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Invited Speakers

GL03

The evolutionary history and phylogeny of mycobacteria

R Brosch¹

1: Institut Pasteur

In the tree of life, the genus *Mycobacterium* is part of the phylum *Actinobacteria* and represents a single entity comprising almost 200 bacterial species, most of them representing environmental saprophytes, and some that can cause infections of different severity in humans and/or animals. Among the latter group, *Mycobacterium tuberculosis* is the causative agent of tuberculosis (TB), one of the deadliest infectious diseases in human history, which remains a huge public health concern in many countries even today. *M. tuberculosis* is part of a group of closely related mycobacteria belonging to the *M. tuberculosis* complex (MTBC), which can be subdivided into several lineages of human- and animal-adapted strains, thought to have shared a last common ancestor emerged by clonal expansion from a pool of recombinogenic *Mycobacterium canettii*-like mycobacteria. A better understanding of how MTBC populations evolved from less virulent mycobacteria may allow for discovering improved TB control strategies and future epidemiologic trends. Here, I will highlight latest insights into the evolution of mycobacteria at the genus level, describing selected milestones in their evolution, with focus on the genomic events that have likely enabled the emergence and dominance of the MTBC. I will also review the various MTBC lineages and highlight their particularities and differences with focus on host preferences and geographic distribution. Finally, I will focus on putative mechanisms driving the evolution of tubercle bacilli and mycobacteria in general, by taking the mycobacteria-specific distributive conjugal transfer as an example.

GL04

Canonical fact versus hypothesis testing to decipher transmission of non-tuberculous and tuberculous mycobacteria: a comparative review

JF Guégan¹ KM Fast^{* 2} C Chevillon^{* 1} M Cobos-Mayo³ A Aliaga Samanez^{1 3}
M Dogbe⁴ M Scott² K Waters⁵ MK Benbow⁶ JL Pechal⁵ JP Receveur⁷
MW Sandel^{2 8} HR Jordan⁴ ME Benbow^{5 9 10 11}

1: MIVEGEC (UMR Université de Montpellier, CNRS, IRD), France 2: Department of Wildlife, Fisheries and Aquaculture, Mississippi State University, USA 3: Grupo de Biogeografía, Diversidad y Conservación, Departamento de Biología Animal, Facultad de Ciencias, Universidad de Málaga, España 4: Department of Biological Sciences, Mississippi State University, USA 5: Department of Entomology, Michigan State University, USA 6: Department of Pediatrics, College of Osteopathic Medicine, Michigan State University, USA 7: Institute for Genome Sciences, University of Maryland, USA 8: Forest and Wildlife Research Center, Mississippi State University, USA 9: Department of Osteopathic 22 Medical Specialties, Michigan State University, USA 10: Ecology,

Despite the clinical relevance of major tuberculous pathogens to domestic animals and humans, the understanding of mycobacterial transmission modes, pathways and interactions in their natural habitats remain very limited. The reason for this is primarily because ecological and evolutionary concepts have not yet been widely applied to the understanding of these bacteria. Most existing research on mycobacterial transmission is not founded on hypothesis testing but rather tends to accept the most recent explanation and turn it into a canonical fact. In this invited conference, we discuss plausible alternative hypotheses against a null hypothesis of environmental origin to intensify research on mycobacterial pathogens and their capacity to spread in the context of global change. We highlight a major bias in perceptions of the transmission of mycobacterial infections, with most work concentrating only on the contagious stage of tuberculous antimicrobial resistant clones, and we suggest broadening the field to include research on environmental non-tuberculous mycobacteria and their life histories. A deeper understanding of mycobacterial ecology and evolution is more important now than ever, considering the vast diversity of known and unknown mycobacterial species in natural ecosystems. Infectious disease medicine, veterinary science and public health surveillance should take a more integrative disease ecology approach to enhance the development of new approaches for control of these animal and human pathogens.

**Equally contributed to the work*

GL07

Early host immune determinants of protection and disease progression in tuberculosis

W Branchett¹

1: Crick Institute, London

The early immune mechanisms determining tuberculosis (TB) outcome are unclear. Laboratory mouse strains exhibit variable susceptibility to TB and can therefore be used to study mechanisms of host protection or disease progression during experimental *M. tuberculosis* infection. The C3HeB/FeJ mouse strain is hypersusceptible to TB and displays lung pathology and blood transcriptional signatures during established disease comparable to those from human TB patients. However, the early host determinants underpinning failed control of *M. tuberculosis* infection in these mice are not known. We now describe distinct early immune response kinetics and lesion organisation in the lungs of TB-susceptible and -resistant mice and highlight common signatures in our parallel analysis of airway cells from TB patients and their contacts.

GL08

Progress and challenges in tuberculosis vaccine development

H McShane ¹

1: *The University of Oxford*

In 2023, more than 10 million people developed TB and more than one million died from this disease. A universally and highly effective vaccine would be game-changing for global TB control. The only licensed vaccine against TB, an attenuated strain of *Mycobacterium bovis*, Bacille Calmette Guerin (BCG) is more than 100 years old. Whilst BCG is effective at preventing disseminated disease in childhood, the efficacy against pulmonary disease is highly variable and very low in TB high burden countries. However to date only one of 4 candidate vaccines evaluated in human efficacy trials has shown any significant efficacy.

Mycobacterium is a complex intracellular pathogen which is adept at evading and subverting host immunity. The uncertain predictive value of preclinical animal models and the lack of validated correlates of protection mean that vaccine R&D is difficult and high risk for investment. In vaccine R&D for other complex pathogens, controlled human infection models have been invaluable in de-risking vaccine R&D in small scale studies prior to committing to large field efficacy studies. It would not be ethical to deliberately infect people with *Mycobacterium tuberculosis*, however attenuated *M.bovis* BCG may be an acceptable surrogate challenge agent. We have established a skin and aerosol BCG human challenge model and validated both models against a BCG vaccine effect in the UK. These models can also be utilised to define the immunobiology of infection in humans. Data from recent studies will be shown.

GL09

Rethinking NTM management: new drugs, new approaches, new hope

N Lorent ¹

1: *KU Leuven*

Nontuberculous mycobacteria (NTM) present a growing clinical challenge due to their intrinsic resistance and the complexity of treatment regimens. This lecture will provide an overview of current therapeutic approaches to NTM infections, encompassing both antibiotic and non-antibiotic strategies. Emphasis will be placed on emerging compounds under investigation and the latest evidence guiding their potential use. Ongoing clinical trials and research gaps will also be highlighted, offering insights into future directions for NTM management.

GL10

Neglected mycobacterial diseases - Continuing challenges for a hopeful future

L Rigouts ¹

1: *Institute of Tropical Medicine, Antwerp*

Buruli ulcer (BU), caused by *Mycobacterium ulcerans*, is the third most significant mycobacterial disease after tuberculosis and leprosy. Though geographically restricted, BU remains endemic in 22 countries, with most cases in Africa and the Western Pacific. Children are predominantly affected in Africa, while adults are more commonly affected in Australia.

The disease primarily targets the skin through the action of **mycolactone**, a toxin that causes painless ulcers by suppressing immune responses and destroying tissue. Diagnosing BU is challenging due to its similarity to other skin conditions. The gold standard is **IS2404-based PCR**, known for its high sensitivity and specificity, but access is often limited in resource-poor settings.

Recent innovations aim to bring diagnostics closer to the point of care. **Mobile qPCR platforms**, like Biomeme, and **LAMP (Loop-mediated isothermal amplification)** assays, including heat-block and pocket-warmer formats, show promise. **P-LAMP**, incorporating a locked nucleic acid probe, enhances specificity and speed, though it requires further validation.

Efforts to detect mycolactone directly through TLC, ELISA, or lateral flow assays are ongoing but limited by technical and biochemical challenges. No validated serological test exists due to weak antibody responses and antigen cross-reactivity.

Treatment has evolved toward shorter, all-oral regimens. **Telacebec (Q203)**, a new drug targeting the bacterial respiratory chain, shows strong potential, especially since *M. ulcerans* often lacks a backup oxidative pathway. Genetic studies suggest low risk of resistance.

In summary, BU diagnostics and treatment are advancing, with molecular tools and novel therapies offering hope for more accessible, effective care in endemic regions.

GL11

Leprosy: back to the future

E Cambau ¹

1: *Mycobacteriology lab, French National reference center for mycobacteria, University Paris Cité, Inserm UMR1137, GHU APHP Nord, Bichat Hospital, Paris, France*

Member of the Technical advisory group of the WHO Global Leprosy programme

Leprosy is one of the oldest infectious diseases, caused by *Mycobacterium leprae* discovered by Gerhard Armauer Hansen in 1873, i.e. before Robert Koch discovered *Mycobacterium tuberculosis*. A second leprosy bacilli, *M. lepromatosis*, was discovered in 2008. Although leprosy appears today as a neglected tropical disease, the infection was all over the world for many centuries before it remains where were concentrated poverty, default in hygiene and lack of medical access, whatever the latitude. Despite significant advances in prevention, treatment, and control, ca. 200 000 new cases of leprosy are notified per year to WHO Global Leprosy Programme. Among these cases, 15% are children below 15 y.o. which demonstrates that transmission of *M. leprae* is still

ongoing in many countries. It is well known that *M. leprae* is not growing *in vitro*, can multiply experimentally in mouse footpads and naturally in nine band-armadillos, resulting in a zoonosis. Bacterial characteristics are the longest bacterial doubling time, estimated as 14 days, growth preference at 33°C, and a small defective genome of 3.27 Mb. This may explain why it multiplies in the skin and peripheral nerves and rarely in other organs. Clinical diagnosis is difficult because the disease manifestations are related to the immune response of the host. Irreversible disabilities are mainly seen when cellular immunity is triggered in tuberculoid forms and leprosy reactions. Strikingly when immune response is low (lepromatous form), clinical signs and symptoms are rare delaying the diagnosis but transmission is the highest. In 2025, diagnosis tools include PCR, WGS and other modern microbiological techniques but still there are no tools for latent infection detection and early diagnosis, especially for neurological forms. Electromyography and nerve echography can help a lot for screening nerve damage, but are still rare in field conditions. Mental health issues affect populations globally, since many countries still have discrimination laws against leprosy patients. Efforts to eliminate leprosy have focused on medical interventions such as multidrug therapy associating dapson, rifampicin and clofazimine, prevention with BCG vaccination and now with the implementation of post exposure chemoprophylaxis (PEP) given to patient contacts. How much the wide distribution of single dose of rifampicin will increase the risk of antimicrobial resistance in leprosy, is still unknown. Surveillance of AMR starts to be organized but needs resources and new user-friendly techniques. Research and financial helps are still concentrated in old non-governmental organisations, when we know so little about the bacteria, the infection, the transmission and when we need new therapies. New antituberculous drugs, such as bedaquiline and telacebec, might ended as antileprosy agents, for the sake of leprosy patients.

GL12

From abyss to Mycobacterial drug discovery

[H Myllykallio](#)¹

1: CNRS, Ecole polytechnique; Palaiseau, France

Despite archaea and mycobacteria being evolutionary unrelated, our bioinformatics and experimental studies using the hyperthermophilic archaeon *Pyrococcus abyssi* have led to the identification of novel thymidylate synthase and mismatch repair enzymes in *Mycobacterium tuberculosis* (*Mtb*). In this talk, I will describe how these unexpected discoveries have contributed to fighting and understanding the emergence of anti-microbial resistance against tuberculosis.

Mtb uses the alternative flavin-dependent thymidylate synthase family, ThyX, for the synthesis of an essential DNA precursor. This enzyme does not exist in humans, and it is structurally and mechanistically distinct from human thymidylate synthase, thus making it an ideal drug target. We have exploited the unique nucleotide binding pocket of ThyX proteins to identify non-substrate-based, tight-binding ThyX inhibitors using a target-based screening. The identified inhibitors selectively inhibited the growth of genetically modified bacteria dependent on thyX in a manner mimicking a genetic knockout of thymidylate synthase. Our inhibitors with anti-mycobacterial activity bind within the conserved active site of the tetrameric ThyX enzyme, at the interface of two monomers, partially overlapping with the dUMP binding pocket. Our studies provide new chemical tools for investigating the ThyX reaction mechanism and establish a novel mechanistic and structural basis for inhibition of thymidylate synthesis in *Mtb*. Currently, we are, in collaboration, developing *Mtb*-specific AI technologies for optimization of ThyX inhibitors, as well as drug repurposing.

The apparent absence of the canonical mismatch repair (MMR) system in many archaea and actinobacteria led to the discovery of a novel mismatch repair enzyme that uniquely corrects mismatches by creating a double-strand break. NucS. We are currently investigating whether this enzyme is involved in a non-canonical mismatch repair (MMR) mechanism in *Mtb*. We have also recently performed a large-scale bioinformatics analysis that causally linked the defects of DNA repair to drug resistance in at least 12.5% of clinical isolates with available genome sequences. Notably, a number of the detected single-nucleotide polymorphisms were positively selected during *Mtb* evolution. Our findings highlight the role of 3 R gene mutations in resistance, emphasizing the need for surveillance to improve early detection and control strategies.

GMeissner

Subclonal *mmpR5* SNPs co-occurring with frameshifts reveal hidden diversity of bedaquiline resistance mechanisms

[JE Phelan](#)¹ S Campino¹ TG Clark¹

1: London School of Hygiene and Tropical Medicine

Resistance to BDQ can emerge through loss-of-function (LOF) mutations in *mmpR5*, a transcriptional repressor of the *mmpL5/mmpS5* efflux pump. In clinical samples, multiple LOF mutations are often found at non-fixed allele frequencies, suggesting independent acquisition events within a single host. While LOF mutations are associated with resistance, the role of single nucleotide polymorphisms (SNPs) remains poorly understood. We hypothesise that SNPs of unknown significance, when co-occurring with *mmpR5* LOF mutations at subclonal frequencies, may represent alternative resistance mechanisms and contribute to resistance. To investigate this, we analysed *mmpR5* mutations in 173,816 *M. tuberculosis* genomes. Candidate SNPs were identified by selecting samples containing both a frameshift mutation and a missense SNP in *mmpR5*, each at subclonal frequencies (<90%). This approach identified 141 unique missense SNPs (observed 284 times) co-occurring with 69 frameshift mutations (303 occurrences) across 246 samples. The missense SNPs were detected in 1 to 59 samples and exhibited allele frequencies ranging from 10% to 97%. Of the candidate SNPs, 45 (31.9%) were observed in more than one sublineage, consistent with repeated selection likely driven by drug exposure. Enrichment analysis revealed that these mutations were significantly associated with multi-drug resistant (MDR; 20.3%) and extensively drug-resistant (XDR; 68.7%) isolates. Our findings suggest that a subset of *mmpR5* SNPs—currently unclassified—may be under positive selection in BDQ- or clofazimine-exposed strains. These mutations warrant further functional characterisation, and clinical isolates carrying them should be carefully evaluated for BDQ/CFZ susceptibility.

Oral Presentations

OR01

Molecular epidemiology of tuberculosis in Ukraine: A nationwide whole-genome sequencing study (2020–2023)

D Butov^{1 2} T Butova³ M Kuzhko⁴ A Rosenthal⁵ C Gerlach² A Grinev⁵
D Hoppes⁵ V Vekshyn⁶ L Abramova⁶ V Dreyer^{* 2} S Niemann^{* 2}

* - equal contribution²

1: Department of Infectious Diseases, Children's Infectious Diseases and Phthiology, Kharkiv National Medical University, Ukraine 2: Molecular and Experimental Mycobacteriology Group, Research Center Borstel, Germany 3: Outpatient Department, Merefa Central District Hospital, Ukraine 4: National Scientific Center of Phthiatry, Pulmonology and Allergology named after F. G. Yanovskyi, National Academy of Medical Sciences of Ukraine 5: Office of Cyber Infrastructure and Computational Biology, U.S. National Institute of Allergy and Infectious Diseases, USA 6: Central Scientific and Research Laboratory, Kharkiv National Medical University, Ukraine

Objective: To study tuberculosis (TB) epidemiology in Ukraine incl. the distribution of strains of different *Mycobacterium tuberculosis* complex (Mtb) lineages and drug resistance rates among pulmonary tuberculosis patients across Ukraine between 2020 and 2023 based on high resolution whole genome sequencing (WGS) analysis.

Methods: We conducted a nationwide study enrolling culture-confirmed TB patients across 18 regions of Ukraine (2020–2023). WGS was performed on 4,332 Mtb isolates using Illumina sequencing platforms. Lineages (L) and genotypic resistance profiles were determined via the bioinformatic pipeline MTBseq. Clinical, microbiological, and epidemiological data were curated in the NIAID TB Portals.

Results: Out of the 4,332 Mtb stains, 3,245 (74.9%) were at least resistant to rifampicin (RR), 2,005 (46.3%) Mtb strains were classified as multidrug resistant (MDR). Resistance to fluoroquinolone was detected in 1,183 (27.3%) Mtb strains, 1,086 strains (33.5% of RR) were classified as pre-extensively drug resistant (XDR). Strains of L2 (Beijing) accounted for 69.9% of the strains investigated, followed by strains of L4 (30.0%). The prevalence of L2 strains significantly increased from 66.9% in 2020 to 71.7% in 2023 ($p \leq 0.05$). L2 strains were dominant in 13 out of 18 regions ($p \leq 0.001$), while L4 strains were more prevalent in a minority of western regions of Ukraine. Furthermore, L2 strains were more frequently MDR, and pre-XDR than L4 strains.

Conclusions: Our findings demonstrate high rates of fluoroquinolone resistance, especially in L2 strains, which potentially compromise the use of the new shorter drug-resistant TB regimens. These results underscore the dynamic molecular landscape of TB in Ukraine and highlight the need for lineage-adapted surveillance and treatment strategies.

OR02

CRISPR and phage footprints reveal an aquatic ancestry for the tuberculosis pathogen

A Ghodousi^{1 2} M Omrani² C Gaudin³ R Antoine³ P Supply³ DM Cirillo²

1: Vita-Salute San Raffaele University 2: San Raffaele Scientific Institute 3: Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, CIL - Center for Infection and Immunity of Lille

The evolutionary origin of the *Mycobacterium tuberculosis* complex (MTBC) remains unresolved, with prevailing theories proposing emergence from terrestrial reservoirs. Here, we investigated the early ecological history of the MTBC progenitor by analyzing molecular imprints of past microbial interactions preserved in the genomes of *M. canettii*, extant members of the MTBC, and MTB-associated phylotype (MTBAP) species. We explored CRISPR spacer repertoires and prophage-associated genomic elements to trace past exposures to mobile genetic elements (MGEs).

Comprehensive BLAST analyses of CRISPR spacers revealed targeted sequences matching prophages and mobile elements predominantly from aquatic mycobacteria, including *M. marinum* and sponge-associated species. Notably, multiple *M. canettii* strains harbored spacers with high-identity matches to prophages infecting aquatic hosts, and a substantial prophage region in *M. canettii* STB-I showed remarkable collinearity with a *M. marinum* phage genome. Parallel analyses of integrated prophage-like regions in MTBAP genomes (*M. lacus*, *M. shinjukuense*, *M. riyadhense*) also highlighted predominant affiliations with aquatic and waterborne bacteria.

The convergence of independent evidence from CRISPR spacer targeting and prophage fossil records strongly suggests that the progenitor of the MTBC evolved within an aquatic environment, with sponges and aquatic microbiomes likely acting as key ecological reservoirs. These findings challenge traditional soil-based hypotheses and offer new perspectives for understanding tuberculosis evolution and potential environmental sources of emerging pathogenic mycobacteria.

OR03

Interred mechanisms of resistance and host immune evasion revealed through network-connectivity analysis of *M. tuberculosis* complex graph pangenome

F Valafar¹ ME Espinoza¹ A Swing¹ A Elghraoui¹ SJ Modlin¹

1: San Diego State University

Mycobacterium tuberculosis complex survives environmental pressures through mechanisms of rapid adaptation which remain poorly understood despite knowledge gained through decades of research. In this study, we used 110 reference-quality, complete *de novo* assembled, long-read sequenced clinical genomes to study patterns of structural adaptation through a graph-based pangenome analysis, elucidating rarely studied mechanisms that enable enhanced clinical phenotypes offering a novel perspective to the species' adaptation. Across isolates, we identified a pangenome of 4,325 genes (3,767 core and 558 accessory), revealing 290 novel genes, and a

substantially more complete account of difficult-to-sequence *esx/pe/pgrs/ppe* genes. Seventy-four percent of core genes were deemed non-essential *in vitro*, 38% of which support the pathogen's survival *in vivo*, suggesting a need to broaden current perspectives on essentiality. Through information-theoretic analysis, we reveal the *ppe* genes that contribute most to the species' diversity—several with known consequences for antigenic variation and immune evasion. Construction of a graph pangenome revealed topological variations that implicate genes known to modulate host immunity (*Rv0071-73*, *Rv2817c*, *cas2*), defense against phages/viruses (*cas2*, *csm6*, and *Rv2817c-2821c*), and others associated with host tissue colonization. Here, the prominent trehalose transport pathway stands out for its involvement in caseous granuloma catabolism and the development of post-primary disease. We show paralogous duplications of genes implicated in bedaquiline (*mmpL5* in all L1 isolates) and ethambutol (*embC-A*) resistance, with a paralogous duplication of its regulator (*embR*) in 96 isolates. We provide hypotheses for novel mechanisms of immune evasion and antibiotic resistance through gene dosing that can escape detection by molecular diagnostics.

OR04

Genome-wide association analysis of *Mycobacterium tuberculosis* lineage 2 reveals key factors for its enhanced epidemic success

N Gharbi^{1 2} E Rousseau^{3 4} M Merker^{4 5} S Niemann^{1 3 4} T Wirth^{1 2}

1: EPHE, PSL University, Paris, France 2: Institut de Systématique, Evolution, Biodiversité, ISYEB, Muséum national d'Histoire naturelle, CNRS, Sorbonne, Université des Antilles, Paris, France

3: Molecular and Experimental Mycobacteriology, Research Center Borstel, Germany 4: Evolution of the Resistome, Research Center Borstel, Germany 5: German Center for Infection Research, Partner site Hamburg-Lübeck-Borstel-Riems, Germany

The *Mycobacterium tuberculosis* (*M.tb*) lineage 2, known as the Beijing genotype, is closely associated with the spread of multidrug-resistant strains and exhibits increased virulence and transmission efficiency compared with other *M.tb* lineages, thereby significantly impacting the global burden of tuberculosis. This study aims to elucidate the biological factors driving the epidemic success of this lineage through genome-wide association (GWAS) and transmission analysis. We implemented an innovative approach combining GWAS and an epidemiological success index, time-scaled haplotypic density (THD), to assess transmission dynamics and underlying mutations. This method was applied to a global whole-genome dataset of 10,646 *M.tb* isolates from 15 geographical regions, encompassing the full diversity of the Beijing genotype. The geographical distribution of subclade diversity shows regional patterns, with a notable overrepresentation of modern sublineages. Among them, the L2.2.M4 sublineage stands out, including several subgroups associated with major local outbreaks, such as the Russian clone W148, the Central Asian outbreak (CAO) and a South African subgroup (L2.2.M4.4), each showing increased epidemic success. Our pangenomic association study found 39 significant loci ($P < 0.05$) in genes related to drug tolerance, virulence, persistence and adaptive mechanisms. A quarter of these loci were either convergent across all modern subclades or specific to highly transmissible groups. Selection analysis using dN/dS ratios indicated that some of these positions were under diversifying selection. Overall, these results highlight the role of specific mutations in the epidemic success of the associated sublineages and contribute to a better understanding of the enhanced adaptive landscape of the Beijing lineage.

OR05

Standardised protocol for the detection and quantification of nontuberculous mycobacteria in tap water and its application in investigating the source of clinical infections

C Allam^{1 2} S Haenn³ E Giraud^{1 2} Z Awad^{1 2} J Robert^{1 4} E Cambau^{1 2} L Moulin³
F Mougari^{1 2}

1: Centre National de Référence des Mycobactéries et de la Résistance aux Antituberculeux (CNR MyrMA) 2: AP-HP Nord, site Bichat; Université Paris Cité, IAME, France 3: Eau de Paris, Direction de la Recherche et du Développement pour la Qualité de l'Eau, R&D Biologie, Ivry sur Seine, France 4: APHP Sorbonne Université, INSERM, Centre d'Immunologie et des Maladies Infectieuses, Paris, France

Nontuberculous mycobacteria (NTM) are emerging pathogens causing pulmonary and extrapulmonary diseases, including healthcare-related infections. Although water is one of the main infection sources, isolating NTM from water samples is rarely done due to the NTM fastidious growth and lack of a standardised method. We set a protocol for NTM cultivation, measured its repeatability (n=20 experiments, 5 for each condition), reproducibility (n=20, 5 for each condition), and performed an inter-laboratory comparison (n=6) using sterile and tap water samples spiked with slowly growing (*M. avium*) and rapidly growing (*M. chelonae*) NTM, plus artificial contamination (*Pseudomonas aeruginosa*). We investigated water-related NTM infections using this protocol from 2014 to 2024. The protocol showed good repeatability, reproducibility, and recovery yield (range: 67–131%) for quantifying NTM in artificial samples. The inter-laboratory agreement was 83%. It was consistent with different growth media and temperatures. The addition of *P. aeruginosa* did not affect NTM recovery. We used the protocol in 13 investigations, involving 24 patients. NTM were detected in 12/13 investigations (92%, 23 patients) with NTM species being the same as patients' isolates in 7/12 (54%, 18 patients). Water was identified as a source of infection in 6/7 investigations (86%, 15 patients) based on matching genotypes. This study provides a standardised protocol for detecting and quantifying NTM colonies in tap water, which allows identifying the source of clinical infections. With an increase in NTM infections, using this method in mycobacteriology and in environmental laboratories would lead to better prevention measures.

OR06

Indoor water systems as reservoirs for clinically relevant non-tuberculous mycobacteria in Germany

M Diricks¹ D Frank² I Friesen¹ S Niemann¹ T Wichelhaus² N Wetzstein²
A NTMscape-ECO study group^{1 2 3}

1: Research Center Borstel 2: Goethe University Frankfurt 3: San Raffael Scientific Institute

Non-tuberculous mycobacteria (NTM) are environmental bacteria increasingly recognized as causes of severe disease, particularly in vulnerable individuals. Yet, key reservoirs and routes of human exposure remain insufficiently characterized. In this study, we investigated the presence and genetic diversity of NTM in 102 indoor water samples collected from private households and

a hospital in Frankfurt am Main, Germany. Isolates were characterized via whole genome sequencing, drug susceptibility testing, and core genome multi-locus sequence typing (cgMLST) to assess potential links with nearly 3,000 global clinical and environmental NTM isolates. NTM were isolated from 19.5% of households and 14.7% of samples, with a higher recovery from shower water (29.3%) compared to tap water (9.2%). Eight different species were identified, including one putative novel species. Rapid and slow-growing NTM were dominated by *M. chelonae* and *M. chimaera*, respectively. None of the isolates were resistant to macrolides or aminoglycosides. Several isolates from household water showed high genetic similarity to clinical strains from Germany and abroad. Strikingly, we detected a *M. abscessus* strain belonging to the dominant circulating clone DCC3 – which is one of the most frequently isolated clones from NTM patients globally – in a shower water sample. Additionally, we describe for the first time dominant circulating clones of *M. chelonae*. These findings reinforce the relevance of indoor water systems as reservoirs for clinically important NTM and emphasize the need for targeted One Health interventions to minimize exposure, particularly among at-risk populations.

OR07

TB through a One Health lens: Challenges in a rural South African setting

D Venter¹ J Musoke² [AL Michel](#)^{1,3}

1: University of Pretoria 2: University of the Free State 3: Utrecht University

Food-producing animals are a source of non-tuberculous mycobacteria (NTM) and, most importantly, of *Mycobacterium bovis* (*M. bovis*), known to cause bovine tuberculosis in cattle and zoonotic TB in humans. The routine approach used by medical laboratories in South Africa to diagnose tuberculosis (TB) in patients is based on the GeneXpert technology, which is unable to speciate *M. bovis*. We implemented an integrated investigation of TB transmission between cattle and humans in a rural setting with a high human TB incidence and endemic *M. bovis* infection in communal cattle herds in northern KwaZulu-Natal, South Africa.

Sputum samples from GeneXpert-positive patients and aspirates from cases with possible extrapulmonary TB were cultured for Mycobacteria using the Bactec MGIT 320 system and Löwenstein-Jensen medium. The TB status of cattle herds owned by TB patients was determined using the Bovigam® assay and culture of milk and nasal swabs.

To date, *M. tuberculosis* was isolated from 28 out of 227 (12.3%) TB patients and NTM from 11 patients (4.8%). *M. bovis* was not isolated. A total of 64 cattle herds owned by TB-confirmed farmers were tested for bovine tuberculosis, revealing a herd prevalence of 10.9%. *M. bovis* was isolated from 5/37 (13.5%) and NTM was isolated from 14 of the 37 (38%) milk samples collected. The preliminary findings corroborate the risk of zoonotic transmission of *M. bovis* and NTM via raw milk, although the analyses to date could not confirm *M. bovis* infection in TB patients in this ongoing study.

OR08

Nontuberculous Mycobacteria in Denmark: Seven decades of clinical occurrence

XES Iversen¹ A Norman¹ DB Folkvardsen¹ EM Rasmussen¹ E Svensson¹
L Rigouts² C Meehan^{2,3} L Jelsbak⁴ T Lillebaek^{1,5}

1: International Reference Laboratory of Mycobacteriology, Statens Serum Institut, Denmark
2: Mycobacteriology/Institute of Tropical Medicine (ITM), Antwerp, Belgium 3: Department of Biosciences, Nottingham Trent University, UK 4: Department of Biotechnology and Biomedicine, Technical University of Denmark 5: Global Health Section, Department of Public Health, University of Copenhagen, Denmark

The prevalence of nontuberculous mycobacteria (NTM) disease continue to rise and remain a challenge worldwide. Taking advantage of the International Reference Laboratory of Mycobacteriology (IRLM)'s collection of freeze-dried mycobacterial isolates, we investigated changes in NTM epidemiology and species distribution in humans over the past seven decades. This study compares historical NTM-designated isolates ($n=666$) from human samples (1948–1978) to contemporary (2013–2022) NTM isolates ($n=1820$) identified in routine diagnostics. Survey of patient metadata showed median ages of 54.9 and 57.4 year for the historical and contemporary isolates, respectively. Species identification was performed using GenoType CM assay, which differentiates among the most common mycobacteria in clinical settings today. For historical isolates, 16 distinct species categories were identified, with the majority remaining unclassified, designated as *M. species* (32.13%). In contrast, recent isolates were dominated by *M. avium* (39.62%), while *M. species* accounted only for 7.8%. Among the most abundant characterizations, *M. avium* and *M. abscessus* showed largest changes and increase in prevalence over time. Further species determination by whole-genome sequencing was performed for most historical isolates characterised as *M. species*, revealing a number of species not usually encountered in diagnostic settings today.

These findings suggest significant changes in the mycobacterial species involved in NTM infections in Denmark over the past seven decades, likely influenced by broader societal and environmental factors. However, definitive conclusions regarding changes in NTM epidemiology cannot be made due to limited information about the criteria for storing the historical isolates.

OR09

Mycobacterium tuberculosis trehalose synthesis pathways as key determinant of tuberculosis disease progression

J Alonso-del-Real^{1,4} M Aleluia^{2,5} E Aledavood^{3,6} M Torres-Puente^{1,4} R Gonçalves^{2,5}
V Furió¹ AI Fernandes^{2,5} C Gil^{3,6} M Saraiva^{2,5} I Comas^{1,4}

1: Instituto de Biomedicina de Valencia (CSIC) 2: Instituto de Investigação e Inovação em Saúde
3: Centro de Investigaciones Biológicas Margarita Salas 4: Instituto de Biomedicina de Valencia, Spanish Research Council 5: Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Portugal 6: Centro de Investigaciones Biológicas Margarita Salas, Spanish Research Council

Antimicrobial resistance is a major contributor to tuberculosis (TB)-related mortality. Host-pathogen interaction-based strategies may offer alternatives that circumvent resistance mechanisms. We investigated early events in *Mycobacterium tuberculosis* (Mtb) infection by RNA-seq, using both an *in vitro* culture model and an *in vivo* murine model resembling human infection. Two clinical Mtb isolates from L4.3/LAM sublineage, obtained from mild and severe TB patients respectively, were included. Despite their close genetic distance (302 bp), the isolates exhibited 1,798 differentially expressed genes under infection conditions. Notably, the severe TB-associated strain showed upregulation of trehalose biosynthesis genes (*treY-treZ*, *treS*), corroborated by metabolomic profiling in infected bone marrow derived macrophages. Given trehalose's role in inducing autophagy via TFEB activation, we propose that this strain may leverage this pathway to reduce IL-1 β production, a key compound in host immune response modulation by Mtb. Our findings highlight potential strain-specific strategies employed by Mtb during early infection. Moreover, targeting trehalose biosynthesis pathways may represent a promising avenue for therapeutic development. Ongoing studies include *in vitro* validation of candidate compounds identified through *in silico* screening.

OR10

Region-specific spatial transcriptomics reveal distinct immunological functions in human tuberculosis granulomas

S Marwitz¹ N Shublazde² S Vashakidze^{2,3} I Khurtsilava² M Shurgaia² A Rosenthal⁴
A Gabrielian⁴ A Grinev⁴ T Goldmann¹ UE Schaible^{5,6} T Dallenga^{5,6}

1: Department of Histology, Research Center Borstel, Germany 2: National Institute for Tuberculosis and Lung Diseases, Tbilisi, Georgia 3: University of Georgia 4: Office of Cyber Infrastructure and Computational Biology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, USA 5: Cellular Microbiology, Research Center Borstel, Germany 6: Thematic Translational Unit Tuberculosis, German Center for Infection Research, Borstel, Germany

A detailed understanding of host-pathogen interactions is essential to develop innovative strategies against multi-drug-resistant tuberculosis (MDR-TB). To investigate pathogenic mechanisms at the site of infection, we assembled a cohort of partial lung resections from patients with drug-susceptible, MDR, and XDR tuberculosis. Lung tissue was stratified based on pathological patterns—such as unaffected areas, diffuse inflammation, cellular granulomas, and necrotic granulomas—and tissue microarrays were constructed to integrate multiple patient samples and lesion types within single paraffin blocks. Using multispectral imaging, we identified key immunological regions at the protein level, including multinucleated giant cell areas, T cell- and macrophage-rich zones, early and late-stage granulomas, and tertiary lymphoid structures. Spatial transcriptomics was then employed to obtain region-specific gene expression profiles, which were correlated with distinct immunological functions. For example, macrophage-rich regions displayed both type I and type II interferon responses, while giant cell areas showed gene signatures related to pH regulation and acidification. The outer rim of granulomas—enriched in T and B cells—were characterized by growth factor signalling and complement activation pathways. These findings reveal distinct molecular signatures within defined morphological niches that are associated with either protective or pathological outcomes. Our results shed light on the immunometabolic landscape of pulmonary TB lesions and may inform the development of targeted, host-directed therapies.

OR11

Reprogramming of human macrophages in response to *Mycobacterium tuberculosis* complex (MTBC) infection

J Schoenfeld² C Utpatel² T Dallenga^{2,4} M Weckmann^{1,2,3} S Niemann^{2,4}

1: University of Lübeck, University Medical Center Schleswig-Holstein, Campus Centrum Lübeck

2: Research Center Borstel Leibniz Lung Center 3: German Center of Lung Research (DZL)

4: German Center for Infection Research (DZIF)

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* complex (MTBC) bacteria, remains a major global health challenge. MTBC has evolved diverse strategies to persist within the human host, including potential adaptation to specific human populations. One such strategy involves epigenetic reprogramming of host cells, particularly cells of the innate immune system. DNA methylation, a key epigenetic mechanism, has been shown to be altered in individuals with TB.

In this study, we investigate the epigenetic response to MTBC infection using an early-stage human macrophage infection model. We also examine long-term epigenetic changes in TB patients from a Gambian cohort. Specifically, we explore how MTBC lineages L4 and L6 influence DNA methylation and gene expression patterns in host cells. These findings are then correlated with DNA methylation profiles of peripheral blood mononuclear cells (PBMCs) and clinical outcomes, including lung function.

By integrating *in vitro* and patient-derived data, we aim to identify lineage-specific host responses and epigenetic signatures associated with disease outcome. Our results can offer new insights into how MTBC modulates the host immune response through epigenetic mechanisms, advancing our understanding of TB pathophysiology and host-pathogen interactions.

OR12

Autologous host-pathogen pairing enhances early immune responses and bacterial control in tuberculosis *ex vivo* granuloma model

C Genestet^{1,2} C Bourg^{1,2} E Hodille^{1,2} O Bahuaud^{1,3} F Ader^{1,3} O Dumitrescu^{1,2}

1: International Center for Infectious Disease Research (CIRI), Lyon, France 2: Lyon University Hospital, Bacteriology Laboratory, France

3: Lyon University Hospital, Department of Infectious and Tropical Diseases, France

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), remains a major global health concern. Granulomas are central to TB pathology, serving as sites of bacterial control and persistence. *In vitro* granuloma models offer a controlled platform to study host-pathogen interactions, yet the impact of Mtb lineage and host variability remains poorly understood. Peripheral blood mononuclear cells (PBMCs) from three active TB patients were used to generate *ex vivo* granulomas, infected with autologous or heterologous Mtb clinical isolates from lineages 1, 2, 3, and 4 (L1-L4), as well as the reference strain H37Rv. Bacterial growth (CFU counting), Mtb dormancy (differentially culturable bacteria) and cytokine production were assessed at 7, 14, and 21 days post-infection (dpi). Bacterial growth and cytokine response were mainly Mtb isolate-dependent. The L4 isolate showed the highest bacterial burden and sustained inflammatory response. In contrast, the L2 isolate showed significant bacterial reduction,

associated with increased dormancy and a weaker cytokine profile. Autologous infections showed reduced bacterial loads at 7-dpi compared to heterologous infections, accompanied by enhanced early Th1-type cytokine responses, including elevated IFN- γ and TNF- α levels, and high IFN- γ /IL-10 ratio. These early advantages did not persist at later time points. Mtb lineage and host-pathogen specificity shape granuloma dynamics, influencing bacterial control and immune responses. These findings underscore the utility of *ex vivo* granuloma models in TB research and highlight the need to account for pathogen diversity and host context. Future studies using single-cell approaches could further unravel early immune mechanisms driving infection outcomes.

OR13

Use of large-scale clinical samples to progressively create a reference catalogue of mycobacterial species

TEA Peto ¹ M Colpus ¹ ER Robinson ² PW Fowler ¹ D Foster ² R Myers ²
C Brown ² H Thieu ¹ R Turner ¹ DW Crook
1: University of Oxford 2: UK Health Security Agency (UKHSA)

Accurate identification of mycobacteria of clinical relevance, whether rare or common, and the ability to detect mixed infections is required. We are analysing competitive mapping of a large and growing number of clinical samples to develop a methodology to classify isolates into clusters, likely to represent independent species. It is, however, currently unclear how many clinical samples are required to create a near-complete catalogue.

Initially, 4443 clinical isolates of non-tuberculous mycobacteria (NTM) from Public Health England (now UKHSA) were sequenced and competitively mapped against 178 reference sequences using a cloud-based pipeline. Isolates were assigned to 87 putative species, and 39 species were in clusters of at least 5 isolates (18 species ≥ 20). For these 39 species, preliminary threshold values were established to distinguish known species, identify possible mixed infections, detect novel species and to identify the need for new reference sequences. The results were compared with the Mykrobe species classifier containing 203 reference species. Also, the pipeline was assessed with a 'truth' set of sequences from 153 reference species sourced from type collections.

Ongoing evaluation of competitive mapping performance on a further >30,000, sequenced by PHE/UKHSA, is being used to confirm the thresholds for assignment of species in clusters, more precise identification of mixed infections, and recognition of new clusters (species). The most current results will be presented. This approach is designed to progressively establish a much enlarged catalogue of species and provide an estimate for the sample size needed to create a near-complete catalogue of mycobacterial species.

