ABSTRACT BOOK

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Invited Speakers

GL08

Unlocking the potential of phylodynamic birth-death models: Opportunities and challenges in TB epidemiology

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Over the past years, the TB field has seen an explosion of genomic sequencing data. These data not only yield insights into which strains are circulating where, but also contain detailed information about past *Mtb*transmission dynamics, including transmission rates, reproductive numbers, and duration of infectiousness. Phylodynamic methods provide an efficient and accurate way of extracting this information, but have so far seen limited use in the field. In this talk, I will focus on phylodynamic birth-death models, showcase their potential for *Mtb* transmission studies, and highlight *Mtb*-related challenges.

GL12

BNT164: Preclinical development of two mRNA-based tuberculosis vaccine candidates

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There is a critical need for new Tuberculosis (TB) vaccines to reduce the incidence and mortality of TB. BNT164a1 and BNT164b1 are two lipid-nanoparticle-formulated mRNA-based TB vaccine candidates. Both candidates encode the same combination of eight *Mycobacterium tuberculosis* (*Mtb*) antigens expressed across different stages of *Mtb* infection and only differ in the type of mRNA used (nucleoside-unmodified RNA or N1-methyl pseudouridine-containing modified RNA). Here, we report the immunogenicity, safety, and efficacy of BNT164a1 and BNT164b1 in preclinical animal models. Following prime-boost immunization, both BNT164 candidates elicited T-cell responses (CD4+ and/or CD8+) against each of the eight *Mtb* antigens in tested mouse strains (C57BL/6, BALB/c, and HLA-A2.1/DR1 humanized mice) as assessed by IFNγ ELISpot. IgG responses were induced against six of the eight target antigens. BNT164 immunization significantly reduced bacterial burden in a low dose aerosol challenge *Mtb* infection model in C57BL/6 mice. In a GLP-compliant toxicology study in rats, the candidates showed a favorable safety profile.

In conclusion, BNT164a1 and BNT164b1 are immunogenic, efficacious, and well tolerated in preclinical models, and are the first mRNA-based TB vaccines to enter Phase I clinical trials (NCT05537038, NCT05547464).

GL15

Tackling the tip of the iceberg – using a one health approach to tuberculosis

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Tuberculosis, caused by infection with *Mycobacterium tuberculosis* complex (MTBC) members, is a significant global health concern for humans and animals. Despite the zoonotic and anthropogenic potential of MTBC, there has historically been little interaction between human and animal health. The “Roadmap to Zoonotic Tuberculosis” (2017) was the first step in recognizing the role of wild and domestic animals in the global plan to eradicate human TB. As scientific advances facilitate improved detection in human and animal populations, there is a growing imperative to revisit the complex epidemiology of this multi-host disease and apply transdisciplinary expertise to construct effective management and control programs. South Africa has one of the highest human TB burdens. People in rural communities often have regular contact with communal livestock, yet there is little information on zoonotic TB or MTBC in animals. The lack of resources for animal health surveillance and intervention, communal herding practices, and traditional use of livestock contribute to maintenance of animal TB in communities. In addition, the historical spillover from infected cattle into wildlife populations has resulted in *Mycobacterium bovis* (*M. bovis*) reported in 25 African wildlife species, some of which have become reservoir hosts. Endemically infected wildlife populations also present a threat of spillback into livestock and people. Although previously understudied, the role of indirect transmission through environmental contamination may be an important factor in inter-species transmission. Using a One Health approach to TB can provide novel insights and improve strategies to control disease in humans and animals.

GL16

One health approaches to trace *M. leprae*'s zoonotic potential through time

V J Schuenemann [1]
1: University of Basel

Leprosy, one of the oldest recorded diseases in human history, is still prevalent in Asia, Africa and South America with over 200,000 cases every year, calling for the integration of new perspectives, such as One Health approaches, essential to enable its characterization, prediction, and eradication. A key element to study the evolution and persistence of zoonotic pathogens are animal hosts. Besides its potential, this concept has often not yet been integrated into studies of diseases in the past, although ancient DNA approaches have helped to uncover the evolutionary

history and prevalence of diseases in the past. Here we will look into the first insights on medieval red squirrels and their connections to leprosy. We will examine two archaeological sites at Winchester, a medieval English city, well-known for its leprosarium and its connections to fur trade, from which we recovered four medieval Mycobacterium leprae genomes, including one from a red squirrel. In combination with historical and archaeological sources this genetic evidence enabled us to reconstruct new details on the transmission of leprosy between humans and squirrels in medieval times including a yet undetected transmission event. Overall, our study represents the first One Health approach on M. leprae's past transmissions, which is centered around a medieval animal host strain, and highlights the feasibility of such approaches to understand the disease's zoonotic past and current potential.

GL18

The (re)discovery of the spectrum of TB

R Houben [1]
1: London School of Hygiene and Tropical Medicine

Since the 1980s, TB has been thought and taught to either present as a 'latent infection' or 'active disease', which informed but also constrained global TB policy and research. After repeated failures of DOTS and other interventions to reduce the burden of TB, the past 15 years have seen a growing push against the classic TB paradigm. Informed by conceptual thinking and empirical evidence from the past and present, we now recognise that TB operates on a spectrum, including non-infectious and subclinical TB, with a wide variety of potential disease pathways.

In his presentation Prof Houben will give an overview of the past decade-plus of turmoil in TB, highlighting how most of this was widely known in the past, where we are now, and explore some of the future consequences of the spectrum for clinical care, research and policy.

GL19

Genomic studies of tuberculosis in archaeological bone

K I Bos [1]
1: Max Planck Institute for Evolutionary Anthropology

The past decade has demonstrated an impressive contribution from molecular methods for understanding the distribution and evolutionary history of a growing number of historical infectious diseases. With genomic analyses quickly becoming standard, analytical techniques have become increasingly specialised. This talk will explore the application next generation sequencing to the study of past disease, and will describe current state of the art techniques for detection of *M. tuberculosis* DNA in archaeological tissues. Using archaeological material from the precontact Americas and post-Medieval Europe as examples, I will offer perspectives on molecular preservation, as well as the challenges and benefits that come with genome-level ancient pathogen reconstruction and analysis.

GL21

Single nucleotide variation catalogue from clinical isolates mapped on tertiary and quaternary structures of ESX-1–related proteins reveal critical regions as putative Mtb therapeutic targets

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Proteins encoded by the ESX-1 genes of interest are essential for full virulence in all *Mycobacterium tuberculosis* complex (Mtbc) lineages, the pathogens causing the highest mortality worldwide. Identifying critical regions in these ESX-1–related proteins could provide preventive or therapeutic targets for Mtb infection, the game changer needed for tuberculosis control. We analysed a compendium of whole genome sequences of clinical Mtb isolates from all lineages from >32,000 patients and identified single nucleotide polymorphisms (SNPs). When mutations corresponding to all non-synonymous single nucleotide polymorphisms (nSNPs) were mapped on structural models of the ESX-1 proteins, fully conserved regions emerged. Some could be assigned to known quaternary structures, whereas others could be predicted to be involved in yet-to-be-discovered interactions. Some mutants had clonally expanded (found in >1% of the isolates): these mutants were mostly located at the surface of globular domains, remote from known intra- and inter-molecular protein–protein interactions. Fully conserved intrinsically disordered regions (IDRs) of proteins were found, suggesting that these regions are crucial for the pathogenicity of the Mtbc. Altogether, our findings highlight fully conserved regions of proteins as attractive vaccine antigens and drug targets to control Mtb virulence. Extending this approach to the whole Mtb genome as well as other microorganisms will enhance vaccine development for various pathogens.

GL22

Data-driven assessment of Mycobacterium tuberculosis transmission in evolving demographic structures

J Sanz [1 2]
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The dynamics of *Mycobacterium tuberculosis* propagation in human populations presents a series of distinctive features that turns the development of accurate transmission models into an especially challenging task. For example, the slow evolution and big inertia of tuberculosis burden trends -that are a consequence of the large prevalence levels of latent tuberculosis infection- demand for model-based simulations that often span for several decades. This fact, coupled with the strong etary dependencies that are observed in many key epidemiological parameters, evidence the need of integrating suitable descriptions of demographic evolution into transmission

models. This is because, in many geographical settings, the demographic structure of the populations is simultaneously a quickly evolving feature and an impactful factor in shaping the transmission dynamics of the pathogen.

In this talk, I will summarize our group's efforts to integrate data-driven descriptions of demographic evolution within TB transmission models, paying special attention to the role of age-mixing patterns and their dependency on (evolving) demographic structures.

Our findings demonstrate that the inclusion of demographic dynamics into the mathematical models we use to produce baseline forecasts for TB burden series, as well as prospective evaluations for epidemiological interventions significantly impact the conclusions of many of these analyses. To illustrate this, we will discuss how the application of these modeling concepts to practical scenarios—such as assessing the expected impact of assorted vaccination campaigns in China, a country characterized by high TB burden levels and rapid demographic aging— yields markedly different implications for public health strategies.

Oral Presentations

OR01

A novel, high-content imaging based approach towards antibiotic drug discovery for non-tuberculous mycobacteria

W Chiu [1] S Cicchini [1] J Schepers [1] M Büyük [1] J Swinnen [1] G J Wijnant [1] E André [1 2]
1: KU Leuven 2: UZ Leuven

Pulmonary non-tuberculous mycobacteria (NTM) diseases are primarily caused by *M.abscessus* and/or *M.avium*. Current treatment regiments are long and ineffective due to the lack of 1) clinical breakpoints for most antibiotics and 2) novel NTM specific antibiotics.

The current gold-standard for drug susceptibility and drug discovery testing relies on determining the minimal inhibitory concentration (MIC), a labour-intensive, slow and biased process. We have developed a novel high-content imaging based assay for the detection of bacterial growth in function of time for *M.abscesssus* and *M.avium*. Bacteria were stained with Syto9 and propidium iodide and treated with different concentrations of antibiotics in a 96-well. By using the Caps-It automation system, plates were transferred from the incubators to the high-content imagers and images were taken every 4 hours for rapid growers and every 12 hours for slow growers. Dose-response growth curves were generated after 32 hours for *M.abscessus* and after 3 days for *M.avium* subsequently IC90-values were computed. We compared a panel of 13 antibiotics using our novel method and the microbroth dilution assay for *M.abscessus* and found concordant results for 12 antibiotics. For *M.avium* we compared 5 antibiotics (rifabutin, clarithromycin, moxifloxacin, rifampicin and amikacin) and obtained concordant results as well. Our novel assay reduces assay duration significantly while yielding more precise and unbiased results by quantifying bacterial growth in function of time. We have validated our assay for *M.abscessus* and implemented it in a high-throughput screening setting for drug discovery. Simultaneously, we believe that we can adjust our assay towards drug susceptibility testing.

OR02

GPAS: evaluation of mycobacterial species identification of 7798 MGIT samples

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The GPAS pipeline is a user-friendly, cloud-based bioinformatic service. It is designed to assemble, variant call and analyse mycobacterial whole genome sequences reporting species, resistance prediction and relatedness.

Here we evaluated its performance in determining the species, sub-species and lineage of 10,000 consecutive mycobacterial samples obtained from MGIT cultures in the Public Health England Lab

Birmingham using Illumina sequencing. 7798 FASTQ files were suitable for analysis. First, human reads were removed, quality checked and trimmed using FastP and filtered by Kraken 2. Only mycobacterial and unclassified reads were further processed by mapping competitively against a target of 179 mycobacteria with published reference genomes, thus, spanning the majority of known species. The reference genome with best coverage was classified as the species and used for subsequent genomic assembly and variant calling. In addition, species was also identified using Mykrobe which recognises unique species/subspecies/lineage determining sequences.

Comparison of competitive mapping and Mikrobe outputs were essentially concordant. 88 species were identified as follows: TB complex
(3,370), intracellulare (1135), avium (925) abscessus (575) chelonae(375). 22 species were only identified once and 13 twice. The analysis was also able to determine the presence of mixed mycobacterial and non-mycobacterial infections (58 cases) and mixed non-mycobacterial cases (6 cases). The detection of TB in a sample was not limited bio-informatically but by laboratory cross contamination. The GPAS pipeline is able to process at least 10,000 samples a day and is suitable for clinical and public health users worldwide.

OR03

Towards restoring antibiotic sensitivity in *Mycobacterium tuberculosis* using bacteriophages

J C Evans [1] E O Johnson [1]
1: The Francis Crick Institute

The global rise in antimicrobial resistance has led to a resurgence of interest in bacteriophage therapies as alternative or adjunctive treatments for bacterial infections. Their therapeutic application is limited, however, by their dependence on predominantly non-essential receptors, enabling bacteria to readily evolve resistance to phage attack. Using an approach analogous to that described for multidrug-resistant *Pseudomonas aeruginosa*, we aim to exploit this limitation in *Mycobacterium tuberculosis* (*Mtb*) by forcing an evolutionary genetic trade-off between acquisition of phage resistance and loss of critical outer membrane components by identifying mycobacteriophages targeting receptors whose loss might potentiate antibiotic activity.

The efflux pump EfpA has been shown to be upregulated in drug-resistant clinical isolates of *Mtb*, and is the target of potent antitubercular compounds in preclinical development. Despite being essential for growth of *Mtb*, *efpA* is dispensable in *M. smegmatis*, enabling us to devise a screening strategy for identifying EfpA-binding mycobacteriophages by testing *efpA* mutants for resistance to phages that lyse wildtype. Using this approach, environmental samples can be rapidly screened to identify mycobacteriophages utilising EfpA as a binding receptor, which can then be further evaluated for their ability to infect and kill *Mtb*.

Although modern phage therapy remains in its infancy, the recent successful use of mycobacteriophages for treatment of incurable *M. abscessus* infections validates their therapeutic utility, and the identification of therapies where bacteriophages exert selection for MDR bacteria to become increasingly sensitised to traditional antibiotics could extend the lifetimes of currently available treatments and reduce the emergence of antibiotic resistant infections.

OR04

Safety of high-dose amikacin in the first week of all-oral rifampicin-resistant tuberculosis treatment for the prevention of acquired resistance

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Effective strategies against rifampicin-resistant tuberculosis (RR-TB) should also prevent resistance. Resistance to bedaquiline (BDQ), a central drug for RR-TB treatment, is emerging, possibly due to the slow onset of BDQ's bactericidal action. We evaluated the safety of two injections of high-dose AMK co-administred with lidocaine to strengthen the first week of treatment.

For this purpose, in a single-arm phase-2 clinical trial, 20 RR-TB patients received two doses of 30mg/kg of intramuscular AMK on the first and fourth day of treatment. The primary endpoint is any grade 3-4 adverse event during the first 2 weeks of treatment related to the use of AMK. Secondary safety endpoints included assessments of ototoxicity and nephrotoxicity, other adverse events and post-injection pain using the Wong-Baker FACES Pain Rating Scale (0-10).

In our study, no grade 3-4 adverse events were observed (95% CI 0-0.139, p0.049). Ototoxicity and nephrotoxicity assessments did not reveal adverse effects. Pain assessment post-AMK injections with lidocaine revealed minimal discomfort, with immediate post-injection pain on day 1 showing a median score of 0 and an interquartile range (IQR) of 0.25 (range 0-5) while subsequent evaluations indicated negligible pain (median 0, IQR 0, range 0-2). As expected, AMK levels in serum were undetectable before the second dose.

In conclusion, the intervention involving two high doses of AMK in the first week of treatment was safe in our small cohort. Post-injection pain was minimal with lidocaine. These safety data will inform a multi-country study evaluating effectiveness to prevent acquired resistance.

OR05

Point-of-care lung ultrasound for the detection of pulmonary tuberculosis in Benin: the TrUST study

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Non-sputum triage tests are prioritised by the WHO to rule out tuberculosis (TB) and identify individuals that require further testing. We investigate the diagnostic performance of lung ultrasound (LUS) in a tertiary outpatient consultation in Benin. This 2-year prospective cohort included adult patients presenting with a lower respiratory tract infection according to the treating physician (October 2021- August 2023). Standardized LUS images and videos were collected. All images were reviewed according to pre-specified categories. TB was defined as a positive GenXpert MTB/RIF® or Xpert Ultra® on sputum. All patients were screened for HIV (Alere Determine® HIV-1/2). We evaluated the association of these ultrasound categories with TB using univariate logistic and multivariate logistic regression. Out of 504 patients included, 192 (38%) were TB positive (TB+) and 312 (62%) TB negative (TB-). Overall, 78 (15%) patients had documented HIV (median CD4 count/mm^3 of 92 [IQR43-358]). TB+ was associated with consolidations larger than 1 cm in any quadrant (p<0.001, odds ratio [OR] 15 95%CI 9.7-23.8), apical consolidations larger than 1 cm (p<0.001, OR 19 95%CI 11.2-32.8) and apical subpleural lesions <1cm (p<0.001, OR 3.6 95%CI2.5-5.3). Multivariate logistic regression score showed an area under the receiver-operating curve of 0.88 to detect TB (sensitivity 90%, specificity 64%, negative predictive value 91% and positive predictive value 60%). LUS is a promising triage tool to exclude TB in the outpatient setting for symptomatic patients in a TB endemic region. Next steps are external validation and computer assisted diagnosis.

OR06

Use of a modified face mask and liquid-based cough aerosol sampling system to measure the infectiousness of drug-resistant tuberculosis patients

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Understanding tuberculosis (TB) patients' infectiousness before and during treatment is crucial to control transmission. The traditional use of sputum smears to define infectiousness is severely limited. We compared two novel aerosol sampling systems 1) gMask (containing a gelatine filter) and 2) liquid cough aerosol sampling system (LCASS) against the established cough aerosol sampling system (CASS) for detecting *Mycobacterium tuberculosis* from aerosols in people with drug-resistant (DR)-TB. gMask sampling times were optimised in ten patients. Forty-five patients enrolled were sampled a total of130 times. LCASS (using two reservoirs) was evaluated on a subset of 14 patients, with samples randomly allocated to MGIT960 or most probable number

(MPN) assays. The latter was done with or without exponential phase cell-free extract (EPCFE)-supplementation to encourage growth of differentially culturable bacilli (DCTB). gMask sampling of 1hr was sufficient to detect TB. Initial visit positivity rates for CASS, gMask, and LCASS were 6/44 (14%), 10/43 (23%), and 4/14 (29%) respectively. Notably, 30% of gMask-positive cases were sputum culture-negative. Only 1/28 (4%) of patients were CASS-positive one week after treatment. Positive gMasks were detected until week 8 (8/82, 10%), while LCASS readouts were largely negative beyond baseline (one positive MPN at week 8). These alternative aerosol sampling methods are simpler and less expensive that the original CASS and may prove useful for the diagnosis, treatment monitoring and transmission intervention strategies. Moreover, aerosols from patients on treatment may harbour DCTB. These findings underscore the need for improved detection techniques to better understand TB infectivity dynamics.

OR07

Pluslife MTBC Card: a new rapid diagnostic tool for management of pulmunary TB patients

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Background
The new MTBC Card (Pluslife, China) is a molecular test for a rapid Tuberculosis (TB) diagnosis, with results available in 30 minutes. The aim of this study is to evaluate the performance of Pluslife MTBC Card on selected Xpert® MTB/RIF Ultra (Cepheid, USA) positive sputum samples.

Materials and Methods
Thirty Xpert® MTB/RIF Ultra (Ultra) positive sputum sediments, previously treated according to the standard guidelines for mycobacteria detection and stored at -20 °C, were included in the study. All thawed specimens have been processed both with Ultra and Pluslife MTBC Card (MTBC-Card) following manufacturers' IFUs.

Results
30 sputum sediment were re-tested by Ultra, with the following semi-quantitative load: High (n=5), Medium (n=5) Low (n=10) and very low (n=10). All samples resulted High and Medium by Ultra were confirmed positive by MTBC-Card with a range time of detection of 12-17 minutes.
9 of 10 (90%) samples with Low result by Ultra were also MTBC-Card positive with a time of detection range of 15-27 minutes.
Among Ultra samples with a Very low load, 3 (30%) were confirmed positive by MTBC-Card with a time of detection range of 21-28 minutes.

Conclusions
These preliminary results showed a full agreement between Ultra and MTBC-Card in smear-positive specimens with significant time savings, whereas MTBC-Card resulted positive in 60% of smear-negative specimens.
In conclusion, this new device appears to be effective in early diagnosis of TB cases, allowing early patient isolation.

OR08

Routine whole genome sequencing of *Mycobacterium tuberculosis* when the consensus in not enough

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The Netherlands whole genome sequencing (WGS) database currently contains almost 6000 *M. tuberculosis* local clinical isolates. The consensus sequence has proven utility to rule out recent transmission, for (sub)species identification and for the prediction of susceptibility. We routinely generate a consensus sequence (SNPs present in >80% of reads called) against the reference genotype to detect any mutations; a short list of SNPs(single nucleotide polymorphisms), including high confidence resistance mutations and clade specific SNPs, are additionally routinely screened for any mutant reads to rule out mixed infections and detect emerging resistance.

We have monitored the noise profile of the SNPs routinely screened for low frequency mutations in detail over time in our database. Notably, the well-known *rpo*B 761152 T>A noise resolved when we switched (Illumina™) sequence provider. In addition to our routine analysis sequence variability detectable in isolates with epidemiological links and serial isolates is analyzed. This more detailed analysis may provide additional insight into epidemiological and disease processes. For example; 1. previous treatment, particularly when treatment has been interrupted or suboptimal fragile regimens have been used, resulting in multiple sub populations of escape mutants; 2. chronic infections or reactivation of a temporally distant infection may yield genetically diversified sub populations within a single individual; 3. emerging mutations can sometimes be detected in isolates within recent clusters, allowing the inference of likely transmission links and direction from sequence data. We present examples of these effects and discuss the possibilities to expand these types of analysis with current and emerging sequencing approaches.

OR09

Genomic epidemiology of tuberculosis using long-read sequencing to increase resolution of transmission clusters

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Genomic epidemiological studies of *Mycobacterium tuberculosis* (Mtb) often use short-read sequencing, which mainly relies on reference mapping based analysis. However, this method only examines 90% of the Mtb genome because the mapping of short reads on repetitive regions is highly inaccurate. To overcome this limitation, we used a long-read whole-genome sequencing to

accurately reconstruct the whole genome of MTBC culture-positive cases over one year (2016) in the Valencia Region (Spain). We aimed to: i) evaluate the added value of long-read sequencing to gain epidemiological resolution; ii) reveal the genetic diversity in repetitive regions at an epidemiological scale.

We sequenced 216/266 MTBC clinical isolates using the PacBio Sequel II platform, obtaining 212 high-quality complete genomes via *de novo* assembly. Short-read sequencing data obtained by the Illumina MiSeq platform and analyzed by reference mapping were available. Pairwise distances between assemblies were consistently higher compared to those obtained from short-read sequencing. At a global level a mean number of 410 SNPs were gained. When focusing on closely related samples (20 SNPs according to Illumina data), we gained a median of 1 SNP. In 60% of pairs, we detected more SNPs with Pacbio data, rising to 70% when considering indels. Most of the SNPs were located in regions that are masked in the Illumina analysis. Additionally, one of these samples presented high genetic diversity in PE-PGRS28, possibly linked to a gene conversion event. In conclusion, our findings indicate a greater genetic diversity between samples, which can provide valuable insights into transmission within clusters.

OR10

Conservation of M72/AS01E vaccine epitopes in 31,428 *Mycobacterium tuberculosis* isolates

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M72/AS01E is a protein subunit vaccine consisting of two *Mycobacterium tuberculosis* (Mtb) proteins, PPE18 and pepA. It is unclear how well M72's epitopes are conserved among clinical strains, which might affect the vaccine's efficacy. Existing studies have used small and/or phylogenetically monotonous datasets. We quantified non-synonymous SNPs (nSNPs) in PPE18 and pepA genes of 31,428 public domain Mtb strains, representative of the near-complete phylogenetic diversity of global clinical strains. We geographically resolved conservation data by Mtb complex lineage and correlated with predicted M72 epitope regions for that geographic region, using high-resolution typing of strains and worldwide MHC-II allele distribution data. All nSNPs at a frequency of more than 0.1% were situated in the PPE18 portion of M72. M72 regions most consistently predicted to be epitopes were mostly situated in the N-terminal portion of pepA. Defining an M72 residue as non-conserved if it is changed in 1% of strains, we found that M72 epitopes were generally well-conserved, with epitope non-conservation rates reaching at most 9% in Southeast Asia and 10% in Australia. MHC-II alleles DRB*11:01 and DRB*13:01 expressed the least number of M72 epitopes. Lineages 4.2.1.2.2.1, 4.2.1.1.1.1.2 and 2.2.1 stood out as having the highest number of nSNPs in epitope regions. We present the most comprehensive epitope conservation study of M72 to date, demonstrating limited loss of M72 epitopes due to non-conservation of epitopes among clinical strains. The ongoing phase 3 trial can test whether these findings predict vaccine failure.

OR11

Deciphering Tuberculosis host-pathogen coevolution using *Drosophila melanogaster* as a model system

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Tuberculosis is an ancient disease that has persisted through the ages and remains a significant global health challenge. Thus, a fundamental question arises: how have both host and pathogen managed to persist?

In this study we used the *Drosophila melanogaster* model to study the host-pathogen coevolution within the context of tuberculosis, using the closely related pathogen *Mycobacterium marinum*. We focused on dissecting the immune response mounted by the flies and the pathogen's evolving strategies for evasion.

We followed the infection over ten generations of *Drosophila* with each generation being reinfected by their own isolated pathogen. Furthermore, we exposed the flies to low doses of a heat-inactivated environmental mycobacterium, *Mycolicibacterium manresensis*, to assess the potential impact that presence of environmental mycobacteria might have to the host-pathogen coevolution. At generations 5 and 10, each group was infected with progressively increasing doses of *M. marinum* recovered from previously infected groups for the tolerance/resistance assays, aimed at determining whether there were changes in the virulence of *M. marinum* after coevolution with the host. We also used non-coevolved flies as control groups.

The results underscore the effectiveness of *D. melanogaster* as a model system for studying host-pathogen coevolution. Results showed that coevolved hosts increased their resistance to the infection with *M. marinum*, while the pathogen diminishes its virulence and its ability to thrive within the host. Moreover, the exposure to the heat-inactivated *M. manresensis* resulted in increased tolerance to the infection, as well as increased resistance, and an even stronger attenuation of the pathogen’s virulence.

OR12

Host and Pathogen Determinants of TB Disease and Transmission in TanzaniaHost and Pathogen Determinants of TB Disease and Transmission in Tanzania

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The outcome of TB infection and disease is highly variable and known to be influenced by host, pathogen and environmental factors. How genomic variation in the *Mycobacterium*

tuberculosis complex (MTBC) interacts with human genetic diversity in shaping these variable outcomes, however, has rarely been studied. Here, we analyzed the human and MTBC genomes from N=1,472 TB patients from Dar es Salaam, Tanzania. Our findings show that the TB epidemic in Dar es Salaam is driven by several genotypes of MTBC Lineages (L) 1, L2, L3 and L4. Phylodynamic modelling suggests that the most prevalent L1 strains have a reduced transmission rate compared to other strains. Yet, we also see that the overall fitness of L1 strains is similar to most other strains. These data suggest longer infectious period in patients infected with L1, raising the possibility of L1 being more likely to cause sub-clinical TB. When adding the human genomic data to our analyses, we find that a high proportion of "Western Bantu" ancestry in patients infected with the most common MTBC genotype in Dar es Salaam is protective against severe lung damage, suggesting a possible mechanism underlying the geographical distribution of some of the most prevalent MTBC genotypes in Africa. In conclusion, we show that i) the MTBC genotypes differ in their life-history traits, possibly reflecting differences in transmission strategies, and ii) the interaction between MTBC and human genomic diversity modulates disease TB outcome. Taken together, our findings might explain some of the phylogeographical characteristics of the human-adapted MTBC.

OR13

Sex and progression towards active TB in an experimental mouse model. Impact of stress-induced glucocorticoids and intermittent fasting

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Tuberculosis (TB) affects mostly males (in a 65:35 proportion). We wanted to assess the impact of chronic stress, linked to glucocorticoid stimulation, and sex in its progression. To do so, we interrogated the active TB experimental mouse model. Adult, 6 months old, C3HeB/FeJ mice were infected with 2x10^4 CFUs of Mycobacterium tuberculosis e.v. Animals were divided in three groups: 'Control' (without stress), 'Restriction' (with daily 5 hours movement restriction), and 'Intermittent-fasting' (with daily 5 hours fasting). Animals were euthanized after 28 days post-infection. We measured pulmonary and spleen bacterial load, lung damaged area, and corticosterone concentration in hair. Both bacillary loads and lung damage were worse in females compared to males of the 'Control' group, with no differences in corticosterone-in-hair levels. However, in the 'Restriction' group females showed lower bacillary loads in lungs and spleen, and less pulmonary damaged area compared with the 'Control' group, linked to higher levels of cortisone-in-hair. Interestingly, there was a significant positive correlation between corticosterone-in-hair and pulmonary damaged area. 'Restriction' had only a minimal impact in males raising slightly corticosterone-in-hair levels. 'Intermittent-fasting' had a positive impact in females in terms of bacillary load and lung damage, non-linked to the modification of corticosterone levels. Our preliminary results highlights the role of chronic stress and the adequate corticosterone production, together with intermittent-fasting to moderate the pro-inflammatory Th17 immune response in females as a possible explanation to understand its protection against progression towards active TB.

OR14

Unravelling host-specific in vitro virulence profiles of Mycobacterium tuberculosis ecotypes

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Tuberculosis is caused by the *Mycobacterium tuberculosis* complex (MTBC), which encompasses 13 distinct lineages or ecotypes. Despite sharing a genetic similarity of over 99%, these ecotypes demonstrate differential host preference, distinguishing between human and animal-associated lineages. Understanding host-pathogen interactions and their compatibility will allow us to decipher the factors that influence *M. tuberculosis* virulence.

Our study hypothesizes that the degree of host compatibility influences the virulence potential of *M. tuberculosis* strains, with increased virulence observed when strains infect their preferred hosts. To investigate this hypothesis, we employed an *in vitro* infection model using human and bovine macrophages (THP1 and BoMac cells) infected with two human-associated *M. tuberculosis* strains belonging to lineages L5 and L6, as well as two strains associated with animals: *Mycobacterium bovis* of lineage A4 and *Chimpanzee bacillus* from the A1 lineage.

Our findings support our hypothesis, revealing an augmented infection ratio in host cells when infected with their preferred strain. Interestingly, our investigation reveals distinct cellular responses across different host-pathogen combinations. Specifically, less compatible combinations exhibited higher cell viability, suggesting potential compromises in host defense mechanisms facilitating enhanced cell survival despite infection. Conversely, more compatible combinations demonstrated elevated levels of apoptotic cells, indicative of a heightened immune response aimed at restricting the infection.

These results underscore the interplay between host compatibility and *M. tuberculosis* virulence, highlighting the importance of host-pathogen interactions in shaping disease outcomes. A comprehensive understanding of these interactions holds promise for the development of targeted therapeutic strategies against tuberculosis

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OR15

Inhibition of menaquinone biosynthesis results in re-sensitization of Bedaquiline resistant *Mycobacterium tuberculosis*

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Multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) pose ongoing challenges to global disease control efforts. BDQ, a diarylquinoline compound, represents a significant breakthrough as the first tuberculosis medication in decades with a unique mode of action, revolutionizing MDR-TB therapy. The current MDR-TB treatment protocol involves a combination of Bedaquiline (BDQ), Pretomanid, Linezolid, and Moxifloxacin. However, the emergence of clinical resistance to BDQ has become a pressing issue, primarily due

to mutations that are disrupting a drug efflux pump repressor, resulting in increased efflux of BDQ.

Mycobacterium tuberculosis relies on oxidative phosphorylation to generate ATP, crucial for both active disease and latent infection. BDQ targets ATP synthase, which results in inhibiting energy metabolism. This enzyme spans the cellular membrane, utilizing proton-motive force (PMF) generated by the electron transport chain (ETC) to produce ATP. Menaquinone facilitates electron transfer along the ETC in *M. tuberculosis*, connecting electron donors like NADH to acceptors. Components of this chain, including MenG, have garnered interest as potential drug targets, as seen with BDQ.

In the menaquinone biosynthesis pathway, MenG serves as one of the enzyme targets. Inhibitors of MenG demonstrate synergistic effects with TB clinical compounds, notably BDQ, validating previous studies on this enzymatic pathway. Our research presents compelling evidence that inhibiting menaquinone biosynthesis alongside BDQ treatment, effectively reverses BDQ resistance due to the drug efflux pump repressor disruption, offering a robust strategy to curb its spread in clinical settings.

