These tests are also helpful for predicting resistance to specific antibiotics. Indeed, new molecular-based rapid antibiotic tests for TB such as the Xpert MTB/RIF test allow for the rapid prediction of resistance to rifampicin [30]. However, there are currently no

rapid phenotypic tests available for TB that can be used for either new drugs or drugs with poorly understood resistance mechanisms. Since phenotypic tests are particularly useful when the mechanism of resistance is unknown, there is a need for the development of new, rapid phenotypic tests that can be used to diagnose and treat TB more effectively.

Another advantage of nanomotion-based AST is that it requires a specimen size of only approximately 100 bacteria [19, 17]. This allows the possibility of testing several antibiotics in parallel using the same positive culture, as well as testing antibiotic combinations. In our previous study on bacteremia, we combined nanomotion technology with a simple method for purifying and concentrating bacteria from positive blood cultures in order to develop a rapid AST against bloodstream infection agents [19]. In the long run, nanomotion technology applied to tuberculosis may reduce MGIT culture incubation times, or even permit AST to begin directly with purified bacteria obtained from a clinical sample such as bronchial aspirate sputum or bronchoalveolar lavage. It is important to ensure that bacteria remain on the cantilever throughout the experiment to avoid signal variation resulting from bacterial loss instead of response to antibiotic treatment. To ensure efficient bacterial adhesion, we validated the efficacy of pDADMAC as a linking agent and visually checked for the presence of bacteria on the cantilever before and after all recordings. Additionally, we monitored resonance frequency variations to confirm bacterial attachment to the cantilever — namely, resonance frequency should not vary if bacterial attachment remains unchanged during experimentation. Furthermore, the method is species agnostic, which makes it an interesting tool for non-TB mycobacteria, such as *M. abscessus*, that also often presents multi-resistance phenotypes [31].

Nanomotion represents a new paradigm for predicting antibiotic susceptibility/resistance [32]. The antibiotic concentrations used for nanomotion AST do not correspond to the critical concentrations of antibiotics as defined by the EUCAST or recommended by the WHO when currently performing classical AST [6, 7]. Due to the very limited antibiotic exposure time (under 24 hours in this study), nanomotion AST may not be fully suitable for MIC determination, even though it can discriminate between sensitive and resistant organisms. Whether nanomotion can distinguish metabolic variations or stress responses is something that would be interesting to explore in future studies. Indeed, bacterial metabolism state (growing versus latent) has been associated with resistance and tolerance mechanisms in MTB [33, 34]. Nanomotion technology enables us to measure bacterial response to antibiotic exposure without relying on bacterial growth-dependent metrics such as MIC or minimum bactericidal concentration (MBC). That said, a correlation has been demonstrated between MIC values and nanomotion signals [15]. As for MBC, it is important to note that 22 hours of incubation may not be sufficient to kill all bacteria [35]. The changes in nanomotion signal observed in this study may be attributed to metabolic changes, something that warrants further investigation in future studies. Further, this technology holds great potential for antibiotic susceptibility testing as it allows for direct measurement of bacterial response to antibiotics without the need for sub-culturing. As such, using nanomotion to test the impact of different antibiotic concentrations may provide new and complementary information on MTB resistance or tolerance to anti-tuberculosis agents. It will be important for the nanomotion technology to be available in multi-channels in the future, to allow for an efficient use in clinical settings or drug discovery. This will increase throughput and enable testing of multiple antibiotics in parallel. The ability to test multiple antibiotics in parallel using

multi-channel technology would greatly improve the speed and efficiency of antibiotic susceptibility testing, particularly in the context of drug discovery where large numbers of compounds need to be screened for potential antimicrobial activity. Furthermore, multi-channel technology could facilitate the testing of antibiotic combinations, which are essential for the treatment of both susceptible tuberculosis and MDR-XDR tuberculosis.

This study presents an innovative growth-independent AST method applicable to BSL-3 conditions, developed first for fast-growing bacteria and then adapted and validated for MTB. This method needs to be further examined in a larger study, as well as tested against low-level and heterogeneous resistant strains to support further development as an in vitro diagnostic test. This approach also represents a new tool for the study of growth-independent drug responses. Our results open the door for further method development and pre-clinical studies using nanomotion technology to examine MTB isolates, working towards the ultimate goal of developing an AST possessing high performance and short time-to-result, thereby combining the advantages of broadly applicable phenotypic ASTs and rapid molecular diagnostic methods.