OR14

SIILTIBCY: A new era in TB diagnosis

U Shaligram¹ P Kulkarni¹ P Nagarkar¹ Z Fneish² R Lothe¹ D Kapse¹ M May²
G Nagar¹ S Poolishwalla¹ P Singh¹ T Westera³

1: Serum Institute of India 2: Serum Life Sciences Europe 3: Bilthoven Biologicals

Tuberculosis (TB) remains a leading cause of death globally. Over the past decade, Europe has fallen short of meeting the WHO End TB Strategy goals, highlighting the need for more specific and sensitive diagnostic tests. SIILTIBCY, also known as Cy-Tb, is a novel skin test that employs two *Mycobacterium tuberculosis*-specific recombinant proteins, rdESAT-6 and rCFP-10. This test was evaluated for safety and efficacy in seven clinical trials, comparing its diagnostic performance to PPD and QFT.

Based on pooled data the sensitivity of SIILTIBCY (76.8% [72.6-81.1]) was higher than QFT (68.0% [64.0-72.0]) and slightly lower than PPD (85.2% [81.6-88.8]) in a microbiologically confirmed TB-positive population. Specificity (92.8% [90.2-95.5]) was similar to QFT (89.5% [86.3-92.6]) and PPD (90.7% [87.5-93.9]) in a TB-negative population. In the pediatric population, SIILTIBCY outperformed QFT in specificity (83.2% vs. 71.7%), particularly in BCG-positive children (85.2% vs. 72.3%), offering a blood draw-free diagnostic tool for this vulnerable group. SIILTIBCY demonstrated high post-test probabilities and low false negatives, confirming its robustness in real world scenarios. Aside from a higher frequency of mild to moderate injection site hematomas, safety profiles of SIILTIBCY and PPD were equivalent.

SIILTIBCY's use of distinct antigens rdESAT-6 and rCFP-10, along with a fixed induration threshold, ensures reliable TB detection across diverse populations, including those with unknown BCG status, unlike PPD. With European Commission's marketing authorization granted in January 2025, SIILTIBCY provides the field-friendly approach of PPD while delivering IGRA-like performance, and can contribute to TB programmes across Europe.

OR15

Genotypic drug susceptibility testing for new tuberculosis treatment regimens: The challenge of comprehensive and reliable WHO catalogue-based sequencing data interpretation

N Hermans^{1,2} J Phelan³ A Iskakova^{4,5} A Slyzkyi¹ K Kremer¹ R Anthony²

1: KNCV tuberculosis foundation, The Hague, The Netherlands 2: National Institute for Public Health and the Environment, Bilthoven, The Netherlands 3: London School of Hygiene & Tropical Medicine, London 4: KNCV Tuberculosis Foundation, Bishkek, Kyrgyzstan 5: National Center for Phthisiology, Bishkek, Kyrgyzstan

The World Health Organization (WHO) "Catalogue of mutations in *Mycobacterium tuberculosis* complex (MTBC)" was updated in 2023. The limited availability of genomic-/phenotypic data restricts the accuracy of genotypic drug susceptibility prediction for newer anti-tuberculosis drugs. To address this, rules were added to the catalogue to provide guidance for these drugs, including pretomanid (PTM) and delamanid (DLM). However, the interpretation of

these rules may be complex. For example, the *M. tuberculosis* H37rv reference genome contains 2.7 (imperfect) repeats at the end of the *fbtC* gene. Yet, we encountered MTBC strains with repeat deletions and insertions. Due to variability in the number of repeats, sequencing platform independent mapping issues were encountered for both Nanopore targeted next-generation- and Illumina short read whole genome sequencing. Reads that were too short to read through the 2.7 repeat area were incorrectly mapped to the *fbtC* reference, triggering the catalogue rule associating loss of function (LoF) of the gene with PTM/DLM resistance. Nevertheless, further analysis revealed that in both single and double repeat deletions, the remaining repeat's stop codon replaced the *fbtC* stop codon leaving the coding region unchanged. Thus, variability in the number of repeats at the end of *fbtC* does not cause LoF nor does it impact susceptibility. Our findings underline the importance of rapid communication of exceptions to the WHO catalogue rules, so data analysis pipelines can be updated and systematic errors eliminated, and the need for external quality control samples or files (allowing digital dissemination), designed to validate the implementation of new rules.

OR16

Complete genomes reveal unprecedented genomic diversity of *Mycobacterium tuberculosis* complex from global to within-host variation

AM García-Marín^{1,8} M Torres-Puente¹ L Martínez-Priego² G De Marco²
MA Moreno-Molina¹ M Hunt^{4,5} Z Iqbal^{4,6} Valencia Region TB Working Group⁷
MG López¹ F González-Candelas^{8,9} J Alonso-del-Real¹ I Comas^{1,9}

1: Instituto de Biomedicina de Valencia - Spanish National Research Council 2: Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunitat Valenciana FISABIO 3: EB House Austria 4: European Bioinformatics Institute 5: Nuffield Department of Medicine, University of Oxford 6: Milner Centre for Evolution, University of Bath 7: Conselleria Sanitat, Generalitat Valenciana 8: Joint Research Unit 'Infección y Salud Pública' FISABIO, University of Valencia- I2SysBio 9: CIBER Epidemiology and Public Health

Sequencing technologies have provided key insights into the epidemiology and pathogenesis of the *Mycobacterium tuberculosis* complex (MTBC), but are hampered by the technical limitations of conventional short-read sequencing and its low genetic diversity. In particular, regions encoding key MTBC host-pathogen interaction genes such as PE/PPE remain poorly characterized, limiting the analysis of host selection on them. We used long-read sequencing of 216 MTBC clinical isolates from Valencia, Spain, to construct high-quality complete genomes. We have been able to generate complete genome sequences solely from long-read HiFi sequencing data.

These genomes revealed previously inaccessible diversity hotspots often linked to *pe/ppe* genes. A median of 312 (-1 to 792) SNPs per pairwise comparison are gained. *pe/ppe* hotspots are driven both by structural variation and point mutations mediated mainly by gene conversion events, contrary to what is observed in the rest of the genome. However not all *pe/ppe* are the same and here we show the diversity and gene conversion landscape across the entire *pe/ppe* repertoire. Paradoxically, we show that the epitopes in *pe/ppe* genes are hyperconserved, with some salient exceptions which happen to be part of vaccine candidates in clinical trials. Furthermore, our results provide new insights to redefine transmission boundaries based on genetic distances. Finally, the use of a complete genome from the same patient as a reference reveals a substantial reduction in non-fixed variation when mapping short reads from

serial isolates, prompting the need to reassess our current estimates of within-host diversity and the dynamics of *Mycobacterium tuberculosis* during infection.

OR17

When non-pathogens kill: *Mycolicibacterium manresensis* as a model for cryptic virulence in Mycobacteria

M Cortacans^{1 2 3 4} M Arch^{1 2 3 4} E Fuentes^{1 2 4} PJ Cardona^{1 2 3 4 5}

1: Unitat de Tuberculosi Experimental, Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol (IGTP), Badalona, Spain 2: Servei de Microbiologia, LCMN, Hospital Universitari Germans Trias i Pujol (HUGTiP), Badalona, Spain 3: Departament de Genètica i Microbiologia, Universitat Autònoma de Barcelona, Spain 4: Centre de Medicina Comparativa i Bioimatge de Catalunya (CMCiB); Badalona, Spain 5: Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBERES), Madrid, Spain

Mycolicibacterium manresensis, an environmental mycobacterium previously regarded as avirulent in mammalian models, displays unexpected lethality in *Drosophila melanogaster*, indicating the presence of cryptic virulence. Using an in vitro infection model with S2 haemocyte cells, we observed both high intra- and extracellular bacterial burdens, alongside a marked decline in host cell viability within 48 hours, assessed by flow cytometry. Live confocal microscopy over 24 hours post-infection further revealed the formation of cord-like aggregates—structures reminiscent of the classical cording phenotype associated with virulent *Mycobacterium tuberculosis*. Preliminary homology searches showed that *M. manresensis* encodes genes with similarity to known cording-associated factors in *M. tuberculosis*, suggesting a conserved molecular basis for this phenotype. In vivo, *M. manresensis* infection elicited a robust activation of the fly's innate immune system, with significantly elevated expression of both Toll and IMD pathway genes compared to infection with the pathogenic *Mycobacterium marinum*. This heightened immune response coincided with high bacterial loads and consistent host mortality within seven days post-infection. Thus, despite its lack of pathogenicity in mice, *M. manresensis* demonstrates virulence traits in flies, suggesting host-specific unmasking of latent pathogenic potential. These findings highlight *D. melanogaster* as a sensitive model organism capable of revealing context-dependent virulence in environmental mycobacteria. Our work contributes to the understanding of the evolutionary continuum of mycobacterial pathogenicity and underscores the value of alternative host models in uncovering hidden facets of environmental microbes traditionally considered benign.

OR18

Investigating the virulence of *Mycobacterium abscessus* using the *Drosophila melanogaster* infection model

M Arch¹ S Buenestado-Serrano² L Perez-Lago² D García de Viedma² PJ Cardona³
1: Institut d'investigació en Ciències de la Salut Germans Trias i Pujol 2: Instituto de Investigación Sanitaria Gregorio Marañón 3: Hospital Universitari Germans Trias i Pujol

Mycobacterium abscessus is a rapidly growing non-tuberculous mycobacterium associated with chronic pulmonary infections known for its intrinsic antibiotic resistance. With its rising clinical relevance, there is an increasing need for in vivo models that enable rapid and scalable assessment of virulence and host-pathogen interactions. In this study, we employed *Drosophila melanogaster* as a genetically tractable model to investigate the pathogenicity of different *M. abscessus* clinical isolates. The selected isolates represent five distinct genotypes collected over a 16-year period from a single patient with a persistent infection, offering a unique perspective on within-host bacterial evolution. Notably, all but one of the genotypes harboured mutations potentially associated with hypermutation, providing an opportunity to explore how genomic changes influence virulence. Adult flies were systemically infected, with survival rates and immune responses monitored over time, and bacterial loads measured at the time of death. Our results revealed distinct virulence strategies among variants: some caused rapid mortality but required higher bacterial loads, while others induced delayed mortality at significantly lower bacterial burdens. These findings suggest that *M. abscessus* may employ diverse mechanisms to subvert host defences and establish infection. This model not only captures key aspects of infection dynamics observed in more complex organisms but also provides a rapid, cost-effective and ethical platform for preclinical screening of virulence factors and potential therapeutics. Overall, the use of *D. melanogaster* as an in vivo model offers valuable insights into *M. abscessus* pathogenesis and within-host evolution.

OR19

Evaluation of immune responses elicited by respiratory mucosal vaccination strategies against TB in a goat model

P Cuenca-Lara¹ M Blay-Benach¹ Z Cervera¹ J Moraleda¹ IA Sevilla² JM Garrido²
S López-Soria¹ E Vidal¹ M Domingo³ B Pérez de Val¹
1: Programa de Sanitat Animal, Centre de Recerca en Sanitat Animal (CReSA), IRTA, Campus de la Universitat Autònoma de Barcelona, Spain 2: Animal Health Department, NEIKER-Instituto Vasco de Investigación y Desarrollo Agrario, Basque Research and Technology Alliance (BRTA), Derio, Basque Country, Spain Development (NEIKER) 3: Departament de Sanitat i Anatomia Animals, Universitat Autònoma de Barcelona, Spain

Currently, Bacille Calmette-Guérin (BCG) is the only approved vaccine for tuberculosis (TB) in humans, and no licensed vaccine is available for livestock. Nevertheless, both BCG and heat-inactivated *Mycobacterium bovis* (HIMB), when administered parenterally, have shown variable levels of protection under experimental and field conditions. A vaccine that effectively prevents

infection and reliable immune correlates of protection are urgently needed. This study evaluated the immunogenicity of mucosal TB vaccination approaches by analysing systemic and local, in the lung mucosa, immune responses of goats. Thirty animals were divided into five groups: 1) intranasal (i.n.) BCG, 2) i.n. HIMB, 3) i.n. HIMB with mucosal adjuvant (HIMBmuc), 4) subcutaneous (s.c.) HIMB prime followed by i.n. HIMBmuc boost, and 5) s.c. BCG prime followed by i.n. HIMBmuc boost. Antigen-specific cytokines and IFN- γ -producing T-cell subsets in blood and bronchoalveolar lavage fluid (BALF) were measured by ELISA and flow cytometry, respectively. Activation and proinflammatory markers in alveolar macrophage (AM) were also analysed. Intranasal BCG and both prime-boost groups induced higher systemic specific IFN- γ levels, increased CD4+ and CD8+ IFN- γ -producing T-cells and greater IFN- γ production in effector and memory CD4+ T-cells in peripheral blood compared to HIMB i.n. groups. Moreover, proinflammatory cytokines (TNF- α , IL-17A and IFN- γ) were elevated in BALF, and greater AM activation and polarization were observed in those groups. These results highlight the potential of intranasal BCG and prime-boost strategies to elicit robust mucosal and systemic immunity against TB, and of intranasal BCG or HIMB booster vaccines for improving protective immunity at the pulmonary mucosal surface.

OR20

Utility of targeted Nanopore sequencing for the detection of drug-resistant tuberculosis in Kyrgyzstan

A Kulzhabaeva¹ A Iskakova^{1,2} G Saparova² F Tilekova² B Myrzaliev^{3,4}
M Ahmatov^{1,3} A Duishekeeva^{1,3} A Soorombaeva¹ A Toktogonova² M Sydykova²
A Slyzkyi⁴ N Hermans^{4,5} A Kadyrov² G Kalmambetova² E Tiemersma⁴ [K Kremer](#)⁴
1: KNCV Tuberculosis Foundation Kyrgyzstan office, Bishkek, Kyrgyzstan 2: National Center for Phthisiology, Bishkek, Kyrgyzstan 3: Kyrgyz State Medical Academy, Bishkek, Kyrgyzstan 4: KNCV Tuberculosis Foundation, The Hague, The Netherlands 5: National Institute for Public Health and the Environment, Bilthoven, The Netherlands

The utility of the Oxford Nanopore Technologies (ONT) early-access targeted next-generation sequencing (tNGS) tuberculosis (TB) assay (OND-CUST-KIT on MinION MK1B) was evaluated in Kyrgyzstan. The assay targets 24 resistance-associated genes, covering 16 anti-TB drugs, including all BPaL drugs.

For validation, 149/155 (95.5%) smear-positive sputum samples were successfully subjected to ONT-tNGS and Deeplex-Myc-TB-tNGS (GenoScreen, on MiSeq); 135 had complete standard of care drug susceptibility test (SOC-DST) results. Concordance was good for all anti-TB drugs, except pyrazinamid and ethambutol.

In a prospective cohort study from January 2024 to February 2025, of 997 enrolled Xpert TB-positive participants aged ≥ 18 years, 597 (60%) had complete tNGS and SOC-DST results. Among these, 267 (45%) had TB resistant to ≥ 1 drug by SOC-DST: 115 (19%) to isoniazid but not rifampicin and 117 (20%) to both rifampicin and isoniazid. Median turnaround times for line probe assay, phenotypic DST and tNGS were 4 (IQR:3–6), 26 (IQR:20–34), and 28 (IQR:15–50) days, respectively. The long turnaround time for tNGS was due to delays in supply of reagents and lack of trained staff during the first months of the study. In times when reagents and staff were available, the turnaround time of tNGS was 6 days (IQR: 5–8). Six patients showed bedaquiline resistance, detected by either tNGS only (n=2), phenotypic DST only (n=1), or by both (n=3). tNGS predicted pretomanid resistance in six cases, while phenotypic DST for pretomanid was not performed.

This ongoing study supports integration of tNGS into routine diagnostic algorithms for accurate and timely treatment decisions.

OR21

Immuno-peptidomics for vaccines against tuberculosis

PJG Bettencourt ^{1 2}

1: Faculty of Medicine, Universidade Católica Portuguesa, Portugal 2: Center for Interdisciplinary Research in Health, Universidade Católica Portuguesa, Portugal

Mycobacterium tuberculosis (M.tb) causes active tuberculosis (TB) leading to death in most cases if left untreated, latent infection, or can be cleared by the host leaving no trace. Protection against TB is strongly dependent on CD4⁺ T-cells, and to some extent CD8⁺ T-cells, as is demonstrated by the absence of protection in immunocompromised individuals with CD4⁺ T-cell lymphopenia. The identification of mycobacterial-specific peptides presented by MHC-I and MHC-II to T-lymphocytes is paramount to understanding the mechanisms of protection against TB.

Using immuno-peptidomics in human macrophages infected with *Bacillus Calmette-Guérin* (BCG) (Bettencourt et al, NPJ vaccines 2020), or M.tb (Almujri et al, in review), 112 BCG and 81 M.tb antigens were identified, respectively. Strikingly, 12 antigens were presented by both BCG and Mtb-infected cells. In the first study, five antigens were expressed in viral vectors, and evaluated as vaccine candidates in a murine aerosol M.tb challenge model. Vaccines containing combinations of three (glfT2, iniB, fas) or five antigens (glfT2, iniB, fas, PPE15, ag85A), as BCG booster, conferred significant protection in mice compared to BCG alone. In the second study three antigens were formulated as protein-in-adjuvant, or DNA vaccines, and one antigen (metE) conferred significant protection in mice. These results constitute a proof-of-concept for this unbiased strategy to identify novel candidate antigens for vaccines against TB.

Finally, the antigens presented by both BCG and Mtb-infected cells, represent the most promising antigen candidates for vaccines to boost BCG. I will describe the selection of combinations of antigens for vaccine design and development.

OR22

Determining the genetic drivers of pathogenicity in the genus *Mycobacterium*

MJ Gebert ^{1 2} W Mulders ³ E Stallard-Olivera ^{1 2} M Diels ^{3 4} P Rupasinghe ^{3 4}

MR Domingo-Sananes ⁵ [O Tzfadia](#) ³ L Rigouts ^{3 4} C Meehan ^{3 5}

1: Cooperative Institute for Research in Environmental Sciences, Boulder, USA 2: Dept. of Ecology and Evolutionary Biology, University of Colorado, USA 3: Unit of Mycobacteriology, Institute of Tropical Medicine, Antwerp, Belgium 4: BCCM/ITM Mycobacteria collection, Institute of Tropical Medicine, Antwerp, Belgium 5: Department of Biosciences, Nottingham Trent University, UK

The genus *Mycobacterium* is made up of over 200 bacterial species which reside in a wide variety of environments and hosts. The full spectrum of pathogenicity (ability to invade a host) is covered within this genus, from environmental species with no reported clinical significance through to

obligate pathogens such as *M. tuberculosis* and *M. leprae*. However, the exact mechanisms which determine if a *Mycobacterium* can infect a host and cause disease are not fully understood.

We gathered a robust dataset of closed, completed genomes that represent over 180 species of *Mycobacterium* to determine the genomic factors which govern the pathogenicity spectrum of this genus. We predicted the presence of phage and plasmids and constructed a genus-wide pangenome, containing a gene presence/absence pattern of each species. We placed species on a four-point pathogenicity scale from completely environmental (0) to obligate human pathogen (4) based on published clinical case reporting for each species. Using supervised machine learning approaches we then determined what gene presence/absence patterns best explain the placement of each species on this scale.

We uncovered an interconnecting pattern of 26 genes which accurately explain the pathogenicity potential of each *Mycobacterium* species. The functions of these genes, many of which allow for survival and proliferation in the host, include 2-methylcitrate dehydratase (*prpR/prpD*) and superoxide dismutase (*sodA*), as well as others of currently unknown function. This work indicates that there are specific genetic factors driving mycobacterial host invasion. Such genes may serve as markers of 'pathogenicity potential' for newly discovered genus members.

OR23

Single-cell RNA sequencing shows that circulating monocytes enriched in IFN signaling are associated with nontuberculous mycobacteria pulmonary disease in cystic fibrosis

NI Lorè¹ S de Pretis¹ A Gramegna² F Nicola¹ F Di Marco¹ S Saldarini¹
C Contarini² C Ferrari¹ S Saliu¹ L Cariani² F Blasi² D Cirillo¹

1: San Raffaele Scientific Institute 2: Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy

Background: People colonized with *Mycobacterium abscessus*, such as people with cystic fibrosis (PwCF) or chronic respiratory diseases, are at risk of nontuberculous mycobacterial pulmonary disease (NTM-PD) and have heterogeneous clinical outcomes. New reliable biomarkers, such as immune signatures, are among the unmet needs for better clinical management of NTM-PD.

Methods: We collected blood samples from PwCF with no history of NTM detection and clinically stable (CF), with NTM-positive isolates and clinically stable (CF-NTM), and with NTM-positive isolates and a clinical diagnosis for risk of pulmonary disease (CF-NTM-PD). We isolated peripheral blood mononuclear cells (PBMCs) and plasma to perform scRNA-sequencing and Luminex assay.

Results: We performed scRNAseq on PBMCs samples, identifying 10 cellular immune populations according to expression levels of canonical markers. We performed differentially expressed gene (DEG) analysis in the three groups in each cell population to identify transcriptomic signatures characterizing CF-NTM-PD. We compared DEGs in samples from people at risk of pulmonary disease (CF-NTM-PD) with those from the other two groups (CF, CF-NTM) and identified unique RNA transcriptomic profiles activated in monocytes that were specifically upregulated in the CD14+ monocyte cluster of CF-NTM-PD. The unique RNA transcriptomic profiles in CD14+ cells

were further validated using a second public bulk RNA dataset from a cohort of individuals with pwCF and NTM-PD.

Conclusions: Our scRNA-sequencing data suggest that monocyte responses are present in PwCF at risk of NTM-PD.

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OR24

International consensus framework for managing treatment failure in Nontuberculous Mycobacterial Pulmonary Disease

D Moreira-Sousa¹ B Martins² A Aguiar^{3,4} M Pinheiro^{3,4,5} O Akkerman^{6,7}
TR Aksamit⁸ S Aliberti^{9,10} C Andrejak¹¹ CL Daley^{12,13,14} J van Ingen¹⁵
C Lange^{16,17,18,19} M Lipman²⁰ MR Loebinger²¹ M Jankovic Makek^{22,23}
K Morimoto^{24,25,26} RM Thomson²⁷ D Wagner^{28,29} KL Winthrop^{30,31} JJ Yim³²
R Duarte^{3,4,33}

1: Pulmonology Department, Local Health Unit Cova da Beira, Covilhã, Portugal 2: Pulmonology Department, Local Health Unit São João, Porto, Portugal 3: EPIUnit ITR, Instituto de Saúde Pública da Universidade do Porto, Portugal 4: Estudo das Populações - Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Portugal 5: Public Health Unit, Local Health Unit Barcelos/Esposende, Barcelos, Portugal 6: Department of Pulmonary Disease and Tuberculosis, University Medical Centre Groningen, University of Groningen, the Netherlands 7: Tuberculosis Centre Beatrixoord, University Medical Centre Groningen, University of Groningen, the Netherlands 8: Mayo Clinic, Rochester, USA 9: Department of Biomedical Sciences, Humanitas University, Milan, Italy 10: IRCCS Humanitas Research Hospital, Respiratory Unit, Milan, Italy 11: Department of Respiratory Diseases, Amiens-Picardie University Hospital, Amiens, France 12: Division of Mycobacterial and Respiratory Infections, Department of Medicine, National Jewish Health, Denver, USA 13: Division of Infectious Diseases, Department of Medicine, University of Colorado, USA 14: Division of Pulmonary Sciences and Critical Care Medicine, Department of Medicine, University of Colorado, USA 15: Radboudumc Community for Infectious Diseases, Department of Medical Microbiology, Radboud University Medical Centre, Nijmegen, The Netherlands 16: Division of Clinical Infectious Diseases, Research Centre Borstel, Germany 17: German Centre for Infection Research (DZIF), Partner Site Borstel-Hamburg-Lübeck-Riems, Germany 18: Respiratory Medicine and International Health, University of Lübeck, Germany 19: Department of Pediatrics, Global and Immigrant Health, Global Tuberculosis Program, Baylor College of Medicine and Texas Children's Hospital, Houston, USA 20: Department of Respiratory Medicine, The Royal Free Hospital NHS Trust, London, & University College London, UK 21: Royal Brompton Hospital and NHLI, Imperial College London, UK 22: Clinic for Respiratory Diseases, University Hospital Centre Zagreb, Croatia 23: University of Zagreb, School of Medicine, Croatia 24: Respiratory Disease Centre, Fukujuji Hospital, Japan Anti-Tuberculosis Association, Japan 25: Department of Clinical Mycobacteriosis, Nagasaki University Graduate School of Biomedical Sciences, Japan 26: Division of Clinical Research, Fukujuji Hospital, Japan Anti-Tuberculosis Association, Japan 27: Gallipoli Medical Research and Greenslopes Clinical Unit, The University of Queensland, Brisbane, Australia 28: Division of Infectious Diseases, Department of Internal Medicine II, Freiburg University Medical Centre, Germany 29: Department of Epidemiology, Helmholtz Centre for Infection Research Braunschweig, Germany 30: Division of Infectious Diseases, Department of Medicine, Oregon Health & Science University, Portland, USA 31: Centre for Infectious Disease Studies, Oregon Health & Science University-Portland State University School of Public Health,

USA 32: Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, Seoul National University Hospital, Seoul National University College of Medicine, Republic of Korea
33: Centro de Saúde Pública Doutor Gonçalves Ferreira – Instituto de Saúde Pública Doutor Ricardo Jorge, INSA Porto, Portugal

Background: Treatment of nontuberculous mycobacterial pulmonary disease (NTM-PD) remains complex, with high failure rates and limited evidence guiding the management of refractory cases.

Objective: This study aimed to develop a consensus-based framework to support clinical decision-making in the context of treatment failure.

Methods: An e-Delphi process was conducted with a panel of 16 international experts in NTM-PD. Initial statements were developed based on responses to an open-ended questionnaire and informed by a systematic literature review. The statements were refined over three iterative rounds using a Likert scale questionnaire and additional written feedback, with the process continuing until consensus was reached across all key domains for NTM-PD management when facing treatment failure.

Results: Treatment failure was defined primarily as the lack of culture conversion after six months of adequate therapy, with clinical or radiological deterioration as supportive, non-essential criteria. Therapeutic decisions should rely on expert consultation. The framework outlines three therapeutic paths: treatment intensification, maintaining curative intention through personalised, species-specific multidrug regimens; de-escalation of treatment with the objective of symptomatic control, through simplified antibiotic schemes, and in selected cases, treatment discontinuation; supportive care for all the patients, including rehabilitation, nutritional and psychological support. Decisions should be guided by patient preference, disease severity, comorbidities, previous antibiotic treatment and documented resistance.

Conclusion: This consensus provides a structured, patient-centred approach for managing refractory NTM-PD. It supports personalised care pathways and standardises decision-making. Further studies are needed to validate this framework in clinical practice and to explore host-directed and alternative therapies.

OR25

Fidaxomicin, a potential new drug for combinatorial therapies against *Mycobacterium abscessus*

C Jacquet¹ M Emeret¹ S Marchand^{1,2} J Clarhaut^{1,2} J Buyck¹

1: Inserm U1070 PHAR2, Pharmacology of Antimicrobial Agents and Antibioresistance 2: Laboratory of Toxicology and Pharmacokinetic, Poitiers University Hospital

Mycobacterium abscessus (Mabs), an opportunistic pathogen, is associated with severe pulmonary outcomes in susceptible population like cystic fibrosis patients. Treatment requires prolonged multidrug regimens (12–18 months) with cure rates below 30%, largely due to intrinsic resistance mechanisms and limited effective antimicrobials. Recently, fidaxomicin—a macrocyclic antibiotic approved for *Clostridium difficile* infections— was identified as a potential candidate against Mabs¹. In this study, we assessed the antimicrobial activity of fidaxomicin against Mabs and explore its interaction with six commonly used antibiotics.

The minimal inhibitory concentration (MIC) of fidaxomicin was determined against *M. abscessus* ATCC 19977T reference strain using the broth microdilution method in Middlebrook

7H09 medium (10% OADC and 0.5% glycerol) (n=4). Results were read after 3 days of incubation at 35 ± 2 °C. Synergistic activity between fidaxomicin and azithromycin, amikacin, apramycin, imipenem, linezolid, or tigecycline was evaluated using the checkerboard method (n=3). The efficacy of each combination was quantified by calculating the Fractional Inhibitory Concentration Index (FICI), with synergy defined as minimal FICI ≤ 0.5 .

The MIC of fidaxomicin was 8 -16 mg/L. Fidaxomicin combined with azithromycin showed a synergistic effect (minimal FICI= 0.375) as well as with imipenem and tigecycline (minimal FICI of 0.375 and 0.5 respectively). No synergistic interaction was found with combination of fidaxomicin with either amikacin, apramycin or linezolid (minimal FICI > 0.5).

The synergistic association of fidaxomicin with azithromycin, imipenem or tigecycline is highlighting the potential of fidaxomicin as a valuable component of combination therapy.

Sun, Q. *et al. J. Med. Microbiol.* **71**, (2022).

OR26

Evaluation of NTM-Profiler for identification and drug resistance prediction in nontuberculous mycobacteria from whole genome sequencing data

S Mok^{1 2} E Roycroft^{1 2} J Phelan³ P Flanagan² ÁO'Halloran¹ J Wagener^{1 2}
B Boyle^{1 2} MM Fitzgibbon^{1 2}

1: Irish Mycobacteria Reference Laboratory, St. James's Hospital, Dublin, Ireland 2: Department of Clinical Microbiology, Trinity College Dublin, the University of Dublin, Ireland 3: Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, London, UK

Global trends of NTM infection and disease are increasing. Accurate identification of NTM to species level and drug susceptibility tests are essential to guide clinical treatment regimens. In this study, we evaluated the performance of NTM-Profiler v0.6.0, a bioinformatic tool for the identification of NTM and prediction of drug resistance from whole genome sequencing data. WGS was performed on 532 NTM isolates (over 40 species) that were collected in the Irish Mycobacteria Reference Laboratory. Species identification and molecular resistance detection was previously performed using Bruker-HAIN line-probe assays (LPA) (GenoType CM/AS/NTM-DR) and/or Sanger sequencing as part of routine diagnostics. Of the 532 NTM isolates analysed with NTM-Profiler, 82% (436/532) were correctly identified to species level. Minor discrepancies were observed in 11% (56/532) of isolates where NTM-profiler further identified the isolate to subspecies level. Major discrepancies were observed in 7% (40/532) of isolates where a different NTM species was identified. Drug resistance prediction for 87 *M. abscessus* isolates showed 100% concordance for macrolides and 99% (86/87) concordance for aminoglycosides when compared to GenoType NTM-DR results. In conclusion, NTM-Profiler is a rapid and robust tool for NTM identification and drug resistance prediction and could potentially be implemented for routine use. Discrepancies were observed mainly among isolates where NTM-profiler had identified a different species or subspecies that is not covered by the LPA. Phylogenetic analysis is required to confirm the species and subspecies identified by NTM-Profiler and further work to evaluate the drug resistance prediction in *M. avium* complex isolates will also be performed.

OR27

Modeling a genetic condition that protects against tuberculosis

AC van der Spoel¹ S Wang² L Carter¹ MA Chowdhury² CP Phenix²
1: Dalhousie University 2: University of Saskatchewan

A genetic condition affecting humans protects against tuberculosis. This condition is Gaucher disease. Zebrafish models of Gaucher disease are resistant to infection with *Mycobacterium marinum* (*M. marinum*), while wild-type fish are not. Also, iPSC-derived macrophages from human Gaucher patients internalize fewer *M. tuberculosis* than isogenic controls. Following uptake in Gaucher macrophages, the mycobacteria do not multiply.

Gaucher disease varies widely in severity and affects tissue macrophages. It is caused by mutations in the *GBA1* gene and subsequent deficiency of its product, the lysosomal enzyme glucocerebrosidase (GCCase). GCCase participates in the degradation of complex glycosphingolipids by cleaving glucose off glucosylceramide and glucosylsphingosine. Mutant *GBA1* alleles associated with Gaucher disease code for enzyme forms that are less active than wild-type GCCase. The deficit in GCCase activity results in lysosomal accumulation of glucosylceramide and glucosylsphingosine. *In vitro*, the latter lipid is toxic to *M. marinum* and *M. tuberculosis*.

The question is now whether chemical models of Gaucher disease also can protect against tuberculosis. This question has been challenging to address due to the lack of GCCase inhibitors that are selective as well as potent. We have developed irreversible inhibitors of GCCase that meet these criteria. Our inhibitors do not affect the non-lysosomal isoenzyme *GBA2* and react with GCCase in minutes at nanomolar levels. These inhibitors can be used to titrate the level of inhibition of GCCase and subsequent glucosylsphingosine accumulation, optimizing the toxicity towards mycobacteria. Altogether, our GCCase inhibitors may offer a novel approach for targeting mycobacteria.

OR28

Preclinical murine models for studying lung infection and antimicrobial treatments of Nontuberculous mycobacteria

F Saliu¹ C Chernichero Martos¹ C Ferrari¹ F Nicola¹ DM Cirillo¹ NI Lorè¹
1: San Raffaele Scientific Institute

Pulmonary infections caused by nontuberculous mycobacteria (NTM) are gaining relevance as a major health concern, especially in individuals with conditions like chronic obstructive pulmonary disease, bronchiectasis, and cystic fibrosis. This rise in NTM infections emphasizes the urgent need for effective antimicrobial treatments, as NTM is often resistant to standard antibiotics. However, progress in developing novel therapies has been hampered by the absence of a standardized and representative murine model of chronic infection.

To address this limitation, we exploited the agar beads method to establish a chronic lung infection with *Mycobacterium abscessus* in immunocompetent mice.

In this model, NTM successfully induced a persistent lung infection with a stable and sustained bacterial load lasting up to two months with minimal systemic dissemination. Histopathological analysis of lung tissue, bacterial localization, and spatial transcriptomics data revealed that in chronically infected mice NTM co-localized with pathological areas, including tissues with infiltrating macrophages and granuloma-like structures, or submucosal inflammatory cells. Moreover, we recently demonstrated that this model can be effectively adapted to test clinical strains and used for the development of new antimicrobial strategies, including both pathogen-directed therapies (Lorè N.I. et al., 2023, *ERJ* 2023; Degiacomi G. et al., *Int J Antimicrob Agents*. 2024) and host-directed therapies (Poerio N. et al., *Microbiol Spectr*. 2022, unpublished).

In conclusion, our refined murine model of chronic lung infection by NTM provides valuable tools for advancing our understanding of bacterial pathogenesis and for evaluating the efficacy of novel antimicrobial therapeutic interventions against NTM lung infection.

OR29

Validating a SNP calling pipeline for *Mycobacterium leprae*

K Cox¹ DJ Whiley¹ AFC Chilengue¹ MR Domingo-Sananes¹ [CJ Meehan](#)^{1 2}
1: Nottingham Trent University 2: Institute of Tropical Medicine, Antwerp

Mycobacterium leprae is the primary cause of leprosy, a chronic infection of the peripheral nerves, causing numbness, muscle weakness, and skin lesions. Each year, 200,000 new cases are reported, predominantly concentrated in a few high-burden countries. Global eradication of leprosy relies on detecting transmission clusters to inform targeted interventions. Molecular epidemiology of *M. leprae* is in its infancy as this bacterium is unculturable, hindering whole genome sequencing efforts. Recent advances in DNA extraction and sequencing have overcome some of these challenges, creating a need for a robust molecular epidemiology framework to enable detection of transmission clusters based on sequence variation.

This project aims to address this challenge by estimating the effectiveness of both a reference-based mapping approach (using the Snippy pipeline) and a reference-free distance estimation (using SKA-2) to approximate the SNP distances between clinical isolates. We use a set of four complete genomes to first estimate the precision of each approach for estimating SNP distances compared to a ground truth estimated by Minimap2 whole genome alignments. We find that both approaches have similar accuracy for estimating SNP distances with almost no false positives and F1 scores over 0.9. This high accuracy is important as the exceptionally low diversity observed in *M. leprae* requires high certainty in each SNP call.

Our findings indicate that a robust clinical pipeline for calling SNPs and reconstructing transmission clusters is possible for *M. leprae*, allowing for molecular epidemiology approaches to be incorporated into leprosy control efforts.

OR30

The diagnostic potential of *Mycobacterium ulcerans* Mycolactone and the surface protein MUL_3720

L Warryn^{1 2} M Gehringer³ P Gersbach³ KH Altmann³ G Pluschke^{1 2}

1: Swiss Tropical and Public Health Institute 2: University of Basel 3: Swiss Federal Institute of Technology Zürich

Early diagnosis is integral to effective Buruli ulcer (BU) control. Consequently, continued efforts are being made to improve the speed of diagnosis at the point-of-care. Mycolactone is integral to *Mycobacterium ulcerans* pathogenesis and is responsible for the main symptoms of the disease. This polyketide exotoxin is unique to *M. ulcerans* and is produced by every known strain of the bacterium, making it an ideal marker for specific BU diagnosis. The surface protein MUL_3720 is highly expressed on clinical *M. ulcerans* strains, and although also found in some environmental mycobacteria, is absent in typical pathogenic mycobacteria. Together, both analytes can constitute complementary biomarkers of *M. ulcerans* presence in clinical samples.

We have generated series of monoclonal antibodies (mAbs) against both analytes and developed antigen capture ELISAs using these mAbs for the specific detection of low nanomolar levels of mycolactone and MUL_3720 in a variety of laboratory and clinical samples. Extensive optimisation of assay parameters, particularly buffer optimisation to counteract matrix effects from serum-rich samples, allows these assays function adequately with crude samples. This makes them more amenable for point-of-care use in the typically low-resource settings that suffer the highest BU prevalence. Additionally, we have converted these ELISAs into an electrochemiluminescent (ECL) format, which is 10 – 50x more sensitive than a typical ELISA. Assessment of these assays with BU lesion samples is planned in the coming months. These assessments will, amongst other things, allow for an estimation of how much of these analytes are present in a typical BU lesion.

OR31

Natural drivers and human-induced pressures shaping the distribution of *Mycobacterium ulcerans* and Buruli ulcer

A Aliaga-Samanez^{1 2} M Cobos-Mayo² C Chevillon¹ M Dogbe³ KM Fast⁴
M Scott⁴ K Waters⁵ JP Receveur⁶ JL Pechal⁵ MW Sandel^{4 7} HR Jordan³
ME Benbow^{5 8 9 10} J-F Guégan¹

1: MIVEGEC (UMR Université de Montpellier, CNRS, IRD), France 2: Grupo de Biogeografía, Diversidad y Conservación, Departamento de Biología Animal, Facultad de Ciencias, Universidad de Málaga, España 3: Department of Biological Sciences, Mississippi State University, USA 4: Department of Wildlife, Fisheries and Aquaculture, Mississippi State University, USA 5: Department of Entomology, Michigan State University, USA 6: Institute for Genome Sciences, University of Maryland, USA 7: Forest and Wildlife Research Center, Mississippi State University, USA 8: Department of Osteopathic 22 Medical Specialties, Michigan State University, USA 9: Ecology, Evolution and Behavior Program, Michigan State University, USA 10: AgBioResearch, Michigan State University, USA

Neglected tropical diseases (NTDs), such as Buruli ulcer, pose a significant challenge to global health, particularly in regions with limited access to diagnosis and treatment. This disease, caused by the environmental mycobacterium, *Mycobacterium ulcerans*, exhibits transmission patterns and geographic distribution strongly linked to environmental, ecological factors.

In this study, we explore how environmental variables such as soil type, mineral and climatic composition, and the richness of animal and plant species, both native and introduced, may influence the distribution of this bacillus. Using pathogeography tools and predictive models, we analyzed environmental, ecological, demographic and socio-economic parameters to identify spatial patterns and associated Buruli ulcer risk factors. Our results indicate that the presence of *M. ulcerans* may be positively influenced by climatic and edaphic factors which limit its spatial range, invasive grass plant species and the presence of gold ores in the ground. Cases of Buruli ulcer and their spatial distribution in Africa are positively influenced by the interaction of UV-B radiation and climatic factors, soil moisture and altitudinal interactions and by a couple of animal and plant species.

These findings underscore the need for an interdisciplinary approach to understand the mechanisms regulating this pathogen distribution and the skin disease it causes. Understanding the environmental dynamics of mycobacteria in animals and humans is crucial for effective control strategies. Collaboration among ecologists, epidemiologists, and public health experts is key to mitigating the impact of these NTDs in the face of global changes.