OR16

In vitro host gene expression profiles in cross-infections of *Mycobacterium tuberculosis* ecotypes

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Pathogen-host interaction is a complex interface where the compatibility between both organisms plays a major role determining the fate of the encounter. *Mycobacterium tuberculosis* is a clonal bacteria with very low genetic diversity, but infecting a great diversity of hosts. The relative impact of the different drivers of this bacteria-host compatibility are not well understood. Here we aim to better understand molecular mechanisms associated with the differences in compatibility by comparing transcriptomic profiles of different bacteria and cell combinations in vitro.

We compared one strain from Lineage A4, typical of cattle infections, and Lineage A1 (the chimpanzee bacillus) with the two closest human lineages Lineage L5 and Lineage L6, infecting human and cattle cellular lines (THP-1 and BoMac). We categorized the infection outcomes as high compatibility (L5 with THP-1 and A4 with BoMac), low compatibility (L5 with BoMac and A4 with THP-1), and intermediate or unknown compatibility (L6 with both THP-1 and BoMac, and A1 with both THP-1 and BoMac). Our analysis identified upregulated and downregulated genes across the pairwise groups, finding synergies of strain-host infections and infection progression. One of our major findings was the identification of upregulated functions involved in intracellular containment of the bacteria and immune evasion in low compatibility combinations. Conversely, we observed functions related to cell replication affecting DNA of the host upregulated in high compatibility combinations. Our approach aims to elucidate molecular determinants of lineage-host preferences and contribute to a better understanding of tuberculosis pathogenesis.

OR17

Democratizing education and research for new generation of medical professionals: TB Portals data and resources

A Rosenthal

The TB Portals program (TBPP) is a crucial educational resource for a new generation of medical professionals, beginning their careers in an era where artificial intelligence (AI) transforms big data into clinical insights, and genomic information enables the prescription of drugs tailored specifically for each patient and pathogen.

TBPP maintains a large patient-centric database, currently comprising 14,000 anonymized records that include socioeconomic, microbiological, genomic, radiological, and clinical treatment data for each patient. This information originates from 19 countries burdened heavily by drug-resistant tuberculosis. In many of these countries, TB remains a disease of the impoverished, making the optimization of treatment and specialized education for doctors and radiologists vitally important.

TB Portals facilitates the sequencing of clinical samples followed by comprehensive bioinformatics analysis of the complete genomic data. For X-rays and CT scans within TB Portals, it is possible to evaluate both manual annotations by professional radiologists and AI predictions. Unique three-dimensional models of lungs, constructed from the CT scans of TBPP patients and enriched with actual clinical data, illustrate the progression of the disease and the effects of treatments.

Thousands of TBPP clinical cases, categorized by drug resistance types, case definitions, comorbidities, treatment outcomes, and radiological findings, are prepared for integration into medical education. They offer a depth and breadth of information unavailable in textbooks and general resources. TB Portals' resources are accessible to students, doctors, and researchers worldwide, democratizing access to TB information, advancing global health equity, and encouraging international collaboration to eradicate tuberculosis.

OR18

Reproducible algorithmic generation of resistance catalogues improves resistance prediction for bedaquiline in *M. tuberculosis*

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Bedaquiline (BDQ) is a recent addition to the WHO recommended treatment regimen for multi-drug resistant tuberculosis, however rising levels of resistance threaten to reduce its efficacy against *Mycobacterium tuberculosis* (Mtb). Catalogues of mutations associated with resistance to bedaquiline are key to detecting resistance genetically. However, the recent second edition of the WHO resistance catalogue, which was built using considerable domain knowledge, assumes a high level of genetic homogeneity, uses complex grading rules and is not reproducible. We will

show that (i) catalogues can be reproducibly and automatically constructed from clinical datasets and (ii) by using a less stringent approach overall performance is improved. We algorithmically applied a less statistically conservative method to the whole genome sequencing data and phenotypic drug susceptibility testing measurements of a dataset of 11,867 Mtb isolates, thereby reproducibly cataloguing genetic variants in known resistance genes and reliably predicting BDQ resistance. Unlike previous approaches, no variant in the *mmpL5* gene is associated with resistance. Our catalogue therefore only considers variation in *Rv0678*, *pepQ*, and *atpE*, achieving a cross-validated sensitivity of 81.8 ± 3.8% for the 97.5 ± 0.3% of samples where a definite prediction can be made. Our results also show that minor *Rv0678* variants are clinically relevant, mirroring published evidence for the fluoroquinolones and analysis done by the WHO, suggesting that bioinformatics thresholds must be lowered to catch them.

OR19

Remission cytochrome spectroscopy reveals the mode of action of bedaquiline in living mycobacteria

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The ATP synthase inhibitor Bedaquiline (BDQ) is the cornerstone of new regimens that have accelerated the treatment multidrug-resistant tuberculosis. Despite its clear importance, its precise bactericidal mechanism continues to debated: specifically whether BDQ binding to ATP synthase disrupts the proton motive force by inducing a leaky 'uncoupled' state, or simply blocks catalysis. To unravel its mechanism of action we applied non-invasive methods to study the effects of BDQ on the oxidative phosphorylation system in live mycobacteria. Via the use of a specialised bioenergetic chamber we are able to monitor changes in cytochrome oxidation states through multi-wavelength remission spectroscopy while measuring the oxygen consumption rate of cells in real time. We find that BDQ's effects do not match those of established uncouplers/ionophores that disrupt the proton motive force. Our results demonstrate BDQ acts as a direct inhibitor of ATP synthase and its effects on oxygen consumption are the result of electrons being routed through cytochrome *bd* oxidase. This strategy by the bacteria limits their susceptibility to back pressure and highlights *bd* oxidase as a key target for future combination therapies to increase the effectiveness of BDQ.

OR20

Nationwide evaluation of Xpert MTB/RIF Ultra reveals challenges in accurate diagnosis of rifampicin-resistant tuberculosis

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Xpert MTB/RIF Ultra (Ultra) was expected to improve accuracy of rifampicin-resistant tuberculosis (RR-TB) detection. To assess the diagnostic performance of Ultra in real-world settings, all RR-TB results in Rwanda had Ultra repeated on a new sample, with *rpoB* target vs whole genome sequencing and phenotypic drug-susceptibility testing as reference standard, with any (hetero)resistant result overriding.

This nationwide observational study from December 2021 to February 2024, included 129 patients initially identified as RR-TB by Ultra. Upon Ultra retesting only 41 (32%) remained classified as RR-TB. Reference testing, available for 40 (98%) patients, confirmed all as true RR-TB. Conversely, of the remaining 88 patients (68%) not classified as RR-TB on retesting, reference methods were available for 61 (69%), identifying only seven (11%) as true RR-TB; the other 54 (89%) were RS. Notably, this high rate of initial false RR affected samples with very low bacillary loads (49/55, 89%). The underlying technical reason has yet to be clarified; melting temperature peaks of the MUT probes did not match with expected values for known mutations, with melt curve analysis ongoing by the manufacturer.

Our study highlights a high rate of false-RR, especially in samples with very low bacillary loads, challenging the expected specificity enhancement in the Ultra system. At least 54 patients with RS-TB (42% of initial RR-TB Ultra) received unnecessary RR-TB treatment. These findings emphasize the necessity to adjust diagnostic strategies to accurately identify true RR-TB cases among paucibacillary patients, and prevent unnecessary treatments.

OR21

Closing the diagnostic gap for the BPaLM treatment regimen with tNGS: Deeplex Myc-TB XL

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The targeted next generation sequencing (tNGS)-based Deeplex Myc-TB assay has been recently endorsed by WHO for rapid diagnosis of tuberculosis (TB) drug resistance, with performance criteria outstandingly met for 10/10 drugs evaluated. However, pretomanid (or delamanid), part of the BPaLM regimen now recommended as the first choice, near-universal treatment for multidrug resistant TB, is not covered. To close the existing BPaLM diagnostic gap, we therefore developed Deeplex Myc-TB XL, based on a single 42-plex PCR amplification (vs 24-plex with Deeplex Myc-TB) followed by deep Illumina sequencing. The test includes all the targets that

harbor mutations associated with resistance/resistance- Interim to recommended anti-TB drugs in the latest WHO mutation catalogue. This comprises a total of 15 targets for BPaLM drugs, with e.g. 6 and 5 full genes for pretomanid/delamanid and bedaquiline, respectively, - and accounting also for known epistasis between mutations in bedaquiline resistance-associated genes -, in addition to optimized design of previous targets. In order to increase culture-free applicability on paucibacillary samples, representing challenges especially for pediatric TB, TB/HIV and TB meningitis, we also worked to improve the limit of detection, with a resulting analytical sensitivity measured at ~10 extracted genome copies. Last but not least, we substantially simplified the DNA library preparation, resulting in a reduction of more than half of the manipulations and hands-on time. This novel, augmented tNGS tool may thus further enable fast, comprehensive diagnosis to guide critical clinical decision-making for treatment of TB, including under its most challenging forms.

OR22

Unveiling the Drivers of *Mycobacterium tuberculosis* Lineage 2 success: An Integrative Approach

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Mycobacterium tuberculosis lineage 2 (L2) presents a significant public health threat, particularly in Eurasia, due to its widespread prevalence and association with multi-drug resistant strains. Despite its dominance in this region, the reasons behind L2's success remain poorly understood. Here, we employed an integrative approach to unveil the factors contributing to this lineage's advantage. Utilizing whole-genome sequencing data from hundreds to thousands of M.tb isolates, we employed Bayesian phylogenomics and demogenetics to reconstruct the evolutionary history of L2. This analysis revealed novel insights into the strength, spread, and timing of major L2 epidemics, confirming its dominance in terms of both antibiotic resistance and overall epidemiological success. Additionally, we investigated the potential influence of major socio-economic events, such as the collapse of the Soviet Union, on ongoing L2 epidemics. To understand the mechanisms underpinning L2's success, we leveraged a suite of complementary techniques. Predictive simulations and epidemicity indices allowed us to quantify the transmission potential of L2 strains. Furthermore, genome-wide association studies (GWAS) were employed to identify genetic factors associated with L2's advantageous traits. Finally, laboratory fluctuation assays revealed higher mutation rates in L2 compared to other lineages, potentially explaining its dominance by enhancing its adaptive landscape and facilitating the acquisition of resistance or compensatory mutations. Our multi-pronged approach provided a comprehensive understanding of the factors driving L2's success. This knowledge is crucial for the development of effective control strategies to combat this concerning lineage of M.tb.

OR23

Delving deeper into the evolution of drug resistance

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Past and present selective presures acting on bacterial organisms result in genetic mutations that leave lasting imprints on their genomes. For obligate pathogenic bacteria like those belonging to the *Mycobacterium tuberculosis* complex, antibiotic treatments represent a significant selective force. In this work, we have made use of a comprehensive dataset generated by the Cryptic consortium, comprising ~11,000 genomes along with phenotypic data (minimum inhibitory concentrations (MIC)) for 13 antitubercular drugs. Our aim was to study the evolutionary factors that have influenced the development of drug-resistant (DR) phenotypes over time.

We utilized innovative phylogeny-based methods to investigate the genes and mutations linked to variations in the MIC values. Genes that were not previously linked to drug resistance, had sparse prior data, or were deemed uncertain in their associations through traditional statistical methods, emerged as potential candidates associated with these phenotypes.

Furthermore, we examined ancestral phylogenetic branches preceding the emergence of drug-resistant mutations to identify mutations that might facilitate the acquisition of these genomic traits. In this instance, we identified previously linked tolerance factors and uncovered novel ones, including regulators of metablic pathways (such as the methylcitrate pathway), genes related with growth and survival, and some others whose prior associations lacked definitive confirmation. Alongside nonsynonymous mutations, synonymous ones, substitutions in intergenic regions, generation of new regulatory regions and even reversions emerged as significant contributors to these phenomenons. We assert that our approach, when combined with other statistical analyses, has the capacity to unveil the determinants of complex DR phenotypes.

OR24

First evidence of pyrazinamide-susceptible *Mycobacterium bovis*, sub-lineage La1.1, in Ireland

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Ireland has a strong agricultural sector. In 2021, the dairy herd alone totalled 1.6 million. *Mycobacterium bovis* is a zoonotic infection that could potentially infect any animal, domestic or wild, already present or introduced into the country. Its incidence is low, but not decreasing at a desirable rate. In 2022/2023, over 5% of human isolates received at the Irish Mycobacteria Reference Laboratory (IMRL) were lineage Bovis. The cattle/wildlife interface has been recognised as a potential source of transmission by the government more notably than the animal/human interface. To date, all human *M bovis* isolated has been intrinsically resistant to pyrazinamide (PZA).

Here, we present the first evidence of PZA-susceptible (La1.1) *M. bovis* in Ireland (May 2023). It was isolated from a sputum sample from an elderly patient in the rural southwest. The patient had no documented occupational history but he had kept some exotic animals in a park at his home. Phenotypic drug susceptibility testing showed that the isolate was susceptible to rifampicin, isoniazid, ethambutol, streptomycin and PZA. Whole genome sequencing was performed and the isolate was compared with other publicly-available global La1.1 strains (reference strain AF212297). It was more closely related to strains from Malawi than those found in Uganda, suggesting the possibility of further delineating this sub-lineage. More strains of La1.1 would be required to investigate this.

Without the collaboration of stakeholders under a One Health policy, the true molecular epidemiology of zoonotic tuberculosis cannot truly be measured. Until then, the threat to humans from *M. bovis* remains.

OR25

Mycobacterium tuberculosis (*Mtb*) transcriptional adaptations to growth arrest: unveiling the role of iron and carbon sources

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Mycobacterium tuberculosis (*Mtb)* adapts to diverse host environments by transitioning from active growth to dormancy. This process is regulated by transcriptional regulatory programs that are triggered, among other factors, by changes in the availability of different carbon sources and key nutrients like iron. While iron deprivation is a well-known trigger of the transcriptional adaptation to dormancy in *Mtb*, our understanding on how it interacts with the environmental availability of different carbon sources remains limited. To shed light on this question, we collected RNA-seq data from *in vitro* cultures of *Mtb* subject to different iron levels and carbon sources (glycerol, dextrose, and fatty acids), from exponential to stationary growth phases. As a result, we found that gene expression was significantly affected by iron deprivation during the stationary phase while showing minimal sensitivity during exponential growth, where only a limited number of genes coding mycobactins showed iron-dependent expression. These phase-dependent effects of iron deprivation on gene expression translate into an iron-dependent modulation of the magnitude of the response to growth arrest. Unexpectedly, a majority of the effects of iron deprivation on the magnitude of growth arrest responses were positive, implying stronger transcriptional changes during the transition to dormancy in iron-deprived media, particularly in cultures lacking fatty acids as a carbon source. Considering that, our results suggest an "OR-like" logic where bacteria integrate the sensing of both iron deprivation and lipid availability as relevant signaling cues to modulate the magnitude of its transcriptional adaptation to dormancy.

OR26

Rifampicin tolerance in *Mycobacterium tuberculosis* complex strains

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The evolution of drug resistance in *Mycobacterium tuberculosis* (Mtb) poses a substantial threat to the successful treatment of tuberculosis (TB) and the global fight against TB. Recent studies indicate that the development of antibiotic tolerance is a stepping stone for resistance acquisition of *Mycobacterium tuberculosis* complex (MTBC) strains. Tolerant bacteria exhibit prolonged survival under antibiotic treatment, facilitated by genetic or transcriptomic mechanisms. We hypothesize that the tolerance capability is greater in strains of some lineages (or sub-lineages), which could explain differences in antibiotic resistance rates as well as epidemiological success.

In this work, we investigate the tolerance of multiple strains representing MTBC lineages 2 (Beijing) and 4 (Haarlem) using time-kill assays under different concentrations of rifampicin (RIF). The results revealed that strains of Lineage 4 displayed a greater tolerance compared to strains of Lineage 2. However, it is noteworthy that one of the Lineage 2 strains exhibited a comparable level of tolerance to that observed in the Lineage 4 strains.

In future research, we will further analyse time-kill assay data to define resilient vs. resistant populations which arose in these experiments. This will be achieved by comparing colony-forming units, live-dead staining and flow cytometry.

This investigation underscores the importance of understanding antibiotic tolerance as a precursor to drug resistance acquisition in the complex phylogeny of MTBC strains.

OR27

Spatial dual host-bacterial gene expression to study pathogenesis and the regulation of virulence factors in tissue during NTM respiratory infections

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Co-localization of spatial transcriptome information of host and pathogen can improve our understanding of microbial pathogenesis. Here, we aimed to demonstrate that customized bacterial probes can be used to simultaneously identify host-pathogen interactions in formalin-fixed-paraffin-embedded (FFPE) tissues with probe-based spatial transcriptomics technology. Host and bacterial spatial transcriptomics profiles were analyzed exploiting a murine lung tissue chronically infected with agar-embedded *Mycobacterium abscessus* beads. Customized mycobacterial probes were generated for the constitutively expressed rpoB gene (an RNA polymerase beta subunit) and the virulence factor precursor lsr2, modulated by oxidative stress. Our investigation shed light on significant disparities in host expression profiles between infected and uninfected lung foci. Infected districts displayed the formation of granuloma-like structures accompanied by an upsurge in spatial inflammatory profiles, distinctly characterizing

the granulomas and their surroundings. We observed that the rpoB expression correlated with bacterial abundance in the airways, while lsr2 virulence factor showed increased expression in lung tissue with high oxidative stress. Furthermore, the application of a deconvolution step reinforced these observations, unveiling how the diverse host expression landscapes surrounding regions positive for bacteria correlated with distinct immune cellular compositions (eg proinflammatory macrophages). This observation highlighted a gradient influenced by the proximity to the pathogen signal, underscoring the complex interplay between cellular components and the presence of the pathogen within the tissue environment. Overall, we demonstrate that dual bacterial and host spatial gene expression assay can pave the way for the simultaneous detection of host and bacterial transcriptomes in pathological tissues.

OR28

Drivers of levofloxacin resistance in a high MDR-TB incidence country

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The VQUIN trial evaluated the safety and efficacy of levofloxacin-based tuberculosis preventative treatment (TPT) in household contacts of patients with RR/MDR-TB in Vietnam. Per trial protocol, contacts of Xpert-positive RR/MDR-TB **index cases** were screened for TB, prior to offering TPT. Individuals with culture-confirmed TB (**co-prevalent cases**) were excluded while Tuberculin Skin Test (TST) positive individuals were randomized to placebo or levofloxacin arm and followed for 24 months for culture conversion (**incident cases**). We investigated whether primary or acquired resistance is driving levofloxacin-resistance amongst MDR-TB strains. Isolates were whole genome sequenced and reads mapped to the H37Rv reference genome using Snippy for variant calling. TB-profiler was used for antibiotic resistance prediction and lineage annotation. A variant threshold was set at 5 as an indicator of transmission in 32 household pairs. We found no cases of acquired resistance amongst levofloxacin naïve strains or in isolates from patients who completed a 6-month levofloxacin-based TPT. Based on the absence of *de novo* resistance in household contacts, we set out to see the geographic and phylogenetic distribution of levofloxacin-resistant strains in Vietnam. Amongst 115 isolates from lab-confirmed RR/MDR-TB patients sampled across Vietnam, 19.1% (n=22) had levofloxacin resistance mutations. Although these strains were primarily found in sublineage 2.2.1 (21/22), genotypes were not phylogenetically or geographically linked. Further, different mutations were observed pointing to independent resistance acquisition events. In conclusion, we found primary resistance to drive levofloxacin-resistance amongst households in the VQUIN trial cohort and acquired resistance to be prominent in levofloxacin-resistant strains in the community.

OR29

Using long term evolution models to define drug resistance mechanisms to DDU209, a novel tuberculosis drug candidate

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Nearly all drugs developed for tuberculosis (TB) treatment lack testing and surveillance strategies when released for commercial use. To better protect new anti-TB drugs and reduce rampant resistance development, molecular and phenotypic assays should be developed alongside the drug and utilized upon commercial use.

We established an *in vitro* evolutionary model which employs low-concentration drug exposure to select mutants with an array of resistant phenotypes which predicated well resistance mechanisms for bedaquiline similar to clinical *Mycobacterium tuberculosis* complex (Mtbc) strains. In the framework of ERA4TB, a large consortium dedicated to the development of new treatment regimens for TB, we applied our method to the novel compound DDU-209, a promising drug candidate developed at Dundee University, which inhibits lysyl-tRNA synthase. We found five genes potentially related to DDU-209 resistance, with the most important resistance determining region (RDR) throughout Rv3598c-Rv3599c. This region is essential to bacterial survival, where single nucleotide polymorphisms, codon deletion, and even gene duplication were identified as modes of resistance. With the mutation catalogues generated, we found phylogenetic SNPs in clinical Mtbc strains in the defined RDR. Finally, with this collection of mutant clones we found no cross-resistance with other anti-TB drugs. With the future publication of this and other work produced by ERA4TB we will have a better standing on surveillance and treatment strategies for DDU-209 and other novel and repurposed drugs.

OR30

Improving the benchmark of *Mycobacterium tuberculosis* variant calling protocols with *in silico* evolved genomes

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Artificial genomes used to assess the performance of variant identification (benchmarking) are set up solely by introducing single nucleotide polymophisms and indels. However, the true evolutionary distance between strains is greater, as genomes undergo structural rearrangements. In *Mycobacterium tuberculosis*, the main structural variants are inserting sequence jumps, chromosomal duplications and large Regions of Deletions.

Using simplistic representation of genomes leads to overestimation and indiscrimination of sensitivity and recall for all variant calling pipelines. To 1) better understand how structural rearrangements impact the performance of alignment and variant calling 2) help the choice of variant calling pipelines, we wanted to build *in silico* evolved genomes with traceable genomic features that mimic the genomic evolution of *M. tuberculosis* lineages.

We provide here Maketube, a framework for building artificial genomes from a reference sequence. The artificial genomes are evolved according to a broad set of characteristics of true strains evolution including structural variants. We compared the properties of these genomes to a set of reference genomes, high quality assemblies and genomes built by other tools for benchmark.

Genomes evolved *in silico* shared numerous properties, such as a greater variability in the fold coverage when aligned to the reference, and such as variants with closer properties to real variants identified in natural genomes. This allows to discriminate between variant calling pipelines where artificial genomes usually used for benchmark fail to help.

OR31

Mutator genotypes drive resistance to antibiotics in natural isolates of Mycobacterium tuberculosis

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The global rise in *Mycobacterium tuberculosis* (Mtb) antimicrobial resistance means we urgently need new approaches to understand how it develops and stops its spread. One of the most common approaches used to counter the evolution of resistance in Mtb is the application of combination antibiotic therapy. Evolving resistance to combinations of drugs should be extremely rare, as it requires multiple mutations to occur in the same genetic background before microbial growth is inhibited. While wild-type bacteria cannot achieve this, mutators (with defects in DNA repair genes) allow multi-drug resistance to evolve easily during single-drug and combination treatments when there is a delay in reaching inhibitory concentrations of antibiotics. Therefore, it is important to identify these mutators to predict how combination therapies will be effective in preventing resistance. Here, our goal was to identify mutators contributing to resistance beyond known resistance genes. We first used a comprehensive bioinformatics approach where we analysed 53589 whole-genome sequences of Mtb clinical isolates, then we performed an association analysis and identified 151 mutator candidates that are associated with drug resistance in all major DNA repair pathways. Next, we used tools for Computational Biology to select different candidates for the experimentation. We finally constructed mutants by introducing point mutations on the chromosome of *M. smegmatis* a model species for Mtb and validated the hyper-mutability of five mutants using frequency tests conferring Rifampicin and Isoniazid. Work to extend these experiments to Mtb is underway to unveil new strategies to predict and combat the development of drug resistance.

Poster Presentations

P001

Unrestrained long-chain fatty acid catabolism kills *Mycobacterium tuberculosis*

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Mycobacterium tuberculosis thrives in infection foci that are notorious for lipid accumulation, setting the selective pressure for preferential usage of lipids, including long-chain free fatty acids (LC-FFA) and cholesterol, as carbon sources. Importantly, this lipid-based diet prompts *M. tuberculosis* to acquire a drug tolerant phenotype. However, host lipids, in specific LC-FFA, are potent antimicrobial agents, leading to the question of how *M. tuberculosis* evades this effect. In previous work, we have identified the respiratory enzyme type-2 NADH dehydrogenase and the membrane oxidoreductase EtfD as necessary for LC-FFA resistance and for full virulence. This illustrates the ability of turning a favorable environment to *M. tuberculosis* into a toxic one. Building on these results, we performed transposon sequencing to identify the full scope of *M. tuberculosis* LC-FFA resistant factors. Sequencing statistical analysis identified 38 genes associated with LC-FFA resistance spanning different metabolic pathways/ cell processes, including cell wall components biosynthesis, cyclic AMP signaling and folate cycle. Next, we performed a functional “deep dive” on the screen’s top hit – a universal stress protein of unknown function. Absence of this protein led to a dramatic increase in LC-FFA catabolism associated with oxidative phosphorylation perturbations and to a pronounced survival defect in the chronic phase of infection in C57BL/6 mice. These results indicate that this universal stress protein is an essential regulator of *M. tuberculosis* metabolism during infection.

P002

Insights into the use of cytochrome Bc inhibitors for future therapeutic strategies for tuberculosis

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The electron transport chain (ETC) of *Mycobacterium tuberculosis* is a complex pathway that has driven the attention for druggable targets since the discovery of bedaquiline (BDQ). Other drugs that also act on the ETC, like clofazimine (CFZ) and pyrazinamide (PZA), have demonstrated clinically their value and individual contribution to regimens. Recently, Telacebec (QcrB inhibitor) demonstrated antimycobacterial activity in a Phase II EBA trial. However, information on the

contribution of QcrB inhibitors to the sterilizing activity or treatment shortening potential in combination therapy is lacking. In this work we have performed several in vivo studies to prove that combination targeting of the ETC has a strong bactericidal effect with faster killing rates than SOC. Furthermore, we report here for the first time the sterilizing effect of QcrB inhibitors and their contribution to treatment shortening. Lastly, we observe that clinical isolates of *Mtb* are more susceptible to QcrB inhibition in monotherapy, suggesting that these relapse data (in H37Rv) might be a worst-case scenario for the true potential of QcrB containing regimens.

P003

X-ray-based 3D histology of the human tongue and its coating using contrast-enhanced microCT imaging

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Tuberculosis (TB) affects millions of people globally, causing significant health and economic burdens. Detecting TB remains, however, challenging. Traditional diagnostic methods still face limitations in terms of safety and ease of access to the samples to be tested. Tongue swabs for taking tongue coating samples, in which the TB bacteria reside, offer a good solution regarding safety and ease of use, showing promising sensitivity and specificity for TB detection.

However, currently used tongue swabs are not designed for TB detection and do not capture sufficient bacterial load per swab. To improve the design of tongue swabs, we aim to get inspiration from the tongue surface anatomy and the spatial distribution of its coating. For this purpose, we performed X-ray-based 3D histology of a human tongue using contrast-enhanced microfocus computed tomography (CECT). Hafnium-Wells Dawson polyoxometalate was applied as X-ray opaque contrast-enhancing staining agent to stain small tissue samples, which were then imaged using microCT. CECT images allowed to differentiate the tongue surface/epithelium from the tongue coating, as confirmed by classical 2D histology.

3D renderings of the tongue surface revealed the different types of papillae between which the TB bacteria could reside. 3D renderings of the tongue coating showed a heterogeneous distribution with clear ‘hot spots’. We use this combined info as inspiration for novel tongue swab designs

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P004

M&Ms | The dependence of bacterial growth phase on Mycobacterium-Macrophage interaction

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Current treatments for nontuberculous mycobacterial infections rely on toxic multi-drug regimens, leading to low patient compliance and success rates. Consequently, there is an urgent need to develop more effective drugs. However, traditional methods used in anti-mycobacterial drug screening do not capture the complexity of these infections, thus failing at predicting clinical effectiveness.

When screening for drug activity against intramacrophagic bacteria, due to practicality and reproducibility issues, macrophages are often infected with frozen bacterial stocks. However, this is far from the in vivo conditions.

We assessed whether the liquid growth phase of Mycobacterium avium and M. abscessus (lag, exponential or stationary) upon infection impacted the host-pathogen interaction and, consequently, the infection outcome. Our results show that, depending on the growth phase, each species has differing intracellular growth and, most importantly, susceptibility to antibiotics. These differences are related to changes in macrophage vesicular trafficking, observed by monitoring the autophagic flux and lysosomal accumulation using live cell imaging.

We are further characterising the macrophage phenotype (e.g., cytokines profile) and performing transcriptomic analysis of the various mycobacterial growth phases to understand the reasons behind the different infection outcomes.

Choosing the right in vitro conditions for screening new compounds against mycobacteria is crucial to increasing assay predictability, which contributes to the success of drug discovery. Our findings emphasise the need to carefully choose these conditions, enabling the most effective and accurate screening process.

This work was financed by Portuguese national funds through Fundação para a Ciência e a Tecnologia, within the project PTDC/BIA-MIC/3458/2020.

P005

Enhancing tuberculosis diagnosis with a mathematical model integrating molecular test histories and patient-specific factors

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Background

TB diagnosis still depends on a multi-test strategy that includes microscopy, PCR, and culture from successive clinical specimens. This approach faces challenges such as high costs and complex data interpretation, and there is a risk of over-relying on these results at the expense of clinical context and patient history. In this study, we aim to develop a model to predict TB, specifically designed to enhance the performance of multiple testing strategies.

Methods

We analyzed a database of 4,179 patients and 9,405 records from 2008 to 2018, assessing the diagnostic performance of individual TB tests across variables like test type, specimen type, and patient demographics. We applied a Hidden Markov Model (HMM) to generate scores indicating a patient's likelihood of TB based on sequential test results. Finally, we evaluated the performance of this predictive model against the historical data.

Results

We calculated TB prevalence, tests sensitivity and specificity across diverse categories, including gender, age, test types (microscopy, GeneXpert, in-house PCR, and culture), and specimen types (sputum, induced sputum, bronchoalveolar lavage and bronchial aspirate). We used PCR test sequences from our cohort to feed into an HMM, which generated a TB likelihood score from 0 to 1. Comparing to a gold standard of culture and clinical data, the HMM achieved 95.7% sensitivity and 97.9% specificity at an optimal score of 0.995.

Conclusions

This work illustrates that a mathematical model can enhance TB diagnosis by integrating molecular test histories with patient-specific factors, improving sensitivity, specificity, and supporting clinical decision-making across various testing stages.

P006

Identification of *Mycobacteroides abscessus complex* subspecies and molecular determination of resistance to clarithromycin and amikacin

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Objectives. *Mycobacteroides abscessus complex* (MABC) is a rapidly growing mycobacterium that encompasses three subspecies: *M. abscessus subsp. abscessus* (MAB), *M. abscessus subsp. bolletii* (MBO) and *M. abscessus subsp. massiliense* (MMA). This study aimed to investigate the diagnostic utility of a commercially available molecular assay that performed on MABC isolates recovered in a University Hospital from 1/2007 to 12/2023.

Methods. We studied 47 MABC non-repetitive clinical isolates (29 MAB, 12 MBO and 6 MMA). For subspecies identification and molecular detection of mutations conferring resistance to clarithromycin and amikacin, we used the reverse-hybridization-based assay Genotype NTM-DR (BRUKER). The MICs of clarithromycin and amikacin, were determined with the standard broth microdilution method using the commercial assay Sensititre™ RAPMYCOI, according to the CLSI recommendations and breakpoints. To investigate possible inducible clarithromycin resistance (CLA-IR), the incubation period was extended up to 14 days. Results of NTM-DR were compared with sequencing results of hsp65, *erm(41)*, *rrl*, and *rrs* genes.

Results. Four isolates were resistant to amikacin and clarithromycin harboring the A to G substitution at positions 1408 and 2508 of the *rrs* and *rrl* genes respectively. All MMA isolates were susceptible to clarithromycin, having a 276-bp deletion in *erm(41)* gene. Thirty-three isolates were CLA-IR and five were susceptible having the T28 and C28 polymorphisms at *erm(41)* gene respectively.

Conclusion. The NTM-DR assay is a useful tool for rapid (turn-around time of only 6 hours) and reliable identification of MABC subspecies and detection of resistance to amikacin and clarithromycin.

P007

Subspecies distribution, and antibiotic susceptibility of *Mycobacteroides abscessus complex* isolates

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Objectives. *Mycobacteroides abscessus* complex (MABC) is a rapidly growing mycobacterium that usually causes infections in both immunosuppressed and immunocompetent persons and encompasses three subspecies: *M. abscessus subsp. abscessus* (MAB), *M. abscessus subsp.*

bolletii (MBO) and *M. abscessus subsp. massiliense* (MMA). This study aimed to investigate the antimicrobial susceptibility of MABC isolates recovered in two University Hospitals.