Declaration of competing interest

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Appendix A. Supplementary data

References

[1] Murray CJL, Ikuta KS, Sharara F, Swetschinski L, Robles Aguilar G, Gray A, et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. The Lancet 2022;399:629-55.

[2] Antimycobacterial Susceptibility Testing G, Georghiou SB, Rodwell TC, Korobitsyn A, Abbadi SH, Ajbani K, et al. Updating the approaches to define susceptibility and resistance to anti-tuberculosis agents: implications for diagnosis and treatment. European Respiratory Journal 2022;59:2200166.

[3] Aghayee S, Benadiba C, Notz J, Kasas S, Dietler G, Longo G. Combination of fluorescence microscopy and nanomotion detection to characterize bacteria. J Mol Recognit 2013;26:590-5.

[4] Acquah R, Furin J. Universal regimens or universal access to drug susceptibility testing for tuberculosis? The Lancet Infectious Diseases 2019;19:224-5.

[5] Antimycobacterial Susceptibility Testing G. Updating the approaches to define susceptibility and resistance to anti-tuberculosis agents: implications for diagnosis and treatment. The European respiratory journal 2022;59:2200166.

[6] Schön T, Werngren J, Machado D, Borroni E, Wijkander M, Lina G, et al. Antimicrobial susceptibility testing of Mycobacterium tuberculosis complex isolates - the EUCAST broth microdilution reference method for MIC determination. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases 2020;26:1488-92.

[7] Schön T, Werngren J, Machado D, Borroni E, Wijkander M, Lina G, et al. Multicentre testing of the EUCAST broth microdilution reference method for MIC determination on Mycobacterium tuberculosis. Clinical Microbiology and Infection 2021;27:288.e1-.e4.

[8] Walker TM, Kohl TA, Omar SV, Hedge J, Del Ojo Elias C, Bradley P, et al. Whole-genome sequencing for prediction of Mycobacterium tuberculosis drug susceptibility and resistance: a retrospective cohort study. The Lancet. Infectious diseases 2015;15:1193-202.

[9] Walker TM, Merker M, Kohl TA, Crook DW, Niemann S, Peto TE. Whole genome sequencing for M/XDR tuberculosis surveillance and for resistance testing. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases 2017;23:161-6.

[10] Walker TM, Miotto P, Köser CU, Fowler PW, Knaggs J, Iqbal Z, et al. The 2021 WHO catalogue of Mycobacterium tuberculosis complex mutations associated with drug resistance: a genotypic analysis. The Lancet Microbe 2022;3:e265-e73.

[11] Alonso-Sarduy L, De Los Rios P, Benedetti F, Vobornik D, Dietler G, Kasas S, et al. Real-time monitoring of protein conformational changes using a nano-mechanical sensor. PloS one 2014;9:e103674.

[12] Kasas S, Ruggeri FS, Benadiba C, Maillard C, Stupar P, Tournu H, et al. Detecting nanoscale vibrations as signature of life. Proc Natl Acad Sci U S A 2015;112:378-81.

[13] Kasas S, Stupar P, Longo G, Dietler G. [Detecting life thanks to the atomic force microscope]. Med Sci (Paris) 2015;31:369-71.

[14] Kasas S, Stupar P, Dietler G. AFM contribution to unveil pro- and eukaryotic cell mechanical properties. Semin Cell Dev Biol 2018;73:177-87.

[15] Longo G, Alonso-Sarduy L, Rio LM, Bizzini A, Trampuz A, Notz J, et al. Rapid detection of bacterial resistance to antibiotics using AFM cantilevers as nanomechanical sensors. Nat Nanotechnol 2013;8:522-6.

[16] Ghaemi N, Nikoobin A, Ashory MR. A Comprehensive Categorization of Micro/Nanomechanical Resonators and Their Practical Applications from an Engineering Perspective: A Review. Advanced Electronic Materials 2022;n/a:2200229.

[17] Al-madani H, Du H, Yao J, Peng H, Yao C, Jiang B, et al. Living Sample Viability Measurement Methods from Traditional Assays to Nanomotion. Biosensors 2022;12:453.