OR32

Solving the transmission enigma of Buruli ulcer (*Mycobacterium ulcerans* infection)

T Stinear¹

1: University of Melbourne

In the 1990s the epidemiology in temperate southeastern Australia of the neglected tropical skin disease, Buruli ulcer (BU) - infection of subcutaneous tissue with *Mycobacterium ulcerans* - changed. From a handful of BU cases reported from rural regions in the eastern area of the state of Victoria, increasing cases began to be reported around the major metropolitan city of Melbourne and the regional centre of Geelong in the state of Victoria, in southeast Australia. Today BU is endemic in these major urban centres, with more than 350 cases reported each year. We have been attempting to address the longstanding question of how *M. ulcerans* is spreading from the environment to humans. Using a combination of extensive field surveillance, pathogen genomics, computational science, and field intervention studies, we have discovered that native possums are a major wildlife reservoir of the pathogen and that mosquitoes are mechanical vectors. These findings have led to the first evidence-based opportunities to intervene in the transmission of BU.

OR33

Implementing large-scale genomics and genomic subtractive strategies to expand the target space in *Mycobacterium tuberculosis*: a strategy for improving target-based drug discovery

P Gomes¹ F Estrada¹ D Mora¹ J Phelan² S Campino² M Viveiros³ T Clark²
I Portugal¹ R Guedes¹ J Perdigão¹

1: Research Institute for Medicines, Faculdade de Farmácia, Universidade de Lisboa, Portugal

2: Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, UK

3: Global Health and Tropical Medicine, GHTM, LA-REAL, Instituto de Higiene e Medicina Tropical, IHMT, Universidade NOVA de Lisboa, Portugal

The current antituberculous drug portfolio remains insufficient in effectively treating drug-resistant tuberculosis (TB). The inclusion of the latest approved drugs bedaquiline, pretomanid and delamanid has already contributed to the emergence of drug resistance mutations capable of rendering these antibiotics ineffective. Hence, expanding the antituberculous drug repertoire is paramount and new strategies are required to accelerate the drug discovery process. As such, a strategy was pursued to identify highly conserved genes in *Mycobacterium tuberculosis* that could serve as potential drug targets. A genome-wide analysis of 12315 clinical strains was performed to investigate the evolutionary dynamics of 4039 mycobacterial genes and predict which genes are under strong purifying selection. Combined with a genomic subtractive workflow, a small sample of genes was selected to further validate their essentiality *in vitro* through CRISPR interference-mediated gene repression. Thirteen genes were selected for having high rates of sites under purifying selection of up to 18% and non-synonymous/synonymous substitution rates ratios as low as 0.07, through statistical inference methodologies and evolutionary models. Most of these genes are responsible for regulating important mycobacterial biosynthetic pathways, such as mycolic acid synthesis, protein synthesis, gene expression, cell division, host-pathogen interactions, and modulating the host immune response. For each gene, recombinant strains of *M. tuberculosis* holding a sequence-specific version of the CRISPR/dCas9 system were produced, gene repression was induced and bacterial viability was measured. Downregulation of approximately 85% of the tested genes led to a complete loss of viability and were therefore considered essential for mycobacterial survival.

OR34

Exploring *in silico* drug repurposing to develop new therapeutic alternatives for tuberculosis

L Rodrigues¹ M Martins² P Cravo¹ M Viveiros¹

1: Global Health and Tropical Medicine, GHTM, LA-REAL, Instituto de Higiene e Medicina Tropical, IHMT, Universidade NOVA de Lisboa, Portugal 2: Department of Microbiology, Moyne Institute of Preventive Medicine, School of Genetics and Microbiology, Trinity College Dublin, The University of Dublin, Ireland

Energy metabolism, particularly the oxidative phosphorylation pathway, has emerged as a novel target pathway in tuberculosis (TB) drug discovery. Inhibitors of bacterial energy metabolism may interfere with several physiological processes, including the activity of efflux pumps involved in

drug resistance. In this work, we used a drug repurposing strategy to find drugs that target energy metabolism and membrane transporters in *Mycobacterium tuberculosis*. A list of *M. tuberculosis* proteins involved in energy metabolism and membrane transport was compiled using the TDR Targets and Mycobrowser databases. Sequence similarity screenings using DrugBank and STITCH 5.0 databases predicted 69 targets associated with 245 approved drugs. Functional regions comparison between the approved drug targets and *M. tuberculosis* targets resulted in 18 potential targets, such as succinate dehydrogenases, sodium-potassium ATPases and NADH-dehydrogenases, that are expected to interact with 23 approved drugs. Some examples are thiabendazole, deslanoside, valproic acid and doxorubicin. Preliminary *in vitro* testing, using *M. smegmatis* as a model, showed that doxorubicin presented the lowest minimum inhibitory concentration (MIC, 0.8 mg/L), while valproic acid promoted a four-fold MIC reduction for clarithromycin. This work has the potential to benefit TB drug discovery by finding drugs that may serve as lead compounds for the development of new therapeutic strategies against TB and multidrug resistant TB and by introducing a new approach that can increase the probability of identifying effective drugs and decrease the bottlenecks of conventional drug discovery.

OR35

DprE1 inhibitors and bedaquiline combination for TB treatment: in-vitro and in-silico analyses

[A Muscetti](#)¹ F Saluzzo¹ A Ghodousi¹ R Sorrentino¹ A Spitaleri² DM Cirillo¹
1: San Raffaele Scientific Institute 2: University of Milan

The bedaquiline (BDQ)-based regimen for multidrug-resistant tuberculosis (MDR-TB), “BPaLM”, is facing limitation due to the increasing presence of Rv0678 mutation that reduce the regulation of mmpL5/S5 efflux pump. DprE1 inhibitors, a new promising class of anti-TB drugs, share the same resistance pathway as BDQ. This highlights the need to analyse their interactions with BDQ when co-administered. EUCAST protocol was used to establish BDQ and BTZ-043 minimal inhibitory concentrations (MICs) in both H37Rv (n=3) and resistant mutants (n=12). An EUCAST-based checkerboard assay was developed to investigate BDQ/BTZ-043 interactions. DMSO-soluble drugs were prepared in 7H9/OADC, and serial dilutions were performed in 96-well plates in 7H9/OADC ensuring a final 0.5% DMSO concentration. Delamanid, expected to be synergic with BDQ, was used as control. A computational analysis was used to explore BDQ/BTZ-043 interactions and mmpL5/S5 efflux pump's role to better understand the *in-vitro* results. Computational analyses reveal that DprE1 inhibitors possess high affinity with an mmpL5/S5 pump's accessory pocket, this affinity increase when in hetero-(with BDQ) or homo-dimers and can be linked to mmpL5/S5 saturation. This suggest that free DprE1 inhibitors (e.g., unbound due to target mutation) may enhance BDQ activity. *In-vitro* experiments indicate that BTZ-043 cooperates with BDQ inducing a MIC shift upon combination, predominantly in strains with high-level resistance to BTZ-043. These results underline the importance of testing combination of anti-TB drugs to predict their interaction before market introduction. The efflux pumps' role will be validated with the use of the FDA-approved efflux pump inhibitor Verapamil in combinatory experiments.

Improved models for drug development against non-tuberculous mycobacteria

MS Gomes¹ CM Bento¹ GS Oliveira¹ T Silva¹

1: *Universidade do Porto*

Infections by non-tuberculous mycobacteria (NTM), notably *M. avium* (Mav) and *M. abscessus* (Mab), have been notoriously increasing worldwide. Mab is currently one of the most challenging human pathogens, often called an “incurable nightmare”. There are no approved drugs specifically for this bacterium. NTM primarily affect immunocompromised patients, as well as individuals with underlying pulmonary diseases like cystic fibrosis. NTM disease requires long, multi-drug treatments that are often associated with poor patient compliance and low success rates. New and more effective treatments are urgently needed. One of the challenges associated with the development of new anti-NTM drugs is the lack of good in vitro and pre-clinical models, with a good capacity to predict clinical activity.

We recently created Mav and Mab reporter strains, for use in drug screening and development. The use of these strains simplifies the screening process, representing a significant advancement from CFU-based methods. With these strains, we created a drug development pipeline, including high throughput axenic and intra-macrophagic assays, as well as biofilm and organoid models. Moreover, we demonstrated that they can be used in vivo, either in larvae or mouse infection models.

Our experimental setting has already been successfully used to identify promising hits from available drug libraries. We are now working on testing these hits on the most complex infection models. The setting can also be used for compound optimization, mechanistic and PK/PD studies, representing a valuable asset for drug discovery against NTM.

Poster Presentations

P001

Modeling co-infection of *Mycobacterium abscessus* and *Pseudomonas aeruginosa* in lab and animal models

V Campo-Pérez ^{1 3} E Torrents ^{1 2} E Julián ³

1: Institut de Bioenginyeria de Catalunya (IBEC) 2: Universitat de Barcelona 3: Universitat Autònoma de Barcelona

The incidence of infection caused by nontuberculous mycobacteria, mainly *Mycobacterium abscessus*, is increasing in patients with cystic fibrosis and other chronic pulmonary illnesses, translating into an accelerated decline in lung function. In most cases, *M. abscessus* co-infects *Pseudomonas aeruginosa*, the most common pathogen in these chronic diseases. However, the interactions between these two bacterial species during infection remain largely unknown.

This study aimed to explore the behaviour of both species in three relevant pathogenic settings. We employed a dual-species biofilm development approach using a recently developed method to monitor individual species within dual-species biofilms. Additionally, we conducted co-infection studies in bronchial epithelial cells using *in vitro* assays and *in vivo* co-infection studies using the *Galleria mellonella* model.

The results revealed that both species can form stable mixed biofilms while reciprocally inhibiting single-species biofilm progression. Co-infections in bronchial epithelial cells were associated with significantly reduced cell viability. Similarly, in *G. mellonella*, co-infections resulted in lower survival rates compared to infections by either species alone. Analysis of the immune response triggered by each bacterium in bronchial epithelial cells and *G. mellonella* larvae demonstrated that *P. aeruginosa* induces the overexpression of the proinflammatory response and activation of the melanization cascade. In contrast, co-infection with *M. abscessus* and *P. aeruginosa* significantly suppressed immune responses in both models, leading to more severe outcomes for the host compared to single-species infections by *P. aeruginosa*.

Overall, the presence of *M. abscessus* compromises the host's immune response, exacerbating infection and resulting in worse outcomes for the host.

P002

Microbiota modulation of mycobacteria-based immunotherapy in the orthotopic murine model of bladder cancer

V Campo-Pérez ¹ S Guallar-Garrido ¹ R Cabrera-Rubio ² P Herrero-Abadía ¹

A Seguí-Moll ¹ M Torres-Puente ³ I Comas ³ E Torrents ^{4 5} E Julián ¹

1: Universitat Autònoma de Barcelona 2: Institute of Agrochemistry and Food Technology-National Research Council (IATA-CSIC), Valencia 3: Instituto de Biomedicina de Valencia (IBV), CSIC

4: Institute for Bioengineering of Catalonia (IBEC) 5: Universitat de Barcelona

The once-held belief that the urinary tract was sterile has been challenged by the discovery of its microbiota. Some studies have observed differences in microbiota between healthy individuals and bladder cancer patients, although results show disparity, and a clear differentiated profile has not been proven. Bladder microbiota may affect tumour progression and response to therapies, including *Mycobacterium bovis* BCG instillations. The interference or synergy mechanisms between bladder microbiota and BCG treatment remain unknown. This study evaluates for the first time the impact of intravesical instillations of BCG and *Mycobacterium brumae*, a non-pathogenic immunomodulatory species, on microbiota composition of bladder tissue and cecal stool in healthy and tumour-bearing mice.

Our results showed that mycobacterial instillations enhanced microbial richness but reduced Actinobacteria abundance across groups in the gut. At the family level, Muribaculaceae and Bacteroidaceae were significantly enriched in tumour-bearing mice, whereas BCG-treated animals showed lower Muribaculaceae and higher Rikenellaceae abundance. Regarding bladder microbiota, Staphylococcaceae dominated in *M. brumae*-treated healthy mice, while Enterococcaceae was abundant in all other groups except BCG-treated tumour-bearing mice. At genus level, *Escherichia* and *Shigella* were notably reduced in mycobacteria-treated tumour-bearing mice, while *Burkholderia* was more prevalent in untreated bladders. Alpha diversity increased in cecal stool following *M. brumae* instillations, regardless of tumour status. In contrast, BCG reduced bladder microbiota richness in tumour-bearing mice. Beta diversity analyses revealed subtle, non-significant clustering among groups.

These findings highlight how intravesical mycobacterial therapy modulates microbiota composition in distinct anatomical sites, underlining the complex interplay between host, tumour, and immunotherapy.

P003

Increased sensitivity for *Mycobacterium tuberculosis* detection with the updated FluoroType MTBDR v2

E Svensson¹ DB Folkvardsen¹ EM Rasmussen¹ A Norman¹ XES Iversen¹
T Lillebaek^{1 2}

1: Statens Serum Institut 2: University of Copenhagen

FluoroType MTBDR v2 (FT; Hain LifeScience) is a multiplex, multi-probe real-time PCR for the simultaneous detection of *Mycobacterium tuberculosis* complex (MTBC) and resistance-conferring mutations for rifampicin (RIF; *rpoB*) and isoniazid (INH; *katG*, *inhA*). The recent kit upgrade (FT-new) improves the MTBC sensitivity by adding the *IS6110* element to the existing *rpoB* in FT-old.

The performance of FT-new to detect MTBC was compared with that of FT-old using MTBC culture as a reference.

In total, 1483 prospective clinical samples of different types were analysed in parallel from the same FluoroLyse DNA extraction. Samples were re-analysed if the FT-old was inhibited or for a first-time PCR-positive patient. PCR results were compared with MTBC culture in MGIT and on Löwenstein-Jensen.

1411 FT-old and 1377 FT-new had results that could be evaluated. The sensitivity and specificity for FT-new were 0.87 and 0.95, respectively; for FT-old, 0.70 and 0.95, respectively. In sputum samples, the sensitivity was 0.92 (FT-new) and 0.81 (FT), respectively. At least one of the three

resistance targets was detected in 39 samples tested with FT-new and 34 samples tested with FT-old.

In conclusion, adding the insertion element *IS6110* as a target for MTBC detection increases the sensitivity considerably. However, the sensitivity of detecting resistance targets does not increase.

P004

Diagnostic accuracy and operational assessment of microscopy and molecular tests for pulmonary tuberculosis in Panama

P Patel¹ M Vergara² L Solis³ J Jurado² I Martinez³ F Acosta¹ D Sambrano¹
J Ku¹ A Goodridge¹

1: *Instituto de Investigaciones Científicas y Servicios de Alta Tecnología de Panama, Ciudad del Saber, Panamá* 2: *Caja de Seguro Social, Colón, Panamá* 3: *Ministerio de Salud, Colón, Panamá*

Accurate and timely tuberculosis diagnosis is essential to reduce transmission and improve patient outcomes. In Panama, despite increased laboratory capacity, delayed diagnosis remains a challenge in high-burden regions. This study analyzed 400 tuberculosis patients diagnosed between 2021 and 2024 in Colón province using Ziehl-Neelsen and Auramine-Rhodamine staining, GeneXpert MTB/RIF Ultra, MGIT culture, and Genotype MTBDRplus. Diagnostic performance, turnaround time, bacillary burden, and treatment outcomes were evaluated. GeneXpert and fluorescence microscopy showed strong concordance ($\kappa = 0.967$) with median turnaround times of 1 day, while MGIT culture and Genotype testing were significantly slower (median 16 and 26 days, respectively). Most cases (64.8%) were diagnosed during hospitalization, with 72% presenting high bacillary load. Among 341 patients with outcome data, 58.8% were cured, 15.9% were lost to follow-up, and 10.3% died, with poorer outcomes linked to delayed diagnosis. Although the regional laboratory demonstrated excellent performance in microscopy and molecular diagnostics, the findings reveal critical gaps in early detection and timely resistance profiling. The results support the need to strengthen primary care-based screening, reduce diagnostic delays, and expand molecular diagnostic access in community settings to improve tuberculosis control in Panama and the Central American region.

P005

Role of the *inhA_S94A* Mutation on drug resistance and the spread of an Isoniazid-Resistant Tuberculosis strain

A Roig-Guill¹ A Gil³ AM García-Marín¹ P Sinisterra-Sebastián¹ C Mariner-Llicer¹
M Torres-Puente¹ P Cano-Jiménez¹ J Alonso-del-Real¹ V Furió¹ I Comas^{1,2}
MG López¹

1: *Tuberculosis Genomics Unit, Instituto de Biomedicina de Valencia, Spain* 2: *CIBER de Epidemiología y Salud Pública, CIBERESP, Madrid, Spain* 3: *Microrbiología Clínica, Hospital Universitario y Politécnico La Fe*

Isoniazid is a key drug for treating drug-susceptible tuberculosis (TB), with strong early bactericidal activity. However, ~5% of isoniazid-resistant (INH-R) cases have unknown resistance mechanisms. The main gene linked to INH-R is *katG*, though mutations in *inhA* have also been associated through mutations, some of them, such as *inhA_S94A*, with limited evidence. This mutation confers low-level resistance, confirmed in allele exchange experiments, but often produces discordant results in phenotypic drug susceptibility testing (pDST). As a result, it is rarely detected in front-line resistance tests, especially since it responds to combined therapy or high-dose isoniazid. In the Valencia Region, a genomic epidemiology study has sequenced ~80% of culture-positive TB cases since 2014. A large cluster of 32 cases carrying *inhA_S94A* has circulated from 2016 to 2024. The role of this mutation in clinical outcomes and the cluster's spread remains unclear. We analyzed this cluster in depth to: (1) confirm the minimum inhibitory concentration linked to *inhA_S94A* using various phenotypic methods and isolates; (2) use PacBio sequencing to improve transmission resolution; (3) apply Bayesian methods to reconstruct transmission trees and estimate the cluster's origin; and (4) analyze clinical, microbiological, and epidemiological data to assess the mutation's impact on TB spread. Results confirm that *inhA_S94A* shows borderline resistance in microdilution tests, while BACTEC has limitations. Although treatment outcomes were not significantly affected, routine diagnostic oversight of this mutation likely contributed to the spread of the mutation, compromising control efforts.

P006

Diagnostic capacity and Biosafety practices for Tuberculosis: Assessment of the Portuguese National Laboratory Network

R Cordeiro ^{1 2} P Gonçalves ³ S Silva ³ I Rodrigues ³ A Pelerito ^{1 2} IL Carvalho ^{1 2}
MS Nuncio ^{1 2} AS Silva ³ R Macedo ³

1: Emergency Response and Biopreparedness Unit, Department of Infectious Diseases, National Institute of Health Doutor Ricardo Jorge, Portugal 2: Institute of Environmental Health, Faculty of Medicine, University of Lisbon, Portugal 3: National Reference Laboratory for Mycobacteria, Department of Infectious Diseases, National Institute of Health Doutor Ricardo Jorge, Portugal

To assess tuberculosis (TB) diagnostic capacity and biosafety practices across the 41 laboratories of the Portuguese TB laboratory network, the National Institute of Health Doutor Ricardo Jorge conducted a survey covering laboratory practices, infrastructures, training, emergency procedures, waste management, occupational health, and diagnostic methodologies. The response rate was 90% (37/41), covering six of the seven Portuguese health regions.

Nearly half (49%) of the laboratories focus exclusively on TB diagnosis. About 32% operate under BSL-3 conditions, though one BSL-1 laboratory reported performing solid-media culture. Most laboratories (70%) have biosafety manuals, yet 16% do not use respiratory protection when handling aerosol-generating infectious materials, despite all reporting the use of class II biological safety cabinets. Additionally, 8% report lacking adequately trained staff, and 57% do not have occupational health programmes implemented.

Microscopy is performed by 97%, and 81% use nucleic acid amplification for *Mycobacterium tuberculosis* complex detection. All but one laboratory performs microbial culture, with 92% using both solid and liquid media. First-line drug susceptibility testing (DST) is carried out by 86% of laboratories, while only 34% extend testing to second-line antibiotics, with just three performing

phenotypic second-line DST. Over half (51%) also test for non-tuberculosis mycobacteria. All laboratories can refer specimens within the network if unable to provide a given service.

The findings reveal strong diagnostic coverage and inter-laboratory articulation at national level, but also highlight considerable gaps in biosafety, training, and occupational health. Continued collaboration and improvement efforts are crucial to improve TB control and ensure quality and laboratory safety.

P007

Speeding up drug susceptibility testing in *Mycobacterium tuberculosis* using RNA biomarkers

M. Maex¹ A. Sury^{1,2} A. Baulard³ F. Sayes⁴ W. Frigui⁴ R. Brosch⁴ L. Rigout⁵
P. Cos² V. Mathys¹ P. Ceysens¹ A. Van den Bossche¹

1: Sciensano, Brussels, Belgium 2: University of Antwerp 3: Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille 4: Institut Pasteur, Université Paris Cité 5: Institute of Tropical Medicine, Antwerp, Belgium

The growing challenge of antibiotic resistance in *Mycobacterium tuberculosis* emphasizes the need for rapid phenotypic drug susceptibility testing (pDST). While DNA-based methods have greatly improved TB diagnostics worldwide, they fundamentally depend on prior pDST data. Moreover, as new drugs are developed and resistance mutations continue to emerge, pDST remains indispensable. Although MGIT is a reliable standard for phenotypic testing of many anti-TB drugs, it is time-consuming and known to miss certain resistant phenotypes. We present TRACeR-TB, a fast, RNA-based pDST method that distinguishes resistant from susceptible strains by measuring transcriptional stress responses triggered by a brief, 6h antibiotic exposure. Using multiplex panels of 8-9 mRNA biomarkers per drug, TRACeR-TB enables accurate resistance profiling directly from crude lysates. Validation on 238 clinical isolates showed higher sensitivity compared to MGIT, with improved performance in detecting borderline-resistant strains, particularly for rifampicin and bedaquiline. TRACeR-TB demonstrated full concordance with MGIT for drugs with reliable MGIT results (moxifloxacin and isoniazid), and proved effective in identifying resistant phenotypes undetected by genotypic methods. Furthermore, the broad applicability of the assay is illustrated through its use in testing novel drug-boosting compounds and its ability to detect intracellular drug-inflicted stress levels, enabling hit-to-lead optimization *in* and *ex vivo*. As a rapid and mutation-independent approach, TRACeR-TB serves not only as a valuable diagnostic tool but also as a platform for investigating resistance mechanisms and supporting the development of novel therapeutic strategies, offering a complementary DST to bridge existing gaps in TB diagnostics and research.

Evaluating the performance of broth microdilution for the detection of rifampicin-resistant tuberculosis caused by borderline *rpoB* mutants

NS Hidayatallah^{1,2} P Rupasinghe¹ J Vereecken¹ N Barreda⁹ L Chingisova⁷

SV Omar⁸ A Ashraf⁶ L Guglielmetti^{3,4,5} L Rigouts^{1,2} BC de Jong¹

1: Institute of Tropical Medicine Antwerp 2: University of Antwerp 3: Medical Department, Médecins Sans Frontières, Paris 4: Sorbonne University, Centre d'Immunologie et des Maladies Infectieuses (Cimi-Paris), Paris 5: AP-HP, Bactériologie-Hygiène, Hôpital Pitié-Salpêtrière, Centre National de Référence des Mycobactéries, Paris 6: The Indus Hospital laboratory, Karachi 7: National Tuberculosis Reference Lab, Almaty 8: National Institute of Communicable Diseases, Johannesburg 9: Socios en Salud, Lima

The clinical significance of borderline *rpoB* mutants was reflected in World Health Organization (WHO) guidance in 2021, when seven such mutations were recognised as causing rifampicin-resistant tuberculosis (RR-TB). Borderline *rpoB* mutations carry fitness costs that impair in vitro growth and result in poor sensitivity of rapid phenotypic drug-susceptibility tests (pDST) such as the liquid culture MGIT 960 system.

The WHO recently recommended broth microdilution (BMD) for pDST in *M. tuberculosis*, providing minimum inhibitory concentration (MIC) values with relatively short turnaround times, and the possibility to extend incubation in case of fastidious growth. We assessed the performance of BMD with borderline *rpoB* mutants, testing 52 strains representing 6 borderline *rpoB* mutations (9-Leu430Pro, 3-His445Leu, 10-His445Asn, 10-Asp435Tyr, 10-Ile491Phe); 26 strains representing 5 of the most common high confidence *rpoB* mutations (5-Asp435Val, 5-His445Asp, 4-His445Tyr, 8-Ser450Leu, 4-Ser450Trp); and 47 wildtype *rpoB* strains.

MIC distributions for rifampicin showed high MICs for high confidence *rpoB* mutations (2 to >16 µg/ml), while borderline *rpoB* mutants showed a wide range of MICs (0.06 to >16 µg/ml), with significant overlap with wildtype MICs (0.06 to 1 µg/ml). The MIC distributions for bedaquiline, clofazimine and linezolid were similar for borderline and common *rpoB* mutations. Extended incubation led to a non-specific increase in MIC values and therefore does not improve borderline detection in BMD.

Our data indicates that although BMD performs well with high confidence *rpoB* mutants, half of the borderline *rpoB* mutants tested gave false rifampicin-susceptible results, despite yielding valid results for other antibiotics.

P009

Treatment of a dog with active tuberculosis (Poland)

E Szacawa¹ N Koziel¹ S Brzezinska² K Kuczynska³ K Szulowski¹
M Krajewska-Wedzina⁴

1: National Veterinary Research Institute in Pulawy, Poland 2: National Tuberculosis and Lung Diseases Research Institute 3: University of Szczecin 4: University of Life Sciences in Lublin, Poland

The aim of the study was to determine the causative factor of a suppurating wound in the area of the hock joint of the left limb of the dog. The material for the study consisted of tissue fragments from the diseased site on the dog's hind limb. Pus from the diseased site and dressing materials used during the surgical procedure were also examined. Laboratory diagnostics were performed according to the methodology of isolating bacteria from the *Mycobacterium* genus. Molecular testing consisted of the Hain test and the spoligotyping method. The isolated strain was classified as *Mycobacterium tuberculosis* with spoligotype T1 926.

The results obtained in both the imaging diagnostics and the microbiological examination allowed for the diagnosis that the dog was suffering from a multi-organ tuberculosis (TB). TB caused by *M. tuberculosis* in a dog is a typical example of anthroozoonosis. In the described case, the infection most likely occurred through the consumption of infected human sputum. Mycobacterial infections in dogs are often overlooked in veterinary practice, despite being a potential source of infection for other animals and humans. This case is the first animal in Poland with diagnosed tuberculosis in which a decision was made to treat it. The dog has been undergoing anti-tuberculosis therapy according to the Engelman et al. regimen for 2 years. Currently, the dog is taking only one anti-tuberculosis drug and the prognosis for this patient is good.

P010

Tuberculosis in alpacas in Poland in 2018-2019

E Szacawa¹ N Koziel¹ S Brzezinska² E Augustynowicz-Kopec² K Szulowski¹
M Krajewska-Wedzina³

1: National Veterinary Research Institute in Pulawy, Poland 2: National Tuberculosis and Lung Diseases Research Institute, Warsaw, Poland 3: University of Life Sciences in Lublin, Poland

Animal tuberculosis is detected mainly in cattle, its etiological factor is the species *Mycobacterium bovis* and *Mycobacterium caprae*. The causative factor for the spread of infection between alpacas is usually the movement of asymptomatic carriers. *In vivo* diagnosis of TB in alpacas, species other than cattle, is difficult to perform due to problems with obtaining appropriate material for testing and the lack of appropriate diagnostic methods. The tuberculin skin test (TST) in European countries, including Poland, is still considered the official screening test for animal infection.

However, performing TST in alpacas does not provide fully reliable results due to the possibility of obtaining false negative results, which was confirmed in studies of alpacas in Poland in 2018-2019. The occurrence of TB was directly related to the import of sick individuals from Great Britain. As a result of molecular epidemiological investigations based on genotyping of the cultured strains, 5 outbreaks of animal tuberculosis were confirmed in Poland in alpacas and a common source of their infection was established. A total of 27 animals died or were put down. The strains obtained in bacterial culture, based on spoligotyping, were classified as *Mycobacterium bovis* with spoligotype SB0666. According to the database of bovine spoligotypes – SB0666 comes from Great Britain and was registered in 2003. Owners of sick animals usually eliminated

them at their own expense, without performing the necessary autopsy, and also without the knowledge of the District Veterinary Officer, in most cases not subjecting the dead animals to thorough microbiological tests.

P011

Culture-free targeted whole genome enrichment of *Mycobacterium tuberculosis* and next-generation sequencing enables rapid surveillance of drug resistance and genomic tracking

M Fosbrink¹ M Zais¹ R Larsen¹ C Andersen¹ L Schauer¹ [D Barbera](#)¹ J Shaffer¹
1: QIAGEN Sciences

Whole-Genome Enrichment (WGE) of *Mycobacterium tuberculosis* (MTB), using hybrid capture technology, offers a powerful approach to elucidate the genetic diversity, evolution and drug resistance of MTB using Next-Generation Sequencing. By enriching the complete genome, WGE overcomes challenges of MTB characterization such as long culture times and low bacterial DNA content in samples while enabling high-resolution analysis of key loci associated with lineage identification, drug resistance, and strain differentiation.

QIAGEN's QIAseq *Mycobacterium tuberculosis* Panel is a hybrid-capture based approach that enriches the complete genome to enable NGS characterization. In this study, we used this product to demonstrate its utility for rapid whole genome analysis. The NGS results suggest the panel covered 93.04% of the genome at 10x base coverage for 300 genome copies of H37Rv that was spiked into 100 ng of sputum DNA. Increasing the input amount to 10,000 genome copies exhibited 95.22% of the genome covered at 100x. Using the dedicated *Mycobacterium tuberculosis* workflow from CLC Microbial Genomics Module, the correct resistance calls with corresponding mutations were determined for strains ATCC 35838 and ATCC 35822. Additionally, the panel was able to identify accessory genes found in H37Ra that are not in the reference sequence of H37Rv.

QIAGEN's QIAseq *Mycobacterium tuberculosis* Panel is a powerful genomic sequencing tool that allows for rapid whole genome sequencing of DNA that was extracted directly from a sample with no culture requirements. Data analysis using CLC Microbial Genomics Module allows for identification of mutations that contribute to drug resistance and variants for tracking transmission.

P012

Detection of first-line and second-line drug-resistance mediating mutations in *Mycobacterium tuberculosis* with FluoroLyse and GenoXtract fleXT extracted DNA from sputum using next-generation sequencing

V Allerheiligen¹ L Wolf¹ M Eckart¹ R Spannaus¹ W Carbone²

1: Hain Lifescience GmbH - A Bruker company 2: ELITechGroup S.p.A. - A Bruker company

Mycobacterium tuberculosis complex (MTBC) is a group of highly transmissible bacterial pathogens that cause significant worldwide morbidity and mortality, particularly among HIV-infected patients. Annually, MTBC is responsible for approximately 1.3 million deaths. The emergence of multi-drug-resistant (MDR) strains within the MTBC has significantly reduced treatment options. Current methods for detecting drug resistance in MTBC can be time-consuming, often requiring up to two months to yield results.

Next-generation sequencing (NGS) has provided valuable insights into the genetic diversity of MTBC, which are crucial for understanding the evolution and transmission of the disease. NGS has also facilitated the identification of drug-resistant strains, enabling rapid and accurate tailoring of treatment. Seven genes have been used for tuberculosis (TB) first-line and second-line drug resistance analysis: *rpoB* (Rifampicin), *katG* and *inhA* (Isoniazid), *rrs* (Amikacin), *rrs* and *eis* (Capreomycin), and *gyrA* and *gyrB* (Levofloxacin, Moxifloxacin).

NGS was performed using DNA from manual extraction (FluoroLyse, Hain Lifescience) or automated extraction (GXT fleXT, Hain Lifescience) from contrived NALC-NAOH decontaminated native sputum. Multiplex PCR targeting the seven resistance genes was employed for targeted NGS using Oxford Nanopore and Illumina technologies. Library preparation incorporated eight distinct barcodes on a single flow cell. Variant calling and annotation were performed using TB-Profiler. Concurrently, extracted DNA was analyzed with FluoroType MTBDR VER 2.0 and LiquidArray MTB-XDR VER 1.0 as references. Analytical sensitivity and sequencing quality were evaluated, demonstrating promising results.

P013

Impact of 'borderline' *rpoB* mutations on the MICs of *Mycobacterium tuberculosis* against new and repurposed anti-tuberculosis drugs

P Rupasinghe¹ NS Hidayatallah¹ F Massou¹ A Dippenaar² M de Diego Fuertes²

A Van Rie² L Guglielmetti^{3 4 5} N Barreda⁶ L Chingisova⁷ N Hirani⁸ PT Hang⁹

DT Ha¹⁰ A Ashraf¹¹ E Ardizzoni¹ L Rigouts^{1 2} C Mitnick^{12 13 14} BC de Jong¹

1: Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp 2: University of Antwerp 3: Medical Department, MSF, Paris, France 4: Sorbonne University, Centre d'Immunologie et des Maladies Infectieuses (Cimi-Paris), France 5: AP-HP, Bactériologie-Hygiène, Hôpital Pitié-Salpêtrière, Centre National de Référence des Mycobactéries, Paris, France 6: Socios en Salud, Lima, Peru 7: National Tuberculosis Reference Lab, Almaty, Kazakhstan 8: Department of Microbiology, Sir JJ Hospital, Mumbai, India 9: Regional Tuberculosis Reference Lab, Ho Chi Minh, Vietnam

10: National Tuberculosis Reference Lab, Hanoi, Vietnam 11: The Indus Hospital laboratory, Karachi, Pakistan 12: Brigham and Women's Hospital, Boston, USA 13: Partners In Health, Boston, USA 14: Department of Global Health and Social Medicine, Harvard Medical School, Boston, USA

Rifampicin-resistance in *Mycobacterium tuberculosis* is associated with mutations in the *rpoB* gene. 'High-confident' *rpoB* mutations, such as *Ser450Leu*, are likely to have concordant, resistant results on phenotypic drug susceptibility test (pDST) in liquid media MGIT. In contrast, 'borderline' *rpoB* mutations, recognized by the World Health Organization (WHO), result in in-vitro growth defects, leading to false-susceptible rifampicin pDST results in MGIT. The effect of these growth defects on liquid-based pDST for other anti-TB drugs is unknown.

We evaluated whether 'borderline' *rpoB* mutations affect minimum inhibitory concentrations (MICs) for bedaquiline, clofazimine, linezolid, and delamanid using 688 isolates (30-lineage-1, 301-lineage-2, 82-lineage-3, 275-lineage-4) from the endTB/Q trials, tested in the EUCAST-derived broth-microdilution method (BMD). MIC distributions of 39 'borderline' *rpoB* mutants (13-*His445Asn*, 9-*Asp435Tyr*, 7-*Leu452Pro*, 5-*His445Leu*, 4-*Leu430Pro*, 1-*His445Ser*) were compared to 649 (508-*Ser450Leu*, 99-*Asp435Val*, 20-*His445Tyr*, 15-*His445Asp*, 7-*Ser450Phe*) 'high-confident' *rpoB* mutants, overall and by lineage. All isolates were from participants unexposed to the tested drugs and were classified as 'likely-susceptible' based on whole-genome sequencing.

Overall median turnaround time for MIC results was 14 days (IQR 10–14) in both groups. Overall, MIC distributions showed no differences between groups for any drug. However, in lineage-1, isolates with 'borderline' mutations had higher median linezolid MICs (1.0µg/ml) than those with "high-confidence" mutations (0.5µg/ml). Our findings suggest that 'borderline' *rpoB* mutations do not affect the validity of pDST results for the drugs tested using the BMD method. Higher linezolid MICs in lineage-1 'borderline' *rpoB* mutants warrant further investigation, considering the small sample size of lineage-1 included in this study.

P015

Overcoming barriers for non-tuberculous Mycobacteria identification: application of a culture-independent targeted sequencing approach

C Ferreira¹ J Isidro² [R Macedo](#)¹

1: National Reference Laboratory for Mycobacteria, Department of Infectious Diseases, National Institute of Health Dr. Ricardo Jorge, Lisbon, Portugal 2: Genomics and Bioinformatics Unit, Department of Infectious Diseases, National Institute of Health Dr. Ricardo Jorge, Lisbon, Portugal

Nontuberculous mycobacteria (NTM) are increasing worldwide and becoming a major public health problem. Among over 180 known NTM species, *Mycobacterium avium* complex (MAC) and *Mycobacterium abscessus* complex (MABC) are the most common cause of human disease, with some subspecies being associated with worse clinical outcomes. Accurate subspecies identification is crucial for guiding effective treatment. However, current laboratory diagnostics are culture-based, delaying diagnosis, and the available commercial kits often lack specificity as they do not take into account the genetic diversity of MAC and MABC subspecies. Here, we developed a culture-independent methodology based on targeted sequencing for correct subspeciation of both MAC and MABC directly from clinical samples.

Based on the current state-of-the-art, we selected 16 genetic regions that allow the identification of key subspecies: *M. bolletii*, *M. massiliensis*, *M. abscessus*, *M. avium spp hominissuis*, *M. intracellulare* and *M. chimaera*. A set of 21 primer pairs, with amplicon sizes ranging from 160-500 bp, were first validated individually with DNA from isolates of each subspecies, and then optimized into two multiplex-PCR reactions under the same cycling conditions. Amplicons were sequenced on an ONT MinION device and sequencing data analysed using the user-friendly bioinformatics platform INSaFLU. Final protocol optimization is ongoing using a set of 30 clinical samples from previously diagnosed patients.

Overall, we present a simple and straightforward approach that enables accurate identification of six clinically relevant NTM subspecies with a rapid turnaround time (~24 hours), offering a practical tool for diagnostic laboratories.

P016

***Mycobacterium avium* bacteremia: direct identification and genotypic susceptibility characterization from positive blood culture bottles**

A Camaggi¹ MG Pellò¹ M Mantovani¹ MS Caroppo¹ S Andreoni¹

1: Laboratory of Microbiology and Virology, University Hospital "Maggiore della Carità", Novara, Italy

Accurate and rapid diagnosis of disseminated mycobacteriosis is critical for the appropriate management of patients. The use of a fully automated method, like the BACTEC-MYCO/F-LYTIC-Becton Dickinson (BD) bottles system, represents a sensitive method for mycobacteremia detection. Unfortunately, Line Probe Assay (LPA) conventional assay for mycobacteria identification cannot be used for samples containing blood (a previous subculture on liquid/solid media is required). In this clinical case we prove the validity of the MBT Sepsityper Kit (Bruker) for the direct mycobacteria identification from positive blood cultures bottles (BCBs) using MALDI-TOF MS Biotyper-Bruker and LPA. A positive BCBs from a 48-year-old HIV-positive patient was sent to our laboratory to confirm the suspicion of disseminated mycobacterial infection. The Kinyoun stain confirmed the presence of acid-fast bacilli. The sample was then subcultured onto solid (Middlebrook 7H10-BD) and liquid (MGIT-BD) media. To evaluate the possibility of direct identification of mycobacteria from BCBs, in addition to the standard protocol, the validated Sepsityper® kit-Bruker and MALDI-TOF MS technology was used. The identification of *M. avium* was obtained in less than two hours. The bacterial biomass obtained with the Sepsityper method was also used for genotyping NTM-DR VER-1.0 LPA (Hain-Bruker). The identification of *M. avium* was confirmed and the genotypic susceptibility profile was rapidly assessed. The results were confirmed with the conventional protocol after 10 days. This clinical case showed that, using the Sepsityper® kit, it may be possible to accelerate the complete diagnosis of mycobacterial bloodstream infections. Same-day results could guarantee more adequate management of patients.