Methods. We studied totally 47 MAB isolates (29 MAB, 12 MBO and 6 MMA) recovered from different patients during a 17-year period (2007 to 2023). Identification to the subspecies level was conducted by sequencing of *hsp65* gene. The MICs (μg/ml) of clarithromycin, moxifloxacin, trimethoprim-sulfamethoxazole, linezolid, amikacin, ciprofloxacin, imipenem, doxycycline, and cefoxitin, were determined with the standard broth microdilution method using the commercial assay Sensititre™ RAPMYCOI assay according to the CLSI recommendations and interpreted based on CLSI breakpoints. To investigate possible inducible clarithromycin resistance, the incubation period was extended up to 14 days.

Results. Forty-one of MABC isolates were recovered from respiratory specimens and six from blood specimens of patients with disseminated disease. Of the isolates, 91.5 and 83% were susceptible to amikacin and linezolid, respectively. All MMA isolates and 5 MAB isolates were susceptible to clarithromycin, 4 were resistant, while the other 32 isolates had inducible clarithromycin resistance. All strains were resistant to the other drugs tested.

Conclusion. All strains were resistant to the majority of drugs tested. The 70% of isolates had inducible clarithromycin resistance and the prolonged incubation period up to 14 days in routine susceptibility testing is efficient for detecting this phenotype.

P008

Mycobacterial carbonic anhydrases as targets for tuberculosis research

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Tuberculosis is amongst the deadliest infectious diseases worldwide with an annual caseload of over 10 million patients. World Health Organization estimates that within high-risk countries, one fifth of the patients develop resistance to at least one tuberculosis drug. The long-lasting anti-tuberculosis regimen burdens the healthcare system, and as the number of multi-drug resistant strains keeps rising, more robust treatment options are needed. One mechanism for developing anti-infectives is to find novel targets amongst the vital proteins of bacteria. A such option would be carbonic anhydrases, which exist in several structurally and functionally differing classes. Mycobacterial genomes encode for three β-carbonic anhydrases, enzymes believed having essential roles in *Mycobacterium tuberculosis* growth and virulence. Thus, the main aim of this research is to develop novel carbonic anhydrase inhibitors to enhance the treatment of tuberculosis when used in combination with first-line treatments. The research is carried out using *Mycobacterium marinum*, a natural pathogen of zebrafish and a well-established model organism for tuberculosis research. Currently, hundreds of carbonic anhydrase inhibitors have been screened against *Mycobacterium marinum*, revealing novel compounds that inhibit the metabolic activity and growth of bacteria even better than the first line drug rifampicin, whilst being safe to zebrafish larvae in toxicity modelling. Furthermore, several inhibitors have shown to possess bactericidal properties in *Mycobacterium marinum* biofilms. Two hits have been further tested in adult zebrafish infection model, showing subtle decrease in bacterial load. These results serve as a proof of concept for designing new anti-tubercular agents targeting the carbonic anhydrases.

P009

Applying whole genome sequencing to predict phenotypic drug resistance in *Mycobacterium tuberculosis*? Leveraging 20 years of nationwide data from Denmark

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Infection with *Mycobacterium tuberculosis* remains one of the biggest causes of death from a single microorganism worldwide, and the continuous emergence of drug resistance aggrevates our ability to cure the disease. New improved resistance detection methods are needed to provide adequate treatment, such as whole genome sequencing (WGS), which has been used increasingly to identify resistance-conferring mutations over the last decade. The steadily increasing knowledge of resistance-conferring mutations increases our ability to predict resistance based on genomic data alone. This study evaluates the performance of WGS to predict *M. tuberculosis complex* resistance. It compares WGS predictions with the phenotypic (culture-based) drug susceptibility results based on 20 years nationwide Danish data. Analysing 6230 WGS-sequenced samples, the sensitivities for isoniazid, rifampicin, ethambutol, and pyrazinamide were 82.5%, 97.3%, 58.0%, and 60.5%, respectively, and specificities were 99.8%, 99.8%, 99.4%, and 99.9%, respectively. A broader range of both sensitivities and specificities was observed for second-line drugs. The results conform with previously reported values and indicate that WGS is reliable for routine resistance detection in resource-rich TB low-incidence and low-resistance settings such as Denmark.

P010

Pharmaco-toxicological profile of sulfonyl hydrazone derivatives with potent antimycobacterial activity

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Tuberculosis (TB) is an infectious disease that continues to pose a significant public health challenge globally. There is an urgent need for new chemotherapeutic agents to address the increasing prevalence of multi-drug resistant tuberculosis (MDR-TB). InhA, the mycobacterial enoyl reductase, stands as one of the few clinically validated targets in tuberculosis drug discovery, prompting extensive efforts to identify direct InhA inhibitors with low toxicity. Here, we present a toxicological profile of two sulfonyl hydrazones, compounds **3g** (MIC 0.0763 μM) and **3k** (MIC 0.0716 μM), which exhibit potent antimycobacterial activity, low toxicity, and high selectivity index (SI=1819 and 2216, respectively). Additionally, we assessed their *in vivo* antioxidant activity and *in vitro* inhibition capacity against enoyl-ACP reductase. Compared to INH, compounds **3g** and **3k** demonstrated lower acute toxicity for intraperitoneal administration, with LD_{50} values of 866 and 1224.7 mg/kg, respectively. Subacute toxicity tests, involving the administration of a single dose of the test samples per day over 14 days, revealed no significant deviations in hematological and biochemical parameters or pathomorphological tissues. The compounds exhibited potent antioxidant capabilities, reducing malondialdehyde (MDA) levels and

increasing reduced glutathione (GSH). Furthermore, **3g** and **3k** demonstrated significant enoyl-ACP reductase inhibitory activity, with IC_{50} values of 18.2 µM and 10.7 µM, respectively. It is suggested that the compounds potentially target InhA. In conclusion, the investigated hydrazones display promising antitubercular drug-like properties and warrant further investigation. This study received support from the Bulgarian National Science Fund (Grant KP-06-H41/3, 2020).

P011

Defining genetic interdependencies essential for *M. tuberculosis* survival

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Mycobacterium tuberculosis, which causes human tuberculosis, is amongst the world's deadliest pathogens. Increasing rates of drug resistance pose challenges for treatment and control, which must be addressed by discovering new drugs with new targets. Conventional target prioritisation focuses only on gene products essential for bacterial survival are targeted, but recently a spectrum of vulnerability was revealed. In polyploid organisms, this recessivity of loss-of-function is caused by the topology of genetic interactions. This topology also determines pathways to resistance in response to inhibitors of a given target by imposing constraints on evolution. To better understand the essentiality spectrum in *M. tuberculosis* and anticipate resistance, we aim to map genetic interdependencies and define gene-gene interactions essential for mycobacterial survival. We developed a dual CRISPRi/dCas9 system to independently titrate transcription of thousands of gene pairs in *M. tuberculosis* in parallel and measure the resulting fitness phenotypes using targeted next-generation sequencing. Alongside understanding the genetic network topology of *M. tuberculosis*, the resulting gene-gene interactions will comprise a reference dataset to assist interpretation of data from PROSPECT, a large-scale chemical-genetic interaction screening platform, to prioritise small molecules with new targets towards developing therapies with reduced selective pressures.

P012

Strain Resolution Revolution: Phylogenetic Reconstruction and Rapid SNP Assay Development for *Mycobacterium bovis* in Ireland

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Bovine Tuberculosis poses significant challenges to the livestock industry and wildlife in Ireland. Monitoring the strains of the causative, slow-evolving pathogen presents its own unique challenges. Here, we reconstruct the evolution of *M. bovis* in Ireland using Next-Generation Sequencing technologies (NGS) and exploit the genomic uniformity of the pathogen for potential epidemiological applications.

We employed a microbial variant calling pipeline on 992 Whole-Genome Sequences of Irish *M. bovis* samples. Hierarchical clade-defining Single-Nucleotide Polymorphisms (SNPs) were

identified. We tested 30 SNPs from the phylogeny using LCG's SNPline plate-based genotyping system on a diverse sample set. Additionally, 12 SNPs representing the MRCAs of multiple sub-clades were identified to confirm ancestry and control delineation errors.
Our method consistently and reliably identified sample lineage to the Most Recent Common Ancestor (MRCA) of specific sub-clades, while excluding descent from other sub-clades' MRCAs. Compared to current methods, hierarchical strain typing offers superior resolution, adaptability, speed, and scalability but without the homoplasy issues that can impede epidemiological investigations.
The use of hierarchical clade-defining SNPs enhances strain-typing resolution to a level only comparable to WGS, but without the significant associated costs, promising significant improvements for track-and-trace systems in both the livestock industry and wildlife disease control.

P013

Combined use of commercial and sequencing analysis methods for the identification of non-tuberculous mycobacteria in a tertiary Hospital of Athens: Seventeen years of experience

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Background: The clinical relevance and the optimal treatment regimens of Non-Tuberculous Mycobacteria (NTM) differs strongly by species. We describe our experience regarding the molecular identification of NTM species by using commercial and more advantaged molecular identification methods as sequence analysis.

Material/methods: Specimens submitted for mycobacterial culture between 12/2006-12/2023 in a University Hospital. The recovered NTM were identified by the commercially available reverse hybridization-based assays Genotype Mycobacterium Common Mycobacteria and Additional Species (Bruker). Sequencing analysis of 16S *rDNA* (1500bp) and *hsp65* (440bp) genes was performed when necessary.

Results: In total, 581 non-repetitive clinical isolates of NTM were recovered; belonged to 35 known *Mycobacterium* species with most frequent the *M. avium* (n= 113) and *M. gordonae* (n=82), while 6 strains were not belonged to any known species representing probably novel *Mycobacterium* species. The 91.2% of strains (18 species) were correctly identified at the species level by the commercial assays. Thirty-two (5.5%) strains identified by the commercial assays only to the genus level. Twenty-six belonged to rare NTM species while six strains had unique sequences. Eleven *M. marseillense* and one *M. mantenii* strains erroneously identified by the commercial assays as *M. intracellulare*. Three *M. kumamotonense* and one *M. nonchromogenicum* strains were identified as *M. celatum* and 3 *M. parascrofulaceum* strains were identified as *M. scrofulaceum*.

Conclusions: Our findings suggest that the combined use of molecular commercial identification tests with sequencing analysis improve the ability to correctly identify the common but also the rare NTM, as well as to detect probably novel mycobacterial species.

P015

Simplified strategy to update tuberculosis transmission situation in Madrid by rationalizing molecular and genomic sequential analysis

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Molecular/genomic strategies provide a precise understanding of *Mycobacterium tuberculosis* transmission dynamics. We aim to evaluate an alternative strategy to faster update the epidemiological situation in Madrid, where systematic molecular/genomic surveillance is not running, through a rationalised use of MIRU-VNTR and whole genome sequencing (WGS). A three-step strategy was performed by sequential application of: i) preliminary screening of potential clusters by a 6 loci-MIRU reduced panel (MIRU6) on TB cases diagnosed in 2019 and 2021, ii) extended genotyping (MIRU24), applied exclusively on the MIRU6-defined clusters and iii) WGS on the MIRU24-defined clusters. The application of MIRU6 on 206 and 248 MTB isolates from 2019 and 2021, respectively, classified 220 (48.5%) as orphan. The remaining 234 cases were analysed by MIRU24; 59 (25%) were considered as involved in likely clusters and, finally, WGS confirmed 43 (73%) of them, with 76.5% of the clusters corresponding to recent transmissions (≤5 SNPs). 15 strain-marker SNPs for eight of the confirmed clusters (1-2 SNPs per cluster) were coupled in a multiplex-PCR and analysed by nanopore sequencing. Prospective targeted sequencing was performed in all the new TB cases along a 6-month period in Madrid (N=207). No new cases involving the targeted strains were identified. The application of a three-step sequential molecular/genomic strategy in Madrid reduced in 87% the strains to be characterized genomically to identify transmission clusters. Targeting strain-marker SNPs by multiplex-PCR and nanopore sequencing could mean an alternative to optimize and accelerate the prospective identification of selected strains.

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P017

Overcoming H37Rv bias: a k-mer based approach to isolate-specific masking

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The *Mycobacterium tuberculosis* complex (MTBC) is monomorphic with low genetic diversity. Genome masking is routinely adopted in MTBC isolate analysis to reduce false positive variant calls in highly variable regions (e.g. PE/PPE genes). Recent studies have shown that H37Rv is likely to be over-masked, leading to the removal of true positive SNPs from analyses. Additionally, such masking is H37Rv specific, making analyses in other lineages difficult. This has led to differences in masking approaches and a lack of masking schemes suitable for other MTBC lineages, confounding comparative analyses. This also has implications for the widely accepted

isolate transmission linkage using 5- and 12-SNP thresholds, as different masking schemes will create different SNP counts.

To address these issues, we have developed an automated pipeline to apply consistent mapping to any genome using a k-mer based approach. By identifying regions of genomes where self 50-mer mappability is poor, we generated isolate-specific masking regions. We calculated the true pairwise SNP distances within a set of 300 complete closed genomes from across the diversity of the MTBC. We then calculated false- and true-positive SNP calls between masked paired isolates, followed by mapping Illumina sequencing reads to these pre-masked genomes.

We show that this pipeline can be applied to any MTBC isolate to create strain-specific masking files. This approach generates a minimum SNP distance between isolates, prioritising the minimisation of false positive SNP calling. This allows for consistent comparisons of MTBC isolates and opens the road towards the use of non-H37Rv MTBC reference genomes.

P018

Genotypic and phenotypic drug susceptibility testing (DST) for Bedaquiline, Delamanid and Pretomanid in South-East Asian multi-drug resistant TB (MDR-TB) isolates

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Conventional treatment of MDR-TB is lengthy, costly and associated with drug toxicity. WHO recommends using a new 6-month oral regimen of bedaquiline (BDQ), pretomanid (Pa), linezolid and moxifloxacin in people infected with MDR/RR-TB or MDR/RR-TB with additional resistance to fluoroquinolones. With increased demand for both phenotypic and genotypic DST for the newer drugs, we surveyed for resistance in high-risk cases while validating the DST methods in our laboratory.

Thirty-nine MDR-TB and 2 pan-susceptible isolates from treatment-naive patients between 2022-2023 were tested against BDQ, Pa and delamanid using the BACTEC MGIT 960 with critical concentrations of 1.0, 1.0 and 0.06 µg/ml respectively. Whole genome sequencing (WGS) was performed (n=32) and the results interpreted using the WHO Catalogue of Mutations (Second Edition). All isolates were susceptible to the drugs, except for 2 isolates that had invalid results for Pa due to poor growth.

No mutations associated with resistance were found in all isolates. For BDQ, there were 2 isolates with Rv0678 mutations: “Uncertain significance” mutation Rv0678_p.Arg96Trp and unclassified mutation Rv0678_p.Ala99Thr; both isolates were resistant to clofazimine at 0.5 µg/ml. “Uncertain significance” mutations in mmpL5, Rv1979c and lpqB also occurred less frequently and were found in both MDR and susceptible isolates. Majority of isolates (>86%) contained mtrB mutations that were not associated with resistance.

Polymorphisms detected for delamanid included one of “Uncertain significance”: fbiA_p.Arg175His. The most frequently encountered polymorphism was fgd1_c.960T>C (76.9% of isolates).

In our study, resistance to the new drugs was absent and the phenotypic and genotypic DST results were concordant.

P019

A *M. abscessus* tenosenovitis case: a diagnostic odyssey

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M. abscessus spp. are known for their potential to cause aggressive skin and tissue infections, often posing a diagnostic challenge, as underscored by following case.

A 47-year-old male farmer presented with a progressive painful swelling of the arm that started 6 months earlier after a banal injury between the first and second finger of the right hand.

Initially, antibiotic therapy under the form of clindamycin and amoxicillin / clavulanic acid was started, but failed to improve symptoms. A tentative diagnosis of reactive tenosynovitis was made, but NSAID's and cortisone infiltrations only temporarily relieved symptoms.

Despite consulting multiple physicians from various disciplines, no conclusive diagnosis could be made. Eventually, a tissue biopsy was performed and cultured, which unexpectedly yielded mycobacterial colonies which were identified as *M. abscessus*. Unfortunately, the significance of this result was initially misclassified as irrelevant contamination, further delaying appropriate treatment.

Finally, another consulted clinician correctly recognised *M. abscessus* as the causative pathogen, and adequate treatment consisting of amikacin, azithromycin, clofazimine and linezolid was initiated. In the reference lab, the NTM was subtyped as *M. abscessus* subspecies *abscessus*, with the antibiogram showing no need to change the antibiotic combination. The patients symptoms steadily improved, and therapy was maintained during 9 months.

NTM skin infections still remain underdiagnosed and due to their atypical presentation, they can pose significant challenges in diagnosis and management, particularly in individuals with compromised immune systems or predisposing skin conditions.

P020

NTM detection in a culture negative skin biopsy using bacterial sequencing: a case report

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In our laboratory, we employ targeted sequencing when other diagnostic methods yield inconclusive results. While 16S sequencing can identify NTM species, its taxonomic resolution is limited. Subsequent hsp65 sequencing enhances species specificity. An important advantage of sequencing is its capacity to directly analyse samples without incubation, conserving crucial turnaround time. Following case illustrates its significant added value in NTM diagnostics.

A 51-year-old male presented with extensive skin lesions on all limbs and his trunk. He had undergone allogeneic stem cell transplantation for myelodysplastic syndrome two months prior. Initial differential diagnoses included acute graft-versus-host disease, erythema nodosum, soft tissue infection, and drug eruption. A skin biopsy revealed septal panniculitis with purulence. Tuberculosis PCR, Mucorales PCR, bacterial and fungal cultures yielded no results. Although

mycobacterial culture also remained negative, direct 16S sequencing of the sample complemented by hsp65 sequencing identified *M. chelonae*.

Two months later, a subsequent skin biopsy was cultured prior to initiating quadruple therapy comprising clarithromycin, doxycycline, moxifloxacin and amikacin. This time, mycobacterial culture (MGIT) grew acid-fast bacilli after 9 days of incubation. 16S and hsp65 sequencing on the MGIT medium confirmed *M. chelonae* once again. Antibiogram revealed no resistance, and the patient's symptoms are steadily improving.

In conclusion, direct sequencing of samples expedites NTM detection, bypassing the need for culture growth, thus saving valuable time. In some cases, sequencing can detect NTM even when mycobacterial cultures remain negative, thus improving diagnostic sensitivity. Additionally, sequencing provides species identification, guiding further clinical management.

P021

Genomic analysis of a *Mycobacterium intracellulare* subsp. *chimaera* variant cluster in a German hospital

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Between 2020 and 2023, lower respiratory tract samples from 63 patients staying in a hospital in West-Germany were tested positive for mycobacteria. The organisms were initially identified as *Mycobacterium vulneris* by 16S rRNA sequencing, and could not be differentiated at the species level by the routinely used GenoType Mycobacterium CM-v2.0 diagnostic test. Due to this unusual high number of cases an investigation with extensive environmental sampling was initiated. Water samples and swabs were collected in the hospital from bronchoscopes, disinfection equipment, drinking water fountains, showers and sinks for further investigation. Whole genome sequencing analysis identified all isolates as belonging to *Mycobacterium intracellulare* subsp. *chimaera*. Subsequent core genome multi-locus sequence typing (based on 3719 genes) showed that the 29 available patient isolates were closely related to each other (1-30 alleles difference; median distance: 9) and to an isolate from a water related source inside the hospital (min. 1 allele difference). When compared to 600 publicly available *M. chimaera* group 1 isolates, they constituted a distinct clade unrelated to the *M. chimaera* strains which caused the global cardiac surgery-related outbreak. However, this separate clade also included 6 closely related isolates from other sites in Germany (min. 3 alleles). The closest non-German isolate was obtained from the United Kingdom (23 alleles distance). After implementing prevention measures, no additional patient isolates were found positive with the clone. Altogether, this suggests the existence of a widespread clone but whether the patients are truly infected, colonized temporarily or samples are merely contaminated is still under investigation.

P022

The effect of diabetes mellitus on mortality during rifampicin-resistant tuberculosis treatment in Indonesia: a retrospective cohort study

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Diabetes increases the risk of tuberculosis (TB) disease, TB deaths and drug-resistant TB. We determined the effect of diabetes on all-cause mortality during rifampicin-resistant TB treatment in Indonesia, which has the second-highest TB burden globally and a rapidly growing diabetes prevalence. The cohort included individuals aged ≥18 years old with Xpert MTB/RIF-confirmed rifampicin-resistant TB treated in a tertiary referral hospital between March 2020 and May 2022. We collected routinely measured hospital data and used baseline HbA1c levels to categorize individuals as diabetes (HbA1c≥6.5%), pre-diabetes (HbA1c≥5.7% and <6.5%), and no diabetes (HbA1c<5.7%). Cox proportional hazards regression was used to analyse the association between diabetes and all-cause mortality. In total, 345 individuals with rifampicin-resistant TB were included (median age 38 (IQR: 28-50), 57% male, 1.7% HIV). Of 276 with further resistance testing, 56% had MDR-TB and 9.1% pre-XDR. Sixty-two died during treatment (18%; 95% CI: 14-22) and diabetes was diagnosed in 96 (28%; 95% CI: 23-33), half of whom had newly diagnosed diabetes. The adjusted hazard rates of all-cause mortality during rifampicin-resistant TB treatment were higher among people living with diabetes (PLWD) compared to those without diabetes (aHR = 1.86 (95% CI: 0.97-3.57)), and especially among underweight PLWD (aHR = 2.63 (95% CI: 1.15 – 6.00)). Moreover, treatment failure was substantial (13%) but not higher among PLWD. In conclusion, diabetes in rifampicin-resistant TB is common and associated with increased all-cause mortality during second-line treatment in Indonesia. More effort is needed to understand this effect, and improve treatment outcomes of diabetes-associated drug-resistant TB.

P023

Characterization of a putative cobalt transport system in *Mycobacterium smegmatis*

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Cobalt is an essential trace metal, incorporated in important metalloproteins, e.g. corronoid proteins. Best-known representatives of this group are cobalamins, such as vitamin B12. 80-90% of all bacteria contain vitamin B12 dependent enzymes, involved in several central metabolic processes and in host-pathogen interaction. Despite this essentiality, only 25-30% are able to produce vitamin B12 *de novo*, including many mycobacteria species. For the production of vitamin B12, cobalt uptake is essential. However, knowledge on cobalt uptake and regulation in Non-tuberculous mycobacteria (NTM) such as the opportunistic pathogen *M. abscessus* and *M. avium* is very limited.

Here we analyzed cobalt homeostasis of *M. smegmatis* (MSMEGwt). A regulator of the ArsR/SmtB family (*msmeg*_2606) associated with a putative metal transporter (*msmeg*_2607-2610) and genes of vitamin B12 synthesis (*msmeg*_2616-2618) was identified by genome mining. The regulon

of *msmeg*_2606 was determined by transcriptome analyses of a MSMEGΔ2606 deletion mutant. 30 genes were >5 fold differentially expressed in the mutant compared to MSMEGwt. Amongst these, expression of *msmeg*_2607-2610 was higher, indicating a role of MSMEG_2606 in regulation. BLAST and TMHMM analyses suggest that *msmeg*_2607-2610 encoded proteins are a CbiMNQO cobalt importer of the Energy-Coupling-Factor (ECF) family. In qRT-PCR experiments *cbi*M (*msmeg*_2607) expression was induced in MSMEGwt grown in cobalt-deficient medium, supporting the putative role of *msmeg*_2607-2610 in cobalt metabolism. A MSMEGΔ*cbi*MN mutant will be used in future growth experiments and reporter studies to analyze regulation by MSMEG_2606 and the metals involved in regulation and transport. These findings might help identifying new antibacterial targets for treating NTM infection.

P024

Illustrating variations in resistance mechanisms of *Mycobacterium tuberculosis* against bedaquiline

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The persistent challenge of drug resistant, and multi-drug-resistant tuberculosis (MDR-TB) continues to undermine current treatment strategies, with nearly half a million cases annually. Several factors including the long-term, complicated and often toxic treatment regimens, delayed diagnostics, and pharmacokinetic interactions facilitate the selection of drug resistant *Mycobacterium tuberculosis* (MTBC) complex strains, which then can be transmitted further. Despite the success of a novel shortened all-oral MDR-TB treatment regiment, the observation of rapid bedaquiline (BDQ) resistance evolution, a pivotal component of this regiment, is of concern. Accordingly, a precise understanding of resistance mechanisms is key for effective diagnostics and treatment.

Nearly all BDQ resistances observed in clinical MTBC strains are linked to variants in *Rv0678*, despite having a moderate resistance profile. To elucidate the effect of different *Rv0678* variants on MTBC strains, the transcriptomic landscape in presence and absence of the target drug BDQ was investigated on lab generated mutants. Initial investigations on the described mechanism of efflux pump mmpS5-mmpL5 revealed moderate expression of these pumps in the *Rv0678* mutants, and enhanced expression under BDQ exposure. Notably, the susceptible wild type ancestor clone also exhibited increased expression of the efflux pumps when compared to untreated conditions. Temporal transcriptional changes indicated metabolic remodelling beyond the described resistance mechanism.

This study challenges our current understanding of *Rv0678* functionality, and advances our understanding of resistance mechanisms of MTBC bacteria. These insights show potential for the development of more effective treatment strategies rooted in evolutionary medicine principles.

P025

In vitro activity of epetraborole, a novel LeuRS inhibitor, alone and in combination against rapidly growing non-tuberculous mycobacteria

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Rapidly growing mycobacteria (RGM) are intrinsically drug resistant, leading to complicated and often unsuccessful long-term treatment for infections due to these pathogens. Treatment generally includes a macrolide, but inducible and mutational resistance are problematic. Epetraborole (EBO) is a boron-containing, oral inhibitor of bacterial leucyl-tRNA synthetase, an essential enzyme in protein synthesis. We determined the MIC values of EBO and comparator agents against several strains of RGM including *Mycobacteriodes abscessus* (MABS), *Mycobacteroides chelonae* (MC) and *Mycolicibacterium fortuitum* (MF). Ten of the MABS isolates were further tested *in vitro* against drug combinations in a checkerboard assay.

Thirty-nine MABS, ten MC and ten MF isolates were tested in cation-adjusted Muller Hinton broth according to CLSI standards. In addition to EBO, clarithromycin (CLR), amikacin (AMK), clofazimine (CFZ), cefoxitin (CFX), linezolid (LZD) and tigecycline (TIG) were tested. EBO was combined with either CLR, AMK, CFZ, CFX, LZD, TIG or imipenem (IMP) in the checkerboard assay. Synergy, additive effects, indifference, or antagonism was characterized using EUCAST criteria.

EBO has potent *in vitro* activity versus RGM with a MIC_{50} and MIC_{90} of 0.06 µg/mL. Also, EBO activity was not affected by resistance to macrolides, aminoglycosides or oxazolidinones. In the checkerboard assay, no antagonisms were observed, and all the combinations tested resulted in indifference when definitive calculations were made. The potent *in vitro* activity of EBO supports further investigation of EBO as a potential therapy for RGM infections.

P026

Tracking cross-border transmission of the Rwanda MDR-TB R3clone

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The use of molecular epidemiology to track circulating strains of *Mycobacterium tuberculosis* is becoming more commonplace in highly endemic settings. However, most such studies are done on a country-by-country basis, which can limit the ability to find cross-border transmissions. Using a WGS dataset of 308 *M. tuberculosis* samples collected from 1992-2018 we found that most MDR-TB in Rwanda was due to a single circulating clone (termed the R3clone). We wished to see if this clone was also present in neighbouring countries to better understand the extent of its spread.

Further WGS in Rwanda revealed this clone is still prevalent in the population. To allow for surveillance of this clone, we determined the spoligotype and drug resistance patterns that characterise these isolates and developed a SNP assay specific for the R3clone. This was undertaken by finding SNPs present in all clone isolates and absent in all others in the Rwanda dataset. This SNP (C25631G) was then validated against the 80,000 isolates within the NCBI SRA database.

We used the spoligotype, DR patterns and SNP assay to search for the presence of this clone in stored isolates from the neighbouring country of Burundi. We found that 25/143 (17%) of RR-TB isolates from Burundi spanning 2011-2013 are R3clone, suggesting sustained cross-border transmission. WGS-based phylogenetics indicated most Burundi isolates derived from a single introduction but there was evidence of additional crossings. This indicates that transmission studies should be undertaken beyond country-specific studies, especially in areas with high levels of both TB and migration.

P027

Using the SpyTag/SpyCatcher system to determine the path of substrates through the mycobacterial type VII secretion system

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Mycobacterium tuberculosis (*Mtb*) possesses a highly impermeable cell envelope (CE), which is composed of an inner membrane (IM) and a specific mycolic acid-containing outer membrane (OM). *Mtb* strictly depends on extracellular proteins to survive and cause infection, as a portion of these proteins mediate crucial host-pathogen interactions. The majority of secreted proteins are exported by type VII secretion systems (T7SSs), of which ESX-1, ESX-2, ESX-3, ESX-4 and ESX-5 are present in *Mtb*. How T7SSs mediate export of proteins while maintaining the permeability barrier of the CE, important for survival during infection, remains unknown. Recently, the high-resolution structure of the ESX-5 inner membrane (IM) complex of *Mtb* has been solved. As the structure reveals a closed channel that resides solely in the IM, the path of substrates through the channel and the remaining layers of the CE remain unclear. In this project we use the SpyTag/SpyCatcher system, a highly versatile technology for irreversible conjugation of recombinant proteins, to determine the path of T7SS substrates through the CE. We use this system (i) to lock substrates in the secretion channel, after which we will co-purify this translocation intermediate with membrane complex for structural analysis, and (ii) to determine whether substrates face the periplasmic space during export. Understanding the mechanism of protein transport through the impermeable mycobacterial CE will provide important insights into the functionality of this complex structure and for the exploitation of T7SSs for better anti-tuberculosis treatments.

P028

Expanding resources for bedaquiline resistance detection: generating heteroresistant mixtures for BCCM/ITM inclusion

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Timely detection of bedaquiline-resistant tuberculosis is critical for effective treatment of rifampicin-resistant tuberculosis, particularly in light of BPaL and BPaLM regimens. Without a rapid molecular diagnostic, bedaquiline resistance is assessed by sequencing the Rv0678 gene. Heteroresistance, the coexistence of drug-susceptible and drug-resistant populations, is reported frequently in clinical isolates and challenges the accurate diagnosis of bedaquiline resistance. We generated bedaquiline heteroresistant mixtures for inclusion in the Belgian Coordinated Collections of Microorganisms (BCCM) at 0.5%-100% resistant allele ratios. We used an Rv0678-wild-type bedaquiline-susceptible *Mycobacterium tuberculosis* (*Mtb*) strain and its *in-vitro* selected isogenic daughter strain with an Rv0678 insertion mutation (192-193_ins_G), a variant reported in clinical isolates. Genotypic assessment of resistant over susceptible populations was done using Deeplex Myc-TB. Bedaquiline minimum inhibitory concentration (MIC) testing was done by the EUCAST broth microdilution method (critical concentration of 0.25 µg/ml). Deeplex accurately identified resistant populations in all mixtures ≥5%, with mutant reads detectable in the BAM files for 0.5%-3% mixtures. Mixtures at 0.5%-5% showed a bedaquiline MIC above the critical concentration, but lower than the MIC of the strain with a 100% resistant variant. Mixtures with a ≥10% resistance allele had the same bedaquiline MIC as the strain with a 100% resistant allele. These findings underscore the need for robust detection of heteroresistance, even at very low allele frequencies. This study enriches the repertoire of drug-resistant *Mtb* mixtures in BCCM/ITM, an important asset for research and development by facilitating the advancement of diagnostic assays for bedaquiline resistance.

P030

Detection a new *Mycobacterium tuberculosis* complex *modern* L2 taxon based on SNPs in the *Rv2983* and *fgd1* gene

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Rv2983 and *fgd1* are involved into pretomanid and delamanid metabolism, both drugs used in new treatment schemes for multidrug resistant tuberculosis (MDR-TB). We look at the sequences

of these two genes in different lineages of *Mycobacterium tuberculosis* complex using NCBI SRAs and the pipeline *TB-Annotator* v2.4, which compiles 112,373 SRAs. Based on the *Rv2983* sequence, we grouped 46 SRAs that share the SNP SPDI NC_000962.3:3339291:A:C. These 46 SRAs share also another unique SNP on *fgd1* at SPDI NC_000962.3:491741:T:C. These genomes were retrospectively tracked back to 20 Bioprojects, most of them concerning MTBC isolates from China (n=14), Canada (British Columbia, n=1) or unknown (n=31). We built a maximum likelihood phylogenetic tree with RAxML on these SRAs and others taken as representative of the L2 diversity. The tree shows these SRAs in a new terminal leaf. The taxon was named as L2-CC-2983-I59L. This branch is structured into two sub-branches C1 (n=3) and C2 (n=43). It belongs to a *modern* L2 sublineage but does not enter into any existing taxonomical classification. We are now investigating the potential loss of function of these two gene products (Rv2983, *fgd1*). It is likely that the detection of this *Rv2983-fgd1* variant antedates the use of pretomanid and delamanid, and just grew to a detectable epidemic signal recently, in relation to the existence of a yet undescribed chinese historical TB genomic diversity reservoir. In conclusions, we describe a new sublineage 2 cluster, made-up of 46 SRAs, that will require search for fitness advantage outside the drug resistance selection.