[18] Kasas S, Malovichko A, Villalba MI, Vela ME, Yantorno O, Willaert RG. Nanomotion Detection-Based Rapid Antibiotic Susceptibility Testing. Antibiotics 2021;10:287.

[19] Stupar P, Opota O, Longo G, Prod'hom G, Dietler G, Greub G, et al. Nanomechanical sensor applied to blood culture pellets: a fast approach to determine the antibiotic susceptibility against agents of bloodstream infections. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases 2017;23:400-5.

[20] Opota O, Senn L, Prod'hom G, Mazza-Stalder J, Tissot F, Greub G, et al. Added value of molecular assay Xpert MTB/RIF compared to sputum smear microscopy to assess the risk of tuberculosis transmission in a low-prevalence country. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases 2016;22:613-9.

[21] Laurent S, Zakham F, Bertelli C, Merz L, Nicod L, Mazza-Stalder J, et al. Genome sequencing of Mycobacterium tuberculosis clinical isolates revealed isoniazid resistance mechanisms undetected by conventional molecular methods. Int J Antimicrob Agents 2020;56:106068.

[22] Opota O, Zakham F, Mazza-Stalder J, Nicod L, Greub G, Jaton K. Added Value of Xpert MTB/RIF Ultra for Diagnosis of Pulmonary Tuberculosis in a Low-Prevalence Setting. Journal of clinical microbiology 2019;57.

[23] Arnal L, Longo G, Stupar P, Castez MF, Cattelan N, Salvarezza RC, et al. Localization of adhesins on the surface of a pathogenic bacterial envelope through atomic force microscopy. Nanoscale 2015;7:17563-72.

[24] Villalba MI, Stupar P, Chomicki W, Bertacchi M, Dietler G, Arnal L, et al. Nanomotion Detection Method for Testing Antibiotic Resistance and Susceptibility of Slow-Growing Bacteria. Small 2018;14.

[25] Mustazzolu A, Venturelli L, Dinarelli S, Brown K, Floto RA, Dietler G, et al. A Rapid Unraveling of the Activity and Antibiotic Susceptibility of Mycobacteria. Antimicrobial agents and chemotherapy 2019;63.

[26] Kadura S, King N, Nakhoul M, Zhu H, Theron G, Köser CU, et al. Systematic review of mutations associated with resistance to the new and repurposed Mycobacterium tuberculosis drugs bedaquiline, clofazimine, linezolid, delamanid and pretomanid. J Antimicrob Chemother 2020;75:2031-43.

[27] Wang L, Yang J, Chen L, Wang W, Yu F, Xiong H. Whole-genome sequencing of Mycobacterium tuberculosis for prediction of drug resistance. Epidemiol Infect 2022;150:e22.

[28] Consortium CR, the GP, Allix-Beguec C, Arandjelovic I, Bi L, Beckert P, et al. Prediction of Susceptibility to First-Line Tuberculosis Drugs by DNA Sequencing. The New England journal of medicine 2018;379:1403-15.
[29] Boehme CC, Nabeta P, Hillemann D, Nicol MP, Shenai S, Krapp F, et al. Rapid molecular detection of tuberculosis and rifampin resistance. The New England journal of medicine 2010;363:1005-15.
[30] Chakravorty S, Simmons AM, Rowneki M, Parmar H, Cao Y, Ryan J, et al. The New Xpert MTB/RIF Ultra: Improving Detection of Mycobacterium tuberculosis and Resistance to Rifampin in an Assay Suitable for Point-of-Care Testing. MBio 2017;8.
[31] Daley CL, Iaccarino JM, Lange C, Cambau E, Wallace RJ, Jr., Andrejak C, et al. Treatment of Nontuberculous Mycobacterial Pulmonary Disease: An Official ATS/ERS/ESCMID/IDSA Clinical Practice Guideline. Clinical Infectious Diseases 2020;71:905-13.
[32] Stojanovic Z, Gonçalves-Carvalho F, Marín A, Abad Capa J, Domínguez J, Latorre I, et al. Advances in diagnostic tools for respiratory tract infections. From tuberculosis to COVID19: changing paradigms? ERJ Open Research 2022;00113-2022.
[33] Smith T, Wolff KA, Nguyen L. Molecular biology of drug resistance in Mycobacterium tuberculosis. Curr Top Microbiol Immunol 2013;374:53-80.
[34] de Steenwinkel JE, de Knegt GJ, ten Kate MT, van Belkum A, Verbrugh HA, Kremer K, et al. Time-kill kinetics of anti-tuberculosis drugs, and emergence of resistance, in relation to metabolic activity of Mycobacterium tuberculosis. J Antimicrob Chemother 2010;65:2582-9.
[35] de Steenwinkel JEM, de Knegt GJ, ten Kate MT, van Belkum A, Verbrugh HA, Kremer K, et al. Time–kill kinetics of anti-tuberculosis drugs, and emergence of resistance, in relation to metabolic activity of Mycobacterium tuberculosis. Journal of Antimicrobial Chemotherapy 2010;65:2582-9.