P017

Isolation of *Mycobacterium nebraskense* in four oncologic patients

A Camaggi¹ MG Pellò¹ A Rossati² MS Caroppo¹ S Andreoni¹

1: Laboratory of Microbiology and Virology, University Hospital "Maggiore della Carità", Novara, Italy 2: Department of Infectious Diseases, University Hospital "Maggiore della Carità", Novara, Italy

Mycobacterium nebraskense is a yellow pigmented, slow growing nontuberculous mycobacterium species with limited documented cases. *M. nebraskense* infections were first described in 2004 in Nebraska (from 5 patients). Only 14 additional cases have been reported in the literature, to the best of our knowledge. Here, we present our 4 cases (between 2009 and 2015) of patients who had *M. nebraskense* isolated from respiratory secretions: 3 were oncologic patients with solid tumours whereas 1 with acute myeloid leukemia. For all patients bronchoalveolar lavage was sent to our laboratory for mycobacteria research. All samples were first decontaminated with NAC-PAC™ Red (AlphaTec®-USA) then submitted to microscopic examination, amplification assays and cultured on solid (Löwenstein-Jensen, Becton Dickinson-BD) and liquid (MGIT 960-BD) medium incubated at 35-37°C, for 56 days. Microscopic examination, after Kinyoun staining, was negative for acid-fast-bacilli (AFB) in all four cases. The amplification test for *M. tuberculosis* complex (BD ProbeTec™) resulted negative in all cases and the culture on Lowenstein-Jensen too. In the four cases the presence of AFB in the liquid medium was detected after 32, 42, 36 and 53 days respectively. The identification of *M. nebraskense* was carried out with the use of the MALDI-TOF MS (Bruker). Species identification were confirmed by gene sequencing (16S rRNA). None of the patients started a treatment because of severe clinical conditions and all died few months later. The exact presentation of infection, disease progression, and treatment have not been described well, continued surveillance will be needed to determine its frequency and optimum treatment.

P018

Widespread preexisting resistance to new and repurposed anti-tuberculosis drugs through loss-of-function

D Conkle-Gutierrez¹ B Gorman¹ N Thosar¹ A Elghraoui¹ SJ Modlin¹ F Valafar¹

1: San Diego State University

Five New and Repurposed Drugs (NRDs) were approved in the last decade for treatment of multi-drug resistant tuberculosis: bedaquiline, clofazimine, linezolid, delamanid, and pretomanid. Unfortunately, resistance to these drugs emerged faster than anticipated, partly from preexisting resistance in naïve strains. Previous investigations into the rapid emergence mostly used short variants. For the first time, we utilize *de novo*-assembled genomes, and systematically include Structural Variations (SV) and heterogeneity to comprehensively study this rapid emergence. First, a systematic literature review revealed 313 NRD resistance variants in 13 genes. Next, 409 globally diverse clinical isolates collected prior to the drugs' programmatic use (308 were MDR, 106 had *de novo* assembled genomes) were utilized to study the 13 genes comprehensively for conventional, structural, and heterogeneous variants. We identified 5 previously reported and 67 novel putative NRD resistance variants. These variants were 2 promoter mutations (in 8/409

isolates), 13 frameshifts (21/409), 6 SVs (9/409), 35 heterogeneous frameshifts (32/409) and 11 heterogeneous SVs (12/106). Delamanid and pretomanid resistance mutations were most prevalent (48/409), while linezolid resistance mutations were least prevalent (8/409). Preexisting NRD resistance was highly prevalent in these strains (85/409, 21%), primarily through loss-of-function mutations in genes responsible for prodrug activation and efflux pump regulation. Loss-of-function mutations were similarly frequent throughout the genome, suggesting a wide bet-hedging strategy. Hence, any future drugs resistible through loss-of-function in non-essential genes may similarly suffer from preexisting resistance, which will then spread rapidly upon programmatic use. This effect might be mitigated at early-stage drug design through comprehensive preexisting-resistance assessment.

P019

Culture-free sequencing of *M. tuberculosis*: evaluating targeted and whole-genome approaches for genotyping and drug-resistance detection

L Iannucci¹ K Moghaddasi¹ F Di Marco¹ P Miotto¹ AM Cabibbe¹ DM Cirillo¹
POR TB Consortium²

1: *San Raffael Scientific Institute* 2: *Statens Serum Institut*

Targeted Next-Generation Sequencing (tNGS) of *Mycobacterium tuberculosis* (MTB) is approved by the World Health Organization for direct drug-resistant tuberculosis (DR-TB) testing of respiratory specimens. The new Deeplex Myc-TB XL assay (Genoscreen) targets several DR-associated loci, including novel key drugs as bedaquiline and pretomanid. Direct whole genome sequencing (dWGS) emerged as an innovative approach for full DR profiling and epidemiological surveillance without prior target selection. The new QIAseq xHYB MTB panel (Qiagen) enables dWGS through hybridization-based MTB genome capture. This study evaluates the detection limits (LOD) and performance for DR and genotyping predictions of the two assays.

We analyzed 89 MTB-positive sputum sediments from a TB vaccine trial with prevention of recurrence design. DNA was extracted and host DNA depleted using the Ultra-Deep Microbiome Prep (Molzym), then quantified by real-time PCR. Deeplex and QIAseq libraries were sequenced on Illumina systems. FASTQ files were processed with adapted MTBseq pipeline and benchmarked against WGS from corresponding cultures.

MTB genome copies (gc) quantification from extracted samples ranged from <10 to >1000 gc/uL. Deeplex yielded complete DR profiles (coverage depth >200x) in 97% of specimens, with LOD of 10 total gc. QIAseq produced high-quality genome coverage (depth >30x; breadth >97%) in 60% of samples, enabling transmission analysis consistent with that of culture-based WGS (concordant clustering for all but one sample). DR profiling was successful in 74% of samples; LOD was >100 total gc. Both platforms achieved >98% sensitivity/specificity for DR mutations identification.

Bacillary load-guided selection of tNGS or dWGS would improve culture-free DR-TB sequencing efficiency.

P020

Big MAC: whole-genome sequencing uncovers novel members of the *Mycobacterium avium* complex unidentified at the species level by line-probe assays

A Norman¹ EM Rasmussen¹ E Svensson¹ T Lillebaek¹

1: *Statens Serum Institut*

Disease caused by nontuberculosis mycobacteria (NTM) is rising worldwide, and in several low-tuberculosis-incidence countries, clinical NTM is approaching the incidence of tuberculosis (TB) disease. Routine species identification still relies on line-probe assays (LPAs) such as GenoType *Mycobacterium* CM/AS, which recognise only the 25–30 most common taxa. Divergent or novel lineages therefore hybridise solely to the genus control band and are reported as “*Mycobacterium* sp.”, obscuring clinically relevant diversity. Targeted sequencing of marker regions (*hsp65*, 16S, ITS, 23S) offers higher resolution but falls short for very closely related taxa, making whole-genome sequencing (WGS) the current gold standard. We analysed every Danish NTM high-quality genome generated in the past decade (n=131) together with representative genomes for all described NTM species in RefSeq. Pair-wise average nucleotide identity (ANI) was calculated and clustered with UPGMA. Eighty-six percent of isolates shared >95% with recognised type strains while the remainder (n=18) did not. Twelve of these grouped within the *M. avium* complex (MAC), forming tight clusters, consistent with at least six novel MAC taxa. As MAC is responsible for >60% of NTM cases in Denmark, we chose to focus on these unknown members of this clinically relevant group. One representative from each cluster underwent Oxford Nanopore sequencing, and hybrid Illumina/Nanopore assembly produced complete, high-quality circular genomes (5.5–6.3 Mbp) for all six candidates, each harbouring one to three plasmids. These findings broaden the known membership of the MAC and highlight the limitations of probe-based diagnostics for emerging NTM pathogens.

P021

Piloting Precision: Implementation of targeted next generation sequencing for antibiotic resistance testing into the diagnostic algorithm of Tuberculosis in Kyrgyzstan

C Utpatel^{1 2} A Iskakova³ H Hoffmann^{4 5} M Sydykova³ A Kadyrov⁶ S Niemann^{1 2}
G Kalmambetova⁶

1: *Research Center Borstel* 2: *German Center for Infection Research* 3: *Republican Tuberculosis Reference Laboratory* 4: *IML red GmbH* 5: *SYNLAB Gauting* 6: *Republican Tuberculosis Center*

Targeted next-generation sequencing (tNGS) is an increasingly valuable tool in tuberculosis diagnostics, offering rapid, comprehensive detection of drug resistance mutations directly from clinical specimens or cultures. Its ability to guide individualized treatment and improve diagnostics makes it particularly beneficial in high-burden settings like Kyrgyzstan, where DR-TB poses major challenges to public health.

Here we report on the first successful implementation of tNGS at the Republican Tuberculosis Reference Laboratory. Using the now WHO-endorsed Deeplex® Myc-TB assay, we conducted a pilot study on sputum samples collected from 2020 to 2022 from 281 people living with TB.

Overall, 266 samples were eligible for tNGS. 238 (89%) yielded full resistance profiles covering 15 drugs. 143/238 (60%) were rifampicin (RIF) resistant. 108 (76%) were MDR, 22 (15%) pre-XDR and 6 (4%) XDR. Bedaquiline resistance was detected in 11/143 (8%) samples which were recovered only from new cases. No resistance to linezolid was detected. Deeplex success rate correlated with smear positivity.

The concordance to phenotypic drug susceptibility testing was 90% for RIF. Noteworthy, 15/19 (79%) of the samples tested phenotypically susceptible towards RIF actually carried low level resistance mutations.

On average, the final tNGS results were available after 53h from the start of DNA extracts.

DR-TB remains a challenge in Kyrgyzstan, particularly due to high numbers of fluoroquinolone and bedaquiline resistance. Our findings support the integration of tNGS into the national diagnostic algorithm as a complementary test to the existing diagnostic methods to improve precise diagnosis of DR-TB.

P022

Acceptance of tongue swabs for tuberculosis screening among healthcare workers in Italy: a qualitative study

R Codsi¹ F Saluzzo² D Nadal¹ D Cirillo² J Cangelosi¹

1: *University of Washington* 2: *San Raffael Scientific Institute*

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. Tongue swab (TS) collection may help mitigate these risks and challenges. This study aims to evaluate HCWs' perceived risks, opportunities, and barriers regarding TS use for TB screening among migrants in Italy.

Purposive sampling was used to enroll HCWs screening migrants for TB, after which they were invited for an in-depth interview. Enrollment started in November of 2023 and will continue until July 2025 or until saturation among themes is reached.

The study employed the COM-B implementation science model across study phases, guiding the development of the semi-structured interview questions exploring participants' capabilities to collect and teach migrants to collect TS samples. Hamilton's Rapid Qualitative Analysis Method was adapted to summarize key findings.

HCWs interviewed (N=6) indicated a preference for TS over sputum to support the scaling up of TB screening in migrants. HCWs shared perceptions that TS reduces their occupational exposure to TB, is safer to perform, is effective on both symptomatic and asymptomatic migrants, and is easier to analyze, with the possibility of pooling samples. HCWs shared that supervision is necessary to collect a high-quality sample and emphasized the need for solutions to language barriers and a lack of educational materials for migrants.

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

P023

Genetic characterisation of *Mycobacterium bovis* strains isolated from cattle in Northern Africa

I Dickie¹ M Khames² J Stiens³ I Nobeli³ D Xia¹ SL Kendall¹

1: The Royal Veterinary College 2: University of Medea 3: Birkbeck, University of London

Mycobacterium bovis is able to cause tuberculosis in a number of mammalian species, including cattle. Bovine tuberculosis (bTB), often controlled by test and slaughter policies, is both an economic and ethical concern for the livestock industry. As a zoonotic pathogen, it remains a threat to public health, particularly in countries unable to support a test and slaughter policy.

Globally circulating *M. bovis* field isolates have been typed using low resolution methods into clonal complexes (Eu1, Eu2, Eu3, Af1 and Af2). High resolution whole genome sequencing (WGS) has classified isolates into lineage La1 with eight sub-lineages (La1.1 to La1.8). Currently, genome sequence databases are skewed geographically and dominated by strains belonging to the Eu1 (La1.8.1) clonal complex. In this study we have applied WGS to gain a better understanding of the genetic diversity in field isolates from Northern Africa, a region under-represented in the global collection.

In 2019 field strains of *M. bovis* were isolated from tissue samples derived from a single abattoir located in the Medea region of Algeria. Genomic DNA was isolated from 10 strains and sequenced using short-read technology. Phylogenetic analysis grouped these strains into an unknown clade (unk7 – La1.8.2) and the better studied Eu2 clade (La 1.7.1). Analysis of single nucleotide polymorphisms (SNPs) is ongoing, including analysis of conservation across immune epitopes, to determine the functional consequences of strain diversity on virulence, zoonotic potential, and sensitivity of diagnostics. Long-read sequencing will be applied for better detection of variants in highly repetitive regions, including PE/PPE genes.

P024

Rapidly and reproducibly building a catalogue of resistance-associated variants using 53,700 *M. tuberculosis* samples with genomes and DST data

D Adlard¹ K Malone² J Westhead¹ H Thai¹ M Colpus¹ R Turner¹ Z Iqbal²

TEA Peto¹ D Crook¹ PW Fowler¹

1: University of Oxford 2: University of Bath

Huge progress towards translating whole genome sequencing (WGS) into tuberculosis diagnostics and surveillance has been made in the last few years with the advent of the WHO catalogues of resistance-associated variants (RAVs) for tuberculosis. The process to build the catalogue is, unfortunately, slow and not reproducible. Here we describe processing 53,700 clinical

tuberculosis samples with WGS data and drug susceptibility testing (DST) results that are publicly available via the CRyPTIC project and automatically generating a catalogue with minimal user intervention. FASTQ files were processed by an online cloud processing platform. Resulting data tables were ingested by catomatic, a freely available Python package, producing a catalogue for 15 drugs. We curated an independent Validation dataset, allowing us to truly assess the performance of the resulting catalogue. The whole process took less than six weeks from FASTQ to sensitivity/specificity graphs. Our catalogue performed similarly to both editions of the WHO catalogue for all drugs without adding any manually-defined expert rules. Since catalogue building is automatic, we were able to vary the proportion of reads needed to identify an RAV and this improved performance, with some drugs (e.g. amikacin) affected more than others. As more *M. tuberculosis* samples are amassed it is becoming possible to generate resistance catalogues rapidly and automatically which will permit us to both respond more quickly and potentially tailor catalogues for specific geographies. Approaches like this could also be applied to common NTM species as WGS/pDST datasets for these become available.

P025

Growth phase-specific DNA methylation bias in *Mycobacterium tuberculosis* clinical isolates

[SJ Modlin](#)¹ M Jeshvaghane¹ D Conkle-Gutierrez¹ F Valafar¹
1: San Diego State University

DNA methylation plays diverse regulatory roles in bacterial pathogens, yet its dynamics remain poorly understood in *Mycobacterium tuberculosis* (*Mtb*). Here, we report intercellular mosaic methylation (IMM) by the orphan Type I methyltransferase MamC in a subset of fast-growing clinical isolates despite having wild-type mamC. Through PacBio long-read sequencing, we identified four *Mtb* complex isolates with anomalous hypomethylation at MamC-targeted sequence motifs. These “MamC-anomalous” isolates exhibited unique methylation heterogeneity, distinct from phase variation, indicating IMM, a phenomenon rarely documented for Type I methyltransferases.

We found that hypomethylation patterns in these isolates were linked to chromosomal replication dynamics. MamC methylation efficiency decreased progressively with distance from the origin of replication, with this positional bias markedly intensified in MamC-anomalous strains. By contrast, other *Mtb* methyltransferases (MamA, MamB) showed strand-specific but less pronounced positional methylation effects.

Using a refined peak-to-trough ratio (PTR) metric adapted for long-read sequencing, we show that MamC-anomalous isolates exhibit elevated and highly stable replication-linked coverage bias, consistent with unusually rapid and synchronized growth. These growth dynamics were not reflected in methylation patterns of MamA or MamB, suggesting a unique sensitivity of MamC activity to bacterial growth phase.

Together, our results uncover a growth phase-associated modulation of methylation efficiency, manifesting as intercellular mosaic methylation, a previously unknown relationship in *Mtb* epigenetics. This advances our understanding of *Mtb* DNA methylation systems and suggests functional links between replication state and epigenetic heterogeneity in clinical *Mtb* isolates with potential implications for adaptive diversification during rapid growth.

Lung ultrasound for the detection of pulmonary tuberculosis using expert- and AI-guided interpretation: a prospective cohort study

V Suttels^{1,2} T Brokowski³ AP Wachinou⁴ J Wolleb³ AR Hada⁴ JD Du Toit⁵
G Makpemikpa⁴ C Bessat¹ E Garcia¹ A Roux¹ T Brahier¹ O Oputa⁶
D Affolabi⁷ MA Hartley² N Boillat-Blanco¹

1: Lausanne University Hospital 2: Intelligent Global Health Research Group, Swiss Institute of Technology (EPFL) 3: Yale school of medicine, Department of Biomedical Informatics & Data Science 4: National Teaching Hospital for Tuberculosis and Pulmonary Diseases (CNHU-PPC) 5: MRC Wits Rural Public Health and Health Transitions Research Unit (Agincourt), Faculty of Health Sciences, University of the Witwatersrand 6: Institute of Microbiology University of Lausanne and University Hospital Centre, Lausanne 7: Laboratoire de référence des mycobactéries (LRM), Cotonou, Benin

Background: Point-of-care lung ultrasound (LUS) is a promising tool for portable sputum-free tuberculosis (TB) triage. We investigate the diagnostic performance of LUS to detect TB using expert and artificial intelligence (AI) guided interpretation. We introduce ULTR-AI (Ultrasound-led TB recognition using AI), a suite of deep learning (DL) models designed to automate TB risk stratification from LUS images.

Methods: In this prospective cohort study in a tertiary center in urban Benin, a standardized 14-point sliding scan LUS protocol was performed for symptomatic patients by a trained operator. LUS images were reviewed by two blinded and independent readers. Same-day single sputum Xpert MTB/RIF Ultra® was the microbiological reference standard. The suite comprises three AI models, ULTR-AI, ULTR-AI[signs] and ULTR-AI[max]. ULTR-AI predicts TB directly from images using DL, ULTR-AI[signs] first generates human-recognizable pathological signs before TB risk prediction in a machine learning (ML) model. Finally, ULTR-AI[max] takes the maximal TB risk score predicted by these two models.

Results: Out of 760 screened, 504 were analyzed. Of these, 192 (38%) had bacteriologically confirmed TB. ULTR-AI[max] reached the recommended WHO requirements for a sputum-free TB triage test of 0.9 sensitivity and 0.7 specificity, achieving a sensitivity of 0.91 (95% CI, 0.90–0.96) and a specificity of 0.85 (95% CI, 0.74–0.88) [AUROC 0.93, 95% 0.92–0.95].

Conclusion: In this cohort, AI-guided lung ultrasound meets the WHO requirements for sputum-free TB triage, enabling point-of-care testing with minimal infrastructure. ULTR-AI could help decentralize TB diagnostics in LMICs, enhancing timely detection. Validation in diverse populations is crucial to confirm clinical utility.

P027

Subpopulations in clinical samples of *M. tuberculosis* can give rise to rifampicin resistance and shed light on how resistance was acquired

VM Brunner¹ PW Fowler¹

1: University of Oxford

The rise of antimicrobial resistance (AMR) necessitates accurate and rapid diagnostics. Especially in *Mycobacterium tuberculosis* infections, whole genome sequencing (WGS) has become an established diagnostic method. But disagreement between the resistance prediction through genotypic, such as WGS, and phenotypic approaches can hinder effective treatment and surveillance. Within-host bacterial diversity, particularly the presence of subpopulations harbouring resistance-conferring alleles, may contribute to these inconsistencies.

This study investigated the impact of incorporating minor mutations in WGS-based rifampicin resistance prediction in a dataset of 38,787 *M. tuberculosis* samples. We examined the presence of resistance mutations in homogenous samples as well as in subpopulations (heteroresistance). The sensitivity and specificity of resistance classification were evaluated with and without the inclusion of minor mutations for prediction.

Identifying resistant minor mutations in the samples led to a significant improvement in sensitivity, with a small trade-off for specificity. Further analysis revealed distinct clusters of samples based on the number of minor variants present, pointing towards different mechanisms of resistance acquisition, i.e. resistance emerging due to within-host evolution or resistance acquired through a secondary infection.

Through our analysis, we have demonstrated the importance of considering within-host diversity when evaluating drug resistance in *M. tuberculosis*. Acknowledging and utilizing the predictive power of resistant minor mutations can significantly improve the accuracy of genotypic resistance classification. This enhanced accuracy can facilitate better-informed treatment decisions and more effective surveillance strategies. These findings raise the question of whether minor alleles should be routinely considered in resistance prediction algorithms.

P028

Emergence and reversion of rifampicin resistance during discontinuous treatment of tuberculosis in an HIV patient

C Genestet^{1 2} B Joannard¹ C Bourg^{1 2} O Bahuaud^{2 3} F Ader^{2 3} E Hodille^{1 2}
O Dumitrescu^{1 2}

1: Lyon University Hospital, Bacteriology Laboratory, France 2: International Center for Infectious Disease Research (CIRI), Lyon, France 3: Lyon University Hospital, Infectious and Tropical Diseases Department, France

We report a unique case of reversible rifampicin resistance in a patient with *Mycobacterium tuberculosis* (Mtb) infection. The patient, HIV-positive at the AIDS stage, received anti-

tuberculosis (TB) treatment over three years with poor adherence. Treatment alternated between rifampicin, isoniazid, pyrazinamide, ethambutol, levofloxacin, and tedizolid. Clinical progression from lymphatic and pulmonary TB to a miliary form was observed. Serial phenotypic drug susceptibility testing (Bactec MGIT 960 System and minimum inhibitory concentration (MIC) determination), PCR (Xpert® MTB/RIF Ultra and Deeplex), and whole genome sequencing (WGS) were performed over time. Initially, Mtb was rifampicin-sensitive, with no resistance-associated mutations. Fifteen months later, PCR detected resistance mutation, and WGS revealed the rpoB H445N mutation, associated with low-level rifampicin resistance. MIC testing showed elevated but subcritical values for rifampicin, as well as for rifapentine and rifabutin. Two years after this episode, the patient was hospitalized for another relapse and phenotypic and genotypic sensitivity testing showed the absence of rifampicin resistance. WGS confirmed that the case represented a true relapse rather than a reinfection, while multiple closely related variants of the same Mtb strain were detected throughout the course of infection. This case is particularly striking as it represents the first documented instance in the literature of mutation reversion during the course of the patient's infection. This raises several important issues: the factors driving the emergence of secondary resistance during discontinuous drug treatment, the role of the immune system in an HIV-co-infected patient in selecting for resistance, and the fitness cost associated with rifampicin-resistance.

P029

Use of large-scale clinical samples to progressively create a reference catalogue of mycobacterial species

TEA Peto ¹ M Colpus ¹ ER Robinson ² PW Fowler ¹ D Foster ² R Myers ²
C Brown ² H Thieu ¹ R Turner ¹ DW Crook
1: *University of Oxford* 2: *UK Health Security Agency (UKHSA)*

Accurate identification of mycobacteria of clinical relevance, whether rare or common, and the ability to detect mixed infections is required. We are analysing competitive mapping of a large and growing number of clinical samples to develop a methodology to classify isolates into clusters, likely to represent independent species. It is, however, currently unclear how many clinical samples are required to create a near-complete catalogue.

Initially, 4443 clinical isolates of non-tuberculous mycobacteria (NTM) from Public Health England (now UKHSA) were sequenced and competitively mapped against 178 reference sequences using a cloud-based pipeline. Isolates were assigned to 87 putative species, and 39 species were in clusters of at least 5 isolates (18 species ≥ 20). For these 39 species, preliminary threshold values were established to distinguish known species, identify possible mixed infections, detect novel species and to identify the need for new reference sequences. The results were compared with the Mykrobe species classifier containing 203 reference species. Also, the pipeline was assessed with a 'truth' set of sequences from 153 reference species sourced from type collections.

Ongoing evaluation of competitive mapping performance on a further >30,000, sequenced by PHE/UKHSA, is being used to confirm the thresholds for assignment of species in clusters, more precise identification of mixed infections, and recognition of new clusters (species). The most current results will be presented. This approach is designed to progressively establish a much enlarged catalogue of species and provide an estimate for the sample size needed to create a near-complete catalogue of mycobacterial species.

P030

Acceptability of tongue swabs for tuberculosis screening in migrant settings: A qualitative study with healthcare workers in Lombardy, Italy

R Codsi¹ F Saluzzo² J Cangelosi¹ D Cirillo² D Nadal¹ GH Wanje¹

1: University of Washington 2: San Raffaele Scientific Institute

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. Tongue swab (TS) collection may mitigate these risks and challenges. Tongue swabbing is now being assessed in community settings worldwide, and plans are underway to seek WHO endorsement. For the latter, evidence is needed to assess the acceptability of TS for TB diagnostics by healthcare workers. This study aimed to evaluate HCWs' perceived risks, opportunities, and barriers regarding TS use for TB screening among migrants in Italy.

Through purposive sampling, we enrolled HCWs experienced with sputum and TS collection for in-depth semi-structured interviews. Enrollment started in November of 2023 and will continue until July 2025 or until thematic saturation is reached. We adapted Hamilton's Rapid Qualitative Analysis Methods to summarize key findings.

HCWs interviewed (N=6) indicated a preference for TS over sputum to support the scaling up of TB screening in migrant settings. HCWs shared perceptions that TS reduces their occupational exposure to TB, is safer to perform, is effective on both symptomatic and asymptomatic migrants, and is easier to analyze, with the possibility of pooling samples. HCWs shared that supervision is necessary to ensure adherence to the protocol. They also emphasized the need for solutions to language barriers and educational materials for migrants.

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

P031

Rapid drug resistance prediction in positive *Mycobacterium tuberculosis* clinical samples using an extensive targeted next-generation sequencing panel

E Rosendal^{1 2} J Isidro³ S Carneiro⁴ JP Gomes^{3 5} R Macedo⁴

1: ECDC Fellowship Programme, Public Health Microbiology path (EUPHEM), European Centre for Disease Prevention and Control (ECDC) 2: Infectious Diseases Department, National Institute of Health Doctor Ricardo Jorge 3: Genomics and Bioinformatics Unit, Infectious Diseases Department, National Institute of Health Doctor Ricardo Jorge 4: National Reference Laboratory for Mycobacteria, Infectious Diseases Department, National Institute of Health Doctor Ricardo Jorge 5: Animal and Veterinary Research Center (CECAV), Faculty of Veterinary Medicine, Lusófona University - Lisbon University Centre

The emergence of drug-resistant *Mycobacterium tuberculosis* strains poses a challenge to TB-control and global eradication efforts. Most methods for drug susceptibility testing (DST) are culture-dependent and time consuming, possibly delaying optimal treatment. This study aimed to develop an extensive targeted next-generation sequencing (tNGS) approach for rapid genotypic DST directly from clinical samples. We designed a tNGS panel comprising 30 amplicons targeting 19 genomic regions associated with resistance to 20 antibiotics. This method was applied to 71 smear-positive (0–3+) pulmonary TB clinical samples collected at the Portuguese National Reference Laboratory. DNA was extracted and amplified using multiplex PCRs, followed by sequencing on Oxford Nanopore Technologies MinION platform. Sequencing data were analysed using the online version of TB-Profiler and the tNGS results compared to phenotypic DST and whole genome sequencing (WGS) data from corresponding isolates. The tNGS demonstrated high concordance with both phenotypic and WGS-based DST across different sample types and smear positivity levels. For first-line drugs, tNGS showed 88% categorical agreement (CA) with pDST, increasing to 97% when excluding undetermined results. Compared to WGS across all analysed antibiotics, tNGS achieved 92% CA, increasing to >99% when excluding undetermined results. Validation of the tNGS panel showed 90% (1,895/2,076) of amplicons reaching >10x depth of coverage at all analysed positions and 43 (61%) samples with all amplicons above this threshold. Non-specific amplification of contaminant bacterial DNA was minimal. In conclusion, this method enables comprehensive resistance prediction directly from clinical samples and may aid in TB diagnostics and resistance monitoring.

P032

Evaluation of new microdilution Plates (FRATMYC1 and FRATMYC2) for drug susceptibility testing to old and new antibiotics against slow growing non-tuberculous mycobacteria

C Poignon^{1 2 4} F Mougari^{2 3} I Hermann² E Gydé^{1 2} E Cambau^{2 3} A Aubry^{1 2 4}

1: AP-HP Sorbonne University 2: Centre National de Référence des Mycobactéries et de la Résistance des Mycobactéries aux Antituberculeux 3: AP-HP GHU Nord 4: UPMC - Paris 6

Drug Susceptibility Testing (DST) is crucial for optimizing therapeutic choices in non-tuberculous mycobacterial (NTM) infection. While broth microdilution is the reference method, commercially available SLOMYCO Sensititre™ plates have two main drawbacks: inappropriate concentration ranges for some antibiotics and the absence of clinically relevant agents, such as bedaquiline (BDQ) and clofazimine (CFZ). To overcome these limitations, we designed customized Sensititre™ plates (FRATMYC1, FRATMYC2) and aimed to (i) test antibiotics missing from standard panels and (ii) evaluate the concordance of first-line antibiotics' MICs measured using SLOMYCO and FRATMYC plates for *Mycobacterium avium* complex (MAC) clinical isolates.

We tested 73 MAC strains (31 *M. avium*, 23 *M. chimaera* and 19 *M. intracellulare*), collected between 2018 and 2024, including the reference strain *M. avium* MAC101 (ATCC700898). Species identification was performed using routine procedures (i.e. line probe assays, GenoType NTM-DR, Bruker, Germany). MICs were determined using all three Sensititre™ plates according to CLSI guidelines. Agreement between methods was defined as MICs within $\pm 1 \log_2$ dilution.

BDQ and CFZ exhibited the lowest MIC90 (0.06 mg/L and 0.12 mg/L, respectively), regardless of susceptibility to clarithromycin (CLR) and amikacin, based on 11 CLR-resistant and 6 amikacin-resistant isolates, including 3 resistant to both. Agreement rates between SLOMYCO and

FRATMYC were 72.2% for CLR and 77.8% for amikacin, with categorical agreement of 98.6% and 90.3%, respectively.

FRATMYC plates provided comparable results to SLOMYCO while expanding DST to clinically relevant agents. BDQ and CFZ demonstrated strong in vitro activity and should be considered in routine DST for slow-growing NTM.

P033

Evaluation of an Oxford Nanopore sequencing workflow for mycobacteria from primary MGIT culture

CS Baker¹ J Gentry² A Hall¹ M Colpus¹ H Webster¹ H Thai¹ R Turner¹
SV Shaheed³ TEA Peto¹ PW Fowler¹ M Morgan² [DW Crook](#)¹

1: The University of Oxford 2: Oxford University Hospitals NHS Foundation Trust 3: National Institute of Communicable Disease, Johannesburg, South Africa

Next-generation sequencing using Illumina has emerged as the standard for Mycobacterial genomic analysis. Mechanical DNA extraction methods have often yielded inadequate concentrations for Oxford Nanopore Technologies (ONT) sequencing kits. We report substantial improvements to the workflow using simplified, automation-aided extraction and a modified Rapid PCR Barcoding protocol, resulting in an ONT-compatible process that enables faster, more cost-effective, high-throughput sequencing.

178 samples collected by a routine NHS laboratory were cultured in mycobacterial growth indicator tubes, then heat-inactivated in a convection oven before removal from containment level 3. Mycobacterial cells were mechanically lysed, and DNA purified through an automated platform. We report on the initial 82 samples sequenced by both Illumina and ONT. One sample failed to amplify through the modified ONT protocol.

All samples had human reads removed prior to processing by an online cloud analysis platform. 80/81 samples were identified as mycobacterial species by both sequencing platforms. One sample (from tissue) failed due to mostly human reads. We identified 17 *M. tuberculosis* and 63 non-tuberculosis mycobacteria; 67/80 were concordant for species and lineage across both sequencing platforms. Eight subspecies differences were observed between platforms, five of which were *M. intracellulare*. Two samples also showed mixed species discordance. Results for all 178 samples will be reported. Clear differences in nucleotide substitutions and indels were observed for the 17 *M. tuberculosis* isolates, which will be further reported in a separate abstract of 400 isolates. These results highlight the need for further robust comparison of ONT and Illumina sequencing.

P034

Comparison of Illumina and Oxford Nanopore Technology sequencing of *Mycobacterium tuberculosis*

M Colpus¹ C Baker¹ HN Hong³ SV Omar² PP Trieu³ DDA Thu³ L Hall¹
PW Fowler¹ T Walker^{3,4} R Spies¹ H Webster¹ J Westhead¹ H Thai¹ R Turner¹
T Peto¹ NL Quang³ NTT Thuong^{3,4} D Crook¹

1: Nuffield Department of Medicine, University of Oxford 2: National Institute for Communicable Diseases, South Africa 3: Oxford University Clinical Research Unit, Centre for Tropical Medicine, Ho Chi Minh city, Vietnam 4: Nuffield Department of Medicine, Centre for Tropical Medicine and Global Health, University of Oxford, UK

Illumina sequencing is the leading sequencing platform for *Mycobacterium tuberculosis*. It has become the standard for determining genomic variation, classifying subspecies, predicting drug resistance, and identifying outbreak clusters.

There have been only a handful of studies comparing the relative performance of long-read sequencing by Oxford Nanopore Technologies (ONT) to Illumina. Here we investigate the differences in performance of 400 genomes sequenced by both Illumina and ONT and initially report on the first 67. Half were South African and half Vietnamese. Sequencing followed standard operating procedures. Genomes were assembled and analysed using an online cloud platform, which found general concordance but also clear differences between ONT and Illumina. Ongoing characterisation and quantification of differences will be reported for all 400.

Illumina samples had a mean total bases 0.67Gbp. ONT produced 0.51Gbp with a mean N50 of 3670bp. 64/67 genomes were assigned identical lineages; 3 samples had a mixed lineage in only one platform. Resistance prediction for 61/67 genomes was identical (across 14 antibiotics), with discrepancies linked to indels with insufficient read support.

For each isolate we calculated the SNP distance between sequencing platforms. Repetitive regions were excluded using masks. The mean difference was 5.6 SNPs. We examined whether platform specific errors or difference in mapping caused this discrepancy and also investigated mask refinement for improving cross-platform detection of clusters. To further aid investigation of platform differences, ONT de novo assembly was performed yielding assemblies of expected total length (12 circularised and 48 consisting of 1-4 contigs).

P035

Stool based diagnostics for TB: who stands the benefit and how far are countries with the implementation

P de Haas¹ E Tiemersma¹ M Mbenga¹ B Puij² V Harawa² A Kebede
1: KNCV TB foundation 2: African Society Laboratory Medicine (ASLM)

Stool-based testing, recommended by the WHO to diagnose TB in children, has been shown to increase TB notification and access to diagnosis. In addition, it showed to benefit adults experiencing difficulties in expectorating sputum. While currently being recommended for the GeneXpert platform only, proof of concept was provided for stool-based testing using the simple one step stool (SOS) method on Xpert-MTB/XDR and the Truenat platform.

To get insights in the extent to which stool-based testing is implemented in Africa we conducted an online survey including representatives of 47/54 African countries. Here, we report the results of this survey.

We received responses for 35 (74%) countries. In 25 (71%), implementation of stool-based testing has started with 11 countries piloting this, and 14 countries engaged in nationwide implementation, while planning the start of stool-based testing is ongoing in 5 more countries. Of 25 countries, in 15, key populations beyond children are included (most often adolescents (n=11), and adults who cannot spontaneously produce sputum (n=10)). In 7 countries (all implementing stool testing nationwide), the Truenat platform was (also) used, while in 9 countries piloting, and in 11 countries with nationwide implementation, the XpertMTB/XDR test was used on stool.

Stool-based testing is widely implemented in the African region and countries do include populations and test (platforms) beyond the WHO-recommendations. Guidance on stool processing methodology for the different assays are developed, so that a bacteriologically confirmed diagnosis including the possibility to obtain a resistance profile becomes in reach for those who cannot easily expectorate sputum.

P036

A rapid DNA extraction method from clinical culture isolates for mycobacteria identification using INNO-LiPA mycobacteria v2

CS Gan^{1,2} KSA Teo² BXJ Tan^{1,2} LH Sng^{1,2}

1: Duke-NUS Medical School 2: Singapore General Hospital

Genomic nucleic acid amplification and species identification of microorganisms require high quality DNA. Since 2011, our lab been using the INNO-LiPA Mycobacteria v2 (INNO-LiPA) to identify nontuberculous mycobacteria isolated from clinical samples. DNA is extracted using Heat-Sonication, which involves a 45min heat-inactivation and a 15min sonication process. With increased number of nontuberculous mycobacteria being isolated, a more rapid DNA extraction method would be advantageous. The GenoLyse (Hain Lifescience, Germany) method only requires a 5min lysis time and a buffer neutralization step. Therefore, this study aimed to compare the INNO-LiPA performance and mycobacteria inactivation efficacy between the Heat-Sonication and GenoLyse extraction methods. A total of 61 bacterial isolates (18 reference strains and 43 clinical isolates) were subjected to DNA extraction, using both methods for a pairwise evaluation. The INNO-LiPA and result interpretation were conducted following the manufacturer's instructions. Although the Heat-Sonication method yielded a 3.35-fold higher (p value 0.0004) DNA concentration than the GenoLyse method, the latter produced a 1.45-fold higher (p value 0.000003) amplicon concentration than the former. Despite the significant differences, analytes amplified from Heat-Sonication and GenoLyse extraction methods demonstrated comparable performances with an assay sensitivity of 96.42% and 98.18%, respectively. The assay specificity was 100% for both extraction methods. The inactivation efficiency was also comparable, with 98.36% (60/61) by Heat-Sonication and 100% (61/61) by the GenoLyse method. In conclusion, the GenoLyse method was shown to be a rapid DNA extraction method that provides comparable INNO-LiPA identification performance when compared to that of Heat-Sonication extraction method.

P037

Exploring patterns of serum CRP level reduction in response to treatment initiation in Latvian patients with pulmonary tuberculosis: the impact of *Mycobacterium tuberculosis* genotype

A Kivrane^{1,2} V Ulanova^{1,2} D Sadovska² A Viksna^{3,4} I Ozere^{3,4} I Bogdanova⁴
I Simanovica⁴ I Norvaisa⁴ R Ranka^{1,2}

1: Pharmacogenetic and Precision Medicine Laboratory, Pharmaceutical Education and Research Centre, Riga Stradins University, Latvia 2: Latvian Biomedical Research and Study Centre, Riga, Latvia 3: Department of Infectiology, Riga Stradins University, Latvia 4: Riga East University Hospital, Centre of Tuberculosis and Lung Diseases, Latvia

In tuberculosis (TB), serum CRP levels vary with patient- and disease-related factors, and failure to achieve a two-fold reduction within the first two weeks may indicate adverse treatment outcomes. Given reported *Mycobacterium tuberculosis* (Mtb) genotype-related variability in immune response and clinical presentation, this study evaluated whether Mtb genotype influences early changes in serum CRP levels. Clinically relevant data, including serum CRP levels at baseline (CRP_b) and 10–12 days after treatment initiation (CRP_{10-12d}), were retrieved from patients' medical records (n=35). Whole-genome sequencing was performed on all patients' Mtb isolates, and TB-Profiler was used to assign genotypes and detect drug resistance-associated genomic variants, interpreted alongside phenotypic drug susceptibility (DS) data. Overall, considerable Mtb genotype diversity was observed: LAM (28.6%), T (25.7%), Beijing (17.1%), Haarlem and Ural (11.4% each), and others (5.8%). Patients infected with Mtb of LAM/Ural genotypes more frequently had lung cavitations, lower body mass index, and higher CRP_b levels than those infected with T/Haarlem or Beijing genotypes, although differences were not statistically significant. DS cases (n=27) were further analysed for treatment-specific effects. In 10–12 days, serum CRP levels significantly declined in the LAM/Ural and T/Haarlem groups (p=0.011 and p=0.025, respectively) but not in the Beijing group (p=1.000). CRP_{10-12d} levels followed a similar pattern to CRP_b, with higher values found in patients infected with LAM/Ural genotypes. In conclusion, the observed trends align with previous reports and suggest that the Mtb genotype may influence early CRP dynamics. The clinical significance of these findings is yet to be established.