P031

Genetic diversity and drug resistance profiles of *Mycobacterium tuberculosis* L2/Beijing isolates in Kazakhstan: Insights from whole-genome sequencing

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Kazakhstan is among the 30 countries with a high burden of multidrug-resistant tuberculosis (MDR-TB). The prevalence of drug-resistant isolates in Kazakhstan is linked to the Central Asian/Russian cluster of the L2/Beijing family of *Mycobacterium tuberculosis*. A total of 102 L2/Beijing clinical isolates of *M. tuberculosis* representing various regions in Kazakhstan, including Aktau and Kostanay (n = 13); Kyzylorda (n = 12); Taldykourgan (n = 11); Semey (n = 9); Aktobe (n = 7); Atyrau, Karaganda, and Pavlodar (n = 6); Stepnogorsk, and Uralsk (n = 5); Almaty, and Taraz (n = 4); and Shymkent (n = 1) were selected for whole-genome sequencing (WGS). The sequenced L2/Beijing isolates were characterized according to a recent SNP-based nomenclature proposed by Thawornwattana. Most of the studied *M. tuberculosis* isolates were assigned to the L2.2.M4.9.1 sublineage (n = 69; 67.64%), also known as the Central Asia outbreak. Other sublineages were represented by Central Asia L2.2.M4.9 (n = 27; 26.47%), Europe/Russia W148 outbreak L2.2.M4.5 (n = 5, 4.90%), and L2.2.M4 sublineage (n = 1; 0.98%). Multidrug-resistant (n = 60) and pre-extensively drug-resistant (n = 7) isolates were prevalent in the sample, based on phenotypic and WGS data. Notably, the Central Asia outbreak and Europe/Russia W148 outbreak strains are associated with the outbreaks of MDR-TB in Central Asia. This ongoing research is focused on drug-resistance profiles, genotypes, and transmission dynamics among different regions of Kazakhstan using next-generation sequencing, followed by bioinformatics analysis employing the TB-Annonator pipeline.

P032

Almost half of the newly described *Mycobacterium* species are published with synonymous genus names

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In 2018, a split of the *Mycobacterium* genus into 5 new genera was proposed: *Mycolicibacterium*, *Mycolicibacter*, *Mycolicibacillus*, *Mycobacteroides*, and a revised *Mycobacterium. [1]* The proposed nomenclature was acknowledged as synonymous for the conservative *Mycobacterium* genus. This led to confusion, notably within clinical microbiology, as lack of traceable names for pathogens can lead to diagnostic confusion.[2]

We searched the PubMed-NCBI and Google Scholar Search databases for newly described species within these genera, between January 2020 and February 2024.

Twenty-three new species were described, of which only 11 with the genus name "*Mycobacterium"*, while another 11 were described as "*Mycolicibacterium* sp" and one as "*Mycolicibacter sp"*. The choice of genus name was not associated with the journal of publication. The "*Mycobacterium"* genus name was used in the conservative way, without reference to the new synonyms. In case the new nomenclature would have been applied, only 4 new species would fall in the revised "*Mycobacterium"* genus, while three would become "*Mycolicibacterium* sp" and four "*Mycolicibacter sp"*.

Five of the six new species with probable or documented human clinical relevance were assigned to "*Mycobacterium"*: four associated with lung disease (*M. hubeiense*, *M. vicinigordonae*, *M. kiyosense*, and *M. senriense)* and one with skin disease *(M. salfingeri*). *M. salfingeri and M. hubeiense* would become "*Mycolicibacterium"* with the synonymous nomenclature.
Also *Mycolicibacterium toneyamachuris* was associated with pulmonary disease.

Despite expressed concerns from mycobacteriologists, new species are often described using the new nomenclature, also clinically relevant species, which may further confuse clinical microbiologists and diagnostic developers.

P033

Limited phenotypic resistance for African clinical *Mycobacterium tuberculosis* isolates harboring variants in genes relevant for bedaquiline and delamanid resistance

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Organization 13: University of Kinshasa 14: National TB Program DRC 15: University of Rwanda 16: University Clinical Research Unit Bamako

We determined minimal inhibitory concentrations (MICs) by the broth microdilution method (BMD) for bedaquiline, clofazimine, delamanid and pretomanid for *Mycobacterium tuberculosis* complex isolates (DIAMA project; nine African countries; 2017-2021; baseline isolates from rifampicin-resistant and -susceptible TB, BDQ/DLM-inexperienced patients; Clinicaltrials.gov, NCT03303963). All isolates underwent whole genome sequencing. Thirteen percent (213/1586) of isolates harbored genetic variants in genes potentially implicated in resistance to these drugs. We prioritized variants that were not mono-phyletic, had uncertain significance or not classified in the WHO Mutation Catalogue V2, and were available at ITM: 5 *atpE*, 10 *mppR5/L5*, 16 *pepQ*, 3 Rv1979c, 13 *ddn*, 22 *fibA*, 24 *fbiB*, 63 *fbiC*, 5 *fbiD* and 43 *fgd1*.

Valid BMD results for 49 isolates with potential delamanid-resistance associated mutations showed a mode of 0.008 μg/ml. Seven (14%) showed an MIC above the cut-off (0.125 μg/ml), and had a mutation in *fbiB/C/D* or *fgd*1. The same seven and two additional isolates had pretomanid-MICs ranging from 0.5 to 2 μg/ml (mode 0.06 μg/ml). Twenty-seven isolates with potential bedaquiline-resistance associated mutations had a mode of 0.06 μg/ml. Four (14%) showed a MIC above the cut-off (0.25 μg/ml): three having a *mmpR5* (1 μg/ml) and one a *pepQ* variant (0.5 μg/ml). The three *mmpR5* variants had a clofazimine MIC of 2 μg/ml, and the *pepQ* variant 0.125 μg/ml. No other isolates were clofazimine resistant.

These preliminary MIC results for mutants of unknown significance reveal resistance to new anti-TB drugs among drug-naïve patients, albeit for only a minority (~14%) of isolates with genetic variants.

P034

Cerebrospinal fluid fatty acid metabolism and tuberculous meningitis mortality

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Dysregulation of tryptophan metabolism contributes to the high mortality of tuberculous meningitis (TBM). We aimed to identify novel metabolic pathways associated with TBM mortality through metabolome-wide analysis, to improve understanding TBM pathophysiology and to identify new therapeutic targets.

We measured 619 metabolites using untargeted liquid chromatography-mass spectrometry in pre-treatment cerebrospinal fluid (CSF) from adults with TBM from Indonesia (n=388; 34 HIV-positive) and Vietnam (n=679; 250 HIV-positive). The primary outcome, 60-day mortality, was modelled using a Cox regression, adjusting for age, and HIV-status in a screening–validation approach. Secondary analysis included hierarchical clustering to classify associated metabolites into subgroups; comparison with non-infectious controls, and correlation of metabolites with TBM patient characteristics, CSF cytokines, blood-CSF barrier leakage, and serum metabolite levels.

Sixty-day mortality, the primary endpoint in the analysis, was 21.6%.We confirmed tryptophan as a predictor and identified nine additional CSF metabolites positively associated with hazard ratios for 60-day TBM mortality of 1.3 and above, grouped into two clusters. The strongest predictive metabolites for TBM mortality (independent of disease severity and CSF tryptophan) was 3-hydroxyoctanoate (FA 8:0; 3OH), part of a cluster of fatty acids, also including hydroxy-isocaproate (FA 6:0; OH), hydroxyisobutyrate (FA 4:0; OH), and C4-OH-carnitine. All four fatty acids correlated weakly with and blood-CSF barrier disruption, but serum fatty acids did not show a concomitant increase.

Concluding, we identified and validated nine new metabolites associated to TBM mortality, independent of HIV-status, disease severity, and tryptophan, pointing to an important role for altered fatty acid beta-oxidation in TBM mortality.

P035

Predominant clones of *Mycobacterium tuberculosis* in Sub-Saharan African Countries

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Tuberculosis (TB) remains a major threat to global public health, with transmission dynamics of *M. tuberculosis* (Mtb) varying considerably across global regions. This study aims to assessing the presence and distribution of Mtb clones and -transmission clusters within and across different sub-Saharan African countries.

In the DIAMA study involving nine Africans countries, we sequenced isolates from consecutive rifampicin-resistant (RR-TB) patients and equal numbers of retreatment patients with RS-TB. Lineages were assigned using the TB Profiler v4.4.2 pipeline. Single nucleotide polymorphisms (SNPs) were identified from VCF files using H37Rv as a reference, and pairwise matrix distances per sub-lineage between samples were calculated using the Lyve-SET pipeline, with a cutoff of 5 SNPs to define clusters.

From 1586 isolates, we identified 49 transmission clusters comprising 1453 isolates, with clusters stratified as follows: Lineage 2 (1), Lineage 3 (3), Lineage 4 (35), Lineage 5 (9), and Lineage 8 (9). We found that 26% of clusters were country-specific, while 78% demonstrated cross-border transmission. Lineage 2 clusters predominated in Guinea (63%) and were confined to West Africa. Lineage 3 clusters were predominant in Ethiopia (79%) and limited to Eastern Africa. Lineage 4 was widespread, although some clusters were country-specific. Culture biases likely caused underestimation of Lineage 5 clusters, primarily found in Benin (41%), affecting West and Central Africa, while Lineage 8 was exclusive to Rwanda.

This comprehensive analysis reveals significant variability in the distribution of Mtb transmission clusters across sub-Saharan Africa. The predominance of multi-country clusters underscores the necessity of regional cooperation in TB control strategies.

P036

Endemic transmission of *Mycobacterium tuberculosis* sublineage Beijing L2.2.M3 within Colon, Panama: A prospective study

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Tuberculosis remains a major public health threat worldwide. We aim to perform a prospective analysis of *Mycobacterium tuberculosis* Beijing sublineage isolates from Colon, Panama. Between 2021 to mid-October 2023 we collected *Mycobacterium tuberculosis* isolates. Drug resistance was determined using GeneXpert and MTBDRplus-Genotype. Genotyping and lineage identification were conducted using allele-specific oligonucleotide PCRs and MIRU-VNTR typing, and whole-genome sequencing. Sequence data were analyzed using the mtb-call2 pipeline and TB-gen tools to predict drug resistance and sublineage, respectively. Genome-wide SNPs were also used for phylogenetic and evolutionary analyses including other Latin American isolates.

Our results show ASO-PCR identified all 66 as Modern Beijing L2.2 isolates. The MIRU-VNTR genotyping revealed eight transmission clusters representing 27/66 (40.9%). WGS analysis with pipeline mtb-call2 confirmed all isolates belong to the L2.2.1 Beijing sublineage, including 96.9% (62/64) pansusceptible and 3.1% (2/64) rifampicin mono-resistant. The strains were genotyped by SNP at position 2078246C>G (gene *gcvB*, locus Rv1832). In contrast, TB-gen genotyped all 64 strains as L.2.2.M3 Beijing sublineage by SNP at position 1219683G>A (gene *coaA*, locus Rv1092c). The phylogenetic analysis revealed a correlation with geographical distribution when compared with other Latin American Beijing isolates. This analysis also confirmed a relatively low evolutionary rate within Panama Beijing isolates and a highly conserved common ancestor shared with Beijing isolates from Peru, Colombia, and Guatemala.

Our findings revealed an endemic transmission of the *Mycobacterium tuberculosis* Beijing sublineage in Colon, Panama. This study highlights the need for combining molecular/genomic with epidemiological data, to design new and reinforce tuberculosis control strategies.

P037

Transcriptomic Insights into Human Macrophage Responses to *Mycobacterium tuberculosis* Complex (MTBC) Strains

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Tuberculosis, caused by various strains of the *Mycobacterium tuberculosis* complex (MTBC), remains a leading cause of mortality globally, necessitating innovative approaches to better understand the host-pathogen interaction. Macrophages play a pivotal role in MTBC infection, serving as both hosts and effectors in the immune response. Results of previous studies indicate that MTBC infection can lead to detrimental epigenetic reprogramming. Understanding the transcriptomic response of human macrophages to different MTBC strains is one step in identifying epigenetic alterations caused by MTBC.

In our study, we aim to investigate the changes in the epigenetic makeup of human macrophages during early-stage MTBC infection. As a first step, we analyze gene expression data of human macrophages infected with diverse MTBC strains to elucidate the differential host-pathogen interactions at the molecular level. This part of the investigation focuses on the variations in gene expression profiles, which could provide insights into strain-specific immune evasion mechanisms. Data analysis involves differential gene expression analysis, pathway enrichment, and network analysis to identify key regulatory mechanisms activated by different MTBC strains.

We anticipate identifying distinct transcriptional signatures associated with different MTBC lineages. These signatures might highlight differences in immune response modulation, providing a deeper understanding of MTBC pathogenesis and host defence strategies.

This study promises to advance our understanding of MTBC-host interactions and tuberculosis pathophysiology.

P038

Non-tuberculous and tuberculous mycobacteria prevalence and drug susceptibility in Italian and foreign subjects: a monocentric observational study

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Mycobacterium tuberculosis (MTB) and non-tuberculous mycobacteria (NTM) can lead to both pulmonary and extra-pulmonary diseases. Despite the increasing identification of NTM, there is limited data on their circulation in Italy. This study aimed to determine the prevalence of mycobacterial infections among patients at Sacro Cuore - Don Calabria Hospital (Verona, Italy)

from January 2020 to December 2023. Patients were categorized by geographic origin and age. Molecular methods were used for MTB and NTM identification, and drug susceptibility was evaluated by the minimum inhibitory concentrations (MICs) test.

Out of 2401 samples analyzed, 84% were from Italian individuals (median age 72 years) and 16% were from foreign individuals (median age 42 years). Real-time PCR revealed that 7.6% of samples tested positive for Mycobacteria: 93 for MTB and 91 for NTM. NTM species identified included: 43 *M. avium*, 14 *M. intracellulare*, 9 *M. gordonae*, 9 *M. chimaera*, 3 *M. xenopi*, 2 *M. fortuitum*, 2 *M. abscessus*, 2 *M. kamsasii*, and 1 each of *M. celatum*, *M. chelonae*, *M. porcinum*, *M. mucogenicum*, *M. simiae*, *M. kumamotonensis, and M. malmoense*. Notably, 35% of MTB and 88% of NTM-positive samples originated from Italian patients, whereas 65% of MTB and 12% of NTM-positive samples were from foreign individuals. The drug susceptibility were then analyzed on MTB and NTM coltures.

Due to the challenge of identifying and treating NTM infections owing to their intrinsic drug resistance, comprehensive monitoring of their epidemiology is crucial to update microbiological and clinical guidelines in our region.

P039

Genomic insights into TB transmission networks: unveiling diagnostic delays or subclinical cases and their impact on secondary cases

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Whole-genome sequencing enhances surveillance of tuberculosis (TB) transmission. A detailed analysis of SNPs distribution and case chronology in genomic networks facilitates differentiation between growing clusters due to recent transmission or reactivations. In this study, we exploit SNPs distribution within these networks to propose candidates of prolonged diagnostic delays or subclinical TB. We support these assignations on clustered cases i) genomically linked with other case/s diagnosed several years before and ii) with sequentially acquired SNPs from the case/s preceding them, suggesting the presence of viable evolving bacteria before diagnosis. We present representative clusters that underscore the differential impact of diagnostic delays on the number of secondary cases caused by them: from no impact, without secondary cases, to causing sequential consecutive infections within a household or being responsible for several community secondary cases, due to likely exposures at different stages along the diagnostic delay. In the household cluster, mono-resistance to fluoroquinolones (FQ) emerged along the diagnostic delay, due to FQ administration to treat other infections, likely causing TB monotherapy. This observation suggested that mono-resistance (monoR) might be a proxy to infer diagnostic delays/subclinical TB. Another eleven cases with monoR (2 to FQ and 9 to INH; 7 of them belonging to 4 clusters) were selected for the study. A revision of their clinical records revealed symptoms/radiological findings compatible with TB, unnoticed for a long time before diagnosis, which was consistent with our hypothesis. Funding: PI21/01823 (ISCIII); CPII20/00001 (Miguel-

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P040

Healthcare workers and migrants user experience with targeted education materials for self-swabbing for TB screening

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TB screening is challenged by a reliance on sputum-based testing and migrants' hesitation to seek care. This study aims to evaluate the feasibility and acceptability of targeted educational materials to guide migrants and healthcare workers in a non-sputum option, supervised tongue swabs (STS) for TB screening using Copan FLOQSwabs®. TS is up to 95% sensitive when laboratory methods are used that are explicitly designed for swab testing. In order to scale TS for screening and population health we need users to be able to swab themselves effectively.

This user experience study uses qualitative research methods with in-depth interviews and purposive sampling of migrants and healthcare workers experiencing screening for TB infection in Milan, Italy. Recruitment began in March 2024 and continues until saturation among meta themes is reached. Hamilton's Rapid Qualitative Analysis Method was adapted to summarize key findings.

Preliminary results from the first 4 people interviewed reveal the barriers and facilitators to using illustrations and videos to teach how to collect a valid tongue swab sample. All the participants reported that the video was the best option but that it needed audio narration. All HCW's shared that the videos and illustrations would guide migrants to swab effectively.

Study explored the usability of educational materials to collect TS for TB screening. Preliminary results indicate that illustrations and videos are acceptable and translated in languages of underserved communities. There is a lack of STS educational materials and health promotion campaigns that are targeted to migrants' needs. This is one solution to these barriers.

P041

Transmission patterns of *Mycobacterium leprae* in the Comoros

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Despite a strong control program since 2008, leprosy remains highly endemic in the Comoros, with ongoing transmission. This study investigates the effectiveness of using multiple-locus variable number of tandem repeats (VNTR) analysis (MLVA) and SNP-typing on a targeted deep sequencing platform, (Deeplex Myc-Lep) to study *Mycobacterium leprae* transmission in the island.

A total of 1403 leprosy patients were enrolled, of which 290 biopsies with the highest bacterial load were processed using Deeplex Myc-Lep. From 256 biopsies, a VNTR-based phylogenetic tree was constructed based on their complete profiles (11 VNTR markers). SNP subtypes 1D (85.9%) and 1A (13.3%) were detected, correlating closely with VNTR-based branches. Two VNTR markers (6-3a and 12-5) lacked variability within the population. Among 97 distinct profiles, 45 (46%) VNTR profiles were clustered and 52 unique, with most of clustered patients living in proximity or self-reporting contact. However, only 50% of known contacts shared the same genotype. In 30 strains from 5 clusters with identical VNTR profiles, we performed whole-genome sequencing (WGS) to get deeper resolution at SNP level. The results showed a median of 5 SNPs (range 2-12) between strains from the same cluster. In this high burden setting, self-reported epidemiological links had low specificity for a transmission link relative to genotyping by VNTR profiling and/or whole genome sequencing. The proportion of leprosy due to recent transmission is underestimated, and improving the limit of detection for genotyping will allow for better transmission cluster definitions and develop new tools to track *M. leprae* spread between communities.

P042

Influence of external potassium on the rifampicin susceptibility of *Mycobacterium*

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Mycobacterium tuberculosis causes tuberculosis (TB) a major human pulmonary infection with around 10 million cases per year. TB treatment requires minimum 6 months of antibiotic treatment, as persistent sub-populations of pathogen are tolerant to antibiotics. In this study we investigate the influence of external potassium concentration on rifampicin susceptibility of *Mycobacterium* species, since rifampicin is a core component of the TB treatment regimen. External potassium in the growth medium increased the rifampicin resistance of the lab model organism *Mycobacterium smegmatis*, increasing the minimum inhibitory concentration by more than 2-fold. Growth curve analysis revealed that potassium affects the lag time but not the growth rate at different rifampicin concentrations. Furthermore, we investigated the influence of potassium on the rifampicin susceptibility of a small set of clinical *M. tuberculosis* isolates. Taken together, our study reveals the influence of external factors such as potassium on the efficacy of rifampicin treatment and *Mycobacterium* growth dynamics. Understanding the molecular mechanism of this interaction will help in exploring possible potentiators of rifampicin action to improve TB treatment.

P043

LiquidArray® technology for the detection of extended drug resistance mutations

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Since 2022, the 6 month BPaL(M) regimen is endorsed by WHO for treating MDR-TB. The global uptake of the regimen has been slow due to drug availability, however the spectrum of the available susceptibility tests is expanding. Up until recently, reliable genotypic drug susceptibility testing (gDST) of new and repurposed drugs, including those used in BPaL(M), was available through sequencing. However, in most EU/EEA countries, sequencing is only available in national laboratories. The LiquidArray® MTB-XDR kit was launched in October, 2023. It is the first moderate complexity assay offering reliable reporting of resistances to linezolid (LZD) amongst other second-line drugs. The aim was to compare the performance of LiquidArray® MTB-XDR with the Xpert MTB/XDR®, the GenoType MTBDRsl ver 2.0 and the reference standard phenotypic DST (pDST) using the MGIT system. The analysis involved 31 TB isolates from the collection of the National Reference Laboratory for Mycobacteriology, Hungary. Regarding fluoroquinolone (FLQ) resistance, GenoType found 8 (25.8%), Xpert detected 7 (22.6%), and the LiquidArray® MTB-XDR showed 6 (19.4%) isolates as FLQ resistant (2 had indeterminate result). With pDST, 7 isolates were reported as FLQ resistant. Regarding amikacin (AMK) resistance, GenoType found 3 (9.7%), Xpert detected 6 (19.4%), and the LiquidArray® MTB-XDR showed 3 (9.7%) isolates as AMK resistant. Phenotipically, 4 (12.9%) isolates were identified as AMK resistant. With regards to LZD, a total of 3 (9.7%) isolates were resistant both pheno- and genotipically. The LiquidArray® technology reliably detects a range of relevant SL drug-resistance mutations.

P044

Mycothione reductase as a potential target in the fight against *Mycobacterium abscessus* infections

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While infections caused by *Mycobacterium abscessus* complex (MABC) are rising worldwide, the current treatment of these infections delivers unsatisfactory cure rates despite long-term use of multidrug regimens. Absence of an optimal treatment regimen and the emergence of multi-drug resistance in clinical isolates necessitates the need for the discovery of better/new drugs to combat these infections. In this study, mycothione reductase (Mtr) was evaluated for its potential as a novel drug target to treat MABC infections since it is a key enzyme needed in the recycling of mycothiol, the main low-molecular-weight thiol protecting the bacteria against reactive oxygen species and other reactive intermediates. For this, a *MabΔmtr* mutant strain was generated, lacking *mtr* expression. First, the *in vitro* sensitivity of *MabΔmtr* to oxidative stress and antimycobacterial drugs was determined. Next, we evaluated the intramacrophage survival and the virulence of *MabΔmtr* in *Galleria mellonella* larvae. We showed that abrogation of *mtr* expression sensitizes the *MabΔmtr* mutant to oxidative stress and antimycobacterial drugs. *MabΔmtr* demonstrated a 39.5-fold reduction in IC90 when exposed to bedaquiline *in vitro*. Finally, the *MabΔmtr* mutant showed a decreased ability to proliferate inside macrophages and

larvae, suggesting that Mtr plays an important role during MABC infection and thus making it a potential target for drug discovery.

P045

Impact of ageing on the immunotherapy effect of mycobacteria against cancer

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Due to adverse events observed in non-muscle invasive bladder cancer (NMIBC) patients treated with *Mycobacterium bovis* BCG, alternative immunomodulators have been evaluated. *Mycobacterium brumae*, a safe mycobacterium with demonstrated *in vitro*, *ex vivo* and *in vivo* antitumor activity emerges as a promising candidate to replace BCG. Immunomodulator studies overlook older patients, despite age is associated with immunosenescence and the target population for NMIBC treatment is mainly men over 60 years old. Here we investigate the immunomodulatory capacity of BCG and *M. brumae* in peripheral blood mononuclear cells (PBMC) from healthy donors of various age groups together with NMIBC patients.

PBMC from young (18-35 years old) and older (60-75) healthy volunteers and NMBC patients (over 65) were stimulated with *M. brumae* or BCG. PBMC phenotyping was performed by flow cytometry. The direct and indirect cytotoxicity of mycobacteria-activated PBMC against bladder tumour cells and the release of a broad cytokines panel were analysed.

A significant age-dependent impact on T-cell differentiation profile was observed. The frequencies of activation-induced markers (CD69 and CD137) on $CD4^+$ and $CD8^+$ T cells stimulated with mycobacteria differed significantly between age groups and between healthy individuals and NMIBC patients. Diminished cytokine production (primary IL-8, IL-17, IFN-γ) in mycobacteria-activated PBMCs was observed from aged individuals, while cytotoxic activity against tumour cells were significantly reduced in NMIBC patients.

The functionality of immune system cells differs according to the intrinsic characteristics of the population group. This heterogeneity can influence the efficacy of activation by immunomodulatory mycobacteria, ultimately impacting antitumor capacity.

P046

Machine learning approaches for the analysis of electrical signals from Oxford Nanopore Technologies to detect pharmaco-resistance in *Mycobacterium tuberculosis*

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Contemporary diagnostic kits predominantly employ short-read analysis to identify drug resistance in *Mycobacterium tuberculosis* (*Mt*). Our study explores alternative methods analyzing

raw electrical signals (ES) from Nanopore Technology. This approach bypasses the base-calling step, enhancing efficiency by avoiding time-consuming processes and improving results' accuracy.

The study examined 104 *Mt* isolates using Nanopore sequencing after amplification employing Deeplex kit. These isolates underwent phenotypic testing against Rifampicin and Isoniazid. ES were extracted from fast5 files and different approaches have been tested to clean and optimize input size and noise-signal ratio. Two distinct neural network (NN) models were developed: the first aimed at identifying ES mapping on genes among the entirety of Nanopore-generated outputs, and the second designed to establish connections between ES from a sample and the observed phenotypes.

The former NN was developed to identify genes from all the reads. Various architectures, employing in different combinations Convolutional 1D, Long Short Term Memory, Fully Connected, Batch Normalization, and Dropout, were implemented. Despite these efforts, none of the models achieved an accuracy exceeding 65%. The second NN, a PointNet-like NN, aimed to identify resistance in the samples, and employed wavelets decomposition for signal cleaning, however, the highest accuracy attained for this task was 50% for Rifampicin and 70% for Isoniazid.

These findings underscore the challenges in accurately discerning genomic features and resistance patterns in *Mt* isolates using the implemented NN models. Further optimization and exploration of alternative methodologies may be crucial for enhancing classification performance in such complex datasets.

P047

Intrabacterial drug metabolism in *Mycobacterium tuberculosis*

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There is an urgent need for new and smarter drugs to shorten TB treatment and end TB. To design these drugs, a key factor is the interaction between the compound and target bacteria, with one aspect being the metabolism of compounds within Mycobacterium tuberculosis (Mtb). That is why this project aims to study intrabacterial drug metabolism (IBDM) in Mtb from two perspectives: first the main chemical changes and second, the involved pathways or genes responsible for this metabolism. Altogether, this knowledge will open up new avenues for developing smarter anti-TB drugs that circumvent intrabacterial drug metabolism or use it to their advantage.

Currently, we are screening known anti-TB compounds to evaluate drug metabolism in Mtb by UHPLC-MS. The first step is to evaluate depletion of the intact drug molecule. Next, for the compounds showing potential metabolism, we perform MS/MS and analyze the data with molecular networking to visualize the biotransformations and elucidate the structures of the metabolic products. With this project we aim to fill the gap in metabolism studies for the ADME-profile of each compound in drug discovery and development which, at the moment, focuses solely on the patient and largely neglects target bacteria.

P048

Are there *blaC* alleles out there that would naturally confer resistance to clavulanic acid?

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The inherent resistance of *Mycobacterium tuberculosis* to β-lactams has meant that the use of these antibiotics to treat tuberculosis has historically not been of clinical significance. Recently BlaC, the chromosomally encoded β-lactamase of *M. tuberculosis* and the primary cause of this resistance, has been recognised as a potential therapeutic target for β-lactamase inhibitors such as clavulanic acid, thereby enabling the action of β-lactams. However, there is concern that resistance to β-lactamase inhibitors may rapidly arise. We have therefore investigated the natural genetic variation present in *blaC* in >75,000 genomes using the CRyPTIC dataset, a data compendium of *M. tuberculosis* samples with mutation and phylogenetic lineage information, and report our findings.

Machine learning models have the potential to predict antimicrobial resistance but require sufficiently large datasets with enough variation – we have therefore also investigated what genetic variation is required for a graph-based neural network (GNN) to begin to learn resistance in *blaC*. This was done by synthetically generating datasets of *M. tuberculosis* BlaC sequences with differing characteristics. Resistant mutations in the datasets were determined from previous *in vitro* studies that identified amino acid substitutions in *M. tuberculosis* BlaC that conferred resistance to clavulanic acid. The GNN used chemistry-based amino acid features and derived its network connectivity from the protein structures of BlaC samples, so that it could infer awareness of the structural and chemical changes arising from the mutations.

P049

The hidden diversity of *Mycobacterium tuberculosis* complex in Africa: the new L10 and the possible diversification histories of the complex

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Africa is home to all known lineages from *Mycobacterium tuberculosis* complex (MTBC) including *Mycobacterium canettii*. Based on the repeated isolation of *M. canettii* from the Horn of Africa, and the isolation of a recent new lineage with deep branching, L9, this region is usually considered as the cradle of tuberculosis disease. In turn, *M. africanum* is inferred to have diversified in western Africa. Yet how these two diversification regions may have connected is unknown.

Using the new TB-annotator database, grouping more than 102,000 SRAs, we identified a new lineage branching between L9 and chimpanzee bacilli. We call this new branch L10. It contained only two representatives in TB-annotator. L10 harbours typical features of *M. africanum* such as absence of RD7-10. L10 strains harbour several distinctive features including a large Region of Deletion encompassing toxin-antitoxin *vapB29/vapC29*, and synonymous SNPs including *gyrA*_G7901T, *recN*_C1920096T, and *dnaG*_C2621730T. The L10 strains also shared

the absence of spacers 7, 9 and 26-43 in standard spoligotyping nomenclature. This feature allowed the identification of a potential third representative of this lineage in SITVIT database.

Interestingly, the only three samples from L10 were all from or in connection with Congo Republic or Democratic Republic of Congo, in central Africa. We further explored the diversity of deeply branching MTBC isolates and their localisation. We discuss the actual diversity of MTBC in Africa and implication on its history of diversification.

P050

Comparative study of two deleted *Mycobacterium bovis* strains in experimental animal models

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Complementary tools such as vaccines could improve the control and eradication of bovine tuberculosis. Two attenuated strains, *M. bovis Δmce2* and *M. bovis Δmce2-phoP*, were inoculated subcutaneously in mice (60 and 90 days, $1{,}10^5$ CFU, n=24) and guinea pigs (178 days, $1{,}10^5$ CFU, n=12), and remained in biosafety facilities (Protocols: 2018/36-2021/33 CICUAL-FCV-UBA). Survival (days), pathology (granulomas), bacteriology and tissue-IS*6110*-PCR (lung, liver, lymph nodes and spleen) led to comprehend its virulence and safety. For the *M. bovis Δmce2* strain, survival was 100% in mice and 66.7% in guinea pigs. In mice, neither lesions nor bacterial isolation were recorded, but a 37.5% positivity for tissue-IS*6110*-PCR was observed. All guinea pigs presented granulomas, 50% positive for tissue-PCR and 66.7% for bacteriology. For the *M. bovis Δmce2-phoP* strain, 100% survival was observed in mice and guinea pigs, with no evidence of granulomas. No viable mycobacteria were recovered in mice, while 16.7% was observed in guinea pigs. However, tissue-IS*6110*-PCR positivity was confirmed in both cases (25% mice, 50% guinea pigs). A higher attenuation degree of *M. bovis Δmce2-phoP* compared to *M. bovis Δmce2* was confirmed, especially evident in a highly susceptible model such as guinea pigs, even when these strains were inoculated subcutaneously.

P052

Treating flies: *Drosophila melanogaster* for screening new therapies against tuberculosis

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Current increase of tuberculosis (TB) caused by COVID19 together with the rising cases of drug-resistant *Mycobacterium tuberculosis* (Mtb), highlights the need for new therapeutic approaches. We have used the *Drosophila melanogaster* (Dm) model for the evaluation of new candidates. Currently in the field there is the active TB experimental model based on the infection of *D. melanogaster* with *Mycobacterium marinum* (Mm). We present an effective treatment protocol in Dm against Mm. Flies were systematically infected with 50 Colony Forming Units (CFUs) of Mm and orally treated by mixing 50 or 500 µg/ml of rifampicin into the standard cornmeal at day 3 post-infection (p.i), when Mm infection is established in the fly, for 7 days. We monitored survival daily and bacillary load at days 0, 10 and 15 post p.i. Results at day 10 showed that 50 µg/ml turned to be suboptimal rifampicin concentration without any difference on survival curves and CFUs between treated and non-treated groups. Treatment with 500 µg/ml rifampicin improved significantly the survival curves and the reduction of the bacillary load. However, there was a bacillary regrowth at day 15, after finishing the treatment, which had not an immediate impact in the survival of the flies, suggesting a tolerance effect. In conclusion, we have established a suitable treatment protocol in the active TB model from a high-throughput screening perspective that can provide insights on the tolerance induced by new therapies. Currently we are adapting this protocol to the latent TB model through Dm infection with Mtb.