Tables and figures

Figure Legends

Figure 1. Principles of a nanomotion-based *M. tuberculosis* AST approach. (A) A laser is emitted on a microcantilever nanomotion sensor and reflected on a position-sensitive photodetector that records its deflection (*Stupar, Opota et al. 2017 Clin Microbiol Infect*). Environmental background noise generates a very low level of vibrations detected on an empty microcantilever, and this is used as the blank control. Live bacteria attached to the microcantilever make nanoscale movements (nanomotion). These vibrations lead to an increased deflection of the microcantilever. Upon exposure to an inhibiting compound such as an effective anti-TB agent, bacterial vibrations from the attached TB strain decrease. The degree of the microcantilever deflection is recorded as the variance. (B) Comparison of nanomotion-based AST and conventional phenotypic AST for tuberculosis. (C) Experimental procedure and preparation of nanomotion AST: i) centrifugation of a positive mycobacteria growth indicator tube (MGIT), ii) resuspension of the bacterial pellet in fresh MGIT medium, iii) MTB strains are attached to functionalized cantilevers by immersing the cantilever in a bacterial suspension for 5 minutes, iv) transfer of the cantilever with attached cells to the nanomotion recording chamber for recording or v) recording and analysis.

Figure 2. Configuration of nanomotion instrument under biosafety cabinet in a biosafety laboratory level 3. **Lower panel:** (a) Biosafety cabinet located in a BSL-3 laboratory (b) nanomotion device; (c) compact laser module, (d) low-profile nanomotion device for easy operation in biosafety cabinet, (e) operator with personal

protective equipment for BSL-3 laboratory , (f) optical microscope with integrated screen, (g) laptop with LabVIEW control software. **Upper left panel:** (h) optical microscope with integrated screen. **Upper right panel:** nanomotion instrument (i) measurement head, (j) protective lid, (k) laser plug, (l) vibration damping unit, (m) control and acquisition unit.

Figure 3. *Mycobacterium tuberculosis* cantilever attachment and subsequent nanomotion variance recordings. Microcantilevers from NANO*SENSORS*™ have the following dimensions: length 120-130 µm, width 33-37 µm, thickness 720-780 nm. Microcantilevers were washed four times with PBS and then incubated with MTB for 5 minutes. Mycobacteria attachment quality was evaluated by microscopy. Images were taken using an EVOS™ XL Core Imaging System (A) before the start of and (B) after the end of nanomotion recording experiments. The pre-experiment image served as the attachment control and showed typical MTB "cords", while the post-experiment image confirmed the absence of any significant detachment or attachment of bacteria on the microcantilever. MTB linical strains obtained from our diagnostic laboratory were selected based on their susceptibility or resistance to isoniazid (INH) or rifampicin (RIF). Upon MTB attachment to the cantilever, we observed a significantly higher nanomotion variance compared to the blank phase. After the addition of INH, both susceptible (C) and resistant (D) strains responded, although they responded differently. Towards the end of the experiment, bacterial vibrational variance was greater in resistant strains than susceptible strains. (E) and (F) MIC distribution for INH (E) and RIF (F) of the MTB strain genotypes used for this validation study.

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504 **Figure 4. Classification performance evaluated with LOOCV at the recording**
505 **level for the isoniazid dataset.** Algorithm complexity increased from a single
506 parameter up to 12 parameters (separation in 1-12 dimensional feature space).