P038

Vitamin D receptor *BsmI* haplotype BB confers lower 25(OH)D levels during tuberculosis: A cross-sectional study

D Sambrano¹ S Martín-Pelaez^{2,3} K Salazar⁴ P Patel¹ F Acosta¹ E Romero^{5,6}
JE Ku¹ I Martínez⁷ Y Cuadra⁷ J Jurado⁷ S Hawkins⁷ O Luque⁸ L Solís⁹
M Vergara⁹ L Garibaldi⁷ A Goodridge¹

1: Instituto de Investigaciones Científicas y Servicios de Alta Tecnología 2: Universidad de Granada 3: CIBER of Epidemiology and Public Health (CIBERESP-Spain) 4: Universidad Latina de Panamá 5: Universidad de Panamá 6: University of Bristol 7: Caja del Seguro Social 8: Ministerio de Salud 9: Laboratorio Regional de Tuberculosis de Colón

This cross-sectional study aimed to evaluate the vitamin D receptor [25(OH)D] (VDR) *FokI*, *TaqI*, and *BsmI* polymorphisms frequency and their relationship with vitamin D levels, metabolites, and ratios (VMR) in patients with tuberculosis (PTB) and healthy controls (HC) in Colon, Panama. We analyzed serum of 80 PTB and 50 HC for polymorphisms by polymerase chain reaction-restriction fragment length (PCR-RFLP) and measured vitamin D metabolites by liquid chromatography-tandem mass spectrometry (LC-MS/MS). In VDR polymorphisms, no significant differences in distribution and relative frequencies of *BsmI*, *FokI*, and *TaqI* were observed when comparing PTB and HC. However, we observed significantly low levels of 25(OH)D in PTB with a *BsmI* BB haplotype (χ^2 test, $p=0.007$). These findings suggest a role of the *BsmI* VDR in 25(OH)D metabolism and immune response during disease. In the case of 25(OH)D₃, we detected a significant difference ($p=0.01$) between PTB and HC, indicating that PTB had significantly lower 25(OH)D₃ levels. For 25(OH)D₂, PTB had lower levels than HC with a significant difference ($p=0.009$), which could be related to an alteration in the metabolism of 25(OH)D₃ and 25(OH)D₂ associated with tuberculosis infection. The 24,25(OH)₂D₃:25(OH)D₃ ratio values revealed that HC had a higher conversion of 25(OH)D₃ to its inactive form 24,25(OH)₂D₃, reflecting a balanced vitamin D metabolism. Conversely, 18 PTB had a lower conversion of 25(OH)D₃ to its inactive form, which could reflect a need for vitamin D supplementation. These results emphasize the importance of individual PTB analysis and establishing MRV values in patients with tuberculosis as predictors of metabolic status.

P039

Chitosan-particle-based cystatin F RNA silencing as a therapeutic strategy to improve the control of intracellular *Mycobacterium tuberculosis*

M.Mandal¹ D Pires^{1,2} AI Matos³ HF Florindo³ JMA Pereira¹ E Anes¹

1: Host-Pathogen Interactions Unit, Research Institute for Medicines, Faculty of Pharmacy, University of Lisbon, Portugal 2: Universidade Católica Portuguesa, Católica Medical School, Center for Interdisciplinary Research in Health, Portugal 3: Drug Delivery and Immunoengineering Unit, Research Institute for Medicines, Faculty of Pharmacy, University of Lisbon, Portugal

Tuberculosis (TB), primarily caused by *Mycobacterium tuberculosis* (Mtb), still continues to exert a tremendous burden on public health concerns and deaths worldwide. There is an urgent need for the development of alternative strategies to control the infection. Targeting cathepsins constitutes one of the potential approaches. We demonstrated that Mtb actively manipulates macrophage lysosomal enzymes with consequent decreased proteolytic activity, and increased pathogen intracellular survival. In addition, the overcome of this enzymatic blockade was made through the manipulation of protease inhibitors “cystatins”. Our recent work demonstrated that the manipulation of cystatin F (CstF) in human macrophages using RNA silencing led to increased proteolytic activity of lysosomal cathepsins, improving Mtb intracellular killing. It also revealed the impact of CstF depletion in macrophages during the coinfection of Mtb-infected phagocytes with lymphocytes infected with HIV. Since the in vitro transfection method used is not suitable for a clinical translation here, a different RNA delivery system was developed (DSs) i.e., nanomedicine based on chitosan (Cs-DS). We functionalized the DS with mannose to potentiate the interaction with the mannose receptor (CD206) expressed on macrophages by promoting receptor-ligand interaction, subsequently improving the delivery of siRNA-targeting CstF to the infected macrophage models. The results indicate a more efficient silencing of CstF mRNA, and an effective impact on the intracellular survival of TB bacilli relatively to the conventional previous transfection method and with less toxicity. Overall, the study suggests the potential use of Cs-DS in adjunctive therapy for TB either in combination with or without antibiotics.

Redefining the role of the signalling by nuclear receptors pathway in Tuberculosis

ML Silva^{1 4} AR Maceiras³ J Couto¹ R Gonçalves^{1 4} M Silva^{1 4} S Macedo¹
D Machado⁵ I Indafa^{6 7} A Sifna^{5 6} C Malaca^{5 6} N Namara^{5 6} L Sanca^{6 7}
PNS Rodrigues^{1 2} V Benes⁸ M Viveiros⁵ F Rudolf^{7 9} C Wejse^{6 10} B Cá^{1 2 6 7}
M Saraiva^{1 2}

1: I3S- instituto de Investigação e Inovação em Saúde 2: IBMC - Instituto de Biologia Molecular e Celular, University of Porto, Portugal 3: Wellcome Sanger Institute, Wellcome Genome Campus, Cambridge, UK 4: Doctoral Program in Molecular and Cell Biology, ICBAS -Instituto de Ciências Biomédicas Abel Salazar, University of Porto, Portugal 5: Global Health and Tropical Medicine, Associate Laboratory in Translation and Innovation Towards Global Health, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Portugal 6: INASA - Instituto Nacional de Saúde Pública da Guiné-Bissau 7: Bandim Health Project, Indepth Network, Bissau, Guinea-Bissau 8: Genomics Core Facility, European Molecular Biology Laboratory, Heidelberg, Germany 9: Dept of Infectious Diseases, Aarhus University Hospital, Denmark 10: GloHAU Center for Global Health, Aarhus University, Denmark

Tuberculosis (TB) continues to pose a major global health challenge, with host immune responses playing a crucial role in shaping disease outcomes. In this study, we revisit the role of the “Signalling by Nuclear Receptors” (SNR) pathway in TB by integrating whole blood transcriptomics from different cohorts of TB patients with experimental validation in preclinical models. We identified the SNR pathway as a transcriptional signature associated with TB severity across independent patient datasets. In mouse models that mirror the immune landscape of human TB, we observed a progressive, time-dependent upregulation of the SNR component liver-X-receptor (LXR) genes in both resistant (C57BL/6) and susceptible (C3HeB/FeJ) strains upon *Mycobacterium tuberculosis* infection. Notably, pharmacological activation of the LXR pathway during later stages of infection significantly reduced pulmonary bacterial burdens and prolonged survival of susceptible mice. This effect was accompanied by enhanced cholesterol efflux and improved macrophage-mediated control of *M. tuberculosis*, suggesting that LXR-driven modulation of host lipid metabolism can restrict bacterial access to a critical nutrient during chronic infection. Importantly, this antibacterial effect occurred independently of major inflammatory changes or tissue damage. Our findings position the SNR/LXR axis as a biomarker of TB severity and also as a promising target for host-directed therapy. By bridging clinical data with mechanistic insight, this study establishes the temporal activation of nuclear receptor signalling as a tractable and potentially transformative strategy to improve TB treatment.

P041

Dueling forces: how glucocorticoids both help and hurt in tuberculosis.

P Soldevilla^{1 2 3 4 5} M Vidal^{1 2 3 4} M Cortacans^{1 2 3 4} E Fuentes^{1 2 4} Y Rosales²
J Díaz² PJ Cardona^{1 2 3 4 5}

1: Unitat de Tuberculosi Experimental, Microbiology Dept. Germans Trias i Pujol Research Institute and Hospital (IGTP-HUGTIP), Badalona, Spain 2: Centre de Medicina Comparativa i Bioimatge de Catalunya (CMCiB), Badalona, Spain 3: Genetics and Microbiology Department, Autonomous University of Barcelona, Spain 4: Servei de Microbiologia, LCMN, Hospital Universitari Germans Trias i Pujol (HUGTiP), Badalona, Spain 5: Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBERES), Madrid, Spain

Tuberculosis (TB) remains one of the deadliest infectious diseases worldwide, causing 1.25 million deaths in 2023. Immune dysregulation is associated with poorer TB outcomes. Intriguingly TB rates are significantly higher in men than in women (with an x1.7 ratio). We investigated the role of stress-response glucocorticoids (GCs) and their intrinsic anti-inflammatory activity, focusing on the influence of age, sex, and psychological stress using the active TB mouse model C3HeB/FeJ.

Mice of different ages were infected intravenously with 2×10^4 colony-forming units (CFUs) of *Mycobacterium tuberculosis* (Mtb), and infection was allowed to progress over four weeks. Hair, lung, and spleen samples were collected to measure corticosterone production and assess infection progression. In a parallel psychological stress experiment, mice were divided into 'Control' (no stress) and 'RST' (daily movement restriction) groups, following the same infection protocol. Weekly blood samples were collected for corticosterone quantification.

Elderly female mice exhibited the highest corticosterone levels. RST animals also showed elevated corticosterone, with females displaying higher levels than males. Elevated corticosterone was associated with reduced pulmonary damage, characterised by lower neutrophil infiltration, without affecting lung bacillary load but increasing bacterial dissemination to the spleen.

These findings suggest a dichotomous effect of GCs: reducing the extracellular bacillary population through dampened inflammatory responses while promoting intracellular bacillary survival by impairing Th1-mediated immunity. Further studies are in progress to explore the sex-specific impact of stress-induced GCs on TB outcomes.

P042

Optimization of 3D culture conditions with five different tumor cell lines to monitor mycobacterial antitumor activity

N Sabando-García¹ N Traba¹ RM Rabanal¹ E Julián¹

1: Universitat Autònoma de Barcelona

Mycobacterium bovis Bacillus Calmette-Guérin (BCG) is the standard treatment for high-risk nonmuscle-invasive bladder cancer (NMIBC). Despite to be the first therapy option for more than 40 years, the mechanism of action is unknown. The interaction between BCG and tumor cells is critical for investigating the antitumoral effects and new strategies for cancer treatment. Until

now, only 2D in vitro cultures have been studied to understand mycobacteria-bladder cancer cell interaction. However, studies using 3D cultures such as spheroids has not been performed. Spheroids are cell aggregates composed of non-progenitor and differentiated cells. Three-dimensional structure enables the formation of oxygen, nutrient, and pH gradients. This gradient variability creates layer heterogeneity and makes spheroids mimic avascular solid tumors more effectively compared to 2D cultures.

In the present study, we have optimized five types of bladder cancer spheroids derived from different cell lines. The interaction between BCG and *Mycobacterium brumae*, a nontuberculous immunomodulatory species safer than BCG, and spheroids has been analyzed. Our results showed that each spheroid type exhibited distinct size, morphology, and growth rate. When treated with mycobacteria, the data indicated that the differentiation grade of the cancer cell lines drives the interaction with mycobacteria modulating their antitumoral effects with corresponding variations in tumor cell proliferation inhibition and induction of cytokines' release. Our work adds evidence of the relevance of culture conditions for unraveling the immunomodulatory and antitumor ability of mycobacteria.

P043

Exploring the role of Mincle in macrophage-mediated antitumoral responses

N Traba¹ E Julián¹

1: *Universitat Autònoma de Barcelona*

Mycobacteria play a relevant role as immunotherapy agents, however the immunological mechanisms behind this effect is not completely understood. This is particularly relevant for *Mycobacterium bovis* BCG, widely used in non-muscle invasive bladder cancer treatment, for which the crosstalk with innate immune cells is crucial to antitumoral efficacy. Mycobacteria modulate immune responses through lipid recognition from their well-characterized lipid-rich cell wall. In this interaction, the C-type lectin receptor Mincle plays an essential role in sensing glycolipids such as trehalose-6,6-dimycolate. However, the function of Mincle-mediated signaling in tumor therapy is unknown.

To investigate the role of glycolipids in the antitumor responses, murine J774A.1 macrophages were stimulated with different glycolipids: glycerol-monomycolate, trehalose-dimycolate, and trehalose-monomycolate purified from *M. bovis* BCG and *Mycobacterium brumae*. Cell cultures were performed under various conditions, including Mincle blockade and/or TLR2/TLR4 activation, to explore the contribution of pattern recognition receptors to macrophage-mediated immune responses. Macrophage activation was assessed through proliferation assays, nitric oxide production, and analysis of cytokines/chemokines released (TNF- α , IL-6, IP-10, IL-12, IL-1 β , RANTES). The antitumoral effect was evaluated by exposing MB49 bladder cancer cells to macrophage-conditioned media.

Certain mycobacterial glycolipids induced robust production of TNF- α , IP-10, IL-6, and nitric oxide in J774A.1 macrophages. Secretion levels varied depending on lipid exposure and receptor modulation. Mincle blockade significantly reduced cytokine and NO production, reinforcing its role in sensing mycobacterial lipids. High levels of TNF- α , IP-10, and NO correlated with decreased MB49 proliferation, suggesting an antitumoral effect of activated macrophages. These results highlight Mincle's role in mycobacteria-driven cancer immunotherapy responses.

P044

In vitro modelling of the tuberculosis granuloma for antimicrobial and host-directed drug screening

B Fontes¹ R Ferreira¹ M Mandal² R Pinheiro¹ E Anes² PJG Bettencourt¹
S David^{2,3} D Pires^{1,2,3}

1: Center for Interdisciplinary Research in Health, Católica Medical School, Universidade Católica Portuguesa, Portugal 2: Host-Pathogen Interactions Unit, Research Institute for Medicines, iMed.Ulisboa, Faculty of Pharmacy, Universidade de Lisboa, Portugal 3: Departamento de Genética Humana, Instituto Nacional de Saúde Doutor Ricardo Jorge (INSA), Lisbon, Portugal

To accelerate the discovery of new antimicrobials or host-directed therapies, we have developed an in vitro 3D cell culture model of the TB granuloma. The model can be easily applied using commercial systems to encapsulate human peripheral blood mononuclear cells (PBMC) infected with *Mycobacterium tuberculosis* H37Rv (Mtb). The 3D granuloma structure exhibited a drastically lower Mtb burden with bacterial growth confined to a restricted number of cells, in contrast to the standard monolayer model. Manipulation of the macrophage and lymphocyte content showed clear distinctions in the ability to control Mtb infection, and introduction of lymphocytes helped control the infection even when the cells were physically separated. Different multiplicities of infection (MOI) didn't affect the ability of Mtb to survive and replicate. Yet, at the lowest MOI, we observed a lower expression of proinflammatory markers and increased expression of anti-inflammatory markers. The model allowed infection assays to last up to one month, with cell viability mainly affected by the bacterial burden. The onset of bacterial replication correlated with increased cell death, primarily in the macrophage population. Some of the granulomas exhibited a distinct region of dead cells at the centre of the structure. The treatment with antibiotics showed greater resistance to isoniazid, followed by rifampicin, and increased susceptibility to pyrazinamide compared to the standard monolayer infection model.

In conclusion, the 3D model resembles some features expected in the TB granuloma, which we will explore in future drug discovery studies.

P045

Phagocytosis of *Mycobacterium fortuitum* by caprine alveolar macrophages is associated with iNOS and proinflammatory marker expression

M Blay-Benach^{1,2} J Repullés^{1,2} P Cuenca-Lara^{1,2} B Pérez de Val^{1,2}

1: Programa de Sanitat Animal, Centre de Recerca en Sanitat Animal (CRESA), IRTA, Campus de la Universitat Autònoma de Barcelona, Spain 2: Unitat Mixta d'Investigació IRTA-UAB en Sanitat Animal, CRESA, Campus UAB, Spain

Phagocytosis mediated by alveolar macrophages (AM) plays a crucial role in host defence against pathogens such as *Mycobacterium tuberculosis*. However, limitations of *in vitro* phagocytosis assays highlight the need for new methods to assess AM functionality. This study aimed to standardize and evaluate a novel fluorescence-based technique for measuring the phagocytosis of *M. fortuitum* by caprine AM and its association with AM activation and proinflammatory markers. AM were stimulated *in vitro* with LPS and heat-inactivated *M. bovis* (HIMB) for 24h. After

stimulation, AM were exposed to fluorescently labelled *M. fortuitum* for 72h and fluorescence kinetics were measured using the Incucyte® SX5 Live-Cell Imaging and Analysis System. This method was compared to a culture-based assay using the MGIT BACTEC system. In addition, AM activation and polarization markers were assessed using flow cytometry, while a multiplex immunoassay was performed to quantify proinflammatory cytokines. The fluorescence intensity kinetics indicated that stimulation with LPS, but not HIMB, resulted in a greater signal reduction compared to unstimulated AM, suggesting a higher bacterial elimination. Similarly, LPS induced a higher percentage of mycobacterial reduction measured by MGIT culture between 2 and 72h after *M. fortuitum* challenge. Moreover, LPS stimulation increased the production of proinflammatory cytokines (IL-1b, IL-6, TNFa), elevated the proportion of cells co-expressing iNOS and MHC-II, and enhanced the expression of these markers within M1-polarized AM, suggesting that LPS induced a more activated proinflammatory phenotype. These findings indicate that enhanced mycobacterial phagocytic capacity of AM correlates with iNOS and MHC-II expression and a proinflammatory profile.

P046

Mycobacteria extracellular vesicles as alternative immunotherapy for Bladder cancer

B Yewew^{1,2} R Sorrentino² C Venegoni³ P Miotto² C Braccia⁴ A Andolfo⁴
A Salonia³ M Alfano³ DM Cirillo^{1,2}

1: Vita-Salute San Raffaele University, Milan, Italy 2: EBPU, IRCCS San Raffaele Scientific Institute, Milan, Italy 3: URI, IRCCS San Raffaele Scientific Institute, Milan, Italy 4: Proteomics and Metabolomics Facility (ProMeFa), IRCCS San Raffaele Scientific Institute, Milan, Italy

Bladder cancer (BC) is the 9th most common cancer worldwide. BCG installation remains the standard treatment for high-grade non-muscle-invasive cases, but its limited efficacy and side effects highlight the need for alternatives. Mycobacterial extracellular vesicles (MEVs) have emerged as promising immunomodulatory agents, providing a unique opportunity for immunoncology applications. Therefore, we assessed the antitumor capability of different MEVs against BC cell lines (BCaCL). MEVs were isolated from OncoTICE BCG, *M. tuberculosis* reference strain H37Rv and environmental *M. gordonae* (MG) by ultracentrifugation; quantified and characterized by Nanosight and BCA assay. Dose-response assays were performed on BCaCLs (T24, 5637) and THP-1-derived macrophages. Cell viability (Calcein-AM) was assessed at 24 and 72 h, and cytokine levels (ELISA) at 24 h post-treatment with MEVs or live BCG. T24 proteins were extracted after 72 h of MEVs treatment (BCG or H37Rv-derived), BCG-infection or untreated cells and analyzed by nLC-MS/MS. MEVs induced dose-dependent cytotoxicity in BCaCLs (12–48% viability reduction) and triggered TNF and IL-12 secretion comparable to BCG infection. IL-6 levels increased by 50–100% relative to BCG-infected cultures. However, MEVs significantly impacted macrophage viability (over 40% reduction). Proteomic analysis identified 228 statistically significant differentially expressed proteins ($p < 0.05$) across the four groups. MEVs demonstrated cytotoxicity and cytokine induction in BCaCLs, like BCG infection. Their impact on macrophage activation remains under investigation. Proteomic analysis revealed limited differences between MEV-treated and BCG-infected cells in terms of immunological activation, supporting further exploration of MEVs as modulators of immune responses in bladder cancer.

P047

Pattern of genotypic mutations and level of MIC for results evaluation of drug susceptibility testing of *M. tuberculosis*

N Ciobanu^{1 2} D Rusu^{1 2} A Codreanu¹ N Turcanu¹ A Buga¹ V Crudu^{1 2}

1: Institute of Pneumology "Chiril Draganiuc"; Chisinau, Moldova 2: State University of Medicine and Pharmacy "nicolae Testemitanu"; Chisinau, Moldova

The aim: the concordances between the level of MIC and pattern of genotypic mutations for evaluation of DST of *M. tb* for correction of treatment.

Methods: In the study 6952 patients with new and previously treated pulmonary TB were examined for 2018-2020 years. Of these 66.2% positive by GeneXpert and 69.98% by culture method. The pDST result of 4865 *M.tb* strains from MGIT were correlated with gDST results obtained by MTBDRplus. The mutation types in the *rpoB* gene, and the presence of *inhA* mutation in the same *M.tb* isolates was correlated correspondingly with the different level of MIC for RIF and INH of individual strains.

Results: INH resistance was found in 64% and RIF resistance in 62% of cases. From 157 isolates with presence of *inhA* mutations only in 31% were susceptible on higher MIC demonstrated by pDST. Among patients with genotypic RIF resistance with D516 mutation only, or without mutations and without wild genes, the phenotypic DST demonstrated that in 28.8% of cases these strains were susceptible to higher MIC.

Discussion: Patients with low level genotypic resistance should have additional information about the level of phenotypic resistance. For reliable DST results, phenotypic and genotypic methods should be used in parallel, to be phased in without loss of existing solid culture and DST capacity.

Conclusion: pDST is still required, for corrective of rapid gDST results, in special from smear-negative specimens for detect XDR-TB. Different levels of phenotypic resistance could take into account in the procedures used by pDST of *M. tb*.

P048

A next-generation LLM-interrogable mycobacterial knowledge base

K Dewaele¹ O Tzfadia¹

1: Institute of Tropical Medicine Antwerp

Many experts foresee smarter-than-human AI in 2027. Already today, Large Language Models (LLMs) can process book volumes of information, can be chained in autonomous, agentic workflows, and perform reasoning tasks - properties expected to enable a revolution in biological hypothesis generation and testing. To leverage these capabilities for mycobacterial research, access to domain-specific knowledge is key. However, existing mycobacterial databases, such as TBDB and Mycobrowser, are defunct. We present a suite of tools to build and dynamically extend a next-generation, LLM-interrogable, mycobacterial knowledge base (KB). This KB is gene-centric, enabling the formulation and testing of hypotheses related to gene function and biological

networks. It is constructed as a knowledge graph, enabling plug-and-play extension with novel data types. Its functionality includes, 1) interrogation of functional protein association databases (comprising co-expression, gene neighbourhood, and co-occurrence data), 2) augmentation of association networks with regulatory data, including transcriptional control and protein-level modulation, 3) labeling of genes and their interactions using an automated literature querying tool that retrieves, stores, and interprets abstracts and full-text open access papers, 4) the addition of functionally relevant genomic markers, including gene conservation, evolutionary patterns (dN/dS, regions-of-difference) and immune-pressure signatures (epitope quantity and conservation). Integrating these diverse data types, we constructed an interactive HTML network visualisation of 1,163 *M. tuberculosis* genes, demonstrating the utility and feasibility of this approach in enabling access to both humans and LLMs. In summary, we present a methodological framework for constructing an LLM-enhanced knowledge base, preparing for mycobacterial research in the AI era.

P049

Comparative analysis of nanopore and illumina sequencing for molecular epidemiology of *M. tuberculosis*

V Szel¹ O Drozdovszky¹ G Jerkovich¹ G Papp-Bernath¹ A Orosz¹ LK Lorinczi¹
1: National Reference Laboratory for Mycobacteriology, Hungary

Timely and accurate molecular epidemiological investigation is critical in controlling *Mycobacterium tuberculosis* (MTB) transmission. Whole-genome sequencing (WGS) has become a key tool in identifying transmission clusters, traditionally relying on short-read platforms, such as Illumina, due to their high accuracy. However, rapid sequencing technologies, like Oxford Nanopore, offer significantly faster turnaround times, making them attractive for real-time surveillance. Despite this advantage, several studies have raised concerns about the specificity and reliability of Nanopore data in the context of MTB genotyping, highlighting the importance for direct comparison.

This study aimed to evaluate whether Nanopore sequencing can identify the same transmission clusters as Illumina WGS when applied to MTB isolates. The analysis involved 20 MTB strains, isolated in Hungary, including epidemiologically linked cases. Each isolate was sequenced using both Illumina and Nanopore platforms. SNP-based phylogenetic analyses and clustering assessments were performed to determine concordance between the technologies.

Our results provide insights into the comparability of these sequencing approaches for MTB cluster identification, highlighting the strengths and limitations of Nanopore sequencing in high-resolution genotyping and underscoring its potential role in complementing traditional methods, especially where rapid decision-making is of primary concern for public health interventions.

P050

Improving the reproducibility of RNA sequencing methodologies in *Mycobacterium tuberculosis*: A pilot study on isoniazid treatment

M Helmsmoortel¹ T Parbhoo² M Ginoux¹ N Boucher¹ M Taha¹ H Hage¹
S Niemann² L Alibaud¹ L Sonnenkalb²

1: BIOASTER, Microbiology Research Institute 2: Research Center Borstel, Leibniz Lung Centre

Standardization and reproducibility of RNA sequencing (RNAseq) data is critical for advancing tuberculosis research, particularly in understanding *Mycobacterium tuberculosis* (Mtb) responses to antibiotics. Within the European Regimen Accelerator for Tuberculosis (ERA4TB) consortium, this study aimed to test the reproducibility of a standardized RNAseq workflow for transcriptomic profiling of Mtb challenged with several concentrations of isoniazid (INH). Identical protocols for bacterial culture and treatment were executed between two independent laboratories followed by deviations in RNA extraction, library preparation, and sequencing depth. Factors of deviation had minimal effect on transcriptional signatures when accounting for batch effects. Therefore, even with cross-laboratory technical variability, consistent biological conclusions across replicates, platforms, and laboratories were achieved.

First, we gained insights on INH dosing, as exposure to half the minimum inhibitory concentration (MIC) of INH, did not affect the transcriptome compared to untreated cultures. Contrastingly, treatment with five-times the MIC of INH, brought about a strong cellular response. In both laboratories we observed an upregulation of previously identified genes involved in mycolic acid synthesis, such as *kasA*, *kasB* and *acpM*, along with the differential expression of 103 other stress and metabolic associated genes. In summary, high cross-laboratory reproducibility confirms RNAseq as a robust tool by providing a critical foundation for identifying novel and emerging mutations, and drug-induced transcriptional changes under varying drug pressures, supporting its utility for preclinical drug evaluation.

This work reflects only the author's views, and the JU is not responsible for any use that may be made of the information it contains.

P051

Transcriptional plasticity of bedaquiline-resistant *Mycobacterium tuberculosis* under drug pressure

T Walz¹ L Sonnenkalb¹ S Niemann¹

1: Research Center Borstel

Drug-resistant and multidrug-resistant tuberculosis (MDR-TB) remains a significant barrier to global tuberculosis (TB) control. Limited treatment access, diagnostic delays, and failures drive the emergence and spread of resistant *Mycobacterium tuberculosis* (MTBC) strains. The recent introduction of an all-oral, shortened MDR-TB regimen centered around bedaquiline (BDQ) has marked a significant advancement for the treatment of MDR-TB patients. However, the rapid evolution of BDQ resistance compromises its long-term efficacy.

A comprehensive understanding of the molecular mechanisms underlying resistance is essential for improving diagnostics, optimizing treatment strategies, and guiding drug development. BDQ-resistant clinical MTBC strains commonly harbour mutations in the *Rv0678* gene, although these typically confer only moderate resistance. To investigate the functional impact of different *Rv0678* mutations, we analysed the transcriptomic profiles of three laboratory-generated BDQ-resistant MTBC clones (harbouring A99V, inversion or 274insA mutations in *Rv0678*) in the presence and absence of BDQ.

At baseline level, 242 differentially expressed genes (DEGs) were identified, with 7 DEGs shared across all three *Rv0678* mutants. This highlights the transcriptomic diversity among clones carrying different mutations in the same resistance gene, emphasizing the variable impact of these mutations even in the absence of drug exposure. Upon BDQ treatment, we observed distinct transcriptional responses suggestive of broader metabolic reprogramming beyond the canonical *Rv0678*-mediated resistance mechanism. These findings reveal the dynamic and multifaceted nature of MTBC adaptation to drug pressure and underscore the importance of considering transcriptional responses when investigating resistance mechanisms. Ultimately, this work enhances our molecular understanding of the transcriptional landscape associated with BDQ resistance in MTBC.

P052

GOMA: an open-source bioinformatics pipeline for genomic characterization of *Mycobacterium tuberculosis* complex in low resource settings

G Schüpbach^{1 2} S Gagneux^{1 2} GA Goig^{1 2} D Brites^{1 2}

1: Swiss Tropical and Public Health Institute 2: University of Basel

The *Mycobacterium tuberculosis* complex (MTBC) causes tuberculosis (TB) disproportionately in resource limited countries. Whole-genome sequencing (WGS) has been key in improving our understanding of drug resistance and of transmission of the MTBC. Yet, the need for expertise guiding WGS implementation and the lack of bioinformatic expertise, are main obstacles hindering the implementation of WGS in low resource settings. We built GOMA (Galaxy Open-source MTBC Genomic Analysis), a bioinformatics pipeline designed to perform DNA variant detection, drug-resistance prediction, MTBC lineage detection, transmission inference and phylogenetic analysis. The pipeline was implemented on the Galaxy platform (<https://usegalaxy.eu>) and is free, cloud-based, accessible through a web-browser and graphical user interfaces, and does not require programming skills. GOMA processes Illumina short reads and can handle single or multiple samples in one run. Its accuracy has been tested using a variety of previously characterized WGS representing; all the human-adapted lineages (L1-L10) and the main animal-adapted MTBC lineages, a wide range of drug-resistant MTBC profiles, and strains involved or not in recent transmission chains. GOMA could accurately detect; i) the main human and animal-adapted MTBC lineages, including mixed infections and contaminations, ii) drug-resistance conferring mutations defined by the WHO and iii) MTBC isolates involved in transmission chains according to several genetic distance thresholds. In summary, we created a pipeline that can aid genomic epidemiology of small-scale projects without the need for a high-performance computing facilities or hardware, nor programming knowledge. Being hosted on the open-source platform Galaxy, this pipeline will benefit especially low-income high-burden TB settings.

P053

Whole-genome sequencing for drug resistance detection and genotyping of clinical *Mycobacterium tuberculosis* isolates in two-year period

E Sodja¹ S Truden¹ I Perko¹ Š Klemen¹

1: University Clinic Golnik

In Slovenia between 2020 and 2021, 145 patients were newly diagnosed with pulmonary or extra-pulmonary tuberculosis. For all *Mycobacterium tuberculosis* (MT) isolates, WGS was performed using Illumina (San Diego, CA, USA) technology. Furthermore, for all of them drug susceptibility (pDST) results to at least first line drugs were available. pDST was performed using BACTEC MGIT 960 System (Becton Dickinson, NJ, USA).

Eight (8/145; 5.5 %) MT isolates were phenotypically resistant to at least one antibiotic. Major resistance pattern (3/8; 37.5 %) was resistance to ethionamide (ETH) and low-level resistance to isoniazid (INH) and could be explained by the presence of -15C>T mutation in *fabG1* in all three cases. One case of pre-XDR tuberculosis was detected using both pDST and WGS. Majority of phenotypic resistance was confirmed with WGS. Only in one case, we observed discrepancy between pDST and WGS; MT isolate was phenotypically sensitive to INH, while using WGS - 796C>T mutation in *inhA* was observed. In that particular case, pDST was performed with lower critical concentration (0.05 µl/ml) and isolate was tested as INH resistant. Large proportion of MT isolates (139/145; 95.86 %) belonged to lineage 4. Other lineages detected were lineage 1 (2/145; 1.38 %), lineage 2 (3/145; 2.07 %) and lineage 3 (1/145; 0.69 %).

WGS proved to be rapid and accurate tool for detecting drug resistance in clinical MT isolates.

P054

Insights into drug resistance in *Mycobacterium avium* pulmonary disease: A retrospective cohort study

XES Iversen¹ VN Dahl^{1 2} A Norman¹ DB Folkvardsen¹ EM Rasmussen¹

E Svensson¹ L Rigouts³ C Meehan^{3 4} L Jelsbak⁵ T Lillebaek^{1 6}

1: International Reference Laboratory of Mycobacteriology, Statens Serum Institute, Denmark

2: Department of Infectious Diseases, Aarhus University Hospital, Denmark

3: Mycobacteriology/Institute of Tropical Medicine, Antwerp, Belgium 4: Department of Biosciences,

Nottingham Trent University, UK 5: Department of Biotechnology and Biomedicine, Technical

University of Denmark, Denmark 6: Global Health Section, Department of Public Health, University of Copenhagen, Denmark

Infections due to nontuberculous mycobacteria (NTM) are increasingly recognized worldwide, with NTM pulmonary disease (NTM-PD) being the most frequent clinical presentation. At the International Reference Laboratory of Mycobacteriology (IRLM), the most consistently identified NTM species is *Mycobacterium avium*, which is also among the most prevalent pathogens responsible for NTM-PD. Treatment is challenging and requires long-term, multidrug therapy, thereby increasing the risk of developing resistance. In this study, we investigate resistance during long-term treatment of *M. avium* patients with follow-up samples received at the IRLM between 2020 and 2024 ($n_{\text{isolates}} = 670$; $n_{\text{patients}} = 98$). Genotypic drug susceptibility testing (gDST) with

GenoType NTM-DR was conducted on 225 *M. avium* isolates from 95 patients, showing resistance to amikacin and clarithromycin in 10.7% and 14.7% isolates, respectively. Phenotypic DST (pDST) using the MGIT 960 system was subsequently carried out on a subset of isolates ($n=39$), revealing high resistance rates to ethambutol (79.5%) and rifampicin (92.3%). Additionally, a pilot study was conducted to investigate the relationship between resistance, treatment, and clinical outcomes in 15 patients diagnosed with NTM-PD. The median patient age was 70 years, and 8 patients had a previous episode of NTM infection. Culture conversion was achieved in 3 patients, while clinical improvement was observed in 6. Most patients (12/15) received antibiotic therapy; however, 4 were deceased during the study period. The observed resistance rates and variable treatment outcomes emphasize the importance of continued research into resistance development and more effective therapeutic approaches.

P055

Benchmarking algorithms for species-level profiling of mycobacteria in shotgun metagenomic samples, and application to the distribution of known mycobacteria in soils

LB Harrison^{1 2} JO Ahmed¹ FJ Veyrier¹

1: Centre Armand-Frappier Sante Biotechnologie - INRS 2: McGill University Health Centre

Mycobacteria inhabit a variety of habitats including soils, water, dusts, and other niches. The distribution of known species has been described to a variable degree by both culture-dependent and culture-independent approaches. Shotgun metagenomics offers promise to delineate mycobacterial diversity of both known and unknown species, but significant questions concerning both wet and dry-lab methodologies remain. In the dry-lab, determining which known species of mycobacteria are present in a given sample of metagenomic sequencing reads is not trivial due to similarity and incompleteness of reference genomes, horizontal gene transfer, among other issues. Three new algorithms addressing these issues have been published to profile microbial communities at the species-level, including at low abundance, using reference databases: metapresence, YACTH and sylph. We benchmarked these tools on their ability to discriminate known species in the family *Mycobacteriaceae* using a curated reference genome database and in silico simulations to create artificial mycobacterial communities. Using precision/recall curves, we demonstrate that sylph and metapresence, when used with an alignment mapping quality filter, provides the best performance, with sylph maintaining high precision, but relatively lower recall/sensitivity at low abundance. Applying these methods to published global datasets of soil shotgun metagenomes ($n = 1284$), relatively few known species are detected per sample (sylph: mean \pm SD 0.24 \pm 0.73 known species per soil metagenome), and at overall low abundances. This likely reflects both the incompleteness of the reference database due to a high proportion of uncharacterized mycobacteria present in soils, and/or extraction protocols not optimized for mycobacteria.

Global genomic landscape of *Mycobacterium avium*: diversity, transmission dynamics, and drug resistance mechanisms

S Matos¹ D Machado² T Clark³ I Couto² M Viveiros² I Portugal¹ J Perdigão¹

1: Research Institute for Medicines, Faculdade de Farmácia, Universidade de Lisboa, Portugal

2: Global Health and Tropical Medicine, GHTM, LA-REAL, Instituto de Higiene e Medicina Tropical,

IHMT, Universidade NOVA de Lisboa, Portugal 3: Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, UK

Non-Tuberculous Mycobacteria (NTM) is estimated to be responsible for over 280 000 deaths worldwide and 9.9 million disability-adjusted life years. Within this group, *Mycobacterium avium* comprises a diverse opportunistic pathogen, frequently associated with pulmonary/extrapulmonary disease, whose genetic diversity and population structure remain insufficiently understood.

This study aimed to characterize the phylogeny and population structure of *M. avium* from diverse geographies. Using large-scale genomics combined with associated metadata for *M. avium* isolates, publicly available data were retrieved and subjected to quality control, reference and *de novo* assembly, mapping and variant calling. Genome-wide phylogenetic reconstruction enabled population structure analysis, cluster identification and analysis of the phylogenetic dispersion of resistance associated variants.

The study includes 2917 genomes from 22 countries, comprising human (n=871), animal (n=1270) and environmental (n=90) isolates. Four main lineages were identified, three of which composed exclusively of *M. avium* subsp. *hominissuis* (MAH) and a fourth that included MAH, *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis* isolates. Sublineages were mostly clustered by geographic location and host. A total of 261 possible transmission clusters were identified (<20 SNPs), most within the same country. Clustering across distinct sources highlight possible transmission between reservoirs. Resistance associated mutations were identified in 169 isolates: *rrs* A1408G (n=58) and *rrl* A2058/A2059 (n=111). These mutations were exclusively found in human MAH isolates and reflect a continued emergence of resistance to aminoglycosides and macrolides, respectively.

These findings highlight the global reach and diversification of *M. avium* along with cross-reservoir transmission and clade-specific proclivity for resistance development.

P057

In-depth analysis of predominant *Mycobacterium tuberculosis* L2.2.M3 strain from Panama, using TB-Annotator

JE Ku¹ F Acosta¹ E Shitikov² C Guyeux³ I Mokrousov⁴ C Sola^{5,6} A Goodridge¹
1: Instituto de Investigaciones Científicas y Servicios de Alta Tecnología de Panamá 2: Lopukhin Federal Research and Clinical Center of Physical-Chemical Medicine of Federal Medical Biological Agency 3: CNRS-Université Franche-Comté (UFC) 4: St. Petersburg Pasteur Institute 5: Université Paris-Saclay 6: Université Paris-Cité, Université Sorbonne Paris-Nord

Mycobacterium tuberculosis lineage 2 (L2) is often associated with increased drug resistance and rapid transmission. Panama recently reported an endemic transmission of drug-susceptible *M. tuberculosis* sublineage L2.2.M3 in Colon. Here, we aim to perform an in-depth analysis of the whole-genome sequences (WGS) of L2.2.M3 Panama isolates to understand how this endemic transmission is connected in a global context. We collected WGS data of 90 L2.2.M3 isolates from Colon, Panama, recovered between 2015 and 2023, and analyzed them using the TB-Annotator, as it contains a collection of >112,000 *M. tuberculosis* strains. Our results indicate that 96.7% (87/90) were classified as pan-susceptible, 2.2% (2/90) as RR-TB (rifampicin and rifampicin/pyrazinamide-resistant), and 1.1% (1/90) as MDR-TB (rifampicin, isoniazid, and ethionamide resistant). The preliminary assessment revealed that all Colon sublineage L2.2.M3 isolates are very homogeneous and share a unique SNP at position 518748 G>A. TB-Annotator results show an interesting clonal complex within a larger tree of 1,568 sequence read archives (SRAs) of sublineage L2.2.M3. Although the Colon sample set does not have enough markers to define it as a sublineage within L2.2.M3, our results suggest that the isolates document a new epidemic branch (internal distance of 35 +/- 17 SNP) that is very endemic in Panama with limited transmission to other countries. A plausible explanation could be that little time has passed (8 years) since the study population was formed. However, this might change as more countries are sequencing and sharing information from current and past L2 tuberculosis cases.