P054

Performance of open-access genomic drug resistance prediction tools for *Mycobacterium tuberculosis*: a systematic review and meta-analysis

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Whole-genome sequencing (WGS) accelerates and simplifies drug-susceptibility testing in *Mycobacterium tuberculosis* (Mtb). Open-access software tools have become available that offer sample-to-answer drug-susceptibility prediction. Their performance and clinical usability has not yet been summarized. We review and meta-analyse the performance of open-access prediction tools for Mtb drug-susceptibility prediction, compared to phenotypic testing as reference. We queried Medline, Embase and Web of Science databases on the 5th of July 2023. Publications that assess prediction performance of any of the following open-access tools were eligible: KvarQ, TGS-TB, CASTB, PhyResSE, ReSeqTB/ReSeqWHO, MTBseq, Mykrobe, TBProfiler,

GenTB, Resistance Sniffer, SAM-TB and MycoVarP. We appraised study quality using the QUADAS-2 tool, and extracted summary performance data per tool. We obtained pooled sensitivity and specificity estimates per tool and per tuberculostatic using a random-effects model. Pooled performance was assessed in 20 studies. Of examined tools, only Mykrobe, TBProfiler, MTBseq and GenTB had been maintained in the three years before the search date. Considering recent versions of these tools, pooled sensitivity generally reaches 90% for isoniazid and ethambutol, and 95% for rifampicin (with specificity reaching 98%). This fulfils criteria set by the WHO. For streptomycin, pyrazinamide, and second-line drugs, sensitivity generally ranges between 70-90%, with specificity approaching or reaching 98%. The performance of open-access drug-susceptibility prediction tools reaches WHO-set criteria for isoniazid, rifampicin and ethambutol, justifying their use as a resistance rule-in and rule-out test for these drugs. For second-line drugs, genomic prediction tools may be used as resistance rule-in tests.

P055

Two cases of *Mycobacterium microti* among primates in a zoo facility

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Mycobacterium (M.) microti, a slow-growing member of the *Mycobacterium tuberculosis* complex, has been found across diverse wildlife species, spanning rodents, carnivores, ungulates, primates, and others. Initially regarded as a pathogen primarily affecting small mammals, recent evidence suggests its potential as an emerging zoonotic threat, with sporadic human infections reported. A study in South Africa even identified *M. microti* as the causative agent in 1.9% of local human tuberculosis cases.

We report two cases of *M. microti* infections in captive primates at a zoo: one in a tamarin (*Saguinus spp.*) and the other in a lemur (*Lemur catta*). Diagnosis initially relied on positive acid-fast staining and PCR results for Mycobacterium tuberculosis complex. Subsequent culturing confirmed *M. microti* in the tamarin case, even though the culture was contaminated. The primary sample identification was consistent with *M. microti* for the lemur, the culture is still ongoing.

Despite confirming infection, culturing *M. microti* from clinical specimens poses significant challenges due to its slow growth rate, necessitating prolonged incubation and specialized media, with a risk of overgrowth by other microorganisms. While culturing was achieved in the tamarin case, efforts continue for the lemur. These challenges emphasize the importance of molecular techniques for swift and accurate diagnosis and the necessity for specialized culture methods. In addition, they underscore the critical need for comprehensive infection control measures and heightened awareness among zoo staff and veterinarians to safeguard both animal and human health.

P056

The effect of *inhA* promoter mutation on iron distribution in *Mycobacterium tuberculosis*

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The *Mycobacterium tuberculosis inhA* operon encodes two mycolic acid synthesis genes, as well as *hemZ*, which encodes a ferrochelatase responsible for incorporating iron into heme. Mutations in the promoter region cause upregulation of this operon resulting in resistance to anti-tuberculosis drugs that target the mycobacterial cell wall. However, the role of *hemZ* as part of the operon is unknown. We hypothesize differential iron availability in response to upregulation of *hemZ*.

We introduced an *inhA* C-15T mutation into the wild-type *M. tuberculosis* H37Rv strain by homologous recombination. The genomic integrity was verified by Illumina whole genome sequencing (WGS); the transcriptional effect of the mutation was determined by RNAseq and FeRhoNox was used to visualize ferrous iron present within cells by super-resolution fluorescence microscopy.

WGS confirmed that two mutant clones were identical and had no off-target changes. RNAseq demonstrated that the only significant transcriptional effect of the mutation was upregulation of *mabA*, *inhA* and *hemZ*. FeRhoNox analysis revealed that absolute iron concentration in mutant strains was higher in long cells vs. short cells while the opposite was true in the wild-type. This was interesting considering wild-type cells were on average longer compared to mutant cells. Short mutant cells had the highest iron concentration at the poles, whereas it was equally distributed in the wild-type and long mutant cells.

Our results demonstrate that the distribution of ferrous iron in mycobacterial cells is not uniform. Further, the *inhA* C-15T mutation affects iron distribution, which may play a role in cell division.

P057

Oral administration of heat-killed *Mycobacterium manresensis* induces an increased response against subsequent infections in the *Drosophila melanogaster* model

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Drosophila melanogaster has shown to be a good experimental model to study tuberculosis (TB) using *Mycobacterium marinum* infection, since flies have homology in 75 % of disease-causing

genes in humans and a complex immune system that relies solely on innate immune responses. *Mycobacterium manresensis* is an environmental mycobacteria that has previously demonstrated high pathogenicity in *D. melanogaster*. This study explored the hypothesis that oral administration of low doses of *M. manresensis* induces the long-term reprogramming of innate immune cells, leading to the establishment of the trained immunity phenomenon. This premise was tested by systemically infecting flies with pathogenic *M. marinum*, *Staphylococcus aureus*, and *Candida albicans* after 24- and 48-hour oral treatments with heat-killed *M. manresensis* (hkMm). Gene expression analysis exhibited that the mere presence of the environmental mycobacteria enhanced the flies' immune response 72 hours after the end of the treatment regimens, leading to an increased response when encountering subsequent challenges. Pathogen load analysis revealed that treatment administration induces a significant reduction in pathogen load in the *S. aureus* and *C. albicans* infection groups in a dose- and sex-dependent manner, indicating a possible increase in resistance to infection in treated flies. Conversely, pathogen load increased in *M. marinum* infection groups, suggesting an increase in tolerance in treated flies since higher CFU counts did not result in higher fly mortality. Hence, the provided data suggest that oral administration of hkMm provides unspecific protection against subsequent infections by modulating the innate immune response of the host.

P058

Epidemiology, genetic diversity and drug susceptibility patterns by whole genome sequencing of *Mycobacterium tuberculosis* complex isolates in Gabon from 2012 to 2022

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Gabon ranks amongst the high tuberculosis (TB) burden countries with more than 700 new cases of rifampicin-resistant (RR)/multidrug-resistant (MDR) TB cases emerging every year. This study aimed to investigate drug resistance profiles of *Mycobacterium tuberculosis* complex (Mtbc) strains isolated from suspected TB patients in Gabon (2012-2022).

Whole Genome Sequencing (WGS) was used for phylogenetic classification, resistance prediction, and cluster analysis (5 SNPs threshold) of 430 Mtbc strains.
Strains of the four Mtbc lineages L4 (n=372; 65%), L5 (n=46; 11%), L2 (n=6; 1,4%), and L6 (n=3; 0,7%) were observed. Interestingly, more than 10% of the strains are represented by *M. africanum* strains (L5 and L6). The proportion of resistance (any resistance to first-line tuberculosis drug) was 30%, with 12% (n = 52) being at least MDR, and six showing additional Fluorochinolone resistance. The overall cluster rate was 64%, which was the same in non-MDR and MDR Mtbc strains. However, the clustered MDR Mtbc strains fall into two large outbreaks, one belonging to the sublineage L4.1.2.1 (n=27) and one to the sublineage L2.2.1 (n=6). Strains belonging to the largest Haarlem MDR outbreak were sampled throughout the whole 10-year study period.

In conclusion, our study provides the first insights into genetic structure of Mtbc strain populations in Gabon. Although the majority of cases are caused by L4 Mtbc strains, *M. africanum* strains contribute also to the TB epidemiology in Gabon. The MDR epidemic is mainly driven by one dominant L4 clone that has been spreading for about a century in the country.

P059

Whole genome sequencing analysis reveals interregional transmission dynamics of tuberculosis in Spain: A study of Catalonia and the Valencian community

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Whole genome sequencing (WGS) of Mycobacterium tuberculosis plays a crucial role in tuberculosis control, particularly in outbreak detection. In Spain, both Catalonia and the Valencian Community utilize WGS to assess tuberculosis transmission. They have observed a substantial rate of intracommunity transmission, with a majority of transmission cases identified through genomic analysis that were missed by corresponding contact studies. The sequencing effort included 2,150 isolates from the Valencian Community and 791 from Catalonia, representing about 70% of positive cultures reported to Public Health. By analyzing the data, we identified 61 mixed genomic clusters between the two regions, encompassing 375 cases. Some of these clusters indicated transmission dating back to 2014, with one cluster showing ongoing transmission since 2015. The largest clusters mostly include cases born in Spain, while among recent transmission groups, one of Moroccan origin stands out. These findings underscore the significant impact of recent transmission between Autonomous Communities, emphasizing the necessity of understanding local transmission dynamics for the formulation of effective tuberculosis control strategies. This research also sheds light on the importance of conducting supraregional tuberculosis surveillance and integrating genomic analysis as a complementary tool.

P060

Serratia sp. dominates the lung microbiome of patients with tuberculosis and non-tuberculous mycobacterial lung diseases

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Pathogenic mycobacteria, such as the *Mycobacterium tuberculosis* complex (Mtbc), and few non-tuberculous mycobacteria (NTMs) can cause severe chronic pulmonary infections. However, not all infected patients develop active disease. Yet, it is unclear whether certain key taxa in the lung microbiome play a role in the pathogenesis of tuberculosis (TB) and NTM lung disease (LD).

We employed 16S rRNA amplicon sequencing (V3-V4) to characterize the baseline microbiome in bronchoalveolar lavage fluid (BALF) from patients diagnosed with TB (n=23), NTM-LD (n=19), or non-infectious inflammatory disease (n=4) prior to the initiation of therapy. The analysis included the depletion of human cells, removal of extracellular DNA, implementation of a decontamination strategy, and exploratory whole-metagenome sequencing (WMS) of selected specimens.

The genera *Serratia* and unclassified *Yersiniaceae* dominated the lung microbiome of all patients with a mean relative abundance of >15% and >70%, respectively. However, at the sub-genus level, as determined by amplicon sequence variants (ASVs), TB-patients exhibited increased community diversity, and TB specific ASV_7 (unclassified *Yersiniaceae*), and ASV_21 (*Serratia*) signatures. Exploratory analysis by WMS and ASV similarity analysis suggested the presence of *Serratia liquefaciens*, *Serratia grimesii*, *Serratia myotis* and/or *Serratia quinivorans* in both TB and NTM-LD patients. Overall, presence/absence of certain *Serratia* ASVs was significantly associated with disease state.

The lung microbiome of TB-patients harbors a distinct, and heterogenous microbiome structure with specific occurrences of certain *Serratia* traits. *Serratia sp.* plays a pivotal role in our understanding of microbial interactions in the lung microbiome of patients infected with Mtbc.

P061

Diversity of nontuberculous mycobacteria in food animals: implications for food safety and one health

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Beyond tuberculosis, leprosy and Buruli ulcer which are well known mycobacterial diseases of public health importance, lurks a myriad of infections caused by atypical mycobacteria commonly referred to as nontuberculous mycobacteria (NTM). NTM are ubiquitous in the environment hence their capacity to cause infections in different hosts via contact, inhalation, or ingestion. In 2014, speciation of mycobacterial isolates from a human population-based nationwide TB prevalence survey in Ghana, revealed that more than 50% were NTM, with *Mycobacterium fortuitum* being the most frequent (21.4%), however published records on NTM infections in animals are still scarce. The objective of this study was to describe the diversity of NTM circulating in food animals in Ghana and the implications for food safety and one health. Overall, 75 NTM isolates were obtained from culture of tissues from 50 cattle, 118 chicken and 516 cultivated fish (catfish and Tilapia) collected between 2019- 2022. Using the Line Probe assay (GenoType Mycobacterium CM/AS), mycobacteria 16S rRNA gene amplification and sequencing, isolates belonged to 20 species were identified. *M. fortuitum* was the most abundant species (18/41, 43.9%) isolated from cattle showing tuberculous-like lesions. In fish, *M. fortuitum* was the most predominant species (12/33, 37%). In poultry only two isolates were recovered belonging to *Mycobacterium mageritense*. These findings suggest that a high diversity of NTM circulates among food producing animals with *M. fortuitum* being a dominant NTM across species. Studies on transmission dynamics are required to unravel the zoonotic potential of NTM and intervention strategies using a one health approach.

P062

A 3D-printed device for reproducible imaging of 96-well broth microdilution plates

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Broth microdilution (BMD) assays are gaining entrance for drug-susceptibility testing in *Mycobacterium tuberculosis*. Interpretation of these assays has limited reproducibility between observers, especially for ambiguous phenotypes (e.g. pinpoint growth). To support reproducible MIC determination, we created a 3D-printed photographic device with a tray for generic 96-well plates. It houses a single-board Linux computer, two cameras (one for each plate halve), and light sources for tangential and trans-illumination. We developed a graphical user interface for image acquisition and minimal inhibitory concentration determination (MIC). Compared to the Thermo Fisher Vizion, a proprietary system for read-out of Sensititre BMD assays, our device has several assets. It has a four times higher pixel density, allowing differentiation of smaller colony features. It provides both darkfield and brightfield images in a single acquisition cycle, while Vizion is limited to either of both. Using a semi-transparent mirror, our device enables manual plate

inspection in addition to imaging, both in standardised lighting conditions. At 400 euros, our device costs a fraction of the price of Vizion. Compared to Vizion, our device suffers more from perspective artifacts in U-bottom plates with a limited volume (50 µL), and from lid condensation artifacts. Our device's performance is yet to be compared to the BIOMIC V3 (Giles Scientific), another recently introduced commercial plate imager. A comparative study of MIC accuracy and reproducibility, using manual plate read-out as a reference, is ongoing. Future iterations of this device will focus on improving perspective and lighting artifacts, as well as implementing automated MIC determination algorithms.

P063

Practical implementation of the WHO 2nd edition mutation catalogue in genotypic drug resistance detection pipelines

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In November 2023 the World Health Organisation released an updated catalogue of mutations found in *M. tuberculosis* clinical isolates and their association to phenotypic drug resistance to enable the use of genome sequencing for the prediction of resistance. This catalogue was published as a combination of Microsoft Excel document, VCF positions file and report (PDF) which included some additional rules, notably a series of epigenetic rules. Here we describe the process to implement this mutation catalogue within two bioinformatic pipelines, TB-Profiler and GPAS.

Firstly, we describe how straightforward it was to parse the various and combined formats provided by the WHO, including implementing additional specific additional grading rules before integrating the catalogue into our pipelines. We note some practical considerations that were not described by the WHO report, such what to do when a mutation in a tier 1 gene not seen in the catalogue is detected. Finally, we describe the development of a genetically-diverse dataset which will aid developers of bioinformatic tools to implement this and future catalogues. The test dataset was developed using publicly available sequences from clinical isolates to cover commonly seen resistance patterns as well as simulated data to cover edge cases that may not yet have been observed yet. We hope this dataset can be utilised by the community to ensure new versions of the catalogue are rapidly integrated into existing pipelines as well as expediting the testing phase of new prediction tools.

P064

Population structure and transmission analysis of drug-resistant *Mycobacterium tuberculosis*' complex strains from Namibia based on whole genome sequencing

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Namibia, characterized as a high tuberculosis (TB) burden country by the World Health Organization (WHO), faces significant challenges in combating TB, including the emergence of drug-resistant *Mycobacterium tuberculosis* complex (Mtbc) strains. Here we investigate the population structure, and transmission dynamics of drug resistant TB (DR-TB) in Namibia. Whole genome sequencing (WGS) was performed on 525 Mtbc strains collected between 2016 to 2023 across the country. Phylogenetic strain classifications, genomic resistance predictions, and core-genome multi-locus sequence typing analysis using SeqSphere (Ridom GmbH, Münster, Germany). Cluster analysis was done using a threshold of 12 alleles. Overall, most Mtbc strains were classified as belonging to the lineage 4 (L4; n=508), 15 belonged to the lineage 2 (L2, Beijing) and the two-remaining belonged to L1 and L3. Following the new WHO definitions, 353 out of 416 multi-drug resistant (84.9%) Mtbc strains were grouped into 56 clusters ranging from 2 to 78 isolates. Twenty five out of 28 pre-extensively drug resistant strains (pre-XDR) (89.3%) were distributed among 9 clusters and 9 XDR (100%) strains were grouped into 4 clusters. All strains of the two largest clusters belonged to the LAM lineage (L4 sublineage). The analysis indicates that transmission of drug resistant Mtbc strains is an important component of the DR-TB epidemiology in Namibia, and that further investigations should be conducted to gain a better understanding of the factors contributing to transmission. Understanding all factors involved will enable the development of targeted TB control strategies, optimizing resource allocation and reducing the spread of DR-TB in Namibia.

P065

Integrating genomic surveillance for tuberculosis control in Catalonia

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Background

The rise in tuberculosis (TB) incidence and drug resistance due to the COVID-19 pandemic makes TB control a major Public Health issue. The integration of genomic surveillance of *Mycobacterium tuberculosis* complex (MTBC) strains into formal epidemiological surveillance activities allows the characterization of transmission clusters and the monitoring of genotypic resistances. We describe the first 1.5 years of genomic surveillance of TB in Catalonia.

Methods

Genomic surveillance was implemented within the TB Control Program in Catalonia in 2022. All cultured strains from a network of 43 laboratories were obtained for centralized WGS. Recent transmission clusters (≤5 SNPs) were identified by phylogenetic analysis, reported to the Public Health Authorities and compared to conventional contact tracing data.

Results

Sequencing and epidemiological data was combined for 816 of 1081 (75.5%) culture-positive notified cases (Jan. 2022-June 2023). Forty-four different lineages were detected, being L4.10 (20.3%) and L4.1.2 (16.3%) the most abundant. 61.4% of sequenced strains corresponded to cases in migrants. 33 (4.0%) cases were monoresistant (8 to INH, 1 to RIF, 4 to FQ, 20 to other). MDR cases accounted for 1.2% and pre-XDR for 0.1%. 76 transmission clusters were identified (clustering ratio 29.5%, 241/816), 9 (11.2%) of which were formed by resistant strains. Conventional contact tracing had identified at least one epidemiological link for 27.8% of clustered cases.

Conclusions

The implementation of genomic surveillance of MTBC strains has evidenced substantial local transmission and can help to detect and stop transmission chains and epidemic outbreaks previously unnoticed by traditional contact tracing strategies.

P066

Establishing a 3D cell culture model of the tuberculosis granuloma as a drug-discovery platform

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The tuberculosis (TB) granuloma is the hallmark cellular structure of latent TB. More comprehensive in vitro models that better resemble the complexity of the granuloma would facilitate the discovery of more active drugs in the granuloma environment and provide a better understanding of bacteria and immune cell interplay.

We aim to generate an in vitro, 3D cell culture model of the TB granuloma that can be easily implemented using readily available commercial reagents and materials. A commercial encapsulation system generated small spheres containing human peripheral blood mononuclear cells (PBMC) infected with GFP-expressing Mtb H37Rv. The results show that human PBMCs readily form 3D cellular aggregates around infected cells. The model could be maintained for several weeks before bacteria-induced cell necrosis. Using different cell types inside or outside the spheres resulted in distinct bacterial replication, demonstrating the contribution of each cell type and suggesting relevant communication between cells inside and outside the spheres to control the infection. The expression of inflammatory genes and the generation of reactive oxygen and nitrogen intermediates increased with the multiplicity of infection. Moreover, bacteria in the 3D model depict increased resistance to isoniazid and rifampicin and increased susceptibility to pyrazinamide when compared to the usual 2D cell culture model.

In conclusion, the 3D infection model resembles some of the structural features and drug-susceptibility profile expected in the TB granuloma and significantly improves the duration of infection experiments. This model shows promise for future use in drug discovery studies.

P067

Drug resistance patterns in pulmonary tuberculosis patients in Namibia based on targeted Next Generation Sequencing

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Drug-resistance tuberculosis (TB) is a major global health problem, and an early detection is important for preventing spread and further resistance acquisition. This study aimed to identify

mycobacterial genetic patterns causing resistance against both first- and second-line anti-TB drugs, in Namibia, a high TB burden country. A total of 185 clinical *M. tuberculosis* strains with rifampicin resistance (RR) detected by the Xpert® MTB/RIF were analysed. Targeted Next-Generation Sequencing (tNGS) was performed locally using the Deeplex® Myc-TB kit on an Illumina iSeq100 platform. Data were analysed on the Deeplex Myc-TB web application. Overall, 69% of the RR isolates were further classified as multi-drug resistant (MDR) TB, (3.8%) as extensively drug resistant (XDR) TB and (1.6%) as pre-XDR-TB as per WHO 2021 definitions. Different rates of resistance were observed to isoniazid (83%), pyrazinamide (46%), ethambutol (49%), streptomycin (29%), fluoroquinolone (5%), bedaquiline (3%) and clofazimine (3%). The most prominent mutations conferring resistance to rifampicin were detected in *rpoB* gene, which are S450L (48%), L430P (11%) and L430P|H445Q (9%). For isoniazid resistance, the mutations *katG* S315T (64%) and *inhA* C-15T (31%) predominated. The most frequent resistance mutations to pyrazinamide, ethambutol and streptomycin observed were *pncA* L35P (17%), *embB* M306I (27%) and M306V (12%) and *rpsL* K43R (28%), respectively. This study confirms that most rifampicin resistant strains are also isoniazid resistant. Resistance to fluoroquinolone and emerging resistance to bedaquiline are a major concern with implications for patient outcomes and warranting investment in surveillance for bedaquiline in all RR-TB patients.

P068

Exploring the adaptative landscape and factors impacting the epidemic success of *Mycobacterium tuberculosis* lineage 2 using simulations and genomic analyses

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Mycobacterium tuberculosis (M.tb) lineage 2, known as the Beijing lineage, stands out for its concerning contribution to the global burden of tuberculosis (TB). Compared to other lineages, it exhibits heightened virulence, drug resistance. To understand the factors propelling the epidemic success of M.tb lineage 2, this study employs a multifaceted approach that combines forward-time genetic simulation, genomic, and epidemiological analyses. Initially, we sought to detect signals of possible mutation rate acceleration between lineage 2 and lineage 4 strains, based on the mutational parameters described by Ford et al. (2015). Using predictive simulations with SLIM and replicating demographic parameters close to recent outbreaks in both lineages, we assessed the ability of Bayesian phylogenomic methods, in particular using the BEAST algorithm, to discern potential rate accelerations during outbreaks, while accounting for demographic influences. By comparing lineages using Bayesian analyses of genomic data from recent epidemics, we demonstrated that the Beijing lineage has significantly higher mutation rates than the L4 lineage. Our subsequent objective was to disentangle the interaction of genetic background and socio-economic factors on the epidemic success of *Mycobacterium tuberculosis* complex (MTBC) strains, using the time-scaled haplotypic density (THD) index. This index was calculated on a comprehensive collection of 12,289 whole *Mycobacterium tuberculosis* genomes from various geographical regions and was compared on a global scale, as well as for lineage 2 only.

P069

Nationwide temporal whole-genome sequencing analysis reveals high rates of clustering among multi-drug resistant *Mycobacterium tuberculosis* strains in South Africa

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South Africa (SA) has the highest per capita rate of multidrug-resistant (MDR) tuberculosis globally. We performed a retrospective temporal analysis of 2,407 whole-genome sequences (WGS) from clinical *Mycobacterium tuberculosis* isolates collected nationally across SA with culture-based drug-susceptibility data for at least one drug. We constructed lineage-specific phylogenies and assessed clustering based on node density and pairwise SNP distance in different drug resistance (DR) groups. Additionally, we used Bayesian molecular dating in BEAST 1.10.4 to estimate the ages of 1,392 most recent common susceptible ancestors across 10,347 instances of resistance to fourteen drugs. Of the 2407 isolates, 1,144 (48.9%) were resistant to rifampicin, and 1,028 (42.7%) were MDR; 782 (32.5%) belonged to 102 clusters with average pairwise distance <25 SNPs. The median ancestral age of these clusters was 34.5 years (IQR 21.6-46.6). Cluster membership was significantly associated with DR across all drugs and DR groups (p-values from 5.23e-04 to 3.08e-195). In simulations, the association between clustering and DR was unlikely to be observed because of random oversampling of DR in our dataset (p<0.0001). The number of independent resistance acquisition events was lower than the number of resistant isolates, both within and outside clusters, indicating ongoing transmission of drug resistance. Our findings suggest that the epidemic of MDR in SA is significantly driven by clonal and persistent transmission of highly fit primary resistant isolates, highlighting the need for enhanced surveillance to control the spread of transmissible MDR strains.

P070

Understanding pathways to chronic disease management for patients with tuberculosis in Haiti: A qualitative study

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Despite the increase in access to tuberculosis treatment globally, patients who have successfully been treated for tuberculosis remain at higher risk of mortality compared with the general population. Multimorbidity with non-communicable diseases (NCD) has been hypothesized to contribute to worsened TB outcomes.

This study is a qualitative study that investigated clinicians' and patients' perspectives on pathways to chronic disease management for patients with TB in a rural hospital network in Haiti. We enrolled twelve clinicians and three patients for semi-structured interviews.

The qualitative analysis found that while a formal pathway to chronic disease management existed, clinicians worked to devise informal workaround to get patients better access to care. Clinicians emphasized the promise of more integrated TB and NCD programs, while also recognizing the potential workload challenges this integration could create.

Health systems must be built to address the synergistic relationship between TB and NCD care together as essential components of universal health care. To truly end TB, health systems must establish supportive, integrated connections between NCD and TB programs to improve patient outcomes without overburdening clinical staff.

P071

TB Portals and the Quest for the Universal Reference Dataset

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The TB Portals database stands at the forefront of tuberculosis (TB) research, offering an invaluable reference dataset, useful for research, education, and clinical studies.

Broad and Deep coverage of clinical cases: TB Portals encompasses a wide array of annotations, including socioeconomic data, clinical treatment and outcomes, imaging (X-rays and CT scans), and genomic sequences.

Customizable data queries to enhance clinical studies: The TB Portals platform allows for tailored data queries, enabling researchers to extract specific datasets relevant to their study objectives, thereby enhancing study design and hypothesis testing.

Hard-to-find data from multiple countries: The database serves as an essential benchmark for developing and testing ML and AI algorithms in TB diagnosis and treatment. TB Portals offer less biased and more diverse dataset, coming from multiple hospitals in 19 countries with heavy DR-TB burden.

Accelerating drug and vaccine development: Access to TB Portals' comprehensive genomics data may help in creating new TB drugs and vaccines by enabling more nuanced analyses of pathogen's evolutionary blueprint against the response to variety of drugs, while taking into account efficiencies of existing genotypic and phenotypic DSTs.

We will present our analysis of TB Portals cohorts, specifically selected to highlight importance of comorbidities (HIV, anemia, diabetes), genomic lineages of M. tuberculosis, and incidence of lesions in lungs. With almost 14,000 clinical cases, it is now possible to perform statistical analysis and machine learning, and enhance many clinical studies with TB Portals data.

P072

Equivalent MIC results are observed by EUCAST BMD and MGIT for levofloxacin, moxifloxacin, D-cycloserine and linezolid

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The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has released a broth microdilution (BMD) Minimum Inhibitory Concentration (MIC) method to be used as a reference against which to calibrate drug susceptibility testing (DST) methods for Mycobacterium tuberculosis complex organisms (MTBC). The BACTEC™ MGIT™ (Mycobacterial Growth Indicator Tube) Automated Mycobacterial Detection System is the gold standard for liquid culture DST of MTBC. In this study the MIC results obtained using the EUCAST BMD reference method were compared to results obtained on the MGIT system for other antituberculosis antibiotics such as bedaquiline (BDQ), levofloxacin (LVX), moxifloxacin (D- cycloserine (DCS) and linezolid (LZD). Sets of MTBC strains known to be resistant and susceptible to each antibiotic by genotypic and/or phenotypic testing were used for this comparison. Essential agreement percentages (EA) between techniques were 100.0, 90.9 and 100.0% for LVX, DCS and LZD respectively, indicating that the two methods provide equivalent results for these antibiotics. However, for BDQ and MFX the EA were of 23.1 and 80.1% respectively. After using correction factors of ~3 and one two-fold dilutions for BDQ and MFX, the EA increased to 92.3% and 100.0% respectively.
In conclusion, the MICs obtained by EUCAST BMD and MGIT system were equivalent for all the antibiotics tested after applying a correction factor if needed. The results described here underscore the importance of understanding the performance of the DST technique per antibiotic tested and how it correlates to a reference method.

P073

UShER for *Mycobacterium tuberculosis*: an evaluation of pandemic-scale tools capacity to perform transmission surveillance

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Whole-Genome Sequencing (WGS) of *Mycobacterium tuberculosis* (MTB) is increasingly standard in health services. The growing data amount allows the reconstruction of phylogenies with a broader genomic context, providing a higher resolution for transmission dynamics but also escalating computational demands. UShER revolutionized SARS-CoV-2 phylogenomics by using sample placement to reconstruct massive phylogenies, and it's been now extended to MTB. We tested its capacity to reconstruct large phylogenies and place new sequences to transmission clusters. To evaluate the robustness of UShER, we first identified transmission clusters in the Valencian Region, Spain, using a population-based dataset collected between 2014-2019. To

recover transmission clusters, we built a regional phylogeny (N=1455), and another including global strains (N=39676), and applied a 10 SNPs threshold. To test the accuracy of USheR to assign strains to their corresponding transmission clusters we removed samples collected in the Valencian Region between 2017-2019 (N=729) from the phylogenies and used phylogenetic placement to reincorporate them in the phylogenies. 212 samples in cluster were placed in the regional phylogeny. 188/212 (88.67%) fell in the correct cluster; 12/212 (5.66%) fell in the cluster neighborhood and 11/212 (5.18%) were misplaced. In the global dataset phylogeny, 209 samples in cluster were placed. 167/209 (79.9%) fell in the correct cluster; 30/209 (14.35%) fell near their cluster and 12/209 (5.74%) were misplaced. UShER places new sequences with high accuracy regardless of the size of the phylogeny, providing a reliable tool for transmission surveillance.

P074

Detection of *Mycobacterium avium* subsp *paratuberculosis* in fecal samples by culture in dairy farms in Panama

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Bovine paratuberculosis poses an economic challenge to dairy farms worldwide. *Mycobacterium avium subsp. paratuberculosis* (MAP) has been identified as an etiological agent. We aimed to evaluate the performance of liquid and solid culture media for isolating MAP from infected animals. We collected stool samples from 14 dairy farms in Chiriquí, Panama. Samples were analyzed using acid-fast bacilli staining; liquid media M7H9C and A7H9J; solid media HEYM-PS, 7H11-M, and 7H9-OP; and confirmation with specific PCR targeting IS900R, F57, ISMAv-2, and Locus 255. We evaluated livestock management practices using a standardized questionnaire. Results show that liquid medium M7H9C yielded a higher bacterial load than A7H9J at sixth week of incubation (1.3 vs. 0.2 clumps/field; $p < 0.05$). Solid medium 7H9-OP yielded a higher bacterial growth than HEYM-PS and 7H11-M starting from the third week of incubation. When using the M7H9C liquid medium, we identified 5.5% (25/452) of animals with acid-fast bacilli (15.9 clumps/field average). Endpoint PCR confirmed MAP in 20% (5/25). Solid medium 7H9-OP identified 1.1% (5/452) animals with acid-fast bacilli (1 CFU each); PCR confirmed MAP in 60% (3/5). A 60% (3/5) of culture-positive farms employed animal purchase as a replacement method, and 40% (2/5) knew their health status. Additionally, 60% (3/5) of the farms housed calves with their mothers after calving. Our findings highlight M7H9C and 7H9-OP media's effectiveness in isolating MAP and the relationship of culture-positive farms with poor management practices. We recommend using culture to aid control measures in the dairy industry and tracking the spread of paratuberculosis.