P058

When bugs spill secrets: Leveraging bacterial genomes and machine learning to identify drivers of recent Tuberculosis transmission in Accra, Ghana

MN Séraphin¹ M Asare-Baah¹ MA Omari³ J Chariker² JS Afriyie-Mensah³
1: University of Florida 2: University of Louisville 3: University of Ghana

Tuberculosis (TB) prevalence in Ghana is estimated at 356 cases per 100,000 people, and efforts to improve case detection are ongoing. Understanding the role of high-risk behaviors in recent TB transmission can help refine intervention strategies. From June 2022 to June 2023, 150 new and previously treated pulmonary TB patients were recruited from a tertiary hospital in the Greater Accra Region. Recent transmission was defined as a bacterial genetic distance of ≤ 12 single nucleotide polymorphisms (SNPs). Bacterial genomes ($n = 296$) from 96 culture-confirmed cases were analyzed using a supervised learning algorithm to estimate transmission probabilities and

individual reproductive numbers (R_i) were calculated. A gamma regression model with a log link was used to assess how social, clinical, and demographic factors were multiplicatively associated with R_i , and model-based population attributable fractions (PAFs) were estimated. The cohort was predominantly male (68%), with 51% in unskilled labor and 15% co-infected with HIV. Unskilled labor was associated with a 1.58-fold increase in R_i compared to skilled labor ($p = 0.014$). HIV co-infection was associated with a 1.67-fold increase in R_i ($p = 0.045$). In contrast, older age and prior TB treatment were associated with 35% and 50% reductions in R_i , respectively ($p = 0.026$ and $p = 0.018$). The estimated PAF showed that unskilled labor contributed 23.7% (95% CI, 4.1%–41.6%) of recent transmission potential. These findings suggest that occupational and clinical factors significantly influence TB transmission dynamics and should be considered in targeted control strategies.

P059

Comparison of DNA-extraction for Illumina and Oxford nanopore sequencing in Mycobacterial Isolates

EM Rasmussen¹ S Mäsiarová² A Norman¹ XES Iversen¹ DB Folkvardsen¹
AB Nielsen¹ E Svensson¹ T Lillebaek¹

1: Statens Serum Institut, Copenhagen, Denmark 2: Jessenius Faculty of Medicine in Martin, Comenius University, Slovakia

Sequencing-based approaches are increasingly used in the identification and characterization of mycobacteria, including both *Mycobacterium tuberculosis* and nontuberculous mycobacteria (NTM). These pathogens are surrounded by a complex, lipid-rich cell-wall, which challenges the outcome and quality of the DNA-extraction step required prior to sequencing. This study aimed to compare the sensitivity of an Illumina-based purification strategy with an optimised method designed for Oxford Nanopore Technologies (ONT). Ten diverse clinical isolates were included: three *M. tuberculosis*, three *M. avium*, one *M. intracellulare*, one *M. heraklionense*, one *M. porcinum* (fortuitum group), and an unclassified member of the *M. avium* complex (MAC).

Illumina sequencing typically requires lower amounts of input genomic DNA for library preparation (1–100 ng depending on the protocol), and the resulting libraries are normalized to very low concentrations (typically 0.2–0.4 ng/ μ l) prior to sequencing. In contrast, ONT protocols such as Rapid Barcoding require a higher total input amount of 200–400 ng per sample. Although the required DNA input differed significantly between DNA-extraction methods, both yielded usable sequence data for all samples for Illumina sequencing, but only the Oxford Nanopore DNA-extraction was useable for ONT. Preliminary analysis indicated that ONT sequencing provided better genome coverage. The use of 400 ng of input DNA per sample further enhanced sequencing depth and coverage uniformity. In contrast, Illumina sequencing demonstrated greater base-calling consistency and lowered error rates. These complementary strengths highlight the advantages of integrating both technologies for comprehensive genomic analysis of mycobacteria.

P060

The function and transcriptional regulation of *M. tuberculosis* efflux pumps

RG Hanson^{1,2} A Roberts² S Freeman-Fox² I Nobeli³ N Underhill³ C Moon²
R Hesp² S Kendall¹ J Bacon²

1: Royal Veterinary College, University of London 2: UK Health Security Agency 3: Birkbeck, University of London

Efflux pumps play a significant role in both intrinsic and acquired drug resistance in *M. tuberculosis*. These transport proteins actively extrude a wide range of compounds and are important for many regular cellular processes, often facilitating resistance to antimicrobial agents incidentally. Studying the regulation, structure, and substrate specificity of efflux pumps expands our understanding of *M. tuberculosis* physiology while informing novel therapeutic strategies, such as efflux pump inhibition. The aims of this project are to further our knowledge of the functions of mycobacterial efflux pumps and to investigate the transcriptional regulation of these export proteins. In many bacterial species, transcription factors in the TetR family of regulators (TFRs) commonly control efflux pump expression. In this study, an *M. tuberculosis* TFR, Rv1255c, hypothesised to regulate an efflux pump gene, *tap*, was recombinantly produced in *E. coli*. Binding assays revealed Rv1255c does not regulate *tap*, but instead binds to a regulatory motif in the promoter region of the adjacent orientated gene *rv1256c* (*cyp130*) and does so as a protein homodimer. In addition to work with the regulator, the physiological role of the Tap efflux pump is also being explored. The impact of CRISPRi-mediated silencing of *tap* on growth and the genome wide transcriptional response was measured. While there was no impact on growth, expression analysis found changes to lipid metabolism genes that could indicate a novel role for Tap.

P061

RAVEN and iIMAGE: An integrated approach to analyse and visualise whole genome data for *Mycobacterium bovis* genomic surveillance and epidemiology

PD Ranasinghe¹ AR Allen¹

1: Agri-Food and Biosciences Institute

The recent SARS-Cov-2 pandemic highlighted the importance of modern genomics in tracking a new global pathogen. Genome sequencing facilitates phylogenetic analysis, epidemiological insights, and the identification of sources for emerging pathogens and lineages worldwide. This was a consciousness-raising event for many policymakers and public health officials, who acknowledged the value of pathogen genome epidemiology and the tools available to improve responses to disease outbreaks. These tools are, however, not just useful for novel, pandemic pathogens. They can readily be applied to endemic, zoonotic pathogens such as *Mycobacterium bovis*, which causes animal tuberculosis.

In the 'One Health' era, the need to apply such methods to pathogens like *M. bovis* has renewed efforts to modernise public, veterinary, and laboratory services. Such laboratories need flexible, modular bioinformatic tools adaptable to different pathogen types and data visualisation

platforms which can present phylogeographic and phylogenetic information in an interactive and user-friendly manner. This is essential for facilitating wider adoption of pathogen genomic surveillance data by computer science and genomics non-specialists who perform key public-facing roles in veterinary and public health.

We present two in-house program solutions to address these issues: Rapid Assembly and Variant Evaluation Network (RAVEN), a pipeline for read and assembly-based pathogen genome characterisation and SNP calling, and interactive *Mycobacterium bovis* Genome Epidemiology (iIMAGE) tool, a Shiny application built in R for visualising phylogenetic and phylogeographic information. As the next step, we aim to bundle our tools in virtual containers to distribute and deploy the application reproducibly on multiple operating systems.

P062

Comparing passed slave migration history to *Mycobacterium tuberculosis* genomic population structure to reconstruct the origins of L1 tuberculosis infections in Brazil

T Morel-Journel¹ C Guyeux² [C Sola](#)^{1,3}

1: IAME, UMR1137 INSERM-Université Paris-Cité, Université Sorbonne Paris-Nord 2: Université de Franche-Comté 3: Université Paris-Saclay

Lineage 1 is an ancestral Lineage of *Mycobacterium tuberculosis* complex (MTBC) made up of at least five sublineages. In a previous study, we suggested that the introduction of a representative of the L1.1.3 sublineage of Lineage 1 found both in Mozambique and in the North of Brazil could have been brought to Brazil through maritime route during the long-term slave history that lasted from the early XVIth to the mid XVIIIth century between Africa and Brazil. In this study, thanks to combination of bacterial genomic and historical human migration data, we reconstitute scenarios of introduction of L1 genotypes from two likely origins in Africa: West Africa (current Angola or Congo region) and/or East-Africa (Mozambique). We present results showing important similarities between population structure of MTBC in Mozambique and in Brazil and suggest that multiple introductions during the XVIth to the XIXth century period could indeed explain the contemporaneous observations of L1 MTBC tuberculosis cases in Northern-Brazil, due to transmission within Brazil after these historical forced slave migrations.

P063

Secondary antibiotic resistance in rifampicin-resistant tuberculosis in a high-burden setting

[OE Solomon](#)^{1,2} VN Nguyen³ CB Nguyen⁶ TA Nguyen^{4,6} BJ Marais⁴ SM Graham⁵
D Menzies^{1,2} GB Marks⁶ GJ Fox^{4,6} MA Behr^{1,2}

1: McGill University 2: Research Institute of the McGill University Health Centre 3: Vietnam National University 4: University of Sydney 5: University of Melbourne 6: Woolcock Institute of Medical Research

Vietnam is a high-burden country of Rifampicin-Resistant/multidrug resistant TB (RR/MDR-TB). Three dominating lineages are endemic to Vietnam, providing a unique *Mycobacterium tuberculosis* (*Mtb*) ecological setting to study drug resistance evolution and the efficacy of chemotherapy.

Using isolates collected from the VQUIN trial, we conducted a descriptive analysis of RR-TB isolates across Vietnam. We genotyped 255 *Mtb* genomes from 9 cities/districts representing north, central, and south Vietnam. Clinical isolates were sequenced on the Illumina Novaseq6000 system and aligned to the reference H37Rv genome for variant calling using Snippy. A maximum-likelihood tree was constructed with IQTREE, annotated by lineage and drug-resistance using TB-Profiler, and visualized using iTOL.

RR-*Mtb* strains were lineage 1 (20, 8%), lineage 2 (226, 88%), and lineage 4 (9, 4%). We found that the positive-predictive value of Xpert for WGS-confirmed RR and MDR was 96.5% and 92.5% respectively. Overall, 97% of all RR-*Mtb* strains in Vietnam were resistant to at least one other anti-mycobacterial drug. One case of BDQ resistance was detected, while no cases of resistance to pretomanid and linezolid was detected. Relevant to the intervention, 20% of RR-TB strains were fluoroquinolone resistant, with no XDR-TB strains detected (RR + resistance to fluoroquinolones and bedaquiline and/or linezolid)

In summary, Lineage 2 was the dominant lineage in RR-TB isolates across Vietnam, consistent with the literature, but there were also lineage 1 and lineage 4 isolates. A high rate of secondary resistance risks complicating the prevention and treatment of drug-resistant TB.

P064

Understanding tuberculosis transmission in Latvia: insights from molecular epidemiology

D Sadovska¹ | Pole^{1,3} | Ozere^{2,3} | Norvaiša³ | R Ranka^{1,2}

1: Latvian Biomedical Research and Study Centre 2: Riga Stradins University 3: Riga East Clinical University Hospital, Centre of Tuberculosis and Lung Diseases

Tuberculosis (TB) remains a public health challenge in Latvia, a low-to-moderate TB incidence country. However, data on local transmission dynamics, particularly in regions outside the capital Riga, are limited. Herein, we analysed all *Mycobacterium tuberculosis* (*Mtb*) isolates obtained from patients with TB diagnosed in 2016-2017 and who resided in Eastern Latvia region, to gain insights into TB transmission patterns. Using Illumina technology, *Mtb* isolates from 144 patients with active TB residing in three cities and 27 counties were subjected to whole-genome sequencing (WGS). WGS-based genotyping via TB-Profiler software revealed three *Mtb* lineages and 10 sub-lineages, with sub-lineages 4.3.3 (LAM) and 4.8 (mainly T) being equally most prevalent (27%, 39/144). Based on phenotypic drug susceptibility testing (DST) and WGS-based DST interpreted using the latest WHO *Mtb* genetic variant catalogue, 31 isolates (22%) were resistant to at least one anti-TB drug; most were classified as isoniazid-resistant (11%, 16/144). A 5-SNV threshold was applied to identify recent transmission events and clusters. In total, 12 recent transmission events and six clusters (3-11 patients) were detected, involving 55 patients (38%) residing in the same or nearby cities or counties. As anticipated, most closely genetically linked isolates belonged to sub-lineages 4.3.3 (13%, 18/144) and 4.8 (10%, 15/144); however, the largest cluster (11 patients) consisted of isolates from sub-lineage 4.1.2 (Euro-American). Notably, only one cluster and one recent transmission event involved drug-resistant isolates. To conclude, local TB transmission in Eastern Latvia appears limited, suggesting many patients may have acquired the infection outside the studied region.

Unexpected diversity in long-term infections by non-tuberculous mycobacteria revealed by longitudinal genomic analysis

S Buenestado-Serrano ^{2 6} M Martínez-Lirola ⁵ M Herranz-Martín ⁶ G Bernal ⁶
MJ Ruiz-Serrano ⁶ MT Cabezas Fernandez ⁵ P Muñoz ^{3 4 6} D García de Viedma ^{3 6}
L Pérez-Lago ⁶

1: *Fundación para la Investigación Biomédica del Hospital General Universitario Gregorio Marañón*
2: *Escuela de Doctorado, Universidad de Alcalá, Alcalá de Henares, Madrid, España* 3: *Centro de Investigación Biomédica en Red (CIBER) de Enfermedades Respiratorias - CIBERES, Instituto de Salud Carlos III, Madrid, España* 4: *Departamento de Medicina, Universidad Complutense, Madrid, España* 5: *Unidad de Gestión de Laboratorios, UGMI, Complejo Hospitalario Torrecárdenas, Almería, España* 6: *Servicio de Microbiología Clínica y Enfermedades Infecciosas, Hospital General Universitario Gregorio Marañón. Instituto de Investigación Sanitaria Gregorio Marañón (IISGM), Madrid, España*

Nontuberculous mycobacteria (NTM) are increasingly recognized as emerging pathogens worldwide, particularly affecting individuals with chronic lung disease or immunosuppression. Gaining insight into the nature and dynamics of prolonged NTM infections is critical to improve patient management and infection control strategies.

We performed genomic analysis on 330 clinical isolates from 35 patients with prolonged NTM infections (involving 13 different NTM species, most belonging to the MAC complex, 83,3%), collected between 2008 to 2024 in Almería, Spain. Infection duration ranged from 4 months to 11 years, with 2-25 isolates per patient.

Genomic analysis allowed us to differentiate between 17 true persistent infections caused by the same strain and 5 reinfections involving different strains of the same species. Three reinfections involving different NTM species were also identified. Based on the genomic patterns identified, persistent infections were further subdivided on: (i) cases without acquisition genomic diversity (0 SNPs between the sequential isolates along the infection); (ii) linear evolution, marked by the sequential accumulation of SNVs; and (iii) divergent evolution, characterized by the emergence of SNVs along different genomic branches. A subset of 6 patients showed more complex infection dynamics, combining co-infections (involving different species and dominance) with prior patterns of persistence and/or reinfection.

Our findings underscore the need for personalized genome-informed interpretation due to the high level of intra-host diversity found in prolonged NTM infections. Longitudinal whole-genome sequencing is essential to differentiate persistence from reinfection, a key aspect for the proper clinical management of cases and to improve our understanding of NTM diversification in real-world clinical settings.

P066

Using the mutant selection window and mathematical modelling to predict resistance selection over long-term *Mycobacterium tuberculosis* treatment in vitro

L Sonnenkalb¹ B Trubenová² RR Regös² M Merker¹ S Niemann¹

1: Research Center Borstel Leibniz Lung Center 2: ETH Zürich

Intra-patient resistance evolution of *Mycobacterium tuberculosis* complex (MTBC) strains is a major player in tuberculosis (TB) treatment failure. Researchers have hypothesized that due to the complex pathology of TB infection, certain niches throughout the body allow for the low concentration exposure of drug to pathogen, fostering resistance selection and proliferation. Here, we aimed to investigate the consequences of long-term exposure to very low concentrations of the important MDR-TB drugs bedaquiline and moxifloxacin. This work provides a framework for understanding resistance selection by examining the resistance mutant selection window.

We performed several in vitro assays to investigate resistant selection and proliferation at sub-inhibitory concentrations, and found resistant mutants arise at concentrations as low as one-eighth the MIC after only five culture passages. We then utilized mathematical modelling to predict the effect of longer treatment durations, which is not as feasible in the lab. The mathematical model accounted for the population genetics and incorporated the experimentally determined pharmacodynamics of bedaquiline and moxifloxacin. These simulations indicated that one-sixteenth of the MIC was enough to enrich mutant populations, even in mutants with a major competitive fitness loss. Further, we estimated that mutant clones with different resistance mechanisms to the same drug, but even mutants with a single variant at different loci within the same gene, had variable mutant selection windows.

This work gives insights on how little drug is likely needed to select resistant MTBC clones, further showing the importance of high-quality treatment design and using drugs which meet their target at therapeutic concentrations.

P067

Investigating antibiotic-induced persistence in *Mycobacterium abscessus*

L Geerts¹ L De Vooght¹ GJ Wijnant² P Delputte¹ E André² N Lorent² P Cos¹

1: University of Antwerp 2: KU Leuven

Among the non-tuberculous mycobacteria (NTM), *Mycobacterium abscessus* (*Mab*) raises substantial concern due to its high pathogenicity and pronounced resistance to current treatment options. It is an emerging pathogen primarily responsible for pulmonary and skin infections. With cure rates below 50 %, it is considered the most difficult to treat pathogen of the NTM. Given the chronic nature and high relapse rate of *Mab* pulmonary disease, there is an increasing interest in understanding the role of persistence in these infections.

Therefore, this study aims to characterize antibiotic-induced persistence in *Mab*, while also considering other triggers of persistence, such as growth rate, nutrient availability and the lung

environment. Firstly, *Mab* ATCC 19977 was treated with different concentrations of amikacin, clarithromycin, moxifloxacin and rifabutin. Based on biphasic killing and heritability assays, persisters were identified under treatment with 100 times the minimal inhibitory concentration (MIC) of moxifloxacin and rifabutin. Experiments were conducted under nutrient-rich and nutrient-starved conditions with preliminary results indicating that nutrient-starvation increases persister formation. To increase clinical relevance, combinations of different antibiotics are currently being tested. Lastly, given that *Mab* predominantly causes disease in cystic fibrosis patients, persister formation is studied in artificial sputum medium designed to mimic these lung conditions and future experiments will also include clinical isolates.

This study seeks to contribute to a deeper understanding of the conditions and mechanisms involved in the formation of *Mab* persistence. This could help clarify why current treatments fail, which could be essential for the identification of new therapeutic targets.

P069

An alternative for high-throughput Illumina-based sequencing: Individualized nanopore analysis of new incident cases coupled to targeted sequencing

SM Saleeb^{1 2 3 4} M López-LLarúa^{1 2} G Bernal^{1 2} S Buenestado-Serrano^{1 2}
A Marcos-Abellán^{1 2 4} M Martínez-Lirola⁶ S Vallejo-Godoy⁵ TC Fernández⁶
A Pardo⁹ L Wang¹⁰ P Muñoz^{1 2 3} B Santiago^{2 7 8} L Pérez-Lago^{1 2}
D García de Viedma^{1 2 3}

1: Department of Clinical Microbiology and Infectious Diseases, Gregorio Marañón General University Hospital, Madrid, Spain 2: Gregorio Marañón Health Research Institute (IiSGM), Madrid, Spain 3: CIBER of Respiratory Diseases (CIBERES), Madrid, Spain 4: Doctoral School, Autonomous University of Madrid, Spain 5: Department of Preventive Medicine, Public Health and Epidemiological Surveillance, Poniente University Hospital, Almería, Spain 6: Microbiology Service, Torrecárdenas Hospital Complex, Almería, Spain 7: Pediatric Infectious Diseases Department, Gregorio Marañón University Hospital, Madrid, Spain 8: Biomedical Research Network Center for Infectious Diseases (CIBERINFEC), Spain 9: CIEMAT, Extremadura Research Centre for Advanced Technologies (CETA-CIEMAT), Trujillo, Spain 10: Faculty of infectious and tropical diseases, London School of Hygiene & Tropical Medicine, UK

Whole genome sequencing (WGS) enables accurate monitoring of tuberculosis transmission. Traditionally, genomic analysis lies on high-throughput Illumina sequencing, which needs to accumulate a high number of isolates per run, leading to delays in identifying new clustered cases. To address these limitations in contexts where epidemiological intervention is linked to genomic analysis, we leveraged the flexibility of nanopore sequencing to analyze 33 consecutive primary cultures on a case-by-case basis. All sequences achieved exploitable genome coverage (>90% at >20X), enabling identification of those associated to existing clusters, within the critical 21-day window when contact tracing remained ongoing. To further accelerate the availability of genomic results we applied the same strategy to sputa from 45 consecutive cases. Following human DNA partial depletion, 24% of the samples yielded exploitable coverage, and an additional 13% showed suboptimal performance but still provided partial information (from 27% at 10X to 49% at 20X). As an additional approach to accelerate the identification of new clustered cases when facing alarming transmission events, we also assessed the usefulness of fast-tracking potentially related cases by targeted sequencing. We applied this strategy in an interterritorial cluster (involving Madrid and Almería), associated with a super-spreader case/event. Sixteen key-marker SNPs of the strain involved were used to design a targeted amplicon-based sequencing approach. This

allowed the rapid analysis of 96 cases from populations epidemiologically related to the involved cases, excluding further expansion of the aforementioned cluster, without the need for complete WGS of the cases.

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P070

An extended genomic analysis reveals unexpected transmissions involving the pediatric population in Madrid with tuberculosis

M López-Llaría¹ G Bernal¹ SM Saleeb^{1 2} M Guida^{3 4} A López Suárez^{3 4}
C Rodríguez-Grande¹ S Buenestado Serrano¹ MJ Ruiz Serrano¹ P Muñoz^{1 2 5}
B Santiago^{3 4} L Pérez Lago¹ D García de Viedma^{1 2}

1: Servicio de Microbiología clínica y Enfermedades Infecciosas, Hospital General Universitario Gregorio Marañón. Instituto de Investigación Sanitaria Gregorio Marañón (IISGM), Madrid, España 2: CIBER Enfermedades Respiratorias (CIBERES), Madrid, España 3: Sección de Enfermedades Infecciosas Pediátricas, Hospital General Universitario Gregorio Marañón. Instituto de Investigación Sanitaria Gregorio Marañón (IISGM), Madrid, España 4: CIBER Enfermedades Infecciosas (CIBERINFEC), Madrid, España 5: Departamento de Medicina, Universidad Complutense, Madrid, España

From an epidemiological perspective, tuberculosis (TB) in the pediatric population is of particular relevance, as children are considered sentinels of TB transmission. Genomic epidemiology enables us to characterize with the highest precision the transmission patterns involving children. Nevertheless, there is a tendency to restrict these analyses to the closest socio-epidemiological circles, involving the children with TB and their index/contact cases. In this study we followed a genomic epidemiology analysis in three sequential layers: i) focusing exclusively on children in Madrid (75 cases) and their suspected index cases (11 cases) years 2018-2025, ii) extending the analysis to compare with other unrelated adult TB cases previously sequenced from Madrid (238 cases, 2018-2025) and iii) from an unrelated population, Almería, located far from Madrid (938 cases, 2003-2025). Isolates were sequenced in the Illumina NextSeq platform with clusters being designated based on the threshold of 12 SNPs. 35% (20/57) of the children in Madrid were included in 14 clusters involving 39 cases. Eleven clusters had <5 SNPs between cases. Only two clusters corresponded to cases with previously suspected epidemiological links. Among the remaining clusters, seven of them involved children with other unrelated adults from Madrid and three included cases from Almería. In summary, most of the clusters involving children with TB in Madrid were linked with cases outside the suspected epidemiological links, which otherwise could have been overlooked. Exhaustive epidemiological interviews must be coupled to genomic analysis to understand the complexity of the transmission involving children.

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P071

Lineage-dependent rifampicin tolerance in *Mycobacterium tuberculosis* complex strains

A Hintz-Rüter¹ L Sonnenkalb¹ S Niemann¹

1: *Research Center Borstel*

Treatment failure remains a critical challenge in the global fight against tuberculosis (TB), leading not only to poor clinical outcomes but also to continued disease transmission. While antibiotic resistance has long been recognised as a major contributor, increasing evidence points to drug tolerance as an additional, underappreciated factor. Drug-tolerant *Mycobacterium tuberculosis* complex (MTBC) bacteria exhibit prolonged survival during antibiotic treatment. We hypothesise that tolerance mechanisms are greater in strains from certain lineages (or sub-lineages), which may explain variations in antibiotic resistance rates as well as epidemiological success.

In this study, we examine the tolerance of 12 strains representing MTBC lineages 2.2.1 (Beijing Central Asia) and 4.1.2.1 (Euro American Haarlem) plus H37Rv, using time-kill assays with increasing concentrations of rifampicin. Our results show that strains of Lineage 4 exhibit slower drug killing, thus greater tolerance as compared to Lineage 2 strains and H37Rv. This suggests that Lineage 4 strains employ a tolerance-based survival strategy, as opposed to the resistance-based strategy observed in Lineage 2 strains.

These findings underscore the need to integrate lineage-specific tolerance profiles into TB treatment strategies and diagnostics.

P072

Genomic diversity of *Mycobacterium abscessus* isolates in Portugal

M Pinto² S Carneiro¹ JP Gomes² R Macedo¹

1: *National Reference Laboratory for Mycobacteria, Department of Infectious Diseases, National Institute of Health Doutor Ricardo Jorge, Lisbon, Portugal* 2: *Genomics and Bioinformatics Unit, Department of Infectious Diseases, National Institute of Health Doutor Ricardo Jorge, Lisbon, Portugal*

Mycobacterium abscessus complex (Mab) is an emerging multidrug-resistant nontuberculous mycobacteria, responsible for a wide spectrum of infections. Recently, dominant circulating clones (DCC) have been identified as clusters of strains associated with increased virulence, worse clinical outcomes and higher levels of antimicrobial resistance (AMR). To implement a genomic based surveillance system for Mab, we adapted the recently published SeqSphere+ cgMLST and agMLST schemas into a dynamic wgMLST schema for chewBBACA. Thirty Mab Portuguese strains were integrated into a public dataset of 6974 genomes and stability analysis was performed using ReporTree. DCC classification could be obtained at 250 allelic differences and was able to classify 12 Portuguese isolates as belonging to distinct DCC. Dynamic wgMLST analysis of 7003 genomes revealed that, at a 100 AD threshold, 24 Portuguese genomes were integrated in eleven distinct clusters, two of which were composed exclusively by Portuguese isolates. AMR results showed that most isolates presented resistance to at least two antibiotics

(23/30). Twenty eight of the thirty isolates presented the macrolide AMR-associated mutation T28 in *erm41*, while just one isolate had the aminoglycoside AMR-associated mutation A1408G in *rrs*. Whole genome analysis allowed us to screen for additional known AMR markers, thus allowing a more complete resistance profile of the strains. This study highlights the genetic diversity of circulating MAB isolates in Portugal and their association with AMR, while standing as the first step towards the implementation of a genomic-based surveillance system for MAB at the Portuguese National Institute of Health.

P073

Genomic insights into *Mycobacterium avium* complex isolates from Portugal reveal extensive genetic diversity

M Pinto² S Carneiro¹ JP Gomes² R Macedo¹

1: National Reference Laboratory for Mycobacteria, Department of Infectious Diseases, National Institute of Health Doutor Ricardo Jorge, Lisbon, Portugal 2: Genomics and Bioinformatics Unit, Department of Infectious Diseases, National Institute of Health Doutor Ricardo Jorge, Lisbon, Portugal

Opportunistic infections caused by nontuberculous mycobacteria (NTM) have become a growing public health concern. Among these, those belonging to the *Mycobacterium avium* complex (MAC) are the most common etiological agents of human disease, including over ten described subspecies, with *M. avium*, *M. intracellulare*, and *M. chimaera* being the most common. To address the current needs in subspeciation and surveillance of MAC disease cases, we evaluated different species classification methodologies (BLASTn-based marker-gene approach, Kraken v2, rMLST and MLST databases) and their congruence with a core-SNP phylogenetic approach, based on whole genome sequencing (WGS) data. For this purpose, we used a collection of 142 MAC isolates from Portuguese patients diagnosed between 2014 and 2022. The marker-gene approach (based on the *rpoB*, *hsp65* and *groEL* genes), showed the best results, allowing the identification of the 142 MAC isolates to the species/subspecies level (*M. avium* subsp. *hominissuis*, *M. intracellulare*, *M. intracellulare* subsp. *chimaera*, *M. intracellulare* subsp. *yongonense*, *M. marseillense* and *M. colombiense*). Using a core-SNP approach, we conducted a detailed phylogenetic analysis within each identified species group. Despite the considerable genetic diversity among MAC species, we successfully differentiated all species and subspecies and identified genetic clusters with epidemiological potential. This study highlights the importance of reliable genotyping methods for accurate species identification towards an effective management of MAC disease. Additionally, it emphasises the need for more comprehensive large-scale WGS data analysis, guided by a One Health perspective, to uncover possible transmission pathways.

P074

Population structure of *Mycobacterium tuberculosis* Lineage 2 in Kazakhstan based on whole-genome sequencing

S Atavliyeva¹ D Auganova¹ A Akisheva² A Tsepke² A Alenova³ Y Skiba¹
C Sola⁴ P Tarlykov¹

1: National Center for Biotechnology, Astana, Kazakhstan 2: City Center for Phthisiopulmonology of the Akimat of Astana, Kazakhstan 3: National Scientific Center for Phthisiopulmonology, Almaty, Kazakhstan 4: Université Paris-Saclay, France

Kazakhstan remains one of the 30 countries with the highest burden of multidrug-resistant tuberculosis (MDR-TB), primarily driven by the circulation of L2/Beijing *M. tuberculosis* strains within the Central Asian/Russian cluster. We conducted whole-genome sequencing (WGS) of 177 clinical L2/Beijing isolates of *M. tuberculosis* obtained from 15 regions of Kazakhstan, including several major urban centers. Whole-genome sequencing and SNP-based classification, according to the nomenclature proposed by Thawornwattana, revealed that the majority of *M. tuberculosis* L2/Beijing isolates belonged to sublineage Central Asia outbreak (L2.2.M4.9.1) (119; 67.23%), followed by L2.2.M4.9 (44; 24.86%), Europe/Russia W148 outbreak (L2.2.M4.5) (11; 6.21%), Clade A (L2.2.M4.9.2) (2; 1.13%), and sublineage L2.2.M4 (1; 0.56%). Phenotypic drug susceptibility testing indicated a high proportion of multidrug-resistant (MDR) isolates (n = 83; 46.89%) and pre-extensively drug-resistant (pre-XDR) isolates (n = 26; 14.69%). Additionally, an extensively drug-resistant (XDR) isolate (n = 1; 0.56%) was detected in the study population. Our findings demonstrate the predominance of the L2.2.M4.9.1 (Central Asia outbreak, CAO) sublineage among circulating L2/Beijing *M. tuberculosis* strains in Kazakhstan and reveal a substantial burden of drug resistance, including MDR, pre-XDR, and XDR phenotypes. These results provide insights into the population structure and resistance patterns of *M. tuberculosis* in the region, emphasizing the importance of integrating genomic surveillance into national TB control strategies.

P075

Do we use the right breakpoints for delamanid susceptibility testing ?

N Lequerré¹ C Poignon^{1 2} E Gyde² J Robert^{1 2} N Veziris^{1 2 3} A Saffarian²
A Aubry^{1 2}

1: UPMC - Paris 6 2: Hôpital Pitié-Salpêtrière 3: Hôpital Saint-Antoine

As delamanid (DLM) is increasingly used in the treatment of drug-resistant tuberculosis (MDR/XDR-TB), accurate DLM drug susceptibility testing (DST) is essential. DST methods recommended by WHO are the proportion method in 7H11 (PM) and the BACTEC™ Mycobacterial Growth Indicator Tube 960™ (MGIT).

We aimed to evaluate the concordance between these methods for DLM and to compare those results to whole genome sequencing data (WGS).

We performed DST following WHO recommendations in MGIT (CC=0.06 mg/L) and in PM (WHO CC=0.016 mg/L + additional CC=0.06 mg/L) against 126 MDR/XDR-TB clinical isolates. Additionally, DLM genotypic resistance markers were identified by WGS (*fbiA-D*, *fgd1*, *ddn*, *Rv2983*).

The agreement between MGIT and PM was moderate using CC^{WHO} and almost perfect using CC^{0.06} (k=0.51 and 0.90, respectively). Among discrepant isolates (n=10 with CC^{WHO}, n=1 with CC^{0.06}), no mutation known to be involved in DLM-R was identified by WGS, but mutations of unknown impact were found in 5/10 using CC^{WHO} and in the strain using CC^{0.06}.

Among strains categorized as DLM-R by both methods (n=6 with CC^{WHO}, n=5 with CC^{0.06}), WGS identified mutations known as involved in DLM-R in 3/6 using CC^{WHO} and 3/5 using CC^{0.06}.

Among strains categorized as DLM-S by both methods (n=110 with CC^{WHO}, n=120 with CC^{0.06}), WGS identified mutations of unknown impact in 54.2% (n=60) using CC^{WHO} and 54.2% (n=65) using CC^{0.06}.

The discrepancies results suggest that increasing PM's CC to 0.06 mg/L allows better concordance with MGIT DST and improving our knowledge regarding genotypic markers for DLM-R is crucial.

P076

Emergence of bedaquiline resistance in samples rifampicin resistant Tuberculosis in Mozambique

T Fernando¹ C Mbebe¹ C Utpatel² I Barilar^{2,3} C Abujate¹ E Muxanga¹
S Chumane¹ A Manhique¹ I Gundane¹ C Madeira¹ B José⁴ C Mutaquiha⁴
T Niemann^{2,3} N Ismael¹ L de Araújo² S Niemann^{2,3} S Viegas

1: Instituto Nacional de Saúde, Mozambique 2: Research Center Borstel, Germany 3: German Center for Infection Research, Partner Site Hamburg-Lübeck-Borstel-Riems, Germany 4: National Tuberculosis Control Program, Ministry of Health, Mozambique

Bedaquiline is currently a key drug for treating MDR/RR-TB. The WHO recently endorsed the use of new regimens based on Bedaquiline (B), Linezolid (L), Moxifloxacin (M), Levofloxacin (Lfx), Pyrazinamide (Z), Clofazimine (C), and Delamanid (D) with the 6-month BPALM regimen as preferred option for most patients with MDR/RR-TB. In Mozambique, BDQ resistance (BDQr) rose from 03-14% from 2016-2021. Emerging evidence suggests that rising BDQr threatens overall effectiveness of rifampicin resistance (RR) treatment. Targeted next-generation-sequencing (tNGS) was performed with Deeplex®-Myc-TB assay from GenoLyse® DNA extracted from sputum submitted to National Tuberculosis Reference Laboratory in Maputo between January 2021 to April 2025. Inclusion criteria was at least isoniazid resistant (INHr) and/or RR. Drug-resistant TB (DR-TB) predictions were successfully made for 279 samples of those 240/279 (86.0%) at least RR-TB. According to tNGS 20.1% of the RR strains were fluoroquinolone resistant (FQr). 11% had an additional BDQr and were classified XDR-TB. Overall, 27% of the RR-TB samples had additional BDQr. Interestingly, 13/240 (5.4%) had the I491F *rpoB* mutation not detected by commercial DR-TB, out of which nine were BDQr. High rates of BDQr identified are alarming and pose a serious threat to DR-TB control efforts in Mozambique, given BDQ's central role MDR/RR-TB treatment regimens. Notably, over 10% of RR strains were classified as XDR, further complicating the rollout of shorter BPAL-based regimens. These findings underscore the urgent need to implement tNGS, to guide the design of

individualized, effective treatment regimens and prevent treatment failure and transmission of highly resistant *Mycobacterium tuberculosis* strains.

P077

Relaunching crypticproject.org: making the MTBC genetic and pDST datasets collated by the CRyPTIC project more available to the wider community

PW Fowler¹ D Adlard¹ J Westhead¹ J Knaggs¹ H Thai¹ M Colpus¹ R Turner¹
TEA Peto¹ DW Crook¹ Z Iqbal² NA Ismail³ TM Walker⁴
1: University of Oxford 2: University of Bath 3: University of the Witwatersrand 4: Oxford
University Clinical Research Unit

Some projects take on a life of their own; the Comprehensive Resistance Prediction for Tuberculosis: an International Consortium (CRyPTIC) project ran from 2017 to 2022 and was one of these. It collected tuberculosis samples with a bias towards MDR-TB. Each sample underwent short-read whole genome sequencing (WGS) and had minimum inhibitory concentrations to 13 antibiotics (including bedaquiline and delamanid) measured using a bespoke 96-well broth microdilution plate. The project also collated and curated existing datasets with WGS and/or pDST data. Following a data freeze in April 2020 several studies were published and this initial dataset was made available via an FTP site (https://ftp.ebi.ac.uk/pub/databases/cryptic/release_june2022/).

The project has, however, continued to amass samples: back in April 2020 a total of 41,130 samples with both WGS and pDST data were included. There are now 53,897, an increase of +31%. Improvements to the quality of the data have also been made: the bioinformatic pipeline has been rewritten (so it e.g. reports all minor alleles in all samples) and the accuracy of the reported MICs improved (by applying a machine learning model to images of the 96-well plates).

At this meeting we shall launch a revamped crypticproject.org to permit researchers to easily get this larger versioned dataset and describe our plans for future expansion through growing a widening network of contributors, including regular data releases, automated (API) access, machine learning competitions and adding samples that have been sequenced using long-read technologies. Finally, we will also describe our efforts to collate non-tuberculous mycobacterial (NTM) samples.

P078

Uncovering hidden complexity in TB infections: A novel WGS-based method for detecting mixed MTBC infections

V Dreyer^{1 2} C Utpatel¹ I Barilar¹ S Niemann^{1 2}

1: Research Center Borstel - Leibniz Lung Center 2: The German Center for Infection Research (DZIF)

Tuberculosis (TB) remains a major global health challenge, particularly due to the rise of multidrug-resistant strains of the *Mycobacterium tuberculosis* complex (MTBC). Mixed infections, where a patient is co-infected with genetically distinct MTBC strains, are increasingly recognized as contributors to treatment failure and misleading results in transmission analyses. However, their true prevalence is likely underestimated due to the limitations of standard genotyping techniques. We developed mixDetector, a novel bioinformatic tool based on the DBSCAN clustering algorithm, designed to detect mixed MTBC infections directly from whole-genome sequencing (WGS) data. The method was validated using synthetic mixtures of known strain combinations and in-silico simulated datasets, then applied to three large TB datasets from Hamburg (Germany), Samara (Russia), and KwaZulu-Natal (South Africa). mixDetector reliably identified mixed infections, including those between closely related strains or with different drug resistance profiles. In the Samara dataset (n = 1,206), 83 samples (6.9%) showed clear evidence of mixed infection. The method achieved high sensitivity (1.0) and specificity (0.97) at a 5% minor allele frequency threshold. Variants below this frequency could not be reliably detected, highlighting limitations in current variant-calling tools. Our findings demonstrate that mixDetector provides a robust and scalable solution for detecting MTBC mixed infections from WGS data without the need for labor-intensive subculturing. This represents a significant advancement for TB diagnostics, treatment strategies, and molecular epidemiology, particularly in high-burden and MDR-endemic settings.

P079

Transcriptomic insight into metabolic adaptations and efflux-based mechanisms underlying bedaquiline resistance

J Snobre¹ L Krausser¹ S de Bock¹ C Meehan² W Mulders¹ A Van Rie³

L Rigouts¹ BC de Jong¹ O Tzfidia¹

1: Institute of Tropical Medicine Antwerp 2: Nottingham Trent University 3: Antwerp University

Bedaquiline (BDQ) is essential for treating rifampicin-resistant tuberculosis (RR-TB), but its long-term efficacy is threatened by emerging resistance. Although clinical resistance is linked to mutations in *mmpR5*, a repressor of the *mmpS5/mmpL5* efflux pump, the genotype-phenotype correlation remains challenging, suggesting additional mechanisms.

To explore the impact of diverse *mmpR5* mutations and identify potential regulatory mechanisms, RNA-sequencing was performed on four BDQ-resistant strains (344delA, L43R, A84T, and the double mutant E138G/M139L) and three BDQ-susceptible strains. Samples were analyzed before and after BDQ exposure (0.5 µg/mL for 30 minutes and 2 hours).

Three of four resistant strains exhibited overexpression of the *mmpS5/mmpL5* efflux system ($p_{adj} < 0.05$). One resistant strain showed no significant upregulation ($p_{adj} > 0.05$), suggesting alternative resistance pathways. Transcriptomic analysis revealed 711 differentially expressed genes ($\log_2FC < -1$ or > 1) in resistant versus susceptible strains prior to BDQ exposure. Changes included upregulation of other efflux genes, increased lipid transport, redox stress tolerance, and respiratory chain reprogramming. Notably, all resistant strains upregulated cytochrome *bd* oxidase, suggesting a compensatory mechanism. At the concentration tested, BDQ exposure did not significantly alter *mmpR5*, *mmpS5*, or *mmpL5* expression or pathway enrichment ($p_{adj} > 0.05$).

These findings suggest BDQ resistance may result from both constitutive *mmpS5/mmpL5* activation and broader metabolic adaptations. Further omics studies are needed to clarify these mechanisms.

P080

Different paths to progress towards a faster and more precise genomic epidemiology in tuberculosis

S Buenestado-Serrano^{1 2 6} [SM Saleeb](#)^{1 2 3 4} S Vallejo-Godoy⁵ G Bernal¹
A Marcos-Abellán^{1 2 4} M Martínez-Lirola⁷ T Cabezas⁷ S Quirós¹⁰ L Herrera⁹
E Abascal⁹ P Muñoz^{1 2 3 8} L Pérez-Lago^{1 2} D García de Viedma^{1 2 3}

1: Department of Clinical Microbiology and Infectious Diseases, Gregorio Marañón General University Hospital, Madrid, Spain 2: Gregorio Marañón Health Research Institute (IISGM), Madrid, Spain
3: CIBER of Respiratory Diseases (CIBERES), Spain 4: Doctoral school, Autonomous University of Madrid, Spain 5: Department of Preventive Medicine, Public Health and Epidemiological Surveillance, Poniente University Hospital, Almería, Spain 6: Doctoral school, University of Alcalá, Spain
7: Microbiology service, Torrecárdenas Hospital Complex, Almería, Spain 8: Department of Medicine, Complutense University of Madrid, Spain 9: National Center for Microbiology, Madrid, Spain
10: Hospital Basurto, Bilbao, Spain

The evolutionary analysis of SNPs within clusters allowed us to distinguish between clusters growing mainly due to reactivations of past exposures and others corresponding to active recent transmission, in which intervention to control is still possible. To optimize the surveillance of active clusters we i) refined the genomic analysis by including repetitive genomic regions and heterozygous calls and ii) accelerated the characterization of new cases by performing nanopore sequencing on primary cultures and/or exploiting suboptimal sequences when sequencing directly on sputa. The usefulness of long-reads in detecting additional diversity was evaluated on a selection of 78 cases from eight long-term clusters. Long-read analysis increased the within cluster diversity (1-22 additional SNPs), thereby enabling the identification of index cases, reconstruct transmission chronologies, and determine the relationships between the cases with greater precision. Secondly, considering not only fixed SNPs but also heterozygous calls (frequency 0.15-0.70) in clusters that demanded higher analytical precision resulted in the proposal of reordering the transmission chronology. Finally, we evaluated whether focusing on key cluster marker-SNPs, we could exploit suboptimal sequences obtained from sputa to rule in/out new cases to pre-existing clusters, when the strain marker-SNPs were sufficiently covered. This rescue strategy enabled not only the assignment of new cases to clusters, but also their location in specific branches of the corresponding genomic networks. The efficacy of this approach was further enhanced by adapting it to a rapid assessment of cases to clusters by calling only specific marker SNPs of the relevant clusters during the nanopore sequencing run.

P081

Characterization of the putative cobalt ECF importer CbiMNQO and its cobalt/nickel dependent regulation by the ArsR/SmtB-like regulator CbiR in *Mycobacterium smegmatis*

E Goethe¹ M Ackermann¹ L Ende¹ M Jarek² RW Goethe¹

1: University of Veterinary Medicine, Hannover 2: Helmholtz Centre for Infection Research

Cobalt is an important transition metal, incorporated e.g. in corrinoid metalloproteins such as the essential vitamin B12. 90% of all bacteria possess vitamin B12 dependent enzymes involved in central metabolic processes and host-pathogen interaction, however only 25-30% are able to produce vitamin B12 *de novo*, including many non-tuberculous mycobacteria (NTM) such as *Mycobacterium abscessus*. For the production of vitamin B12, cobalt uptake is essential. An important system for cobalt import, e.g. in *Salmonella* Typhimurium, is the energy-dependent ECF transporter CbiMNQO. Genes are present in many other bacteria. Associated regulators of this system are unknown. No cobalt importers including CbiMNQO have been analyzed in mycobacteria.

We analyzed an operon encoding for a putative CbiMNQO transporter (*msmeg_2607-2610*) in the non-pathogenic model organism *M. smegmatis* (MSwt), associated with an ArsR/SmtB family regulator (*msmeg_2606*, designated CbiR) and genes involved in vitamin B12 biosynthesis (*msmeg_2616-2618*). By transcriptome analyses of MSwt and an Δ *cbiR* mutant, we identified the CbiR regulon, including *cbiMNQO*, and a CbiR binding motif. A cobalt/nickel (Co/Ni) dependent regulation of *cbiMNQO* by CbiR was shown by RT-PCR, reporter studies and ChIP-Seq analyses of cultures grown in metal depleted Sauton's medium or supplemented with different cations. A Δ *cbiMN* transporter mutant was analyzed for metal dependent growth and vitamin B12 production. Compared to MSwt no difference was observed. However, this is the first report of a regulator involved in Co/Ni dependent regulation of a putative Co/Ni importer CbiMNQO in mycobacteria. These findings might help understanding cobalt/nickel homeostasis in other mycobacteria and identifying potential NTM-treatment strategies.

P082

Improving the benchmark of variant calling of *M. tuberculosis* using more complex *in silico* genomes

A Le Meur¹ RC Rodríguez de la Vega¹ G Refrégier¹

1: University Paris - Saclay

The democratization of Whole Genome Sequencing (WGS) methods require robust benchmark methodologies. Existing benchmark studies for *M. tuberculosis* rely either on simplistic *in silico* genomes evolved from the H37Rv reference genome, or on *de novo* assembled genomes which require an additional complete genome alignment step. We introduce maketube, a new method for building more realistic evolved genomes using the extensive literature describing *M. tuberculosis* structural variation. This includes the transposition of large Inserting Sequences (IS), large deletions and duplications. These genomes can be built from any reference, and the comprehensive evolutionary steps can be traced back to this reference. We show that maketube

genomes are realistic and more representative of the diversity found in different lineages of *M. tuberculosis*. Using these genomes, we show that genome to genome alignment tools miss 5% of variants, introducing noise to the performance evaluation. We evaluated the performance of two recently published variant calling methodology and our in-house genomic pipeline genotube. As traditional benchmarks overestimate tools performance, methodologies with precision and recall previously described with near perfect precision and recall missed up to 10% of variants on maketube genome. At last we studied the local impact of structural variants and found that structural variations with sequence homology negatively affected the precision and recall of variant callers.

P083

The nature and consequence of epistasis between drug resistance mutations in *Mycobacterium tuberculosis*

S Borrell^{1 2} A Banaei-Esfahani^{1 2} S Bouaouina^{1 2} S Gagneux^{1 2}
1: Swiss Tropical and Public Health Institute 2: University of Basel

Studies in model organisms have shown that mutations conferring resistance to different antibiotics can interact epistatically. However, the nature and epidemiological consequences of such epistatic interactions have rarely been studied in human pathogens. Here we show that in *Mycobacterium tuberculosis*, one third of strains carrying mutations in RpoB and GyrA causing resistance to rifampicin and fluoroquinolones, respectively, interacted epistatically *in vitro*. In two instances, the double-mutants exhibited a higher fitness than the corresponding single mutants — a phenomenon known as positive sign epistasis. These two double-mutant genotypes together accounted for the large majority of highly drug-resistant *M. tuberculosis* in clinical settings, and exhibited a reduced proteome perturbation compared to less epidemiologically successful genotypes. Taken together, our findings highlight the relevance of epistasis for the evolution and spread of antimicrobial resistance.

P084

Genomic description of routinely laboratory-diagnosed XDR-TB strains from 2010 to 2019 in the Western Cape Province, South Africa

JT Ngom¹ J Loubser¹ E Maasdorp¹ Y Ghebrekritos^{1 2} CJ Opperman^{1 2}
M Klopper¹ RM Warren¹ EM Streicher¹
1: Stellenbosch University 2: National Health Laboratory Service, South Africa

Extensively drug-resistant tuberculosis (XDR-TB) is a global health threat, being expensive and difficult to treat, with high mortality rates. Drug resistance genotypes and transmission present substantial regional variability. Thus, a better understanding of genetic diversity and the factors related to transmission can aid in prioritizing resources to effectively target high-risk populations and regions that are disproportionately affected. We aimed to describe genetic diversity and drug resistance profiles of XDR-TB strains in the Western Cape Province over a 10 year period.

We included 729 XDR-TB samples, identified through routine diagnosis from 2010-2019, from six healthcare districts (HCDs) in the province. Genomic DNA from cultured isolates was sequenced using the Illumina platform. Sequences were analysed for strain type, drug resistance mutations, and genomic clustering using TBProfiler and MTBseq pipelines. We conducted logistic regression to identify potential factors associated with genomic traits related to the spread of XDR-TB.

Of the 729 XDR-TB strains, sublineage 2.2.2 (Atypical Beijing: n=378, 58.79%) strains were predominant, followed by sublineage 2.2.1 (Typical Beijing: n=260, 40.43%). L2.2.2 were more likely to cluster than L2.2.1. Most of the clusters were small, with a few large and very large clusters, and the very large clusters (primarily L2.2.2.) were more likely to be found within 3 HCDs. Some L2.2.2 were found resistant to new and repurposed drugs recently introduced in the WHO treatment guidelines.

Near-untreatable Atypical Beijing strains are prevalent in the WCP. Hence, hotspot areas for clustering in 3 HCDs should be prioritized for targeted intervention to prevent ongoing XDR-TB transmission.

P085

Deciphering NucS interactions to explore anti-evolution strategies against *Mycobacterium tuberculosis*

[R Zein Eddine](#)¹ [S Skouloubris](#)^{1,2} [G Refrégier](#)³ [A Ignas](#)¹ [H Myllykallio](#)¹
1: Laboratoire d'Optique et Biosciences (LOB), Ecole Polytechnique, Inserm U1182, Institut Polytechnique de Paris, France 2: Université Paris-Saclay, Gif-sur-Yvette, France 3: CNRS, AgroParisTech, Ecologie Société Evolution, Université Paris-Saclay, France

The rise of drug-resistant *Mycobacterium tuberculosis* (*Mtb*), including multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains, poses a serious global health threat. Bedaquiline, the only new tuberculosis (TB) drug approved in the past 50 years, is already facing resistance, with up to 47% of post-treatment strains showing acquired mutations. Unlike many pathogens, *Mtb* develops antimicrobial resistance (AMR) solely through spontaneous mutations, not horizontal gene transfer. Understanding the mechanisms underlying this mutagenesis is crucial to designing new intervention strategies. This study focuses on DNA repair, a key process balancing genome stability and mutation-driven adaptation. Our long-term goal is to assess the endonuclease NucS, part of the Mismatch Repair (MMR) system, as a potential target for “anti-evolution” strategies to slow resistance emergence. As a first step, we identified and characterized NucS interaction partners and their functional role in *Mtb*'s DNA repair pathways. Using a yeast two-hybrid system screening, we identified two NucS partners, Mku and UvrA. We then constructed single and double gene deletion mutants in *Mycobacterium smegmatis*, a model for *Mtb*, and assessed mutation rates and competitive fitness to validate their interplay. Our results suggest NucS functions in two distinct contexts: in coordination with Mku, it likely couples MMR with double-strand break repair; with UvrA, it appears to support nucleotide excision repair of UV-induced lesions. These findings highlight the central role of NucS and its partners in maintaining genomic stability. They also support the feasibility of targeting DNA repair to limit AMR evolution, offering a novel avenue for durable TB control strategies.

P087

Strategies to detect emerging SNPs in clustered *Mycobacterium tuberculosis* isolates for precision epidemiology

RM Anthony¹ M Kamst-van Agterveld¹ A Mulder¹ J van den Dool¹ R de Zwaan¹

1: National Institute for Public Health and the Environment

The detection of emerging SNPs within clustered isolates (genetic drift) could help determine transmission dynamics and support epidemiological investigations. Tools for the routine detection of non-fixed SNPs in resistance genes and as well for emerging SNPs are being developed. To interpret emerging SNPs throughout the genome calls need to be checked for quality and false calls removed otherwise any real signal is masked. We present a validated strategy to detect epidemiologically informative (emerging) variation in clustered isolates and present examples of how this can be used to support epidemiological investigations. Clusters are first identified using a SNP threshold then all potentially informative SNPs not present in all members of the cluster are determined. The positions where these potentially informative SNPs occur are then interrogated with respect to their distribution in a large collection of isolates and the average quality of sequencing data at these sites (to detect platform specific sequencing noise). In this way noisy or non-informative SNPs can be identified and exclude. The remaining informative SNPs are examined in all members of the cluster for to identify non-fixed / emerging SNPs. In eight clusters of epidemiologically linked cases investigated in detail so far emerging SNPs were identified in seven clusters which were fixed in other members of the cluster, suggesting a direct link and transmission direction between cases. Emerging SNPs are often associated with high bacterial load and transmission as other groups have observed.

P088

Evolutionary dynamics of MDR-TB and the escalation of drug resistance and clinical impact: a case study from Portugal

P Gomes¹ P Paixão¹ F Maltez² L Brum³ J Phelan⁴ S Campino⁴ T Clark⁴

M Viveiros⁵ I Portugal¹ J Perdigão¹

1: Research Institute for Medicines, Faculdade de Farmácia, Universidade de Lisboa, Portugal

2: Serviço de Doenças Infecciosas, Hospital Curry Cabral, Centro Hospitalar Universitário de Lisboa

Central, Portugal 3: SYNLAB Portugal, Lisboa, Portugal 4: Faculty of Infectious and Tropical

Diseases, London School of Hygiene and Tropical Medicine, UK 5: Global Health and Tropical

Medicine, GHTM, LA-REAL, Instituto de Higiene e Medicina Tropical, IHMT, Universidade NOVA de Lisboa, Portugal

Globally, endemic multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis* pose a significant risk to tuberculosis (TB) management and control. Its continued circulation enables compensatory evolution and accumulation of additional mutations, potentially impacting resistance levels and clinical outcome. In Portugal, MDR-TB has been historically linked with two unusual endemic clades: Lisboa3 and Q1, whose microevolutionary trajectory over the last five decades is herein studied and integrated into pharmacokinetic models. This study comprises 40

representative clinical isolates, all of which were subjected to whole-genome sequencing and for which the minimum inhibitory concentration (MIC) of 12 anti-TB drugs was determined by broth microdilution. Pharmacokinetic (PK) models were generated to ascertain the maximum concentration to which each drug remains efficacious according to previously established targets. Drug resistance phenotypes were quantified and compared across distinct allelic configurations. Strains harboring dual mutations in *inhA* and *embA/B* loci exhibited significantly elevated MICs for isoniazid and ethambutol, respectively, relative to isolates with single-site mutations. Statistically significant intergroup differences in resistance levels were observed across phylogenetic lineages for rifamycin, streptomycin, and ethionamide, largely attributable to the differential distribution of high-level resistance-conferring genotypes. Pharmacokinetic/pharmacodynamic (PK/PD) simulations for isoniazid and moxifloxacin indicated that increased dosing regimens are unlikely to overcome resistance in strains possessing double *inhA* mutations or *gyrA/B* alterations. Only cycloserine and para-aminosalicylic acid maintained predicted efficacy against most clinical isolates. Comprehensive genotypic profiling of resistance-associated mutations is essential for optimizing therapeutic strategies in TB, as discrete allelic profiles markedly influence resistance levels and treatment outcomes.

P089

Whole-genome sequencing-based surveillance system for *Mycobacterium tuberculosis* in Portugal

M Pinto² R Macedo¹

1: National Reference Laboratory for Mycobacteria, Department of Infectious Diseases, National Institute of Health Doutor Ricardo Jorge, Lisbon, Portugal 2: Genomics and Bioinformatics Unit, Department of Infectious Diseases, National Institute of Health Doctor Ricardo Jorge, Lisbon, Portugal

Portugal is a medium incidence tuberculosis (TB) country, with 14.9 cases per 100 000 inhabitants reported in 2023. In an effort to improve TB surveillance and diagnosis, the Portuguese National Reference Laboratory (NRL) began implementing whole-genome sequencing (WGS) for all RR/MDR-TB cases in 2019. Since 2020, this approach has been expanded to include all isolates received by the NRL, and has undergone upgrades towards antimicrobial resistance (AMR) prediction and epidemiological analysis.

Here we aim to describe our current WGS-based surveillance system, which relies on three main steps: QC/QA and contamination assessment, with a novel data filtering step (Kraken_v2); genotyping and AMR prediction (tb-profiler_v6 plus WHO 2024 catalogue); and dynamic SNP-based approach, that maximizes the number of variable sites under analysis (Reportree_v2). Among the pipeline upgrades, we highlight: i) the novel filtering step that allowed the improvement of 111 out of 147 contaminated samples, reducing false positive results in AMR typing, without hampering SNP-based analysis; ii) the inclusion of 5% missing data per site threshold (Reportree) which more than doubled the resolution of the core variable sites under analysis (~40000 vs 20000 sites for 100% core-SNPs); iii) the automatic maximization of the number of shared variable sites (zoom-in/Reportree) for in-depth cluster analysis, which is key to consolidate genetic links for epidemiological investigation. The advantages of these novel features will be presented in light of data from the last five years (n=869) framed with retrospective data (n=302). Ultimately, the implemented system has significantly contributed towards strengthening TB surveillance and diagnosis in Portugal.

P091

Endemic transmission of a *Mycobacterium tuberculosis* L2.2.M3 sublineage of the L2 lineage within Colon, Panama: a prospective study

F Acosta¹ D Candanedo^{1,2} P Patel¹ A Llanes¹ J Elizabeth Ku¹ K Salazar^{1,2}
M Morán¹ D Sambrano¹ J Jurado³ I Martínez⁴ L Garibaldi³ M Delgado³
L Solís⁴ O Luque⁴ K Da Silva⁵ J Andrews⁵ A Goodridge¹

1: Instituto de Investigaciones Científicas y Servicios de Alta Tecnología Panama City 2: Universidad Latina de Panamá, Ciudad de Panamá 3: Caja de Seguro Social, Colón, Panamá 4: Programa de Control de Tuberculosis, Ministerio de Salud, Colón, Panamá 5: Division of Infectious Diseases & Geographic Medicine, Stanford University, USA

Mycobacterium tuberculosis lineage 2 (L2) remains a globally significant lineage associated with increased drug resistance and rapid transmission. The L2 lineage exhibits a hotspot for genetic diversity and evolution in Panama, requiring an in-depth analysis. We conducted a prospective analysis of 274 *Mycobacterium tuberculosis* L2 isolates from Colon City between January 2021 and October 2023. Drug resistance was determined using GeneXpert and MTBDRplus-Genotype assays, strain lineage was determined by strain-specific PCR (ASO-PCR), and whole-genome sequencing was conducted for phylogenetic and evolutionary analyses. Sequencing data were analyzed using the mtb-call2 pipeline and TB-gen tools to predict drug resistance and sublineage, respectively. [ASO-PCR](#) results identified all 31.7% (86/271) isolates as Modern L2.2. WGS analysis of 66 strains confirmed all isolates belonged to the L2.2.1 sublineage. Sixty-four strains were analyzed in depth, with 96.9% (62/64) classified as pan-susceptible and 3.1% (2/64) as rifampicin/pyrazinamide-resistant. The sublineage analysis based on SNPs using the TB-gen tool identified a SNP at position 1219683G>A, which genotyped all 64 strains as L2.2.M3 sublineage. Phylogenetic analysis revealed a correlation with geographical distribution compared to other Latin American L2 isolates. Transmission clusters (≤ 12 SNPs) were identified and used to determine recent transmission events or TB transmission clusters. These analyses also confirmed a relatively low evolutionary rate within Panama L2 isolates in Colon province. These findings suggest endemic transmission of the *Mycobacterium tuberculosis* L2.2.M3 sublineage in Colon, Panama. We recommend combining genomic information with epidemiological data to accurately track and identify the source hotspot for the L2.2.M3 sublineage and focus control measures.

P092

Genomic analysis of a novel nontuberculous mycobacterial isolate causing persistent, disseminated infection after cardiac transplantation

C Mariner Llicer¹ AR Menon² AM Xet-Mull² MG López¹ N Alavian² EK Maziarz²
MJ Lee² JE Stout² DM Tobin² I Comas¹

1: Instituto de Biomedicina de Valencia (IBV-CSIC) 2: Duke University School of Medicine

A 71-year-old male presented 6 years after orthotopic heart transplantation with 4 months of fatigue and night sweats accompanied by a 30-pound weight loss and acute renal failure due to hypercalcemia. PET/CT demonstrated diffuse, hypermetabolic lymphadenopathy and splenomegaly. Excisional biopsy of a cervical lymph node demonstrated abundant non-

necrotizing granulomas containing acid-fast bacilli. Blood cultures grew an organism initially identified as *Mycobacterium nebraskense* by 16S rRNA sequencing; 16S PCR of lymph node tissue demonstrated the same organism. The patient had persistent bacteraemia with this organism over 2.5-years and clarithromycin resistance emerged (MIC ≤ 0.06 to >64 $\mu\text{g}/\text{mL}$) after 8.5 months of azithromycin-based therapy. Whole genome sequencing later revealed that the mycobacterial isolate was not *M. nebraskense* (90.0% average nucleotide identity despite 99.8% nucleotide match by 16S), matching an isolate designated as SMC-2 (99.95% average nucleotide identity). This organism, most closely related to *M. paraseoulense*, has not been previously reported in human disease. Long-read sequencing of the initial isolate with short-read sequencing of the initial and subsequent isolates revealed several differences from the reference strain including the lack of a plasmid and absence of several prophage sequences. Preliminary results did not reveal emergence of the expected mutation in 23S rRNA gene after emergence of phenotypic clarithromycin resistance, suggesting a potentially novel resistance mechanism. One low-frequency, nonsynonymous mutation of unclear significance emerged and persisted in isolates obtained after emergence of clarithromycin resistance. Whole genome sequencing provided more accurate species identification and provided insight into within-host mycobacterial evolution under antibiotic and immune pressure.

P094

Phylogenetics and ESX-1 characterisation of a new human pathotype in the *Mycobacterium conceptionense* / *Mycobacterium senegalense* clade

A Piek^{1,2} MA Fliss^{1,2} L Gard^{1,2} E Bathoorn^{1,2}

1: University Medical Center Groningen 2: University of Groningen

Mycobacterium fortuitum group (MFG) comprises rapidly growing species, generally of low virulence. However, invasive infections have been reported. The ESX-1 gene complex is a system for excretion of virulence factors. Here, we describe the phylogenetics and ESX-1 comparisons of MFG from invasive infections in two unrelated immunocompetent patients. UMCG_patient1 was isolated from blood from a patient with septic catheter-related thrombophlebitis and UMCG_patient2 from a patient with infected implanted artificial material. First, Miseq (Illumina) was used to perform short-read sequencing. BLAST comparison of the *16s rRNA* gene showed 100% identity to both *M. conceptionense* and *M. senegalense*. Next, whole genome-based phylogeny was performed using TypeStrain. This showed that both isolates cluster together as a separate branch within the *M. conceptionense* / *M. senegalense* clade. Genome BLAST Distance Phylogeny analysis showed that UMCG_patient1 and UMCG_patient2 were related to *M. conceptionense* CCUG 50187 with a dDDH of 81.7% and 78.4%, respectively, and to *M. senegalense* DSM 43656 with a dDDH of 87.9% and 83.2%. This indicates that a definite classification was not possible. Finally, ESX-complexes were identified using RAST. The nucleotide content of ESX-1 of UMCG_patient1 was 99.98% identical to UMCG_patient2, whereas identities to closest related type strains were 98.38% (75% cover) to *M. conceptionense* and 98.15% to *M. senegalense*. Altogether, this study shows two isolates from acute infections, representing a new human pathotype. The isolates had identical ESX-1 complexes, which were variable between species. ESX-1 composition may act as driver of speciation, and could be useful to define pathotypes in MFG.

P095

Twenty-three years surveillance of antimicrobial resistance in leprosy

E Cambau^{1 2} A Chauffour^{1 3} M Nait-Chabane^{1 2} F Mougari^{1 2} J Robert^{1 3}
V Jarlier^{1 3} A Aubry^{1 3}

1: Centre national de référence des mycobactéries et résistance aux antituberculeux (CNR-MyRMA)
2: APHP Nord-Bichat Hospital, Université Paris Cité, IAME UMR1137 Inserm 3: 3APHP Sorbonne Université, INSERM, Centre d'Immunologie et des Maladies Infectieuses, Paris, France

Antimicrobial resistance (AMR) surveillance is a main WHO objective for leprosy. Since *Mycobacterium leprae* cannot grow *in vitro*, AMR surveillance is done by molecular detection of mutations conferring resistance to rifampicin (RIF, *rpoB*), dapsone (DAP, *folP1*) and fluoroquinolones (FQ, *gyrA*).

From 2001 to 2023, skin samples from patients suspected of leprosy were subjected to microscopy and RLEP PCR to confirm or rule out the diagnosis. When positive, samples were further analysed by PCR sequencing or the test GenoType LepraeDR® to detect AMR.

Among 1359 skin samples from 1008 patients suspected of leprosy, 474 were positive (34.9%) corresponding to 378 patients (37.5%); 150 (39.7%) living in France metropolitan and 228 (60.3%) in French overseas territories. AMR detection was interpretable for 293 (77.5%) patients (256 new and 37 relapse cases); 272 being susceptible *M. leprae* strains while 22 (5.6%) harboured resistance to at least one antimicrobial. There were 13 primary resistance cases (11 DAP, 2 FQ) and 9 secondary resistance cases (5 DAP, 4 RIF). The primary resistance rate (5.1%) was significantly lower ($p < 0,001$) than the secondary resistance rate (24%). The mutations observed were as follows: P55L (n=10), T53I (n=5) T53A (n=1) for *FolP1*; S456L (n=2) and S456F (n=2) for *RPOB*; and A91V (n=2) in *GyrA*.

AMR surveillance in leprosy is crucial for detecting resistance patterns that may compromise the efficacy of antibiotic treatments and impede cure. It is essential to establish laboratory capacity and organisational frameworks to sustain long-term surveillance in both relapse and new case populations.

P096

The drinking water-gut microbiome axis in NTM disease

I Cravo Roxo^{1 2} S Alarico¹ A Maranhã¹ C Fernandes¹ M Alves³ C Dias⁴
A Santos Silva⁴ I Ladeira⁴ I Tiago⁵ R Duarte^{3 6} N Empadinhas¹

1: Molecular Microbiology & Microbiome Group, CNC - Center for Neurosciences and Cell Biology and CIBB - Centre for Innovative Biomedicine and Biotechnology, University of Coimbra 2: PDBEB - Ph.D. Programme in Biomedicine and Experimental Biology, Institute for Interdisciplinary Research, University of Coimbra 3: ISPUP - Instituto de Saúde Pública da Universidade do Porto, and ICBAS - Instituto de Ciências Biomédicas Abel Salazar, University of Porto 4: CDP Gaia and CHVNGE - Centro Hospitalar de Vila Nova de Gaia/Espinho, Unidade Local de Saúde de Gaia e Espinho, Vila Nova de Gaia 5: Department of Life Sciences, University of Coimbra 6: INSA - Instituto de Saúde Pública Doutor Ricardo Jorge, INSA Porto

Drinking water as a route for nontuberculous mycobacteria (NTM) infections is an emerging concern. Chlorine-based disinfection selects chlorine-resistant microorganisms, including opportunistic NTM, still unregulated by water-quality directives. In plumbing, NTM persist as biofilms on pipes, taps, and showerheads, niches for accumulation and aerosol/ingestion-mediated dissemination. The absence of routine point-of-use monitoring increases the risk that these resistant populations will colonize water fittings and reach susceptible individuals.

We collected running-water and biofilm samples from taps and showerheads in 46 homes of NTM-infected patients and 9 control households across Portugal's North and Centre regions. Clinical isolates were dominated by *M. avium* (34.8%) and *M. intracellulare* (32.6%) with other species occurring at lower frequencies. From environmental samples, we recovered 340 isolates of 21 NTM species, mainly *M. phocaicum*/*M. mucogenicum*, *M. chelonae*, *M. abscessus*, *M. stephanolepidis*, *M. gordonae*. High-throughput sequencing of biofilm communities partially corroborated the findings, with *M. mucogenicum* and *M. gordonae* dominating at taps and showerheads, and *M. avium* sequences detected sporadically.

Chlorine-selected microbiota surviving stomach passage may interact with resident microbiota. Prolonged antibiotic therapies deplete diversity and disrupt balance, possibly compromising mucosal immunity and increasing reinfection risk. Fecal analysis of NTM patients showed loss of alpha-diversity and shifts in immune-regulating taxa. By linking chlorine-driven selection in drinking water, domestic microbiota assembly, and gut resilience, our study highlights the need to revise water-safety protocols. Adding monitoring and alternative disinfection could curb resistant biofilms, reduce exposure, and protect gut-microbiome integrity, lowering NTM burden in vulnerable populations.

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P097

***Mycobacterium appelbergii* sp. nov., a novel species isolated from a drinking water fountain in a rural community**

S Alarico¹ IC Roxo^{1,2} A Fonseca¹ D Machado³ A Maranhã¹ I Tiago⁴
R Duarte^{5,6,7} N Empadinhas¹

1: CNC-UC - Center for Neuroscience and Cell Biology and CIBB - Centre for Innovative Biomedicine and Biotechnology, University of Coimbra, Portugal 2: PDBEB - PhD Program in Experimental Biology and Biomedicine, Institute for Interdisciplinary Research, University of Coimbra, Portugal 3: CHVNGE - Centro Hospitalar de Vila Nova de Gaia/Espinho, Unidade Local de Saúde de Gaia e Espinho, Vila Nova de Gaia, Portugal 4: Department of Life Sciences, University of Coimbra, Portugal 5: ISPUP - Instituto de Saúde Pública da Universidade do Porto, Universidade do Porto, Portugal 6: ICBAS - Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Portugal 7: INSA - Instituto Nacional de Saúde Doutor Ricardo Jorge, INSA Porto, Portugal

The identification and characterization of novel nontuberculous mycobacteria species are of increasing environmental and clinical relevance, as opportunistic infections by these opportunistic pathogens are on the rise globally. Three isolates of a novel, rapidly growing, non-pigmented *Mycobacterium* species were recovered from the water and runoff of a public fountain in a rural village in Central Portugal. High-quality draft genome sequencing, in silico DNA-DNA

hybridization, and phylogenetic analyses confirmed that the isolated strains 21AC1^T, 21AC14, and 21AC21 represent a previously undescribed species within the genus *Mycobacterium*, forming a distinct phylogenetic lineage closely related to *Mycobacterium wolinskyi*, *Mycobacterium goodii*, and *Mycobacterium smegmatis*. MALDI-TOF MS analysis of the type strain 21AC1^T revealed a unique spectral profile. A comprehensive polyphasic characterization was performed, including chemotaxonomic analyses of fatty acid and mycolic acid composition, as well as an extensive biochemical characterization. The antimicrobial susceptibility of the three strains to 12 antimicrobials was also assessed.

Based on our findings, we propose that strains 21AC1^T, 21AC14, and 21AC21 represent a novel species within the genus *Mycobacterium*, for which we propose the name *Mycobacterium appelbergii* sp. nov. This name is given in honour of Prof. Rui Appelberg, in recognition of his outstanding scientific contributions to the understanding of immune responses, vaccine development, and immunopathology in the context of *Mycobacterium avium* and *Mycobacterium tuberculosis* infections.

Strains deposit: 21AC1^T (=BCCM/ITM 501212 = DSM 113570), 21AC14 (=BCCM/ITM 501447 = DSM 118402) and 21AC21 (=BCCM/ITM 501448 = DSM 118403).

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P098

Analyzing the mycolic acids (MAs) and lipids from the cell wall of *Mycobacterium bovis* strains isolated from Zacatecas and nearby regions

JMFH Favela-Hernandez ^{1 2} A Martinez Romero ¹ GG Guerrero Manriquez ³

1: Facultad de Química. Universidad Juárez del Edo de Durango 2: Instituto Multidisciplinario de Ciencias AVICENA, Torreon, Coahuila, Mexico 3: Unidad Academica de Ciencias Biológicas. Universidad Autónoma de Zacatecas

Bovine Tuberculosis (bTB) is a zoonotic disease caused by *Mycobacterium bovis* that affects cattle and wild animal species, thus contributing to its dissemination and transmission. It is a threat to ecology, agriculture, and human health. The problem aggravates because diagnostic tools have been based on classic methods, the tuberculin test (PPD) and histopathology, due to the high costs of the molecular test implemented in some reference laboratories. The composition of the cell wall is among the factors that play a role in antimicrobial resistance. The waxy cell wall of pathogenic mycobacteria of *Mtb* is structured of three structural types of MAs: 1) the alpha-, 2) the methoxy, and c) the keto mycolic acids (alpha-, M-, and K-MAs). The peculiarity of these types of MAs is that both the keto and methoxy derivatives not only enhance the pathogenicity of *Mtb* but also allow it to modulate the host immune response. In previous work, we isolated *Mycobacterium bovis* from the nostrils of cattle in the region of Zacatecas, Mexico. From the strains of *M. bovis* analyzed, mostly they have type I mycolates, polar and apolar lipids. Therefore, we sought to dissect in detail the types of MAs and lipids of the cell wall of *M. bovis* isolates from nearby regions, using, in addition to thin layer chromatography (TLC), spectroscopic techniques (eg, RMN, mass spectrometry).

P099

Computational identification of broadly protective *Mycobacterium tuberculosis* vaccine candidate

SS Almuji¹ H Turki¹ M Mubarak¹ A Ahmad¹

1: College of Pharmacy, King Khalid University

Mycobacterium tuberculosis (*M.tb*), the causative agent of tuberculosis, continues to pose a significant global health burden, exacerbated by the variable protection offered by the only licensed TB vaccine, Bacillus Calmette-Guérin (BCG), and the rise in antimicrobial resistance. These challenges highlight an urgent need for novel and broadly protective vaccines. Protective immunity against *M.tb* primarily involves T cells recognising epitopes presented via the human leukocyte antigen (HLA) complex; however, cumulative evidence also suggests a significant role for antibodies. This study employed immunoinformatics tools to identify promising vaccine candidates by predicting and analysing T-cell-associated epitopes from 30 highly expressed *M.tb* antigens previously reported. These antigens were computationally screened against a comprehensive range of globally prevalent HLA alleles, including HLA class I (A, B, C) and class II (DR, DQ, DP). Ten antigens consistently predicted to bind across all assessed alleles were selected, ensuring extensive population coverage. Subsequent computational simulations using C-ImmSim indicated that these antigens have the potential to induce robust cellular and humoral immune responses, highlighting their promise as candidates for next-generation TB vaccines. These findings provide a compelling rationale for further preclinical studies to validate their immunogenicity and protective efficacy.

P100

Identification of *Mycobacterium avium* peptides presented by major histocompatibility complex through immunopeptidomics

H Mateus^{1,2} G Bianchi^{1,3} AQ Figueiredo^{2,4,5} R Ferreira^{1,6} R Pinheiro¹ D Pires^{1,6,7}

H M Santos^{2,4,5} PJG Bettencourt^{1,6}

1: Faculty of Medicine, Universidade Católica Portuguesa, Portugal 2: OMICS and Analytical Development Group, and BIOSCOPE Research Group, LAQV-REQUIMTE, Department of Chemistry, NOVA School of Science and Technology, Universidade NOVA de Lisboa, Portugal 3: Department of Medicine, University of Perugia, Italy 4: PROTEOMASS Scientific Society, Portugal 5: Department of Pathology, University of Pittsburgh Medical Center, Pittsburgh, USA 6: Center for Interdisciplinary Research in Health, Universidade Católica Portuguesa, Portugal 7: Host-Pathogen Interactions Unit, Research Institute for Medicines, iMed-ULisboa, Faculty of Pharmacy, Universidade de Lisboa, Portugal

Mycobacterium avium (*M.a*) is a slow growing Nontuberculous Mycobacteria (NTM). It is an opportunistic intracellular pathogen, which may affect the lungs of patients with Acquired Immunodeficiency Syndrome (AIDS), Cystic Fibrosis (CF), Chronic Obstructive Pulmonary Diseases (COPD), and others. *M.a* infections can be dramatic in patients with co-morbidities. Treatments are available with modest therapeutic efficacy, and there is no vaccine available. In AIDS patients, low counts of CD4⁺ T cells can associate with the onset of *M.a* Complex infection, and CD8⁺ T cells may contribute to a protective role against this pathogen. With the purpose to

discover M.a antigens for the development of a new vaccine candidate, we used immunopeptidomics to identify peptides presented by MHC-I and MHC-II in M.a-infected human macrophages.

We have adapted and optimized mycobacterial growth conditions for M.a, characterized the kinetics of M.a survival in macrophages, and assessed the innate immune response of macrophages upon M.a infection. Following, we applied a well-established protocol of immunopeptidomics, based on immunoprecipitation of MHC-complexes, peptide purification, mass spectrometry, and peptide-spectrum match data analysis using Peaks 12.0 software.

We have identified 32 MHC-I and 58 MHC-II M.a peptides, with a Peaks Score higher than 15 (equivalent to a p-value < 0.05). Using NetMHCpan, we characterized the peptide binding affinity relative to the macrophage's HLA alleles. Finally, combining Peaks Score hierarchization with peptide binding affinity, we shortlisted a number of peptides currently being evaluated for its capacity to stimulate T-cell responses for vaccine design and development.

P101

Detected cases of *Mycobacterium tuberculosis* infection in domestic and companion animals – indicators of epidemiological trends and health risks

S Špicic¹ I Reil¹ L Žmak^{2,3} M Zdelar-Tuk¹ M Obrovac² M Dopud¹ S Duvnjak¹
1: National Reference Laboratory for Animal Tuberculosis, Department for Bacteriology and Parasitology, Croatian Veterinary Institute, Zagreb, Croatia 2: National/Supranational Reference Laboratory for Tuberculosis, Croatian Institute of Public Health, Zagreb, Croatia 3: University of Zagreb, School of Medicine, Zagreb, Croatia

Zoonotic tuberculosis cases in the EU and Croatia are mainly limited to the species *Mycobacterium (M.) bovis* and *M. caprae*. The primary sources of infection are unpasteurized dairy products and occupational exposure in slaughterhouses and diagnostic laboratories. Companion animals, especially dogs, become infected through direct or indirect contact with infected humans and can pass on *Mycobacterium tuberculosis*, the main causative agent of tuberculosis in humans. The Croatian National Reference Laboratory for Animal tuberculosis has detected *M. tuberculosis* infection in two cattle (2008 and 2010) and two dogs (2006 and 2018) in the last 20 years. Both cases were diagnosed in cattle through the national control program based on annual tuberculin skin tests. Tuberculosis in dogs was incidentally diagnosed following a fatal outcome after non-tuberculosis related long-term therapy. The epidemiological facts in all four cases followed a different scenario, indicating different sources and routes of infection. The identification of unique strains in cattle and dogs could be related to a new global socioeconomic era and health risks linked to intensive human migration, suggesting that public health resources are unprepared or unable to address preventive control of tuberculosis in economic migrants. Also, often misdiagnoses of clinical tuberculosis, particularly in companion animals, are a dangerous result of public unawareness. These facts pose a big challenge for disease control, as they indicate potential cross-species transmission and the need for more comprehensive surveillance and control measures.

P102

GenEpi-BioTrain: Interdisciplinary training in genomic epidemiology and public health bioinformatics: 5th edition (tuberculosis focus)

I Barilar¹ V Dreyer¹ C Utpatel¹ T Enkirch² L Freschi² S Niemann¹

1: Research Center Borstel Leibniz Lung Center 2: European Centre for Disease Prevention and Control

An intensive two-week training on genomic epidemiology and public health bioinformatics was held from 31 March to 11 April 2025 in Borstel, Germany, bringing together 30 professionals from 10 European countries. Each participating country was represented by an interdisciplinary team comprising a bioinformatician, an epidemiologist and a microbiologist, reflecting the collaborative nature of modern public health practice. The course, funded by the European Centre for Disease Prevention and Control (ECDC), aimed to strengthen participants' capacity to apply next-generation sequencing (NGS) data for infectious disease surveillance and control, with a focus on *Mycobacterium tuberculosis*. Through a combination of lectures, hands-on exercises with real-world data and group work, participants enhanced their understanding of genomic epidemiology and bioinformatics, gained experience with tools for integrated analysis and visualisation of NGS and epidemiological data, and developed skills for interdisciplinary interpretation of results. The training also fostered collaboration among disciplines within and across countries, promoting more integrated approaches to public health action. Designed for professionals working in public health institutions, the course welcomed both those with experience in computational biology and bioinformatics and those from epidemiology or microbiology backgrounds who are increasingly required to interpret genomic data. This fifth edition of the course confirmed the importance of building interdisciplinary expertise to ensure the effective use of NGS data in public health, particularly for priority pathogens such as *M. tuberculosis*.