P075

Genotyping of *Mycobacterium leprae* in humans and armadillos in a highly endemic area for leprosy in Brazil

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Mossoro is a city in Rio Grande do Norte State (RN) in Northeast Brazil, hyperendemic for leprosy and known for hunting armadillos and consuming their meat. In our earlier study, we demonstrated high anti-PGL-1 and PCR positivity for *M. leprae* specific sequences in 20 *Euphractus sexcinctus* (six-banded armadillos), living in rural areas surrounding Mossoró/RN (doi: 10.1016/j.cimid.2019.101397). We now show data on VNTR-based genotyping on liver or spleen samples from these animals and although we could not obtain complete 16 VNTR-based genotypes for all animals, seven genotypes were considered for cluster analysis for having at least 9/10 informative alleles. Although we were unable to generate their SNP type, their combined 27-5/12-5 indicated SNP-type 4. All VNTRs observed in animal isolates presented the same copy number so we considered seven animals as belonging to a single cluster, indicating high transmissibility between animals. To compare *M. leprae* genotypes to those from human leprosy patients, we studied slit skin smear (SSS) samples from 54 multibacillary cases, residents of Mossoró. SNP typing could be performed for 32 patients with SNP type 4 being dominant (72%). Besides, 35 patients rendered genotypes with at least 10 amplified VNTRs and were considered for cluster analysis. We observed a single cluster of eight strains but no clustering of human and animal genotypes was observed, suggesting lack of transmission between the two species. The main limitation of this study was that sampling occurred in different ecological niches and we have no information about invasion of humans of this armadillo territory.

P076

From research to surveillance: Leveraging the state of whole genome sequencing for precision public health in a high-burden tuberculosis setting

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Tuberculosis (TB) continues to pose a significant public health challenge in 30 high-burden TB countries, including Brazil. Whole-genome sequencing (WGS) offers promising prospects in the realm of precision public health. By facilitating the pinpointing of individuals involved in recent TB transmission chains, WGS emerges as a valuable asset for TB surveillance. Through a literature review, we aimed to assess all *Mycobacterium tuberculosis* complex genomes isolated in Brazil that are publicly accessible to evaluate TB transmission and drug resistance. We employed the MAGMA pipeline utilizing two distinct cut-off analyses involving 5 and 12 Single Nucleotide Polymorphisms (SNPs). Drug resistance prefiling (DRP) was obtained using TB-Profiler v.6.2.0. From 23 studies, we obtained 2182 FASTQ paired-end files, of which 65 failed quality control. Of the 2118 genomes analysed, 1340 were identified within 333 clusters using a 12 SNP cut-off, while 789 genomes clustered into 286 clusters with a 5 SNP cut-off. Based on 12 SNPs, 21 clusters harboured strains from different states and regions: Southeast-Southeast (5), Southeast-South (5), South-Mideast (5), Southeast-Midwest (4), South-South (1), and Southeast-Southeast-Midwest (1). This suggests transmission chains across these Brazilian regions. Lineage 4 (1994/94.15%) was the most frequent. The DRP was: pan-susceptible (1364/64.40%), multidrug-resistant (298/14.07%), mono-isoniazid (149/7.03%), pre-extensively resistant (76/3.59%), mono-rifampicin (46/2.17%) and others (185/8.73%). No resistance was detected to para-aminosalicylic acid, clofazimine, linezolid, bedaquiline, or delamanid. This is preliminary data that will be accessible for research and TB surveillance purposes in Brazil within the GEMIBRA (Genome of Mycobacteria in Brazil) web platform v.2 which is currently under development.

P077

TOAST - A Tool for the design of targeted gene amplicons for the application of high throughput sequencing in genomic studies of tuberculosis

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The targeting of *Mycobacterium tuberculosis* genes linked to drug resistance and strain-types through amplicon sequencing is a cost-effective approach to profiling infections and personalising clinical management of tuberculosis disease. However, such assays need to be updated with growing knowledge of loci and mutations involved in important phenotypes, including drug resistance and strain-types. Here we present a software tool called *TOAST* (Tuberculosis Optimized Amplicon Sequencing Tool), which optimises the design of amplicons across platform (e.g., Illumina and Oxford Nanopore Technology) and read length, drug resistance loci, the spoligotyping region; all informed by a growing database of >200K mutations across >100K *M. tuberculosis* strains. TOAST provides an optimal number and set of primers, across a user-specified amplicon size (based on sequencing platform), coverage, and selected genome-wide loci (e.g., genes). We have validated the outputted primers experimentally, and the tool can be readily modified for the design of amplicons for other important pathogens or species.

P078

Analyzing the cell wall lipids of *Bacillus Calmette Güerin* (BCG) strains and pathogenic strains of the complex of *Mycobacterium tuberculosis* isolated from humans

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Pathogenic Mycobacteria of the complex *Mycobacterium* tuberculosis *(MTb*) are the causative agents of human Tuberculosis. Current reports after the COVID-19 pandemic continue to show that pathogenic mycobacteria constitutes a health public problem that is worsened by the increasing multidrug-resistance strains (MDR), by the co-morbidities and by the lack of long-term memory of the actual and official approved vaccine BCG. Approximately 1.7 million deaths annually and morbidity of 10.6 million. While most of the infected individuals remain not symptomatic, a percentage (5%) develop active disease. During most of their life, only 5 % develop active disease. Molecular diagnostics based on real-time PCR, and whole genome sequencing have made significant improvements to early detection and specificity sensibility. Transcriptomics has contributed also in terms of the biomarkers of the course and progression of the disease. In previous work, we isolated *M. bovis/M tuberculosis* of exudate from humans and characterized the serological reactivity and genic expression profile with encouraging results. Furthermore, several studies have highlighted that cell wall lipids of pathogenic mycobacteria play a role as a factor of virulence and are recognized by the innate immune cells, resulting in effective connection with the adaptive immune system, especially those by B and T lymphocytes. Therefore, we pursue to characterize and update the status of BCG strains and utmost of pathogenic mycobacteria of the complex of *Mycobacterium tuberculosis*, in terms of the lipids profile using chromatographic techniques. We agree that lipids can be harnessed and targeted for the improvement of the BCG vaccine.

P079

Can emergence of canonical mechanisms of isoniazid resistance in tuberculosis be predicted?

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The emergence of drug resistance in *M. tuberculosis*, seems to follow an order. In most cases resistance to isoniazid (INH) emerges first. Prevention of emergence of INH resistance can potentially prevent the emergence of resistance to other drugs. Here we present the prognostic value of specific mutations in predicting emergence of INH resistance through canonical mutations (katG315, inhA-15, and inhA-8).

In our approach, we used genomic and phenotypic data from over 16,000 samples collected by two projects, the TB Portals and the CRyPTIC consortium. We evaluated the potential of prediction canonical INH resistance using each mutation and combination of two mutations associated with phenotypic resistance.

Our best performing model used a combination of two genomic markers with an estimated prognostic accuracy of 73%, sensitivity of 55%, specificity of 84%, PPV of 69%, and an NPV of 75% for correctly predicting the emergence of the three canonical INH resistance mutations. These results are high enough to be demonstrate the potential of the approach. The relatively lower sensitivity is partly a reflection of different evolutionary paths to the eventual emergence of the three canonical mutations. This exercise only evaluates the predictability of the evolutionary paths that involve the prognostic mutation(s) used for building the model. This work also presents evidence that resistance to INH follows a stepwise evolutionary trajectory which can be exploited for prediction and avoidance of INH resistance.

Additional time course samples and analysis will uncover prognostic markers for other evolutionary trajectories that lead to resistance.

P080

Predictive AI models for personalized prognosis of tuberculosis treatment

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Evolutionary trajectory of a pathogen partially depends on the immune response and drug pressure. This is also true for *M. tuberculosis*. The ability to predict this evolutionary trajectory, enables prognosis of the disease and potential course-change to avoid emergence of resistance. In this abstract we report a model that predicts the emergence of canonical isoniazid (INH) mechanisms of resistance (*katG*315, *inhA*-15, and *inhA*-8).

To develop such a predictive model, we extensively experimented with Logistic Regression (LR) and Deep Neural Models (DNMs) (multi-layered deep neural networks). All models were trained to takes an exhaustive combination of specific genotypes of the *M. tuberculosis* genomes (collected prior to the onset of resistance during the course of the treatment) as their input, and provide a prediction of the likelihood of emergence of one of the listed three canonical mutations. Data for training and testing of these models were downloaded from the CRyPTIC consortium and the TB Portals.

Our best performing model was a DNM with an estimated prognostic accuracy of 73% with a sensitivity of 55%, specificity of 84%, a PPV of 69%, and an NPV of 75% for correctly predicting the emergence of the three canonical INH resistance mutations. These results are high enough to demonstrate the potential of DNMs as a prognostic tool at least for TB. We expect that larger genotypic/phenotypic data sets allows the training of such models to achieve higher sensitivity, specificity, and prognostic accuracy.

P081

Xpert MTB/XDR assay: Rapid TB drug resistance detection

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We aimed to evaluate the effectiveness of the Xpert MTB/XDR assay in rapidly detecting resistance to isoniazid, fluoroquinolones, ethionamide, and second-line injectable drugs among tuberculosis (TB) patients. Between August 2020 and July 2021, we enrolled samples from TB suspected patients at a tertiary care center for our investigation. Our study involved conducting mycobacterial culture and phenotypic drug susceptibility testing (DST) using the proportion method in liquid culture at WHO-recommended concentrations, alongside the Line Probe Assay. Concurrently, we performed the Index test, Xpert MTB/XDR, following the manufacturer's guidelines. Among 356 samples, 97 were excluded due to incomplete information. We found resistance to isoniazid, levofloxacin, and moxifloxacin in 45/251, 21/251, and 20/251 samples, respectively. The diagnostic accuracy of the Index test, with phenotypic DST as the reference standard, was 95.8%, 99.04%, and 99.05% for isoniazid, levofloxacin, and moxifloxacin, respectively. The Index test demonstrated a specificity of 99.1% for detecting second-line injectable drug resistance, resulting in a diagnostic accuracy of 99.2%. Compared to the line probe assays, the Index test showed improved sensitivity and specificity. Overall, the Index test showed promising results in identifying resistance to isoniazid and fluoroquinolones, outperforming the line probe assay, which could be crucial for promptly initiating treatment in cases of drug-resistant TB

P082

Susceptibility patterns of *Mycobacterium avium complex* isolates recovered in two Greek University Hospitals

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Objective. *Mycobacterium avium complex* (MAC) consists a group of mycobacterial pathogens that causes disease in susceptible hosts. In this study, we investigated the *in vitro* drug susceptibility patterns of individual MAC species in antimicrobial agents commonly used to treat MAC infections.

Materials/methods: We studied 119 *M. avium (MAV)* and 103 *M. intracellulare* (MIN) non-repetitive clinical isolates. The minimum inhibitory concentrations (MICs) for clarithromycin, amikacin, moxifloxacin, linezolid, rifampicin and rifabutin were determined with broth microdilution method using the commercial assay Sensititre™ SLOMYCOI according to the CLSI recommendations and interpreted based on CLSI breakpoints for clarithromycin (S ≤ 8 mg/l) and amikacin (S ≤ 16 mg/l) and tentative breakpoints for moxifloxacin (S ≤ 1 mg/l) and linezolid (S ≤ 8 mg/l).

Results. Overall, 208 MAC strains (93.6 %) were susceptible to clarithromycin while only 11 (9.2%) *MAV* and 3 (2.9%) *MIN* strains were resistant (MIC > 64 mg/l). For amikacin, moxifloxacin and linezolid the CLSI breakpoints split the wild-type populations for both species. Only 67.6% of *MAC* isolates were categorized as susceptible to amikacin and 5.4% of isolates were categorized as susceptible to moxifloxacin and linezolid. The rifampicin WT distribution for both species was truncated at the upper end (>8 mg/L) while for rifabutin, was instead truncated at the lower end (≤0.25 mg/L).

Conclusions. The *in vitro* drug susceptibility patterns of the different MAC species studied are comparable to each other. Except for clarithromycin, current breakpoints for MAC categorization should be reevaluated.

P083

Broth microdilution plate-based minimal inhibitory concentration testing using MGIT-positive cultures

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The current WHO-endorsed 96-well-plate-based broth microdilution testing (BMD) for minimum inhibitory concentration (MIC) testing of *Mycobacterium tuberculosis* complex (MTBc) relies on inoculum prepared from a solid-medium culture, which requires long incubation, limiting its use in clinical settings. We aimed to develop a protocol for BMD-MIC testing from freshly positive, actively growing MGIT cultures.

Bacterial suspensions were prepared by resuspending well-dispersed pellets obtained by centrifuging the contents of purity-confirmed, 3-5 days old positive MGIT cultures in sterile distilled water (SDW). The bacterial suspensions with optical density (OD) ≤McF0.5, were diluted 1:100 in supplemented 7H9 broth to be used as the inoculum for BMD-MIC testing while those with OD >McF0.5 were diluted in SDW to obtain an OD of Mcf0.5 before diluting 1:100 in 7H9. MICs obtained for moxifloxacin, levofloxacin, clofazimine, bedaquiline, linezolid, and delamanid using an MGIT inoculum were compared to the MICs obtained using a solid medium inoculum.

We tested 16 MTBc isolates, yielding 96 MICs: 10/16 MGIT inocula achieved an OD of McF0.5, the OD of the remaining six ranged between McF 0.38-0.47. All 16 isolates had interpretable MICs to all drugs tested with an average turnaround time of 16.5 days since MGIT positivity. The difference between the MICs obtained by the two methods was within the acceptable range for 90/96 MICs: zero for 33 MICs and +/-1 dilution for 67. Few (6/96 MICs) differed by 2 drug dilutions.

Our results suggest that standardized MGIT inocula yield comparable BMD-MICs to that of solid medium inoculum while reducing the turnaround time.

P084

Antimycobacterial activity of Bulgarian essential oils from *Rosa* species against reference and clinical *Mycobacterium tuberculosis* strains

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Based on the valuable biological properties of rose essential oils, the aim of this study was to evaluate the antimycobacterial activity of four Bulgarian oil-bearing roses: Rosa damascena Mill., R. alba L., R. centifolia L., and R. gallica L.. against *M. tuberculosis H37Rv* and *clinical Beijing and LAM genotype*. The chemical composition of the essential oils was determined by Gas chromatography (GC-FID/MS). Minimal inhibitory concentrations (MIC) were determined using the REMA method. R. alba oil showed the highest inhibitory activity against all M. tuberculosis strains with MIC in the range of 0.16-0.31 mg/ml, while R. gallica oil was the least active (MIC 0.62-1.25 mg/ml). The obtained results show heterogeneity of rose oil action on different mycobacterial strains. Strain Beijing 396 was relatively more susceptible to the rose oils probably due to multiple and likely deleterious mutations in its efflux pump genes. This study allows us to hypothesize that the combined level of geraniol and nerol is a key factor that underlies the antimycobacterial action of the Rose oils. Further investigations are needed to show a possible synergistic action of the new-generation anti-TB drugs and the most promising rose oil extract on the large panel of different strains. This study was supported by the Bulgarian National Science Fund (Grant KP-06-H41/3, 2020 and KP-06-H36/17, 2019).

P085

Reference-free clustering as an epidemiological tool for *Mycobacterium tuberculosis* strain typing

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Whole genome sequence (WGS) analysis of *M. tuberculosis* employing a 5-SNP cut-off is a robust tool for surveillance and detection of recent transmission events. This approach has shown many advantages in clinical and epidemiology studies. However, it requires significant computational resources, making it challenging to perform in many low-resource, high-incidence environments, where most tuberculosis cases occur. To address this problem, we explored reference-free tools for clustering genomes to make transmission tracking feasible in settings with limited computational resources.

We analysed a dataset of global clinical isolates from across the lineage diversity of *M. tuberculosis* and a local transmission dataset from Rwanda. We used PopPunk (Population Partitioning Using Nucleotide *k-mers*), Mash (Fast genome and metagenome distance estimation tool using MinHash) and SKA2 (Split *K-mers* Analysis), reference-free tools for population analysis and clustering genomes. We assessed each approach for accuracy in defining and recovering

strain types (e.g. lineages and sub-lineages) and compared genome distance distributions with the standard SNP distance matrices to find correlations.

Our analysis revealed that reference-free tools have the potential to detect new *M. tuberculosis* strains and transmission clusters. These tools can delineate *M. tuberculosis* lineages efficiently, though they are not all consistently accurate across all sub-lineages. SKA2 allows for lineage-defining split *k-mers* distance cut-offs. However, the accuracy of reference-free distances for detecting recent transmission clusters requires further investigation to fully explore the utility of *M. tuberculosis* molecular epidemiology. Such advancements could significantly enhance WGS analysis of all lineages of *M. tuberculosis*, particularly in low-resource environments.

P086

Diagnostic accuracy of the LiquidArray MTB-XDR VER1.0 for the detection of *Mycobacterium tuberculosis* complex and fluoroquinolone, amikacin, ethambutol, and linezolid resistance

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Rapid drug susceptibility testing (DST) for fluoroquinolones and linezolid is crucial to confirm eligibility for the new shorter regimens for rifampicin-resistant tuberculosis (TB). We assessed the diagnostic accuracy of Bruker/Hain Lifescience LiquidArray MTB-XDR (LA-XDR) for the detection of *Mycobacterium tuberculosis* complex (MTBC) and mutations associated with resistance to fluoroquinolones, linezolid, ethambutol and amikacin. For evaluation we used residual diagnostic specimens from people with presumptive TB in South Africa and well characterised drug-resistant specimens from the FIND Specimen Bank. Liquid culture was used as reference standard for MTBC detection, while phenotypic DST and Sanger sequencing were used as composite reference standard for resistance detection.

In total 720 specimens were available for the evaluation. LA-XDR showed an overall sensitivity of 85% (95% CI, 80-89) and specificity of 99% (95% CI, 98-100) for the detection of MTBC. In smear-negative specimens, sensitivity was 79% (95% CI, 71-85). For fluoroquinolone and ethambutol resistance detection, LA-XDR sensitivity was 94% (95% CI, 86-98) and 85% (95% CI, 75-91), respectively. Sensitivity for amikacin resistance detection was 55% (95% CI, 34-74), due to the inclusion of specimens in which resistance was conferred by *eis* promoter mutations, which are not included in the LA-XDR design. LA-XDR was able to detect linezolid resistance conferring mutations in 6/7 linezolid phenotypic-resistant cultured isolates.

LA-XDR met minimal WHO TPP criteria for the detection of MTBC and has the potential to provide rapid DST for two key second-line drugs, linezolid and fluoroquinolone, which may allow for rapid initiation of appropriate regimens to improve treatment outcomes.

P087

NGS for the identification of less frequently encountered mycobacteria species in a clinical laboratory

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Nontuberculous mycobacteria (NTM) are increasingly becoming a major cause of pulmonary and extrapulmonary infections. CTBL is a reference laboratory for identification of mycobacteria and unidentifiable species are frequently encountered in clinical samples. We assessed an in-house NGS workflow as a single assay for speciating mycobacteria unidentified by routine diagnostic methods. Clinical isolates (171 NTM, 5 *M. tuberculosis*), 1 NTM from heater-cooler water and 19 reference strains were sequenced using Illumina MiSeq. Majority of the NTM were unidentifiable or poorly identified by Vitek MS-RUO and line-probe assays; or had conflicting results. A CLC Genomics workflow program was used map the paired end reads to 4 in-house custom reference sequences made by concatenating 1) housekeeping genes: 16SrRNA-23SrRNA-*rpoB* and *hsp65*, 2) ITS, 3) *M. avium* complex IS and DT1 sequences and 4) *erm41* sequences for *M. abscessus* complex; and identified via in-house BLAST using reference sequences. Definitive identification was assigned if at least 2 genes met the match criteria (a combination of the identification score and difference from the 2nd match). Isolates that remained unidentifiable or with ambiguous results were further analysed by BLAST searches of the mapped reads on NCBI. The agreement with isolate morphology for conventional tests was 64.8 versus 80.1% for NGS, and 91.3% for NGS with additional NCBI BLAST. The raw concordance between NGS and conventional tests was 55.1%, which improved to 69.4% after including manual correlation with phenotypic tests and online BLAST. NGS is potentially a suitable single assay for the identification of mycobacteria in the clinical laboratory.

P088

Pre-treat or Not pre-treat? Evaluation of DNA extraction pre-treatment procedures for *Mycobacterium tuberculosis* whole genome sequencing of clinical primary MGIT cultures

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Contaminant DNA often remains present in *Mycobacterium tuberculosis* (*Mtb*) clinical primary MGIT cultures (CPCs) despite sputum decontamination and the use of PANTA™. To evaluate pre-

treatment procedures for whole genome sequencing (WGS), we generated triplicates of 5 mL aliquots of CPCs using pooled decontaminated *Mtb*-positive sputum sediments, which were cultured and heat-inactivated at 80°C. Pre-treatment procedures evaluated were: no pre-treatment; water, DNAse treatment with or without DNAse I enzymatic digestion (30 min, 1h or 2h); 1M-NaOH with and without heating at 65°C; and benzonase (30 min, 1h, or 2h). For all samples, DNA extraction was performed using InstaGene™Matrix/FastPrep. Total and *Mtb* (*Rv2341* qPCR) DNA quantity was assessed. Illumina WGS data was analysed by the MAGMA pipeline. No pre-treatment showed the highest median total DNA yield (51 ng/uL) and benzonase (30min) and the lowest (8.51 ng/uL). Benzonase (1h) showed the highest *Mtb* DNA yield (597859 copies/ng) and DNAse (30min) the lowest (178094 copies/ng). The average insert (313.7), mapped percentage (81.7%) and adjusted coverage (113.5) of WGS data obtained from samples without pre-treatment did not differ from that of pre-treated samples. No pre-treatment was the fastest method (35 min). Benzonase was the slowest method (>5h) and NaOH resulted in an unexpectedly long average insert size (±1400bp). The results obtained by these highly controlled experiments suggest that the increased time-to-result imposed by a pre-treatment step is not offset by decreased contamination of WGS data. Further research should confirm whether pre-treatment can be avoided without impacting the WGS data quality obtained from CPCs.

P089

Whole genome sequencing analysis of M. tuberculosis from direct specimens: a hybrid capture approach

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Targeted Next Generation Sequencing (tNGS) approaches efficiently interrogate M. tuberculosis drug resistance in respiratory samples, matching modern treatment regimens, but are limited by pre-selection of target genes and drugs. In addressing these issues, the Qiagen QIAseq xHYB assay on MTB is developed which employs a hybrid capture panel able to enrich the whole MTB genome sequence from indexed libraries.

Sixty-two DNA samples from sputum sediments, previously analysed with a tNGS-based Deeplex MycTB assay, were resequenced using the QIAseq xHYB library preparation assay on an Illumina Nextseq 500 instrument. Briefly, the genomic DNA was fragmented, amplified, and pooled for hybrid capture, then hybridized to M. tuberculosis probes, further amplified, and pooled for sequencing. Obtained Fastqs were analysed using the Kraken2 + Bracken for contamination check and MTBseq for variant calling adopting H37Rv genome as reference.

The analysis showed consistency between mean depth coverage of the QIAseq genome and Xpert MTB/RIF Ct values: 101.35x (Xpert High, Ct < 16, 35 samples), 32.92x (Medium, Ct 16-22, 21 samples), 7.28x (Low, Ct 22-28, 5 samples) and 21.34x (Very low, Ct > 28, 1 sample). The 70% of study samples achieved full coverage of genome breadth suitable for extended analyses such as strain relatedness. We also obtained 100% concordance in drug-resistance–associated target calls when compared with the Deeplex MycTB assay.

The Qiagen assay permits direct MTB sequencing without culturing delay and at full genome resolution. This salient aspect ensures a comprehensive and accurate detection of genome variants supporting outbreak investigation beyond drug resistance detection.

P090

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P091

The novel combination alpibectir/ethionamide (AlpE) is active on drug-sensitive and drug-resistant clinical isolates of Mycobacterium tuberculosis

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Tuberculosis (TB) is one of the world's top infectious disease killer. It is estimated that 10.6 million people developed TB in 2022, and despite being a preventable and curable disease, 1.3 million died from it. Over the last decades, treatments for TB have improved in efficacy, toxicity and length duration with the WHO new recommendation for drug-susceptible TB being a 4-drug cocktail (isoniazid (INH), rifapentine, moxifloxacin and pyrazinamide) for 4 months. Meanwhile, antimicrobial resistance (AMR) is increasing, and it is estimated that, in 2022, 410 000 people developed MDR (multidrug-resistant)/RR (rifampicin-resistant)-TB. The current treatment for

MDR/RR-TB is a combination of bedaquiline, pretomanid, linezolid and moxifloxacin (BPaL±M, 6 months) but many patients developed severe adverse effects (AEs) associated to the long-term administration of linezolid, reinforcing the need for new medications.

Developed by BioVersys, GSK, The Pasteur Institute Lille and Lille University, the clinical candidate alpibectir is a new chemical potentiator of ethionamide (Eto), a pro-drug used in MDR-TB for decades. Alpibectir enhances the activation of Eto in its active form allowing for a safer and better tolerated dose of Eto.

AlpE, the combination of alpibectir and Eto, is rapidly bactericidal, active on drug-susceptible and MDR-TB isolates. AlpE keeps robust activity against Eto-resistant, INH-resistant and MDR strains. AlpE is also active on bedaquiline and pretomanid-resistant strains. AlpE emerges as a promising candidate for an essential companion drug to treat INH mono-resistant and MDR-TB patients.

P093

Evaluation of a new molecular diagnostic tool for the identification of NTM/MTB in positive MIGIT cultures

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To better control the spread of tuberculosis (TB) and *Mycobacterium tuberculosis complex* (MTB), WHO has recommended rapid molecular tests as the initial diagnostic step for tuberculosis. In recent years, the spreading of non-tuberculous mycobacteria (NTM) has also increased. In countries with low tuberculosis incidence such as Italy, the rapid differentiation between NTM and MTB is useful for mycobacterial disease management, to allow appropriate and timely therapeutic decisions.

This study evaluates the diagnostic capability of a new rapid molecular assay (Standard M10 MTB/NTM, SD Biosensor), to detect the presence of MTB, NTM mycobacteria or co-infection (MTB/NTM), in positive liquid medium cultures (MIGIT,BD). The assay was validated using 100 positive and 50 negative MIGIT cultures, all confirmed by a commercial real-time PCR kit (MDR/MTB MGB Kit, ELITechGrop) and Sanger identification.

After evaluation of the best dilution culture samples to use, and assessment of potential interference from co-occurring MTB/ NTM species, we confirmed excellent sample stability (up to 4 hours) after sample pre-treatment with the indicated reagent, highlighting the flexibility of this test for laboratory organization. In terms of specificity, the M10 MTB/NTM system showed excellent performance (all 50 negative samples were confirmed) and excellent sensitivity, identifying all 50 MTB and 48 on 50 NTM samples. Notably, one *M. celatum* positive culture was not identified and one *M. fortuitum* culture sample was identified as a co-infection with MTB. These two discordant cases will be investigated further. Moreover, the "early call" result, could reduce the diagnostic response time being useful for mycobacterial disease management.

P094

Excellent specificity and moderate to robust sensitivity of Xpert MTB/XDR for TB drug-susceptibility testing compared against Deeplex Myc-TB in nine African countries

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The GeneXpert MTB/XDR® cartridge (Cepheid, USA) is a near point-of-care tool for rapid drug-susceptibility testing directly on clinical samples for isoniazid (INH), fluoroquinolones (FQ), ethionamide (ETH), and amikacin (AMK).

We evaluated Xpert-XDR under field conditions against Deeplex Myc-TB® (Genoscreen, France), a targeted next generation sequencing test, using samples from the DIAMA project (equal number of rifampicin-resistant and -susceptible patients collected between 2017 and 2021 in nine African countries). Both tests were conducted on the same sputum sample.

A total of 1138 samples were included in this evaluation. The sensitivity (Se) and specificity (Sp) with 95% confidence intervals for the Xpert-XDR cartridge were:

INH: Se = 93% [91-95], Sp = 96% [95-97]

FQ: Se = 65% [48-79], Sp = 100% [99-100]

ETH: Se = 54% [48-60], Sp = 99% [99-100]

AMK: Sp = 100% [99-100]

Part of mutations missed by Xpert-XDR were attributable to heteroresistance: 11/45 for INH and 10/12 for FQ. For ETH, 130 mutants were not detected: 94% had mutations in regions not covered by the test, and the remaining 6% all shared the same mutation (S94A) in the *inhA* promoter. Sensitivity for AMK-resistance detection could not be assessed within our sample.

The Xpert-XDR demonstrated a high specificity across all tested drugs and robust sensitivity for INH, highlighting its utility in rapid detection of second-line TB drug resistance, despite the limited ability in detecting heteroresistance and mutations outside targeted regions. Extended evaluations in regions representing other resistance profiles is warranted.

P095

Preliminary study on STANDARD™ M10 MTB/NTM, a RT-PCR multiplex test for management of TB and NTM patients.

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Background
The STANDARD™ M10 MTB/NTM (SD BIOSENSOR, KR) is a new cartridge-based RT-PCR multiplex able to detect *M. tuberculosis complex* (MTB) and Nontuberculous Mycobacteria (NTM) positive pulmonary samples.
The aim of this preliminary study is to evaluate the performance of this test on selected Xpert® MTB/RIF Ultra (Cepheid, USA) positive respiratory specimens.

Materials and Methods
Forty-five decontaminated frozen respiratory samples, which had previously resulted MTB-positive on GeneXpert® system were included in the study: 10 were detected with “high” DNA load, 10 “medium” and 10 “low”, all with MTB culture-positive. In addiction we included 15 MTB culture-negative specimens, of which 10 were detected as “very low” and 5 “trace” on GeneXpert®. All samples were mixed in a 2:1 ratio with a “sputum pretreatment solution” and loaded into the cartridge according to the manufacturer’s instruction.

Results
All samples obtained “High”, “Medium” and “Low” load on GeneXpert® were confirmed MTB-positive on STANDARD™ M10 system.
Among the 15 MTB culture-negative specimens with “very low” and “trace” load on GeneXpert®, two of them were detected MTB-positive on STANDARD™ M10.

Conclusions
In this preliminary study we observed a full agreement between GeneXpert® and STANDARD™ M10 system in samples with MTB culture-positive pulmonary samples, while among culture-negative and MTB DNA poor load samples, only 13% were detected positive by STANDARD™ M10 MTB/NTM test. These data should be confirmed in a study with a larger sample size and NTM positive samples, however this new device appears to be suitable in the diagnosis of TB cases.

P096

Growth Units in the MGIT below the instrument threshold to call resistance may be predictive of *Rv0678* conferring resistance mutations

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For drug-susceptibility testing (DST), MGIT™960 interprets mycobacterial growth units (GU) in drug-containing tubes as resistant (R) when ≥100 GUs are detected (commonly reaching 400), and susceptible (S) when <100 (typically 0), without differentiating GUs between 0-100 or 101-400.

At ITM, *Mycobacterium tuberculosis* isolates from MSF projects are tested for bedaquiline (Bdq) susceptibility with MGIT-DST, minimal-inhibitory concentration (MIC) determination, and *Rv0678* sequencing by Deeplex® Myc-TB or whole genome sequencing, subsequently analysed by the MAGMA pipeline. Bdq-resistance-associated variants with ≥95% allele frequency are considered fixed.

Of 100 isolates with Bdq-MGIT, MIC and *Rv0678* sequencing results available, 50 showed *Rv0678* variants. Of these, 30 were MGIT-R: 21 with GU=400 and 9 with GU between 111-337. For both groups, Bdq-MIC ranged between 0.25µg/ml and 0.5µg/ml, with unfixed variants equally represented (n=9 and n=4). The remaining 20 isolates with variants were MGIT-S: 12 with GU=0 and 8 with GU between 4-84. In both groups, Bdq-MIC ranged between ≥0.125 and ≤0.5µg/ml, and 4 unfixed variants in each group.

All 50 WT isolates were MGIT-S, 48 showed GU=0 and Bdq-MIC ≥0.0155 and ≤0.25µg/ml, and two GU=13 and GU=100, with MIC=0.06µg/ml. MIC sensitivity to detect variants was 39% and specificity 100%. If MGIT GUs between 1 and 100 were classified as MGIT-R, sensitivity would increase from 58% to 74%, decreasing specificity from 100% to 96%.

Currently applied critical concentrations do not detect all *Rv0678* mutants. While unfixed-variants do not affect MGIT sensitivity, GU between 1-100 in MGIT-S results may indicate the presence of variants associated with Bdq-resistance.

P097

Collision of three global pandemics: the effect of tuberculosis and HIV on the epidemiological, clinical, virological, and immunological trajectory of Covid-19 in household contacts

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Covid-19 emerged as global pandemic with an unprecedented impact on public health. SARS-CoV-2 epidemiology was poorly understood, especially in the African context. A particular gap in knowledge was the effect of HIV and tuberculosis (TB) on the outcomes of Covid-19 disease. We implemented a research study that addressed critical questions concerning Covid-19 disease epidemiology in the context of low resource countries with high burden of poverty, and high rates of TB and HIV.

Recruitment commenced in July 2022; we followed a two-pronged approach: first, all primary healthcare facility (PHC) attendees were tested for TB infection, TB disease, Covid-19 and HIV screening. Second, we followed-up Covid-19 patients as diagnosed by the Ministries of Health, and tested these index cases and their households for TB infection, TB disease, Covid-19 and HIV.

Preliminary results for the household transmission component: we enrolled 197 participants of whom 64 are index cases and 133 household contacts. Of the index cases, 39/64 (61%) were male and 9/64 (14%) HIV infected. Of the household contacts, 72/133 (54%) were male and 15/113 (13%) HIV infected. The Covid-19 household secondary attack rate was 32% (95%CI 25-41). No-one had active TB but of the index cases 30/64 (47%) and 59/133 (44%) of the contacts had latent TB infection.

Future analyses will include investigating risk factors associated with these differential rates. Our findings will contribute to the growing literature on Covid-19 household transmission in high burden TB/HIV settings, and to the understanding of the interaction between Covid-19, TB, and HIV.

P098

Collision of three global pandemics: the effect of tuberculosis and HIV on the epidemiological, clinical, virological, and immunological trajectory of COVID-19 in Botswana and Namibia at primary healthcare facilities

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Covid-19 emerged as global pandemic during the past three years, with an unprecedented impact on public health. SARS-CoV-2 epidemiology was poorly understood, especially in the African context. A particular gap in knowledge was the effect of HIV and tuberculosis (TB) on the outcomes of Covid-19 disease. We implemented a research study that addressed critical questions concerning Covid-19 disease epidemiology in the context of low resource countries with high burden of poverty, and high rates of TB and HIV.

Recruitment commenced in July 2022; we followed a two-pronged approach: First, all primary healthcare facility (PHC) attendees were tested for TB infection, TB disease, Covid-19 and HIV. Second, we followed-up Covid-19 patients as diagnosed by the Ministries of Health, and tested these index cases and their households for TB infection, TB disease, Covid-19 and HIV.

Preliminary results are shown for the PHC attendees. To note: (i) the high TB infection rate in Namibia (72%, 95%UI 67-76) vs. Botswana (44%, 95%UI 41-47, p<0.001), (ii) the high TB disease rate in Namibia (3.8%, 95UI 2.4-5.9) vs. Botswana (1.6%, 95UI 0.8-2.8, p=0.01), and (iii) the proportion of the total number of participants who had TB and SARS-CoV-2 co-infection (155/1500, 10.3%).

Future analyses will include investigating risk factors associated with these differential rates. We believe our findings will contribute to the growing literature on enhanced case finding at PHC through universal TB screening, and to the understanding of the interaction between Covid-19, TB, and HIV.

P099

Better care through better quality control: Why quality control of DNA libraries is crucial for real-time sequencing of clinical cultures

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'Real-time' whole-genome sequencing (WGS) of *Mycobacterium tuberculosis* (*Mtb*) from clinical primary-MIGT culture (CPC) is a powerful tool for patient care. CPC library quality control (QC)

parameters are not stated in manuals. There is no guidance on which genomic libraries can be successfully sequenced. We evaluated the genomic libraries QCs of 286 patient samples that were MGIT cultured and assessed for presence of *Mtb*. DNA was extracted using CTAB protocol and assesses for quantity and quality using Qubit and nanodrop. Libraries were prepared with Illumina DNA-Library Prep Kit with 0.23-20 ng/µl of total DNA input, using 10 PCR cycles. Library fragments were assessed with LabChip® and TapeStation. A library pool of 3-5 samples were sequenced on mid-output MiniSeq cartridge. The MAGMA-pipeline was used for WGS analysis. Of 103 patient baseline samples, 81 were Mtb-positive, 16 culture-negative and 6 Mtb-negative. Of the 81 Mtb-positive, 78 passed DNA QC. Of these 78 libraries, 25 were visually categorised as “excellent”(single peak, no tailing/shoulder), 32 as “good”(single peak, visible shoulders and/or tailing), 4 as “borderline”(single or multiple peaks with shoulders and/or tailing), 9 as “critical”(small peak, no shoulders and/or tailing) and 8 as “failed” (no peak, or peak below 350 or above 1200 bp). All 70 samples not classified as failed were successfully sequenced. A model was constructed using Amazon-Recognition, which had an F1-score of 0.857 for sequencing failures using a training dataset of 125 TapeStation images. Standardizing the CPC library fragment sizes range (350-1200 bp) and an automated visual-processing-tool can help streamline real-time WGS.

P100

Rapid and sensitive detection of viable mycobacteria in bovine faeces and blood by a novel phagomagnetic separation (PhMS)-qPCR assay

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Mycobacterium avium subsp. *paratuberculosis* (MAP) and *Mycobacterium bovis* are slow-growing mycobacterial pathogens that cause Johne’s disease (Paratuberculosis) and Bovine Tuberculosis, respectively. Previously we developed a rapid and sensitive phage-based PhMS-qPCR assay to detect viable MAP in bovine milk within 24 h. The potential of this new test to detect viable MAP and/or *M. bovis* in bovine faeces and blood was investigated. Following careful optimization of sample preparation protocols, faeces samples (n=376) from calves and adult cattle on several Johne’s affected farms, and blood samples (n=174) from TB skin test reactor and routine slaughter <30 month old cattle collected at point of slaughter, were tested by PhMS-qPCR and culture. Results of faecal testing clearly demonstrate that the novel PhMS-qPCR assay can detect calves or cattle shedding viable MAP, i.e. infectious animals. Overall, 119 (31.6%) faeces samples tested PhMS-qPCR positive and there was slight agreement (Kappa 0.160) with culture results. Results of blood testing revealed that viable MAP and/or *M. bovis* mycobacteraemias were readily detectable in cattle at slaughter in Northern Ireland, with co-infections (both MAP and *M. bovis* detected in same animal) frequently encountered. Detection sensitivity by PhMS-qPCR was also greater than culture when blood was tested (78 and 45 of 174 blood samples positive for viable *M. bovis*, respectively). Collectively, these results indicate that the novel PhMS-qPCR assays rapidly and sensitively detects viable MAP or *M. bovis* in bovine faeces and blood. Therefore, they may represent new diagnostic tests for Johne’s disease and bovine Tuberculosis in the future.

P101

Novel duplex qPCR and LAMP assays suitable for differentiating *Mycobacterium bovis* from other *Mycobacterium tuberculosis* complex species in MGIT sputum cultures

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Mycobacterium bovis causes zoonotic Tuberculosis (TB) in humans, mainly in developing countries. It differs from *Mycobacterium tuberculosis*, the predominant cause of human TB, by being pyrazinamide resistant, making it more difficult to treat. Otherwise, the species are similar phenotypically and genetically. Currently spoligotyping or whole genome sequencing are required to distinguish these two species after isolation from patient cultures. Our aim was to develop a duplex qPCR or LAMP assay that would permit rapid and easy differentiation of *M. bovis* from *M. tuberculosis* in positive MGIT cultures. We selected multiple published qPCR and LAMP primer sets claiming specificity for *M. bovis*, *M. tuberculosis or M. tuberculosis* complex, and designed several new primer sets in-house. All primers were screened to assess detection sensitivity and specificity using DNA derived from 10-fold dilution series of *M. bovis* NCTC 1333 and *M. tuberculosis* H37Rv broth cultures (10^6 to 10^1 CFU/ml). Then, the most sensitive and specific qPCR and LAMP primer sets for each target species were tested together, to check if they amplified successfully without detection sensitivity for either species being adversely impacted. Ultimately, both a duplex qPCR and a duplex LAMP assay were achieved. When a panel of gDNAs extracted from growth positive MGIT cultures was tested, both new tests correctly differentiated 17 *M. bovis* positive samples from *M. tuberculosis* (n=123), *M. africanum* (n=2) and *M. abscessus* (n=17) positive samples. The developed duplex assays take < 90 min after MGIT culture, so they could expedite identification of the causative agent of TB infections.

P102

Evaluation of the cobas® MTB Real-Time PCR plus MTB-RIF/INH assay in a low incidence country

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The cobas® MTB Real-Time PCR assay is intended for the rapid detection of MTBC in clinical specimens with a reflex-test, the cobas® MTB-RIF/INH assay, to detect resistance to isoniazid (INH) and rifampicin (RMP) in MTBC positive specimens. WHO recommended this as one of the moderate complexity assays to rapidly detect MTBC and additional drug resistance. We evaluated this assay for its applicability in a low incidence country with low resistance rates. For this purpose, 500 sputum specimens were analysed in parallel by culture and by the cobas® assay. MTBC positive specimens were subsequently analysed for INH and RMP resistance. To evaluate the ability of the cobas® MTB-RIF/INH assay to detect different mutations causing resistance to INH and RMP a collection of 34 TB strains with different well-described mutations in the rpoB, inhA or katG genes was deployed. In total, the cobas® MTB Real-Time PCR assay presented a

high sensitivity (91.8%), even in smear negative specimens (85.1%), and the detection of all smear positive cases (100%). All analysable positive samples were correctly identified as susceptible to INH and RMP. Using the strain collection with selected mutations, 41 of 45 cases of drug resistance were detected with the cobas® MTB-RIF/INH assay. Resistances not recognized by the assay were caused by very rare mutations, according to the WHO catalogue of mutations. Overall, the cobas® MTB Real-Time PCR plus MTB-RIF/INH assay enables a largely automated testing for the detection of TB and drug resistance with the possibility of a high throughput capacity.

P103

Resolution of phylogenetic typing in *Mycobacterium tuberculosis*: a comparison between WGS and tNGS

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Strain typing is crucial for public health interventions. For 60 patients, we processed a sputum sediment by Deeplex® Myc-TB (Deeplex) and DNA from the corresponding primary liquid culture by Illumina whole genome sequencing (WGS) with MAGMA pipeline analysis. Of the 60 samples, Deeplex was unsuccessful for 7 (11.7%), reported “Other than lineage 4.9” (undefined) for 20 (33.3%), a lineage for 35 (58.3%), and sub-lineage for only 11 (18.3%) samples. MAGMA reported lineage plus sub-lineage for 59 (98%) samples. Mixed infection (minority strain ≥3% for Deeplex, any level for MAGMA) occurred in 4 samples. One sample was reported as 91% *Mtb* 2.2.1 + 8% *Mtb* 4.4.1.1 by MAGMA, and 87% *M. bovis* + 39% undefined *Mtb* lineage by Deeplex. One sample was 99% NTM + 1% *Mtb* by MAGMA but 100% *Mtb* 4.3 by Deeplex. Another sample contained 5 *Mtb* lineages, animal strains and *M. canettii* (4%) + undefined *Mtb* lineage (96%) in Deeplex and 100% *Mtb* 4.1.1.3 in MAGMA. The fourth sample contained two undefined lineages at 11% and 89% in Deeplex but 100% *Mtb* 2.2.1 in MAGMA. Discordances between spoligotype and lineage assignment in Deeplex were observed. Most (62%) samples shared an identical spoligotype (7 groups of size 2-19). In contrast, only 3.4% samples were part of a WGS cluster when using a 12 SNP cut-off. Only 1 of 7 spoligotype groups had a SNP distance <12. Our results suggest that the tNGS Deeplex assay has poor phylogenetic resolution, limiting its ability to inform public health interventions.

P104

Low pH, high-throughput assay as a clinically relevant screening effort in *Mycobacterium tuberculosis* (H37Rv)

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We conducted an extensive screening of 1 million compounds targeting Mycobacterium tuberculosis under low pH conditions. Initially, we optimized a high-throughput screening assay for low pH by utilizing 1536-well plates and incorporating a resazurin read-out, which enhanced our throughput capabilities. Furthermore, we refined the protocol to shorten the incubation time

and enhance assay reproducibility. The screening process involved evaluating the inhibitory effects of 1 million compounds at low pH using a single-dose approach. Compounds exhibiting inhibition above 30% underwent confirmation screening, where their activity was assessed in dose-response experiments under both low pH and normal conditions. Promising compounds underwent further in vitro evaluation to determine their bactericidal efficacy across additional relevant culture conditions, such as cholesterol and hypoxia. Simultaneously, in silico methodologies aided in hit clustering based on chemical structure and predicted compound liabilities using machine learning algorithms.

P105

The upstream region of *Mycobacterium abscessus whiB7* as a culprit for poor treatment outcome?

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Mycobacterium abscessus infections can be considered as an antibiotic nightmare. Treatment efficacy is hampered by its intrinsic and acquired drug resistance, and relapse or reinfection occur frequently. Prolonged multidrug regimes with different antibiotic classes are recommended to eradicate surviving bacteria and improve treatment outcomes. While different stress responses and mechanisms have been implicated in *M. abscessus* drug survival, underlying genetic causes remain poorly understood.

The aim of the current study was to explore these causes in *M. abscessus* by identifying and characterizing genetic mutations that affect survival during *in vitro* evolution experiments and to investigate their role in resistance. To this end, bacterial cultures were exposed to amikacin and rifabutin for 7 days, after which surviving cells were washed, passaged and treated again with the same antibiotic combination. Clonal and bulk populations were subjected to whole genome sequencing to identify mutations and genes involved in drug survival.

One frameshift mutation in *mab_3509c* was found to induce a stress response similar to the previously described macrolide-induced stress response. Interestingly, the frameshift results both in increased survival in the drug combination treatment and increased minimal inhibitory concentrations of amikacin and clarithromycin. It is located upstream of the *mab_3508c* gene encoding transcriptional regulator WhiB7, a well-known determinant of multi-drug resistance. Our findings highlight the importance of the upstream region in inducing the mycobacterial *whiB7* stress response. More insight in the regulation of drug resistance will be instrumental in devising future therapies to treat *M. abscessus* infections.

P106

Dialysis waters: should we look for Mycobacteria?

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Nontuberculous mycobacteria (NTM) are common environmental contaminants and, as opportunistic pathogens, they could colonize or infect immunocompromised patients.

Consecutive ultrapure dialysis fluid samples were collected during one year in the Emilia Romagna region, and processed by Microbiology Units at IRCCS University Hospital of Bologna and at The Great Romagna Hub Laboratory, Pievesestina.

Concentrated samples were cultured for 42 days. Mycobacterium saskatchewanense was identified in positive cultures by MALDI-TOF technique and isolates were sent to the National Institute for Infectious Diseases “L. Spallanzani” (INMI) for epidemiological investigation by Whole Genome Sequencing (WGS) using Illumina system. Sequences were analysed with SeqSphere+ RIDOM software, by core genome Multi-Locus-Sequence-Typing (cgMLST of 5090 alleles), upon alignment to the reference genome (ID Genbank NZ_AP022573.1).

Thirty-five strains of M. saskatchawanense were isolated from 722 dialysis samples. The WGS analysis allowed the creation of a phylogenetic tree [Minimum Spanning Tree (MST)]. Genetic distances between the strains were below 15 alleles. Raw sequence reads are available under the BioProject accession number PRJNA1055936.

This explorative analysis showed the presence of M. saskatchawanense genetically close strains in fluid samples from dialysis systems, raising the question whether this and other mycobacteria should be routinely searched for in sanitary waters. Furthermore, alternative disinfection methods of the devices should be explored, in order to efficaciously eliminate potential sources of human infection. A potential explanation for the role of NTMs in medical devices could be their ability to form biofilms, thus further investigation in this area is warranted.

P107

Optimization of *Mycobacterial tuberculosis* complex sample processing for whole genome sequencing

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Mycobacterial tuberculosis complex (MTBC) sample processing for whole genome sequencing (WGS) is usually performed on (MGIT) subcultures with subsequent enzymatic and/or chemical lysis, often providing suboptimal DNA yield. In 2022, we included bead beating in our routine DNA extraction protocol, which increased DNA yield 60-fold from subcultures. So, we now investigated the effect of culture-based enrichment and bead-beating-based DNA extraction for MTBC Illumina-based WGS from primary received cultures. Additionally, we investigated the impact of bead beating on read length N50 and coverage depth for Nanopore sequencing. Bead-

beating-based DNA extraction from primary received cultures was performed from July 2023-November 2023 (n=174). Using bead beating, the DNA yield was 11.0 ng/µl, and the sequencing success rate was 80%. Two culture-based enrichment experiments were performed to demonstrate WGS is also possible from enriched-pre-positive MGIT cultures using bead-beating-based DNA extraction. From early-pre-positive MGIT cultures, a 36x average coverage depth was achieved eight days before culture positivity (approx. 2.72*10^3 CFU/ml). The effect of bead-beating-based DNA extraction on Nanopore sequencing, read length and coverage depth was also assessed. After optimization, the read length N50 increased from 1.4 to 2.9 kb and coverage depth from 87x to 217x.Bead beating significantly increases DNA yield and allows sequencing from primary cultures. Experimental work demonstrated that sequencing from enriched-pre-positive MGIT cultures is also possible. These optimized DNA extraction protocols help reduce the turnaround time for tuberculosis WGS-based diagnostics. In our laboratory, sequencing is now performed routinely from primary cultures and only in the event of failure from sub-cultured bacteria.

P108

Phenotyping resistance to bedaquiline and emerging medicines assessment by a fast RNA-based drug susceptibility test: TRACeR-TB

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Since bedaquiline (BDQ) has become a core drug in drug-resistant tuberculosis therapy, rapid and accurate drug-susceptibility testing (DST) is of major importance. Currently, molecular mechanisms leading to BDQ resistance have not been fully elucidated, and only provisional MIC breakpoints and critical concentrations exist for pDST. To dramatically speed up pDST for *M. tuberculosis*, we previously developed TRACeR-TB, an assay based on the quantification of antibiotic-specific RNA biomarkers, based on the principle that antibiotic exposure triggers transcriptional stress responses in susceptible but not in resistant microbes, enabling the distinction between resistant and susceptible strains in only a few days. By focusing on a general stress response rather than the resistance mechanism itself, TRACeR-TB is an efficient tool to complete the knowledge on genotypic-phenotypic associations. At ESM, we will present TRACeR-TB results that outcompetes MGIT-DST for BDQ. In combination with WGS, this fast assay can be of great value to enrich or curate drug-resistance/WGS databases (e.g. WHO Catalogue of mutations). In this study, we compared TRACeR-TB outcomes from strains carrying mutations known to be associated with resistance or of uncertain significance in three key genes: *pepQ*, *Rv0678* and *atpE*. All mutants were identified as low or moderate/high-level BDQ-resistant, while MGIT-DST found some to be BDQ susceptible. Our tool can therefore give new insights into resistance conferring mutations. In addition, we will present how TRACeR-TB can be

applied to investigate novel medicines that boost antibiotic efficiency or to assess *in vivo* drug responses in a macrophage model.

P109

The first evaluation of targeted nanopore-based sequencing for tuberculosis drug resistance detection

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We investigated the performance of the targeted Next Generation Sequencing (tNGS)-based Oxford Nanopore Diagnostics (OND) AmPORE TB assay, recently approved by the World Health Organization (WHO) for clinical use on tuberculosis (TB)-positive respiratory samples.

A total of 105 DNA samples from Xpert MT/RIF and smear-positive TB sputum samples were tested with the AmPORE TB kit, using the Genoscreen Deeplex Myc-TB as comparative assay. For AmPORE TB, the samples were divided into five sequencing runs on MinION device. Data analysis was performed using proprietary software. The WHO catalogue of mutations was used for drug resistance interpretation.

The AmPORE TB turnaround time was approximately 5–6 hours from the extracted DNA to the tNGS report for batches of 22 individual samples. The assay achieved a high validity rate of 98% (103/105 samples), homogeneous mean reads coverage across TB-positive samples, and 100% Positive and Negative Agreements for detecting mutations associated with resistance to rifampicin, pyrazinamide, fluoroquinolones, ethambutol and capreomycin, compared to Deeplex MycTB. The main discrepancies for the remaining drugs were attributable to the different assay panel designs.

The AmPORE TB assay drastically reduced the time to tNGS reporting from days to hours and showed a performance largely equivalent to that of the Deeplex Myc-TB kit.

P110

Emergence of bedaquiline resistance in drug-resistant tuberculosis samples from Mozambique

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Bedaquiline (BDQ) is a key component of the World Health Organization endorsed 6-month BPaLM regimen (BDQ, pretomanid, linezolid and moxifloxacin) for treatment of patients with rifampicin resistant (RR) or multi-drug-resistant (MDR, isoniazid and rifampicin resistance [INHr, RR]) tuberculosis (TB). Recent data from Mozambique indicated an increase of BDQ resistance

(BDQr) from 3% to 14% from 2016-2021, suggesting that current RR/MDR-TB treatment regimens are not able to prevent BDQr development at population level. To study current resistance levels, we performed targeted Next-Generation-Sequencing using Deeplex®-Myc-TB from GenoLyse® DNAs extracted from 164 INHr and/or RR (classified by Bruker-Hain GenoTypeMTBDR*plus*) clinical and cultured samples submitted to the National Tuberculosis Reference Laboratory in Maputo between January/2021 and April/2024. A total of 148 samples were classified RR, 124 at least MDR/RR, 27 fluoroquinolone resistant (16%, FQr), 12 pre-XDR (7%, MDR+FQr), and 10 XDR, (6%, MDR+FQr+BDQr). 33 were BDQr (20%): two INHr+BDQr, two INHr+FQr+BDQr, two RR+FQr+BDQr, 17MDR+BDQr, and 10 XDR. Seven samples had the *rpoB* I491F mutation as sole RR marker (four MDR+BDQ, two XDR). Alarmingly, these samples were classified rifampicin susceptible by GenoTypeMTBDR*plus*. Increasing BDQr rates found are alarming and potentially jeopardize DR-TB control in the country. BDQr is observed in a broad spectrum of resistance combinations. “Diagnostic escape” *Mycobacterium tuberculosis* strains with *rpoB* I491F RR mutation, not detected by commercial molecular drug resistance assays such as Xpert® MTB/RIF and GenoTypeMTBDR*plus*, cause an additional challenge for the current DR-TB test algorithms, and underline the urgent need for implementation of rapid comprehensive resistance testing in country.

P111

Evaluation of sample pre-treatment protocols for detection of mycobacterial DNA directly in sputum samples

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Strategies to increase sensitivity for rapid detection of mycobacterial DNA are needed. Current methods are commonly used on decontaminated samples and necessary for culture but may also reduce mycobacterial DNA in sputum samples. The aim of this study was to optimize pre-treatment for increased yield of mycobacterial DNA.

Pooled sputum samples were spiked with 500 and 10^4 CFU/ml of M. tuberculosis H37Rv (Mtb). Each concentration with and without decontamination was pre-treated by either inactivation in 80°C for 20 minutes, a saponin-based protocol or with sample reagent (SR) solution (GeneXpert, Cepheid, USA). The DNA yield was measured by Ct-values (*m*2000; Abbott, USA).

In total, 180 samples were processed including 18 for each pre-treatment with or without decontamination. Ct- values were was significantly lower without decontamination (mean Ct 27,9 at 10^4 CFU/ml and 32,7 at 500 CFU/ml) compared to decontaminated samples (mean Ct 31,9 and 35,6 respectively). Highest DNA yield was obtained from samples heated to 80°C for 20 minutes without decontamination (mean Ct 24,3 at 10^4 CFU/ml and 29,4 at 500 CFU/ml) compared to decontaminated samples (mean Ct 31,0 and 35,4 respectively). Compared to heating without decontamination, SR solution had lower DNA yield (Ct 33,3 at 500 CFU/ml) but higher than the saponin-based protocol (35,5 at 500 CFU/ml).

Overall, decontamination decreased mycobacterial DNA in sputum samples. Samples without decontamination in combination with heating at 80°C gave the highest Mtb DNA yield. Further

optimization of pre-treatment including DNA extraction, consideration of human DNA influence in combination with targeted sequencing methods is planned.

P112

Clinical characteristics and treatment of non-tuberculous mycobacterial skin and soft tissue infections: a retrospective case series

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The incidence of skin and soft tissue infections (SSTIs) caused by non-tuberculous mycobacteria (NTM) is increasing. NTM SSTI antibiotic treatment is challenging because of its long duration and toxicity. Little is known about the clinical characteristics, treatment and outcome of NTM SSTI. We describe the clinical management of adult NTM SSTI patients in two Dutch tertiary referral institutes, using electronic patient data from patients diagnosed between 2017 and 2023. We identified 55 NTM SSTI patients of which 62% were male, with a median age of 62 years (IQR 52-73). Prior to presentation, 56% of patients had received immunosuppressive medication. Corticosteroids were the most common medication, followed by TNF-α inhibitors. *Mycobacterium chelonae* (29%) was most frequently isolated, followed by *Mycobacterium avium* complex (15%) and *Mycobacterium marinum* (15%). Disseminated disease was exclusively observed in immunocompromised patients. Antibiotic treatment was given for a median of 28 weeks (IQR 19 - 33) in immunocompetent patients, while this was 42 weeks (IQR 35 - 52) in the context of immunosuppression. Side effects led to treatment regimen changes in 73% of cases, with gastro-intestinal complaints and ototoxicity being the most reported. Surgical debridement was performed in 53% of patients. All but four patients, who were all immunocompromised, achieved clinical cure. In conclusion, the use of immunosuppressive medication is a major risk factor for NTM SSTI. The multidrug antibiotic regimens commonly lead to side effects. Adjuvant surgical treatment to reduce the mycobacterial load could be an additional strategy to achieve clinical cure sooner.

P113

Validating the second edition of the WHO tuberculosis resistance catalogue using GPAS, a cloud platform for processing pathogen genetic data

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Whole genome sequencing is faster, cheaper and, for many drugs, as accurate as traditional phenotypic testing for tuberculosis and is increasingly being adopted by Public Health Agencies and hospitals. This shift is being encouraged by the WHO – they have recently released the second edition of their catalogue of resistance associated genetic variants, yet it remains difficult

in practice to apply the information contained within the WHO catalogue. One consequence is it is challenging for pipeline and tool developers to assure that their implementation of the WHO catalogue is faithful to the original.

Here we calculate the performance for all the drugs in the WHO catalogue using two datasets; one derived from the CRyPTIC project (described in another abstract) and a second complementary dataset that "fills in" the missing compounds. Our results are comparable to those reported by the WHO on their training set, which is pleasing if a little surprising given the datasets are different. We then analyse the discrepancies between the genotype and phenotype in our dataset but are unable to explore the discrepancies with the reported WHO figures since their dataset is not publicly available. Finally, we describe the key design principles, such as ensuring no identifiable information in the cloud, and the architectural designs that underpin GPAS, for example the use of Kubernetes to grow the number of samples that can be processed concurrently, as demand allows.

P114

Non-tuberculous mycobacteria among previously treated tuberculosis patients with microscopy-positive and Xpert-negative results in Niger

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Disease caused by non-tuberculous mycobacteria (NTMs) shares many clinical features with tuberculosis (TB), challenging differential diagnosis. GeneXpert currently used for TB screening only detects *Mycobacterium tuberculosis* complex. This study explored the causes of microscopy-positive but Xpert-negative results in patients with chronic lung disease.

Descriptive cross-sectional study was conducted between July 2022 and June 2023 in four tuberculosis facilities in Niger. It included any patient previously treated for TB presenting with a microscopy-positive but Xpert-negative result, for whom sputum samples were cultured on solid medium. All sputum samples collected had undergone culture and direct *rrs* PCR sequencing. Positive cultures also had *rrs* PCR and Sanger sequencing.

We included 59 patients, predominantly male (sex ratio=5.5), aged 49.3 (±14.8) years, with mean BMI 17.4 (±3.15) kg/m2. Respiratory symptoms were found in 94.9% of patients, and majority (93%) had abnormal chest X-rays. Half (45.8%) of samples grew mycobacteria, while 84.7% were positive by direct PCR: 13 were only positive by direct PCR, three only by culture, and seven by both. Twenty-two different NTM species were identified, majority belonging to the *M. intracellulare* complex, followed by *M. palustre*. One patient was positive for *M. tuberculosis* complex by PCR and culture. On multivariate logistic analysis, no risk factor was associated with detection of NTMs. Programmatic monitoring is needed to assess their clinical relevance.

NTMs are increasingly isolated from previously treated TB patients in Niger. It is important to isolate, identify and assess the clinical relevance of these mycobacteria, as treatment strategies for TB and respiratory NTM infections differ.

P115

Non-tuberculous mycobacteria isolated at the end of the world

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Non-tuberculous mycobacteria (NTM) are a group of mycobacteria widely distributed in the environment (e.g. soil and water). NTM can also cause infections in different mammals, mostly in immunocompromised individuals. Moreover, they may interfere with the *ante-mortem* diagnosis of bovine tuberculosis (BTB) by the tuberculin skin test. In this work, two isolates of NTM species were obtained from soil of Tierra del Fuego, the only province free of BTB and brucellosis in Argentina. Molecular typing of the isolates was performed by gene sequencing of the 16S rRNA, *hsp*65 and *rpo*B genes. The criterion for species identification was concordance of identity of at least two of these genes after blast comparison. *Mycolicibacterium diernhoferi* (*M. diernhoferi)* and *Mycolicibacterium novocastrense* (*M. novocastrense*) were identified in the two isolates. Furthermore, in this study the presence of *esx*A, *esx*B and *esp*C genes was investigated by PCR. None of the three genes were detected in either *M. diernhoferi* or *M. novocastrense*. In the future, whole genome sequencing of these strains will be performed to compare them with the reference genome of *M. bovis* AF2122/97 to detect antimicrobial resistance, virulence genes and other genes related to BTB diagnosis. Knowledge of the presence and distribution of NTM in the environment and the evaluation of the specificity of the genes used in the diagnosis of BTB can contribute to better control of the disease, especially in areas declared free of the disease.

P116

Deeplex® Myc-TB assay: Informing genotypic/phenotypic associations in *Mycobacterium tuberculosis* isolates

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The World Health Organization (WHO) endorsed the Deeplex Myc-TB assay (GenoScreen), which utilizes targeted next-generation sequencing (tNGS) to predict *Mycobacterium tuberculosis* antibiotic resistance, including drugs in the BPaLM regimen (bedaquiline [BDQ], linezolid, and moxifloxacin).

We initiated a pilot study (TS ELiOT) in collaboration with the National Health Laboratory Services to determine the feasibility of implementing tNGS into the routine standard of care for

rifampicin resistant (RR-)TB. We successfully sequenced 343 clinical specimens (consecutive sampling) using the Deeplex assay between April and August 2023.

Resistance-associated or uncharacterized variants we identified from sequence data using the automated Deeplex web app. Phenotypic drug susceptibility testing was done on isolates with variants in the *mmR5* gene to confirm BDQ resistance.

A total of 29/343 (8.5%) isolates harboured variants in *mmR5* of which 18/29 (62%) were deemed resistant to BDQ by tNGS, while 11/29 (38%) harboured uncharacterised variants. pDST confirmed BDQ resistance to 9/11 (82%) uncharacterised variants. 5 of the 11 uncharacterised variants were listed as uncertain significance in the WHO catalogue while 6 were not listed. Out of the 18/29 isolates deemed resistant to BDQ by tNGS, pDST confirmed resistance to 15 isolates.

We document a relatively high proportion of BDQ resistance in an unselected sample of patients with RR-TB before the formal roll-out of BPaLM. Scale-up of tNGS may be critical to inform clinical decision-making and preserve the utility of keystone drugs like BDQ. tNGS data must contribute to the WHO catalogue to ensure the utility of genetic drug susceptibility testing.

P117

Investigating the resistance rate and transmission of *Mycobacterium abscessus* among cystic fibrosis and non-cystic fibrosis patients

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Mycobacterium (*M.*) *abscessus* is the most prevalent and clinically relevant rapidly growing NTM characterized for its capacity to induce severe pulmonary disease, which can be devastating for individuals with cystic fibrosis (CF). Moreover, the presence of this bacteria before the transplant may be a contraindation due to concerns for increased morbidity, mortality, and risk of post-transplant transmission. Hence, investigations into the epidemiology and transmission of *M. abscessus* and accurate antibiotic susceptibility data are essential for effective treatment of infections caused by this pathogen. This retrospective nationwide study included all clinical *M. abscessus* isolates (n=59) from 29 patients. Whole genome sequencing (WGS) was performed to identify clusters and classify isolates into predominant circulating clones (DCC). Subspecies identification of unique isolates showed subspecies *abscessus* as the most prevalent (65.5%). 65.5% of all isolates were resistant to at least 3 antibiotics tested. Utilizing WGS to evaluate genotypic drug sensitivity to macrolides and aminoglycosides revealed 100% sensitivity and specificity, when compared to the GenoType NTM-DR test. When compared to phenotypic

sensitivity testing, the results showed 100% sensitivity and 68.75% specificity for macrolides, and 100% sensitivity and 95.83% specificity for aminoglycosides. WGS identified 7 clusters (including two cross-border) comprising CF and non-CF patients, with a total clustering rate of 48.3%. Furthermore, we identified representatives of all major DCCs. The results shown high discriminatory power of WGS in molecular-epidemiology of *M. abscessus* and provide supporting evidence of direct or indirect cross-transmission of subspecies *massiliense* among both CF and non-CF patients.

P118

DprE1 inhibitors and bedaquiline combination for the treatment of TB: *in vitro* effect on MIC of susceptible and resistant *M. tuberculosis* strains

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The efficacy of the new, short regimen, for drug resistant tuberculosis (DR-TB) treatment "BPaLM" is currently threatened by the increasing resistance to bedaquiline (BDQ). Resistance to BDQ is often driven by mutations on Rv0678, a transcriptional repressor that regulates the efflux pump MmpS5-MmpL5. It's reported that DprE1 inhibitors, the latest developed anti-TB drugs, share the same resistance pattern. A systematic review was performed by our group, to identify the molecular bases of DprE1 inhibitors resistance mechanisms. Our analysis underscored the importance of testing drug synergism, particularly for drugs that may share the same resistance mechanism. Accordingly, we developed a checkerboard assay based on the established EUCAST protocol for determining MICs in *M. tuberculosis* complex. We tested the DprE1 inhibitor BTZ-043 and BDQ or delamanid (DLM) through double serial dilutions, assessing their concentrations individually and in combination to determine individual drug effects as well as synergistic effects. To set our assay, we included 7 crossed dilutions per drug combination (BTZ-043 and DLM 0,004 mg/L±3 dilution, BDQ 0,032 mg/L±3 dilution) all tested against the H37Rv reference strain. Initial findings indicate a MIC shift between the BTZ-043/DLM control combination and the BTZ-043/BDQ combination. As anticipated, there was no evident synergistic effect observed for the BDQ/DLM combination. Whereas, these findings suggest a possible synergistic or additive effect of BDQ and DprE1 inhibitors, in H37Rv strain. Currently, we are in the process of validating these findings on a larger cohort, encompassing both BDQ-resistant any susceptible clinical isolates.

P119

Tuberculosis screening among migrants: a qualitative study on healthcare seeking behaviour and acceptance of tongue swabs

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TB screening in migrants is challenged by a reliance on sputum-based testing and hesitation to seek care. In Italy, a key country of the migration route to Europe, almost half of TB cases are of foreign origin. The need for more comprehensive and accepted TB screening methods serving these migrants' communities emerged. This study aims to evaluate the feasibility and acceptability of a non-sputum option, supervised self-swabbing (SSS) for TB screening within the context of migrants' health care seeking behaviours. SSS have a reported sensitivity up to 95% when paired with appropriate laboratory methods.

This user experience study uses qualitative research methods with in-depth interviews and purposive sampling. Recruitment of migrants experiencing TB screening began in November 2023 in Milan, Italy and continues until saturation among themes is reached. Hamilton's Rapid Qualitative Analysis Method was adapted to summarize key findings.

Preliminary results from the first 12 people interviewed reveal barriers and facilitators to seeking care and using SSS. All migrants had limited knowledge of the TB screening process and TB signs and symptoms. All participants were able to self-perform the tongue swab even if they had limited or no experience of self-testing. Most participants (8/12) indicated STS was easier than sputum while (3/12) had no differential preference. Only one participant from Mali preferred sputum over SSS.

These preliminary results indicate that SSS is feasible and acceptable. There is a lack of SSS educational materials and health promotion campaigns that are targeted to migrants' needs.

P120

Possible host genetic factors influencing leprosy susceptibility in a multiplex family from Anjouan, Comoros

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Leprosy remains hyperendemic in the Comoros, specifically on Anjouan, despite continuous efforts in disease control by the National Tuberculosis and Leprosy Control Programme. The continued prevalence of leprosy led to the hypothesis that host genetic factors may contribute to an increased susceptibility to *Mycobacterium leprae* in this area. In a pilot study we recruited eight members of a multiplex leprosy family, including seven multibacillary leprosy patients, who donated whole blood for genomic DNA extraction. Employing whole genome sequencing analysis, we searched for candidate mutations with strong impact on leprosy susceptibility.

We prioritized our search on protein-altering variants with predicted functional or regulatory impact at the protein level. Further, we assumed recessive and dominant models of inheritance of variants to identify three candidate leprosy susceptibility genes: *IL12RB1*, *HLA-A* and *MALT1*. All affected family members were homozygous for p.P40L, an expression quantitative trait locus (eQTL) downregulating the *IL12RB1* expression. This might lead to an IL12RB deficiency,

impairing Th1-mediated immunity and reducing control of *M. leprae*. Additionally, all affected participants were heterozygous for p.I641V in *MALT1*, while the healthy control did not have the variant. MALT1 is involved in the formation of the noncanonical inflammasome and IL1-beta processing. By inferring the *HLA-A* alleles, we found that 6/7 affected family members were homozygous for the *HLA-A*30:02* allele, with a potentially altered *M. leprae* antigen recognition mechanism.

These promising candidates have to undergo additional investigations, including wider genomic analyses of the population in the endemic areas and functional confirmation of their influence on leprosy susceptibility.

P121

Comparison of DRB3.2 alleles among false-negative and positive tuberculin skin test cattle with bovine tuberculosis

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Mycobacterium bovis is worldwide distributed mainly affecting livestock. Tuberculin skin test (TST), the primary screening tool, detect the infection in cattle. Some animals remain undetected (false-negatives) limiting sanitation programs success. Bovine lecukocyte antigen (BoLA) DRB3 gene is involved in antigenic presentation. BoLA-DRB3.2 polimorpysms were identified in false-negative TST cattle, comparing to TST+ bovines. Twenty-nine TST+ and 18 false-negative dairy bovines were studied. DNA extraction from blood (PuriPrep-S kit, InbioHigway®), PCR amplification of BoLA-DRB3.2 (319bp, primers DRB3FRW/DRB3REV) and genotyping by sequencing (BigDye ® v3.1, Applied Biosystems Inc.) were performed. Genotypes were assigned using Haplofinder and allelic frequency was compared using Epidat 3.1. Among TST+, *0101, *1501 and *1001 were the most frequent alleles (86.3%, accumulated frecuency) followed by *1101, *0902, *0601 and *1701. Among false-negatives, *1501, *1101, *0101, *4401 and *2703 were the most frequent alleles (71.5%, accumulated frequency), followed by *3701, *1001, *0902, *0601,*1701,*14011 and *1602. The *0101 allele was more prevalent in TST+ (p=0.01), while the frequency associated to the other allelles were similar among both, false-negative and TST+ groups (p>0.05). Alelles *1601, *3701, *4401, *14011 and *2703 were only detected in false-negatives, but in a frequency ≤ 8.3%. In this preliminary study, a higher diversity of BoLA-DRB3.2 alleles was observed in false-negative cattle comparing to TST+; with some alleles only identified among false-negatives bovines, although in low proportion. Further studies are needed to really comprenhend the implicancies of the DRB3 diversity on the false negative TSTphenotype.

P122

70 % ethanol preserves mycobacterial RNA from culture more efficiently than GTC-TCEP

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The efficient preservation of mycobacterial RNA molecules is crucial both for research and clinical applications. For *Mycobacterium tuberculosis* RNA-based viability assessment, e.g. RS ratio, has a substantially faster turnaround time than conventional phenotypic drug susceptibility testing. Searching for an alternative to the widely used RNA fixating buffer GTC-TCEP which requires storage at ultra-low temperatures, we compared it to 70 % ethanol fixation. For this, a suspension of cultured Mtb H37Ra was mixed with either 70 % ethanol or 4.5 M GTC-TCEP and stored in triplicate for up to 12 months at -80 °C, -20 °C, 4 °C or 30 °C. RNA extracts were analysed in terms of purity and integrity and reverse transcribed before the targets *icl*, *esxA*, *16S* and *sigA* were quantified by Mtb specific qPCR. Results show that both quantity and integrity of RNA were consistently higher when cultured Mtb was stored in 70 % ethanol. The normalised RNA quantity determined by qPCR remained comparable to fresh samples for up to 12 months at 4 °C when stored in 70 % ethanol. Storage at 30 °C resulted in heavily degraded RNA regardless of the fixative. Based on these findings we propose 70 % ethanol and 4 °C as adequate storage conditions for RNA preservation in cultured Mtb for up to six months. This could be a possible low-cost alternative for the preservation of samples collected in field conditions. However, a confirmation of these findings for clinical samples is still necessary to translate the findings to a clinical setting.

P123

Demonstration of targeted next-generation sequencing using the Oxford Nanopore Technologies tuberculosis assay for the detection of drug resistant tuberculosis in Kyrgyzstan

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We present the first results of an ongoing study that validates the Oxford Nanopore Technologies (ONT) targeted next-generation sequencing (tNGS) tuberculosis (TB) assay and compares its use to standard of care (SOC) drug susceptibility testing (DST) in Kyrgyzstan.

For validation, 82 smear-positive sputum samples were subjected to both the ONT tNGS TB assay (OND-CUST-KIT on MinION MK1B) and the Deeplex Myc-TB kit (GenoScreen, on MiSeq

(Illumina)). 6/82 samples had indeterminate results. Among the remaining 76 samples, concordance was 100% for isoniazid/rifampicin/amikacin/kanamycin; 98.6% for fluoroquinolones/capreomycin/linezolid, 96.0% for bedaquiline/clofazimine/ethambutol/streptomycin; 93.4% for pyrazinamide, and 92.1% for ethionamide. High concordance was observed between the two assays for high-confidence-graded mutations.

For the comparative study that will enrol 782 Xpert-TB-positive patients, sputum samples of participants from Kyrgyzstan, aged >=18 years with signs and symptoms of pulmonary TB, are being collected for testing with the ONT assay and SOC-DST (i.e. Xpert, line probe assay and phenotypic DST). From January-April 2024, 776 patients were enrolled, of which 140 (18%) had Xpert MTB/RIF detected. Of these, 75 were sequenced and 21 also had SOC-DST-results. SOC-DST revealed isoniazid but not rifampicin resistance in nine (43%) patients and rifampicin plus isoniazid resistance in ten (48%) patients. Generally, the ONT tNGS assay had results concordant with SOC-DST, but identified additional resistance, including to levofloxacin and moxifloxacin, in 18 patients.

This study shows the usefulness of ONT tNGS for the diagnosis of TB in Kyrgyzstan and highlights the potential of incorporating tNGS into routine TB diagnostic algorithms for accurate and timely treatment decisions.

P124

Establishing of drug susceptibility testing to Pretomanid

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Pretomanid has recently been proposed as a new anti TB-drug. Together with Bedaquiline, Linezolid, and Moxifloxacin it is one component of the revolutionary new six-months treatment regimen (BPaLM) recommended by WHO. Meanwhile this regimen is implemented in several regional treatment guidelines, as in Germany. To guide clinicians and to be prepared for surveillance of drug resistance to Pretomanid, we aimed to establish a robust drug susceptibility testing (DST) in our laboratory. We used the BACTEC MGIT 960 system in combination with the EpiCenter™/TB eXiST™software, that enables rapid drug susceptibility testing using any drug and drug concentration.

For establishing DST to Pretomanid we obtained pure drug from the The Global Alliance for TB

Drug Development. For preliminary MIC testing drug concentrations from 0.03 to 4.0 µg/ml in 2-fold dilutions were chosen, based on the study of Bateson et al. The H37Rv strain was included as control strain. Since differences in MIC values have been described for the various *M. tuberculosis* lineages as well as the animal adapted TB strains and *M. canetti*, we included TB strains from lineages 1 to 4 as well as *M. bovis* and *M. africanum*. Furthermore, we compared MIC values starting from solid culture (Löwenstein-Jensen) vs. MGIT liquid culture. We could confirm differences in MIC values between lineage 1 and the other TB strains and we additionally established a protocol to start from liquid MGIT medium to be able to include the Pretomanid DST into the routine DST together with other second line drugs.

P125

Experiences from a demonstration study on Nanopore sequencing for the diagnosis of drug-resistant tuberculosis in low-middle-income countries

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We are implementing a project to investigate the utility of nanopore sequencing for the diagnosis and surveillance of drug-resistant tuberculosis (DR-TB) and other lung diseases in national and decentralized laboratories in low-middle-income countries. We aim to generate evidence on the performance, patient-important outcomes, feasibility, acceptability, implementation costs and budget impact of using nanopore sequencing for this purpose. Here we present our first experiences with the implementation of this study.

The Oxford Nanopore Technologies (ONT, United Kingdom) targeted next-generation sequencing (tNGS) TB assay (OND-CUST-KIT) on MinION MK1B sequencers with wf-tb-amr (v2.0.0-beta.2) EPI2ME analysis workflow was implemented in Kyrgyzstan and Tanzania. Implementation is planned for Vietnam. Two two-weeks trainings were provided to the laboratory staff.

Sequencing is ongoing in Kyrgyzstan and Tanzania for validation purposes (n=200 samples tested) and for the investigation of patient-important outcomes in Kyrgyzstan (75/782 planned Xpert-positive TB patients enrolled). Implementation was delayed due to lengthy procedures for agreements, procurement and importation, and delays in delivery of equipment and reagents. Practical challenges included transcription errors in the preparation of the sample sheet for EPI2ME software analysis, lower sequencing capacity/flow cell than anticipated, and difficulties with updating the EPI2ME software (due to unstable internet connection).

Implementation of the ONT tNGS TB assay proved to be feasible with limited training. The recent workflow update improved the functionality and display of results of the EPI2ME analysis pipeline. The challenges and critical issues highlighted in this study will guide future development and scale-up of Nanopore sequencing for the detection of DR-TB.

P126

Xpert MTB/RIF Ultra versus Xpert MTB/RIF for the diagnosis of extrapulmonary tuberculosis

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Diagnosis of extrapulmonary tuberculosis (EPTB) is difficult due to the different clinical presentations, the need for invasive procedures to secure appropriate samples and the paucibacillary load in such samples. In recent decades, molecular techniques have been developed to improve its diagnosis. One of them is the GeneXpert platform. The aim of this study was to compare Xpert ultra versus Xpert MTB/RIF in samples from patients with suspected extrapulmonary tuberculosis.

A total of 2.186 samples from 1.924 patients was studied. All samples were collected between 2014 and 2022. The samples were decontaminated using the method described by Kent and Kubica. Following decontamination and concentration, the sediment of the specimens was used for microscopic examination, culture, and GenXpert MTB/RIF (Cepheid, Sunnyvale, CA) or Xpert Ultra during the years 2014-2019 and 2020-2022, respectively.

The overall sensitivity and specificity of the Xpert MTB/RIF and Xpert Ultra assay were 67.8%, 97.9% and 81,2%, 98,3% respectively (p=0.026). The samples studied with Xpert MTB/RIF and Xpert Ultra were 1.232 (56.3%) and 954 (43.6%) respectivelly. Smear microscopy was positive in 25 (1.14%) samples and negative in 2.161 (98.8%) samples. The largest number of samples studied corresponded to lymph node biopsies (766/35%), pleural fluid (389/17.7%) and cerebrospinal fluid (335/15.3%). The sensitivity with the Xpert Ultra for lymph node biopsies and pleural fluid with smear microscopy negative was 75% and 100% while with the Xpert MTB/RIF was 62.8% and 46.15% respectively. This increase in sensitivity of the Xpert Ultra was statistically significant ($p<0.05$).

Xpert Ultra has improved the diagnosis of extrapulmonary tuberculosis.

P127

Using targeted Next Generation Sequencing directly from sputum for comprehensive drug resistance prediction of Mycobacterium tuberculosis strains in Namibia

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In high tuberculosis (TB) burden countries access to drug susceptibility testing is a major bottleneck. Targeted Next Generation Sequencing (tNGS) is a promising technology for rapid resistance detection. This study assessed the performance of tNGS for detection of drug-resistant TB (DR-TB) directly from sputum in a university lab in Namibia. A total of 34 sputum samples (13 rifampicin resistant [RR] and 21 sensitive based on Xpert® MTB/RIF) from confirmed TB patients were subjected to tNGS using the Deeplex® Myc-TB kit and sequenced using the Illumina iSeq100 platform. Data were analysed on the Deeplex Myc-TB web application. 20 out of 34 (59%) were successfully sequenced, of which 19 had a complete resistance profile for all 13 anti-TB drugs evaluated by Deeplex® assay and one sample was reported as NTM. Of the successfully sequenced samples, tNGS identified 9 pan-susceptible, 3 mono-resistant, 6 multi-drug resistant TB and one extensively drug resistant (XDR)-TB strain. Seven out of 13 RR by Xpert® MTB/RIF were confirm using tNGS, of this 6 had additional resistance to one or more first-line drugs. With tNGS, resistance to bedaquiline was detected in one sample, resulting in an XDR-TB re-classification, which would have been missed by the current diagnostic tools in the country. Moreover, three samples that were initially defined as rifampicin sensitive, had mutations conferring resistance to Isoniazid, Pyrazinamide and Streptomycin. In conclusion, our data show that tNGS can be used to complement in the diagnosis of DR-TB and for the design of a timely introduced individualised treatment regimen.

P128

GPAS pipeline relatedness service for TB

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Whole genome sequencing (WGS) of *Mycobacterium tuberculosis complex* (MTBC) provides data which can be used to investigate the relatedness between groups of isolates with high resolution. Global Pathogen Analysis Service (GPAS) is a user-friendly, cloud-based bioinformatics tool which assembles and analyses MTBCs WGS data reporting relatedness.

To investigate relatedness, we used the 11 well described MIRU-VNTR-defined clusters reported in the Lancet Infectious Diseases 2013 https://doi.org/10.1016/S1473-3099(12)70277-3. GPAS generated a “Relatedness” csv file containing a pairwise matrix of all uploaded sequences within 20 single nucleotide polymorphisms (SNPs) of each other. Additional outputs included a table displaying all samples within 20 SNPs of the reference sample; a bar graph demonstrating the number of samples at each SNP distance from the reference sample (up until 20 SNPs) and a neighbor-joining phylogenetic tree consisting of samples within 20 SNPs of the reference sample. The table, bar graph and phylogenetic tree included interactive links, enabling detailed inspection of related sequences at an individual level. Using the GPAS outputs we were able to reconstruct the previously described cluster, demonstrating very close agreement with the originally reported SNP distances.

In conclusion, GPAS is an effective and user-friendly tool for analyzing the relatedness between MTBC whole genome sequences. The tool’s relatedness function may be particularly valuable in public health, facilitating detailed outbreak investigation and enhancing epidemiological surveillance capabilities.

P129

BTZ-043 exposure *in vitro* selects efflux pump mutants and BTZ-043, bedaquiline and clofazimine resistance in *Mycobacterium tuberculosis*

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Background

With the emergence of drug-resistant strains of *Mycobacterium tuberculosis* complex (MTBC), substantial efforts have been directed towards the development of innovative drugs for Tuberculosis treatment. Among the most encouraging lead compounds is the benzothiazinones BTZ-043 which is presently undergoing phase IIb clinical trials.

Methods

In light of the potential clinical significance of BTZ-043, we aimed to pinpoint potential synergistic interactions and novel resistance mechanisms using a stepwise approach, exposing wild-type strain of *M. tuberculosis H37Rv* ATCC 27294 to escalating concentrations of BTZ-043, and the generated resistance-associated variants (RAVs) were identified using the whole genome sequencing method. Mutant MIC values were confirmed for BTZ-043 in broth microdilution and for bedaquiline/clofazimine in MGIT960.

Results and conclusion

Genomic analysis of these BTZ-043-resistant mutants revealed the emergence of mutations in *Rv0678*, the negative regulator of the mmpS5/L5 drug efflux pump, and *dprE1*. Mutant MIC values showed a 4-16 fold and a 1000 fold increase compared to baseline for *Rv0678* and *dprE1* mutants, respectively. Moreover, all *Rv0678* mutants were found to be resistant to both bedaquiline and clofazimine.

Our experiments confirmed that *in vitro* generated *Rv0678* mutations confer a low-level cross-resistance to BTZ-043. While it remains uncertain whether *Rv0678* mutations would render benzothiazinones ineffective in TB treatment, these results underscore the significance of monitoring the clinically prevalent *Rv0678* mutations during the ongoing BTZ043 and other benzothiazinones clinical trials.

P130

Clusters of intrinsically delamanid/pretomanid cross-resistant strains of *Mycobacterium tuberculosis* in eastern Europe and central Asia

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Multidrug-resistant tuberculosis (MDR-TB) is a major public health issue, especially in many eastern European and central Asian countries. Using whole genome sequence data from Georgia, Kazakhstan, Moldova, Tajikistan, and Ukraine we identified a phylogenetically-cohesive cluster of 19 MDR-TB strains that shared the same mutation conferring cross-resistance to delamanid and pretomanid, *ddn* W88*. Estimation of the date of the most recent common ancestor of these samples indicated that the mutation in this lineage predates the use of both drugs. An additional 121 samples from other studies and multiple European reference laboratories yielded more examples from patients who appear to have travelled across Europe. These data also included samples collected before the clinical use of delamanid, further supporting a natural emergence of this cluster. In fact, *ddn* W88* evolved at least three times, once in lineage 2 and twice in lineage 4. The lineage 4 samples were from sublineages 4.1 and 4.8 and had different nucleotide mutations leading to the same amino acid change. The lineage 2 samples were from the widespread B0/W148/European/Russia outbreak lineage. To estimate the prevalence of delamanid/pretomanid cross-resistance due to this mutation, we analysed genomic data from published comprehensive surveys of culture-positive samples over proscribed time spans in Georgia and Moldova. Although the prevalence of *ddn* W88* was found to be low in these two countries, this still underscores the need to scale up the capacity for pretomanid drug-susceptibility testing across the WHO European Region to accompany the rollout of BPaL(M).

P131

Cathelidicin, Vitamin D isoforms levels during the first month of anti-tuberculosis therapy

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Vitamin D has preventive functions against infectious diseases, including tuberculosis. The active form acts as an immunomodulator against *Mycobacterium tuberculosis* infections. Here, we aim to correlate the vitamin D and isoforms levels and receptor genetics with the dietary patterns of patients on the first month of anti-tuberculosis therapy in Colón, Panama. A total of 80 tuberculosis patients and 50 healthy controls were recruited. A total of 25(OH)D and the Fok1,

Tak1, and Bsm1 haplotypes of the vitamin D receptors were determined by ELISA and RFLP-PCR, respectively. Levels of values of isoforms 25(OH)D, 25(OH)D2, 25(OH)D3, and 24,25(OH)2 were obtained by LC-MS/MS. Our results show that 63% (50/80) of tuberculosis patients have sufficient levels of total vitamin D, compared with 54% (27/50) of healthy controls. We observed a significantly higher level of total vitamin D in tuberculosis patients (41.7 ng/mL) compared to healthy controls (32.8 ng/mL) (Wilcoxon test $p<0.005$) in the first month of anti-TB therapy. When looking at vitamin D isoforms 25(OH)D2, 25(OH)D3, and 24,25(OH)D2, we found 0.22, 55.94, and 5.75 ng/mL, respectively, during the first month of anti-tuberculosis therapy. No significant differences were observed in cathelicidin levels between tuberculosis patients (58.7 ng/ml) and healthy controls (53.4 ng/ml). Only the *Bsm1* homozygote BB haplotype resulted in a significantly lower total vitamin D level when compared to healthy controls (32.0 vs 53.3 mg/mL $p<0.001$), respectively. We recommend a complete evaluation of the patient's nutritional and vitamin biochemistry before using vitamin D supplementation during anti-tuberculosis therapy treatment to enhance treatment outcomes.

P132

Acceptance of oral swabs for tuberculosis screening among healthcare workers: a qualitative study

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Reliance on sputum-based assays for the diagnosis of tuberculosis (TB) presents challenges, including occupational infection risk for healthcare workers (HCWs) and the need to collect samples from a symptomatic population. Oral swab (OS) collection may help mitigate these risks and challenges. This study aims to evaluate HCWs' perceived risks, opportunities, and barriers regarding OS use for TB screening among migrants in Italy.

Purposive sampling was used to enrol HCWs experienced with sputum and OS collection after which they were invited for an in-depth interview. Enrolment started in November of 2023 and will continue until July 2024 or until saturation among themes is reached. We adapted Hamilton's Rapid Qualitative Analysis Methods to summarise key findings.

All HCWs interviewed so far (N=4) indicated a preference for OS over sputum to support the scaling up of TB screening in migrants. All HCWs shared perceptions that OS reduces their occupational exposure to TB, is safer to perform, effective on both symptomatic and asymptomatic migrants, and is easier to analyse, with the possibility of pooling samples. All HCWs shared that supervision is necessary to collect a high-quality sample. All HCWs emphasised the need for solutions to language barriers and educational materials for migrants.

Preliminary results indicate that OS represents a safe tool to perform TB screening if complemented with educational resources tailored for the migrant populations.

P133

Routine investigations for tuberculosis on bronchoalveolar fluid lavage in a low-incidence setting: is it worth it?

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Italy has a low-incidence of tuberculosis (TB) (4.6/100.000 population); most cases occur in foreign-born residents from high-incidence countries. The value of routine investigations for TB on bronchoalveolar lavage fluid (BALF) in this setting is unclear.

In the Interventional Bronchoscopy Unit of a tertiary hospital in northern Italy screening diagnostics for TB on all BALF samples was implemented from 2016 with PCR (Xpert Ultra from 09/2018), microscopy and culture. We retrospectively analyzed all bronchoscopies performed from 01/09/2018 to 31/12/2023, assessing the rate of TB diagnosis (Xpert Ultra result as the index test) and the number of cases in which TB was not suspected (absence of: previous investigations for TB, written suspect of TB, IGRA test/tuberculin skin test performed).

In the study period, 1694 BALF samples were investigated for TB, n=47(2.8%) were Xpert Ultra positive. Of these, n=40(85%) were requested with TB suspect from either an infectious diseases specialist (n=34, 85%), or from a pneumologist/internal medicine specialist (n=6,15%). In seven cases, there was no clear TB suspect at the time of bronchoscopy (0.4% of all bronchoscopies performed without TB suspect). All patients but one were Italian-born, mean age was 75.5 years (SD 9.5). The underlying suspect was lung malignancy in three, atypical pneumonia in four. In all cases, typical radiological findings and symptoms were absent.

The positivity rate of routine TB investigations on BALF was low, but it led to seven unexpected TB diagnoses, which is non-negligible in a low-incidence setting. TB should be suspected in elderly patients presenting with atypical pneumonia/query of pulmonary malignancy.

P134

LiquidArray MTB-XDR VER 1.0 - a powerful molecular genetic tool for rapid diagnosis of extensively drug-resistant tuberculosis

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1: Bruker-Hain

Introduction: Extensively drug-resistant tuberculosis (XDR-TB) and pre-XDR-TB are a major challenge to TB control due to their complex diagnosis and obstacles in treatment. With LiquidArray® MTB-XDR VER 1.0 Hain Lifescience provides an assay for rapid diagnosis of XDR-

TB. Besides detection of Mycobacterium tuberculosis complex (MTBC) and its mutations conferring resistance to fluoroquinolones, amikacin, and ethambutol, the assay can also detect the most significant mutations conferring resistance to the group A drug Linezolid.

Methods: As starting material patient specimens and cultivated samples are used. DNA extraction is performed manually or automated with the GenoXtract® fleXT nucleic acid extraction system allowing high sample throughput with minimal hands-on time. Amplification, detection, and automated result interpretation is performed in the FluoroCycler® XT system within one single tube.

Results: An excellent performance of the assay could be demonstrated in the analytical study for all parameters investigated, including analytical sensitivity and analytical specificity. In an extensive clinical study, which focused on resistance detection for fluoroquinolones, amikacin, ethambutol and linezolid a high sensitivity and excellent specificity for MTBC and resistance detection was shown from sputum and culture samples.

Summary: LiquidArray MTB-XDR VER1.0 is a powerful tool for reliable detection of resistances related to XDR-TB.

P135

LiquidArray® *Mycobacteria direct*: Detection of MTBC and NTM directly from patient specimens

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Introduction:

Mycobacterial infections pose a major global public health challenge. Within the genus Mycobacteria, with over 200 different species, *M. tuberculosis* takes center stage as the main cause of tuberculosis. In addition, other nontuberculous mycobacteria (NTM) species like *M. avium* complex (MAC) also contribute to pulmonary infections. The varying severity and clinical presentations of mycobacterial infections emphasize the need for tailored treatment strategies based on the specific mycobacterial species.

We are developing the LiquidArray® *Mycobacteria direct* assay as an diagnostic tool for the detection and differentiation of clinically significant mycobacterial species in sputum samples.

Methods:

The assay is powered by LiquidArray® technology, which utilizes multiplexed PCR to amplify target DNA sequences, using the FluoroCycler® XT for simultaneous detection across 5 fluorescence channels. The LiquidArray® technology uniquely integrates fluorescent probes that enable the detection of multiple targets in a single reaction to save time, resources and sample material.

Results:

The application of target-specific melting curve analysis enhances the assay's performance, distinguishing between closely related species. The LiquidArray® *Mycobacteria direct* can detect and distinguish between several different species from over 200 different mycobacteria in one reaction. In addition, all other mycobacteria species are detected at the *Mycobacterium* genus level. Including DNA isolation, reliable and automatically evaluated results are obtained within 3 hours.

Summary:

The LiquidArray® *Mycobacteria direct* will be a powerful tool for the rapid identification of clinically relevant mycobacterial species directly from patient specimen paving the way for fast specific treatment and preventive measures.

P136

Analytical and Clinical performance evaluation of the FluoroType® MTBDR VER 2.0 with Dual Target Detection of *Mycobacterium tuberculosis* Complex from Native Sputum and Culture

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1: Bruker-Hain

Introduction

The WHO-endorsed FluoroType® MTBDR VER 2.0 is an important tool in diagnosis of TB and its resistance-mediating mutations against first-line antibiotics rifampicin and isoniazid. The multi-copy target IS*6110* was integrated into the FluoroType MTBDR VER 2.0 as a second target for *M. tuberculosis* complex (MTBC) detection. In this study we determined the analytical and clinical performance of the modified assay.

Methods

The modified FluoroType MTBDR VER 2.0 in combination the Liquefaction Set VER 1.0 allows easy and simultaneous detection of MTBC and its resistance to rifampicin and isoniazid directly from native sputum specimens. In total 8 workflows for manual and automated DNA extraction from native sputum and culture samples have been evaluated using the GenoXtract® and GenoXtract® fleXT instruments in combination with the FluoroCycler® XT.

Results

Integration of IS*6110* leads to a high sensitivity of MTBC detection. The FluoroType MTBDR VER 2.0 allows reliable detection and identification of most relevant mutations in *rpoB*, *katG* and the *inhA* promoter region responsible for resistance to rifampicin and isoniazid. It also identifies silent mutations within *rpoB*. The Liquefaction Set VER 1.0 inactivates and liquifies native sputum very effectively in one step. The excellent performance for MTBC and resistance detection was also demonstrated in a clinical study with ~600 patient samples.

Summary

The modified FluoroType MTBDR VER 2.0 enables sensitive detection of MTBC and identification of mutations directly from native sputum specimens. Additionally, NALC-NaOH decontaminated sputum samples and culture samples can be analyzed.

P137

Whole Genome Sequence of *Mycolicibacterium parafortuitum* Panama NTM 1 isolated from a cattle farm in Panama

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Non-tuberculosis mycobacteria (NTM) are environmental organisms that can infect humans and animals. They can be found in soil, dust, and water. *Mycolicibacterium parafortuitum* is a rapidly growing NTM. We aim to characterize and annotate a *de novo* genome assembly of *Mycolicibacterium parafortuitum* isolated from a cattle farm in Panama.

The feces, perianal zone, and milk samples were decontaminated with (N-acetylcysteine + NaOH 2%) and cultivated on Lowenstein-Jensen medium. The isolate was analyzed using biochemical and microbiological methods. Next-generation sequencing was performed using Illumina´s platform. Phylogenetic analysis was conducted using 16S rRNA sequence. Our results confirmed the isolate's identity as *Mycolicibacterium parafortuitum* Panama NTM 1 strain. Phylogenetic analysis demonstrated a close relationship with *Mycolicibacterium parafortuitum* strain JCM 6367. We observed a high conservation in gene content, including synteny between their genomes.

These results allowed us to confirm the identity of the *Mycolicibacterium parafortuitum* NTM 1 strain at the genomic level. The genomic characteristic provides novel information on the isolate of *Mycolicibacterium parafortuitum* NTM 1 in Panama. In addition, it provides the basis for future research on pathogenicity, evolution, and molecular biomarker studies of veterinary and public health importance.