P103

The impact of environmental factors on tuberculosis in Albania, 2023–2024

D Mema¹ A Ylli¹ A Fico¹ B Kodra¹

1: Institute of Public Health, Tirana, Albania

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains a global public health challenge. Beyond known risk factors such as malnutrition, smoking, alcohol use, and comorbidities, environmental determinants significantly influence both disease transmission and progression. This study investigates the impact of environmental factors—including air pollution, climate variability, migration, overcrowding, inadequate housing, and poor ventilation—on TB risk and geographic distribution. Data were collected from the Albanian National TB Program and the University Hospital “Shefqet Ndroqi” during 2023–2024. Approximately 400 hospitalized TB patients were interviewed using a structured questionnaire assessing living conditions and environmental exposures. Of the respondents, 66.5% were male and 33.5% female; 62% resided in urban areas and 38% in rural regions. High air pollution affected 55% of participants, while 31% reported using wood for heating or cooking, particularly in rural zones. Dampness or mold was

present in 28% of homes, and 19% of patients lived in poorly ventilated dwellings. Notably, 21% shared a room with at least three other individuals, a condition that facilitates airborne transmission. Migrants constituted 10% of cases, with many residing in overcrowded, unsanitary refugee settings. Furthermore, 25% of patients came from high-incidence areas, underscoring the association between environmental conditions and TB spread. Although Albania maintains a relatively low TB incidence (8.7 per 100,000), these findings underscore the need for an integrated strategy that addresses both health and environmental determinants. Sustainable TB control requires intersectoral collaboration and policy alignment to reduce exposure risks and improve population health outcomes

P104

Linking long-term and short-term *Mycobacterium tuberculosis* transmission dynamics inferred from 15 years study in Orizaba, Mexico

P Sinisterra-Sebastián¹ FJ Martínez-Martínez¹ L Varón-Pozuelo¹ P Cano-Jiménez¹
V Furió¹ M Torres-Puente¹ G Delgado-Sánchez² L Ferreyra-Reyes²
M Bobadilla Del Valle³ A Ordaz-Vázquez³ N Mongua-Rodríguez² L García-García²
I Comas^{1,4} MG López¹

1: Tuberculosis Genomics Unit, Instituto de Biomedicina de Valencia, CSIC, Valencia, Spain 2: Centro de Investigación sobre Enfermedades Infecciosas, Instituto Nacional de Salud Pública, Cuernavaca, México 3: Laboratorio de Microbiología, Instituto Nacional de Ciencias Médicas y Nutrición "Salvador Zubirán", México 4: CIBER de Epidemiología y Salud Pública, CIBERESP, Madrid, Spain

Transmission is the main reason for the persistence of the tuberculosis (TB) pandemic. Although genomic epidemiology of *Mycobacterium tuberculosis* (*Mtb*) is increasingly used in public health surveillance, long-term transmission dynamics remain poorly understood, limiting insights into transmission drivers. From 1995 to 2010, passive case-finding was implemented in Orizaba, Veracruz (Mexico), a low TB-burden district. A total of 1,257 TB patients were recruited, with 845 (67.22%) culture-positive samples sequenced using Illumina technology. By combining genomic, evolutionary, and epidemiological data, we aimed to (1) describe the local *Mtb* population structure; (2) investigate transmission at different levels and time spans, beyond recent genomic clustering, and (3) estimate the origins and long-term transmission dynamics of key genotypes in Orizaba.

In the short term, a high clustering rate was observed, with 48.6% and 61.0% of cases grouped using 5- and 10-SNP threshold, respectively. Furthermore, we unveil the role in transmission of cases not usually prioritized in public health investigations, such as those with negative sputum smear microscopy results. In the long term, a consistent clustering pattern was observed, indicating exponential expansion of the pandemic beginning at the turn of the 20th century. A large-scale global phylodynamic analysis revealed that local genotypes have been expanding for decades, contributing to today's transmission.

In conclusion, Orizaba exhibits high transmission levels driven by locally circulating *Mtb* genotypes with decades-long persistence. Our findings suggest disease burden poorly predicts transmission. Furthermore, short-term genomic clustering is better interpreted within the long-term transmission dynamics context.

Tuberculosis Alert: a board game designed to promote tuberculosis awareness and fight stigma

P Soldevilla^{1 2 3 4 5} M Cortacans^{1 2 3 4} D Aznar^{1 2} M Vidal^{1 2 3 4} KL Fonseca^{1 5}
C Vilaplana^{1 2 4 5}

1: *Unitat de Tuberculosi Experimental, Microbiology Dept. Germans Trias i Pujol Research Institute and Hospital (IGTP-HUGTIP), Badalona, Spain* 2: *Genetics and Microbiology Department, Autonomous University of Barcelona, Spain* 3: *Centre de Medicina Comparativa i Bioimatge de Catalunya (CMCiB), Badalona, Spain* 4: *Servei de Microbiologia, LCMN, Hospital Universitari Germans Trias i Pujol (HUGTiP), Spain* 5: *Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBERES), Madrid, Spain*

To debunk myths and stigma related to tuberculosis (TB) we have developed 'Tuberculosis Alert', a role-playing board game designed to improve public understanding of tuberculosis (TB). The game introduces both basic and specific concepts about the disease, including what TB is, how it is transmitted, its symptoms, the current treatment, and the concepts of drug resistance and latency.

Supported by the Department of Research and Universities of the Generalitat of Catalonia and a charitable campaign by Condis supermarkets, we have 8 copies of the game available in four languages (Catalan, Spanish, English, and French). Its innovation lies in being the first board game specifically dedicated to TB education, with flexible game dynamics that adapt to audience feedback. To measure the game's impact on knowledge, we visited schools where, apart from playing, the students also received a knowledge assessment tool before and after gameplay. According to test results, all students improved their knowledge about the disease by about 30%, with a sharper effect noted in the basic-knowledge questions.

Tuberculosis Alert has proved to be an effective tool for promoting tuberculosis awareness in a format that is not only entertaining but also impactful. Our future plans include continuing to establish collaborations with multiple high schools across Catalonia in various geographic and socio-economic contexts, as well as working with public health institutions to visit schools with a reported case of TB. In addition, we plan to scale up production to ensure availability for national and international institutions.

Genomic Analysis of *Mycobacterium chelonae* isolates associated with heater-cooler units: a potential risk of infection during cardiac surgery

S Mok^{1 2} TK Teoh^{2 3} E Roycroft^{1 2} P Urwyler⁴ P Schlaepfer⁵ PM Keller⁶
S Tschudin Sutter⁴ TR Rogers² J Wagener^{1 2} MM Fitzgibbon^{1 2}

1: *Irish Mycobacteria Reference Laboratory, St. James's Hospital, Dublin, Ireland* 2: *Department of Clinical Microbiology, Trinity College Dublin, the University of Dublin, Ireland* 3: *Public Health Laboratory, Health Service Executive in Dublin, Cherry Orchard Hospital, Ireland* 4: *Division of Infectious Diseases and Hospital Epidemiology, University Hospital Basel, Switzerland* 5: *Laboratory*

Routine surveillance of heater cooler-units has been performed in Ireland since 2015 due to the risk of *Mycobacterium chimaera* cardiovascular infection associated with contaminated heater-cooler units (HCU) manufactured by LivaNova. This also led to the replacement of these devices with another HCU brand, Maquet (Getinge), in nine Irish cardiac surgery centres. Here we report the detection of *Mycobacterium chelonae* contamination in Maquet HCU and LivaNova HCU used in five centres over a nine-year period. Whole genome sequencing was performed on 41 *M. chelonae* isolates from various types of HCU brands, unrelated environmental sources and unrelated patients to investigate the source of contamination. We also included five *M. chelonae* isolates associated with Maquet HCU from the University Hospital of Basel in Switzerland. Phylogenetic analysis based on WGS showed that *M. chelonae* isolates from various HCU brands were identified in the same group. *M. chelonae* isolates from Maquet HCU in Ireland and Switzerland were closely related (median pairwise distance of 5 SNPs) and were unrelated to *M. chelonae* isolates from LivaNova HCU and other sources. In conclusion, WGS of *M. chelonae* isolates from Maquet HCU from Ireland and Switzerland suggests that there is a common source of contamination in Maquet HCU, potentially at the manufacturers site or with products commonly used with these HCU's, which poses a potential risk of infection to patients undergoing cardiac surgery. Other NTM including *Mycobacterium gordonae* were also frequently identified in Maquet HCU's and further work is required to investigate the origin of contamination.

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Applications and challenges of prospective whole genome sequencing for tuberculosis public health investigations: a systematic review

DR Wang¹ A Talwar¹ B Marais^{2,3} V Sintchenko^{2,3} J Denholm⁴ MD Kirk¹

1: Australian National University National Center for Epidemiology and Population Health 2: The University of Sydney, Sydney Infectious Diseases Institute 3: NSW Health Pathology - Institute of Clinical Pathology and Medical Research 4: Victorian Tuberculosis Program, the Royal Melbourne Hospital

Whole genome sequencing (WGS) is increasingly being adopted for the management of tuberculosis (TB). However, questions remain regarding its utility in different settings and barriers for implementation. We conducted a systematic review to explore the applications and documented challenges in using WGS for TB detection and response. We only included studies that prospectively obtained WGS data and actively linked it with epidemiological data. 54 studies met our inclusion criteria. Most of the studies (36/54, 66.7%) were published after 2022. 41 were surveillance studies, 6 were outbreak investigations, and 7 had a research focus. Most studies (38/54, 70.4%) investigated TB in World Bank low to upper-moderate TB burden countries (<100 per 100,000 population). Sequencing was primarily performed in high-income countries (31/54, 57.4%); no sequencing was conducted in low-income countries. Most studies used WGS to identify transmission/clusters (50/54, 92.6%). Drug-resistant TB was the focus of 20/54 (37.0%) studies. Only 16/54 (29.6%) studies reported using WGS to inform public health decisions, most commonly to prompt intensified epidemiological investigation or guide active case detection of genomically-clustered patients. Reported challenges around WGS implementation included lack of culture-confirmed cases (cultures rarely done in resource-limited settings), long delays in getting WGS results, high costs, and limited reported engagement of the local TB control team to

act on results. This review investigates the utility of WGS for public health, including in detecting transmission links and drug resistance, but also highlights challenges around implementation and the need for increased access to WGS for public health agencies in lower-income settings.

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Recent updates to the SITVIT databases: ongoing development of the SITVITEXTEND and SITVITGeno projects

D Couvin^{1 2} D Cazenave¹ N Rastogi¹

1: Institut Pasteur 2: Université des Antilles

Databases and software tools that have been developed at the Institut Pasteur de la Guadeloupe, over the last two decades, contributed to a better understanding of *Mycobacterium tuberculosis* complex (MTBC) isolates circulating worldwide. Several databases have been introduced by the Institut Pasteur de la Guadeloupe over the last several years. The two most recently released databases are SITVIT2 (<http://www.pasteur-guadeloupe.fr:8081/SITVIT2/>) and SITVITBovis (http://www.pasteur-guadeloupe.fr:8081/SITVIT_Bovis/). The most recent version under construction (SITVITEXTEND), with genotyping and demographic information on >128,000 MTBC strains, contains tools for prediction and data analysis allowing phylogeographical mapping and statistical analyses based on: year and place of isolation, genotyping profile, drug-resistant patterns, gender and age of patients, etc.). In addition, dedicated tools for machine learning-based classification or phylogenetic analyses, such as SpollLineages (<http://www.pasteur-guadeloupe.fr:8081/SpollLineages/>) and simpITB (<https://github.com/dcouvin/simpiTB>), today allow the prediction of the MTBC lineages using classical and machine-learning methods. Ongoing development of updated SITVIT databases will allow further analyses on circulating MTBC isolates. Furthermore, inclusion of data based on whole genome sequencing (WGS), will improve our understanding of genomic diversity, transmission characteristics and variation of various MTBC isolates. A novel database, tentatively called SITVITGeno, that will be dedicated to WGS data, is also under construction in our lab. Finally, scientists wishing to submit their data to the database will be able to contact us for the registration of their data.

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The impact, implementation, and ethics of whole genome sequencing for tuberculosis public health: staff perspectives from an outbreak in Victoria, Australia

DR Wang¹ A Ferdinand² B Marais^{4 5} V Sintchenko^{4 5} MD Kirk¹ J Denholm³

1: National Center for Epidemiology and Population Health, Australian National University

2: Microbiological Diagnostic Unit Public Health Laboratory, the Royal Melbourne Hospital

3: Victorian Tuberculosis Program, the Royal Melbourne Hospital 4: Sydney Infectious Diseases

Institute, The University of Sydney 5: Institute of Clinical Pathology and Medical Research, NSW Health Pathology

Whole genome sequencing (WGS) is increasingly being adopted for tuberculosis (TB) public health investigations. To ensure effective translation into practice, it is essential to evaluate the utility, implementation, and ethical implications of WGS. We explore these factors in the context of a TB outbreak amongst a Pasifika community in Australia. We used descriptive epidemiology to characterise the outbreak and conducted semi-structured interviews with 10 laboratory and public health staff to examine perspectives on WGS impact, implementation, and ethics. Between 14 February 2017 to 28 July 2023, 36 cases were linked to the outbreak. Interviewees reported that WGS helped to identify transmission links and clusters, enabling more efficient public health action including additional screening and directing contact tracing questioning. Others described a lack of impact due to long sequencing turnaround times and limited translation into action. These diverging experiences highlights the importance of continued program evaluation and developing facilitators to support translation into practice. Interviewees identified patient privacy as a key ethical concern when using WGS, particularly due to TB stigma in the community. They raised the importance of building trust through transparency, engagement, and non-stigmatising language. However, uncertainties around operationalising these principles indicates a gap for clearer frameworks around managing and using WGS information. Conflicting experiences around the utility of TB WGS highlights the importance of program evaluation and translation with a focus on public health outcomes. This study also identifies ethical concerns that require additional strategies to support operational staff and improve public trust.

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‘I was in a place where I was going to be healed. Let others think what they want’: A qualitative study on the experience of having tuberculosis in a sample of the Portuguese population

P Barbosa¹ T Silva³ A Aguiar^{1,2} M Marques⁴ A Gomes³ M Pinto⁵ R Duarte^{1,2,6}
1: EPIUnit ITR, Instituto de Saúde Pública da Universidade do Porto, Portugal 2: Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto, Portugal 3: Unidade de Saúde Pública da ULS Tâmega e Sousa, Pólo Vale Sousa Norte, Paços de Ferreira, Portugal 4: Unidade Local de Saúde de Gaia/Espinho, EPE, Vila Nova de Gaia, Portugal 5: Faculdade de Psicologia e Ciências da Educação da Universidade do Porto, Portugal 6: Instituto Nacional de Saúde Doutor Ricardo Jorge, INSA-Porto, Portugal

Background: Tuberculosis (TB) remains a global health concern with significant impacts on physical and emotional well-being. This study explores the perceptions of individuals with TB regarding their diagnosis and its effects on their daily lives.

Methods: A qualitative approach was employed to collect and analyze the experiences of 22 individuals diagnosed with TB. Data were obtained through semi-structured interviews, focusing on reactions to the diagnosis, disclosure practices, stigma, and social support.

Results: Reactions to the diagnosis varied, ranging from relief and trust in treatment to shock and sadness. Most participants felt comfortable disclosing their diagnosis to social networks, recognizing its importance for awareness and prevention. However, some reported fear of stigma, leading to limited disclosure and self-isolation. Stigma was associated with fear of contagion, social distancing, and emotional distress, including loneliness and frustration. Support from family and friends was identified as a key factor in mitigating stigma and emotional difficulties. Negative experiences, including stigmatization in social and healthcare settings, were also reported.

Discussion: TB-related stigma has a complex impact, influencing disclosure, mental health, and social interactions. While social support serves as a protective factor, stigma exacerbates emotional challenges and may hinder care-seeking. Holistic TB care should integrate strategies to reduce stigma, promote mental health, and strengthen social networks to improve patient outcomes.

P1111

A multinational Delphi consensus on TB screening of migrants in Europe

A Aguiar^{1 2} M Pinheiro^{1 2 3 4} DN Moreira³ OW Akkerman^{5 6} Z Al-Suwaidi⁷
JWC Alffenaar^{8 9 10} I Arandjelovic¹¹ U Brito¹² P de Colombani¹³ R Curcic¹⁴
AL Garcia-Basteiro¹⁵ D Goletti¹⁶ G Günther¹⁷ E Ibraim¹⁸ N Kapata¹⁹
C Lange^{20 21 22 23} M Lipman²⁴ M Jankovic Makek²⁵ BJ Marais²⁶ A Mariandyshev²⁷
C Magis-Escorra²⁸ GB Migliori²⁹ A Sánchez Montalvá^{30 31 32 33} Z Nanovic^{34 35}
DJ Palmero³⁶ M Priwitzer³⁷ MCB Raviglione¹³ D Rossato Silva³⁸ HJF Salzer^{39 40 41}
C Schwarzbach⁴² I Spruijt⁴³ KL Winthrop⁴⁴ Z Udwadia⁴⁵ T Vasankari⁴⁶
C Vilaplana^{47 48 49} R Duarte^{1 2 50}

1: EPIUnit ITR, Instituto de Saúde Pública da Universidade do Porto, Portugal 2: Estudos das Populações - Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Portugal
3: Barcelos/Esposende Local Health Unit, Public Health Unit Barcelos/Esposende, Portugal
4: Departamento de Ciências Químicas, Faculdade Farmácia, Universidade do Porto, Portugal
5: UMCG dept of pulmonary diseases and tuberculosis, University of Groningen, The Netherlands
6: University of Groningen, University Medical Center, TB center Beatrixoord, Haren, The Netherlands
7: Head of National TB Reference Laboratory, Hamad Medical Corporation, Qatar
8: Sydney Institute for Infectious Diseases (Sydney ID), The University of Sydney, Australia 9: School of Pharmacy, Faculty of Medicine and Health, The University of Sydney, Australia 10: Westmead Hospital, Sydney, Australia 11: Institute of microbiology and immunology, Faculty of Medicine, University of Belgrade, Serbia 12: Pulmonology Department, Centro Hospitalar e Universitário do Algarve, Faro, Portugal 13: Centre for Multidisciplinary Research in Health Science (MACH), Università di Milano, Italy 14: Municipal institute for lung disease and TB, Belgrade, Serbia
15: Centro de Investigação em Saúde de Manhiça, Maputo Mozambique & ISGlobal (Hospital Clínic - Universitat de Barcelona) & Centro de Investigación Biomédica en Red de Enfermedades Infecciosas (CIBERINFEC), Spain 16: Department of Pulmonology, Allergology and Clinical Immunology, National Institute for Infectious Diseases L. Spallanzani-IRCCS, Italy 17: Inselspital, Bern University Hospital, University of Bern, Switzerland 18: Marius Nasta Institute of Pneumophthiziology in Bucharest, Romania 19: Zambia National Public Health Institute, Zambia 20: Clinical Infectious Diseases, Research Center Borstel, Leibniz Lung Center, Germany 21: Respiratory Medicine and International Health, University of Lübeck, Germany 22: Baylor College of Medicine and Texas Children's Hospital, Global TB Program, USA 23: Clinical Tuberculosis Unit, German Center for Infection Research (DZIF), Hamburg-Lübeck-Borstel-Riems, Germany 24: Royal Free Hospital & University College London, UK 25: University of Zagreb School of Medicine, Croatia; University Hospital Centre Zagreb, Croatia 26: Sydney Institute for Infectious Diseases (Sydney ID) and the WHO Collaborating Centre in Tuberculosis, The University of Sydney, Australia 27: Northern State Medical University, Arkhangelsk, Russia 28: Department of Pulmonary Diseases, Radboud University Medical Center-Dekkerswald, Nijmegen, The Netherlands 29: Servizio di Epidemiologia Clinica delle Malattie Respiratorie, Istituti Clinici Scientifici Maugeri IRCCS, Tradate, Italy 30: Unidad de Salud Internacional Vall d'Hebron-Drassanes, Servicio de Infecciosas, Hospital Universitario Vall d'Hebron, PROSICS Barcelona, Spain 31: Universidad Autónoma de Barcelona, Medicine department, Spain 32: Grupo de Estudio de Infecciones por Micobacterias (GEIM) de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC), Madrid, Spain 33: Centro de Investigación Biomédica en Red de

Enfermedades Infecciosas (CIBERINFEC), Instituto de Salud Carlos III, Madrid, Spain 34: Faculty of Medical Sciences, Goce Delcev University, Stip, Republic of North Macedonia 35: Institute for Lung Diseases and Tuberculosis, Skopje, Republic of North Macedonia 36: Hospital Muñiz, Universidad de Buenos Aires, Argentina 37: Landeshauptstadt Stuttgart, Public Health Department, Germany 38: Faculdade de Medicina, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil 39: Division of Infectious Diseases and Tropical Medicine, Department of Internal Medicine 4 - Pneumology, Kepler University Hospital, Linz, Austria 40: Medical Faculty, Johannes Kepler University Linz, Austria 41: Ignaz-Semmelweis-Institute, Interuniversity Institute for Infection Research, Vienna, Austria 42: Hamburg Tuberculosis Control Unit, District Public Health Department Hamburg-Mitte, Germany 43: KNCV Tuberculosis Foundation, The Hague, The Netherlands 44: Division of Infectious Diseases, Oregon Health and Science University, USA 45: Hinduja Hospital & Research Centre, Mumbai, India 46: Filha ry, Helsinki and University of Turku, Finland 47: Experimental Tuberculosis Unit. Microbiology Department. Germans Trias i Pujol Research Institute (IGTP) and Hospital (HUGTIP), Badalona, Spain 48: CIBER Enfermedades Respiratorias, Madrid, Spain 49: Universitat Autònoma de Barcdelona, Spain 50: Centro de Saúde Pública Doutor Gonçalves Ferreira – Instituto de Saúde Pública Doutor Ricardo Jorge, INSA Porto, Portugal

Background: The disproportionate burden of tuberculosis (TB) among migrants in the WHO European Region highlights the urgent need to address the public health challenges linked to global migration. Current screening procedures and recommendations for active TB and TB infection (TBI) vary widely across European countries, underscoring the necessity for standardized practices and coordinated responses.

Methods: This study utilized the Delphi method to convene a multidisciplinary panel of 33 TB experts from academia, healthcare, non-governmental organizations, and government agencies across 22 European countries. The goal was to develop consensus-based recommendations to harmonize migrant TB screening practices across the region.

Results: The panel formulated 19 consensus statements and 36 actionable recommendations directed at governments, health systems, and other relevant stakeholders. These recommendations span four key domains: (1) policy; (2) health systems and health professionals; (3) screening procedures and target populations; and (4) treatment and continued care. Notably, 7 statements and 19 recommendations were unanimously supported, while 8 recommendations had more than 10% disagreement.

Discussion: The study provides the first harmonized guidance for TB screening among migrants in Europe. Key recommendations emphasize the need for culturally inclusive, evidence-based, and feasible approaches that balance immediate health system improvements with long-term public health goals. These consensus outcomes offer a framework for addressing current inconsistencies and advancing the elimination of TB as a public health threat across the European Region.

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Determining epidemiological cut-off values for BTZ-043 and Ganfeborole using the EUCAST reference method

A Ghodousi^{1,2} S Andres³ E Terschlüsen⁴ F Saluzzo¹ C Kallenberg³ I Friesen³
J van Ingen⁴ DM Cirillo²

1: University Vita-Salute San Raffaele 2: San Raffaele Scientific Institute 3: National and World Health Organization Supranational Reference Laboratory for Mycobacteria, Research Center Borstel, Leibniz Lung Center, Germany 4: Radboud University Medical Center

Background: Tuberculosis (TB) remains a significant global health challenge, particularly with the rise of drug-resistant strains. The introduction of novel anti-TB drugs like BTZ-043 and Ganfaborole offers promising treatment options. Both drugs are currently in Phase 2B clinical trials, and establishing reliable susceptibility thresholds before their introduction into clinical use is crucial. The EUCAST reference method enables the determination of Epidemiological Cut-Off Values (ECOFFs) to differentiate wild-type strains from resistant strains of *Mycobacterium tuberculosis complex* (MTBC).

Objective: To determine the tentative ECOFFs for BTZ-043 and Ganfaborole using the EUCAST reference method, based on the H37Rv reference strain and clinical wild-type MTBC strains, tested across three laboratories.

Methods: The study was conducted across three laboratories for both Ganfaborole and BTZ-043. A total of 124 independent cultures of H37Rv reference strain and 130 clinical MTBC isolates were tested using the EUCAST broth microdilution method to determine the Minimum Inhibitory Concentration (MIC) for each drug. ECOFFs were calculated by analyzing MIC distributions and applying statistical methods, including ECOFFinder, to distinguish wild-type from non-wild-type strains.

Results: The tentative ECOFFs for Ganfaborole were 0.0625 µg/mL (95% confidence) and 0.125 µg/mL (99.9% confidence). For BTZ-043, the tentative ECOFFs were 0.004 µg/mL (95% confidence) and 0.008 µg/mL (99.9% confidence).

Conclusion: The established tentative ECOFFs provide essential thresholds for susceptibility testing and are crucial for monitoring TB drug resistance. These values will support the clinical application of these agents, ensuring that a reliable reference method for performing drug susceptibility testing is available upon their clinical use.

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Genomic surveillance uncovers ongoing Tuberculosis transmission in Catalonia (2022–2024)

V Saludes^{1 2} E Sicart-Torres³ AE Bordoy¹ MG López⁴ PJ Hesketh-Best¹ L Soler¹
A Antuori¹ A París¹ D Panisello-Yagüe¹ S González-Gómez¹ S Esteban-Cucó⁵
E Vicente⁵ G Tudó^{6 7} J González-Martín^{6 7 8} MT Tórtola^{8 9} T Soler¹²
MD Guerrero¹² I Prats¹³ F Alcaide^{6 14} L Fernández-Delgado¹⁴ E Cuchí¹⁵
M Garrigó¹⁶ P Costa¹⁷ A Casabella¹⁸ A Pulido¹⁹ E Picó-Plana²⁰ J López²¹
G Trujillo²¹ N Torrellas²² X Casas¹¹ L Oliver²³ O González-Moreno²³ JR Agüera²⁴
P Ciruela^{2 3} M Bach³ P Gomà³ P Bach³ M Carol³ P Cano³ L Clotet³
A Despuig³ L Curto³ J Ferràs³ G Ferris³ N Follia³ M Sabater³ JP Millet^{2 10 11}
R Prieto¹⁰ E Plasencia³ T Pérez-Porcuna³ À Tarrés³ M López³ H Martínez³
O Pérez-Lallave¹ G Clarà¹ AC Pelegrín¹ M Piñana¹ M Torres-Puente⁴ I Comas^{2 4}
S Pequeño³ L Gavaldà-Mestre³ E Martró^{1 2} J Mendioroz³ PJ Cardona^{1 25 26}

1: Servei de Microbiologia, Laboratori Clínic Metropolitana Nord. Institut de Recerca i Hospital Germans Trias i Pujol (IGTP), Badalona 2: CIBER en Epidemiología y Salud Pública (CIBERESP), Madrid
3: Agència de Salut Pública de Catalunya, Departament de Salut, Generalitat de Catalunya, Barcelona 4: Tuberculosis Genomics Unit, Instituto de Biomedicina de Valencia (IBV)-CSIC
5: Laboratori de Referència de Catalunya, El Prat de Llobregat, Barcelona 6: Universitat de Barcelona
7: Hospital Clínic de Barcelona-ISGlobal 8: CIBER en Enfermedades Infecciosas (CIBERINFEC), Madrid 9: Hospital Universitari Vall d'Hebron, Barcelona 10: Programa de Prevenció i Control de la Tuberculosis de Barcelona (PPCTB) 11: Serveis Clínics, Barcelona 12: Consorci del Laboratori

Intercomarcal (CLILAB) de l'Alt Penedès, l'Anoia i el Garraf, Vilafranca del Penedès 13: Hospital Universitari Arnau de Vilanova, Lleida 14: Hospital Universitari de Bellvitge-IDIBELL, L'Hospitalet de Llobregat 15: CATLAB-Centre Analítiques Terrassa AIE, Terrassa 16: Hospital de la Santa Creu i Sant Pau, Barcelona 17: Laboratori Clínic Territorial de Girona, Hospital Universitari de Girona Dr. Josep Trueta 18: Àrea de Microbiologia, Servei de Laboratoris Clínics, Parc Taulí Hospital Universitari, Institut d'Investigació i Innovació Parc Taulí (I3PT-CERCA), Universitat Autònoma de Barcelona, Sabadell 19: Hospital General de Granollers 20: Hospital Universitari Joan XXIII, Tarragona 21: Fundació Althaia. Hospital Sant Joan de Déu, Manresa 22: Laboratori Fundació Hospital de Palamós 23: Departamento de Microbiología y Parasitología. SYNLAB Diagnósticos Globales, Laboratorio central - Barcelona 24: Laboratorio Echevarne - Sant Cugat del Vallès 25: CIBER en Enfermedades Respiratorias (CIBERES, Madrid 26: Departament de Genètica i Microbiologia, Universitat Autònoma de Barcelona

Tuberculosis (TB) incidence in Catalonia has remained stable in recent years, highlighting the need to strengthen surveillance efforts. In 2022, a population-based genomic surveillance system for *Mycobacterium tuberculosis* complex (MTBC) was integrated into the Catalan TB Control Programme to characterise transmission patterns and monitor drug resistance. Whole-genome sequencing (WGS) of MTBC isolates was performed at the Microbiology Service of Hospital Germans Trias i Pujol using Illumina technology on cultures collected from 17 laboratories. Lineages, sublineages, and resistance-associated mutations were identified using TB-Profiler, while genomic distances (based on single nucleotide polymorphisms, SNPs) were assessed with MTBseq, excluding repetitive and resistance-related regions. Recent transmission clusters were defined as groups of ≥ 2 monophyletic cases within 0–5 SNPs. Between 2022 and 2024, 2,293 cultures were received, of which 1,754 met inclusion criteria. Sequencing results were obtained for 1,593 isolates (90.8%). A total of 68 (sub)lineages were identified, with L4.1.2.1 (22%), L4.3.2 (13.4%), and L4.8 (12.4%) being most prevalent. No resistance mutations were found in 82% of isolates. Monoresistance was observed in 8.5%, multidrug resistance in 1%, and pre-extensively drug-resistant TB in 0.3%. Overall, 148 recent transmission clusters were detected, comprising 32.4% of sequenced cases, with 16.9% involving resistant strains. Epidemiological links were confirmed in approximately 25% of clustered cases through contact investigations. A multidisciplinary review group has been established to prioritise and evaluate relevant clusters. These findings confirm sustained community transmission of TB in Catalonia and demonstrate the added value of genomic data to inform and enhance public health interventions.

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Exploring LL-37-Based synthetic peptides to combat multidrug-resistant Tuberculosis: A step toward innovative therapies

PA Santos-Ruiz¹ CA Cruz-Baquero¹ LV Triana¹ M Maya-Hoyos² LM Salazar² CY Soto-Ospina²

1: Grupo Relaciones Microbianas y Epidemiológicas Aplicadas al Laboratorio Clínico y Molecular (REMA), Facultad de Ciencias de la Salud, Universidad Colegio Mayor de Cundinamarca, Bogotá, Colombia 2: Grupo Bioquímica y Biología Molecular de las Micobacterias (BBMM), Departamento de Química, Facultad de Ciencias, Universidad Nacional de Colombia, Bogotá, Colombia

The emergence of multi-drug resistant (MDR) and extensively drug resistant (XDR) *Mycobacterium tuberculosis* strains (MTB) has driven the finding of more effective anti-tuberculosis drugs. Currently, antimicrobial peptides (AMPs) are considered interesting molecules with broad-spectrum antimicrobial activity due to their unique mode of action, which is based on

their ability to bind to the cell membrane without requiring specific receptors. In this work, we studied the antimycobacterial activity of two synthetic peptides derived from LL-37 and modified by bioinformatic tools (LL37-1 and D-LL37) against MTB, by determining the minimum inhibitory concentration; as well as the evaluation of their direct effect on membrane permeability and cell death, by fluorescence microscopy and fluorometry experiments. We found that LL37-1 peptide inhibited MTB growth from a concentration of 12.5 μ M and D-LL37 peptide from 6.25 μ M, with no significant cytotoxic or hemolytic activity observed at these concentrations. The direct effect of both PAMs on the bacterial cell membrane was also identified. The results obtained could significantly contribute to the pharmacological field in an attempt to promote modified PAMs in anti-tuberculosis therapies. In addition, further research can be planned to understand how MTB would respond to modified synthetic PAMs, leading to a better understanding of the mechanism of action of these molecules on bacterial virulence.

Keywords: Antimicrobial peptides (AMPs), LL-37-derived peptides, Membrane permeability, Anti-tuberculosis therapy innovation.

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Validation of a cost-effective *Drosophila melanogaster* platform to identify novel treatments against tuberculosis

M Vidal^{1 2 4} M Arch^{1 4} E Fuentes^{1 3 4} PJ Cardona^{1 2 3 4 5}

1: Unitat de Tuberculosi Experimental, Microbiology Dept. Germans Trias i Pujol Research Institute and Hospital (IGTP-HUGTIP), Badalona, Spain 2: Genetics and Microbiology Department, Autonomous University of Barcelona, Spain 3: Centre de Medicina Comparativa i Bioimatge de Catalunya (CMCiB), Badalona, Spain 4: Servei de Microbiologia, LCMN, Hospital Universitari Germans Trias i Pujol (HUGTiP), Badalona, Spain 5: Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBERES), Madrid, Spain

Antimicrobial-resistant infections are an escalating public health issue, underscoring the urgent need for new drug discovery. Current methods often rely on in vitro cell cultures, resulting in many low-quality compounds progressing to mammal models. *D. melanogaster* (Dm) is a valuable model for studying host-pathogen interactions and treatment efficacy. Acting as a bridge between in vitro and mammal models, Dm may overcome the time and cost challenges of drug screening. However, further refinements are needed to establish it as a standard tool in this process. In this study, we validated a cost-effective treatment protocol using Dm infected with *Mycobacterium marinum* (Mm). We tested five standard tuberculosis drugs, the current standard of care; five repurposed host-directed therapies; and two anti-virulence treatments. Flies were systematically infected with 50 Colony Forming Units (CFUs) of Mm and orally treated with a mixture of each compound in a yeast paste on day 3 post-infection (p.i.), after the infection was established. The treatment lasted 7 days, with survival monitored daily and bacillary load measured at days 3 and 10 p.i. By day 10, treated flies showed significantly higher survival rates and reduced bacillary load than controls, except for pyrazinamide, isoniazid, and doramapimod-treated flies, possibly due to the model's physiological characteristics. In conclusion, this study validates the Dm infection model as proof of concept for drug screening, offering a cost-effective method while acknowledging the limitations for those compounds that need to be metabolized inside flies and those with structural targets not present in *D. melanogaster*.

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Building a large dataset of genome mutations associated with antibiotic resistance in *Mycobacterium tuberculosis*

J Al Akl^{4 5} C Sola^{1 2 3} C Guyeux⁴ D Laiymani⁴ C Abou Jaoude⁵ Z Al Chami⁵
1: INSERM, Université Paris-Saclay 2: IAME, Université Paris-Cite 3: UMR1137, Université Sorbonne Paris-Nord 4: FEMTO-ST Institute, CNRS, University of Marie & Louis Pasteur, Belfort 5: Ticket Lab, Antonine University, Lebanon

Antibiotic-resistant *Mycobacterium tuberculosis* (Mtb) is a growing threat, and clinicians need clear genetic clues to spot it early. We collected and cleaned a very large set of Mtb genomes tied to drug-susceptibility results—over 15,000 isolates—by merging three sources: (i) public datasets such as CRyPTIC and a recent *Nature*-published study, (ii) a light AI text-mining system that sifted through more than 20,000 papers to pull extra Sequence Read Archive (SRA) numbers linked to resistance, and (iii) the PATRIC database, which lists thousands of strains with tested drug responses. After matching strain names to SRA records at the National Center for Biotechnology Information, we created a single, standard resource covering first-line, second-line, and newer drugs, and capturing both well-known and newly reported resistance mutations. Early machine-learning runs already confirm classic genotype-phenotype links and point to emerging patterns such as rare *rpoB* combinations that raise rifampicin MICs. By sharing this dataset and code, we give mycobacteriologists a practical tool to validate suspected resistance mutations, track multidrug-resistant TB, and guide new treatment strategies while keeping the helpful speed of AI in the workflow.

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In vitro activity of MRX-6038, a novel Leucyl-tRNA Synthetase inhibitor, against *Mycobacterium abscessus*

MH Cynamon¹ MS DeStefano¹ CM Shoen¹ W Wang²
1: Veteran's Health Research Institute, Syracuse, USA 2: MicuRx Pharmaceuticals, Inc., Foster City, USA

Infections caused by nontuberculous mycobacteria (NTM) have risen world-wide. Increased numbers of immunocompromised individuals, longer life expectancies for cystic fibrosis patients, better diagnostics and changes in climate are thought to be contributing factors. *Mycobacterium abscessus* (MABS) is the second most frequently diagnosed NTM infection. MABS infection is extremely difficult to treat due to its intrinsic drug resistance that generally leads to complicated and largely unsuccessful long-term treatment. Treatment is often guided by susceptibility testing due to the lack of a standardized regimen. Multidrug regimens commonly include a macrolide and amikacin, but inducible and mutational resistance are problematic.

MRX-5, a prodrug, releases MRX-6038 *in vivo* to exert anti-MABS activity. MRX-6038, the active drug, is a novel boron-containing inhibitor of bacterial leucyl-tRNA synthetase, an essential enzyme in protein synthesis. We evaluated the *in vitro* activity of MRX-6038 against a panel of 36 MABS clinical isolates. Clarithromycin (CLR), amikacin (AMK), clofazimine (CFZ), omadacycline (OMD), and cefoxitin (CFX) were also evaluated. MICs were determined in a broth dilution assay using CLSI methodology.

MRX-6038 demonstrated potent activity against MABS, with MIC₉₀ and MIC₅₀ 0.25µg/ml and 0.125µg/ml, respectively. The MIC₉₀ and MIC₅₀ for all other compounds (µg/ml) were CLR (4, 0.5), AMK (16, 8), CFZ (4, 2), OMD (1, 0.5), and CFX (32, 8). Previously MRX-5 efficacy was demonstrated in a mouse model of pulmonary MABS infection, suggesting its potential for further development. Inherent drug resistance, lack of effective treatment, and growing numbers of infections highlight the need for new therapies to treat MABS infection.

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Microbial antagonism against nontuberculous Mycobacteria

A Maranhã^{1 2} LR Baptista^{1 2} A Dias^{1 2} S Alarico^{1 2} I Tiago^{3 4} N Empadinhas^{1 2}

1: CNC - Centre for Neuroscience and Cell Biology, University of Coimbra, Portugal 2: CIBB - Centre for Innovative Biomedicine and Biotechnology, University of Coimbra, Portugal 3: Department of Life Sciences, University of Coimbra, Portugal 4: TimeUp Lda, IPN incubator, Coimbra, Portugal

Nontuberculous mycobacteria (NTM) are opportunistic pathogens of growing public health concern, owing to their intrinsic antibiotic resistance and persistence in human-engineered environments. This study explores microbial interactions involving NTM from both environmental and clinical origins, aiming to uncover competitive relationships and identify potential sources of antimycobacterial compounds. We investigated interactions between NTM and methylobacteria, co-inhabitants of drinking water systems, as well as with skin-associated bacteria. Co-culture and mixed biofilm assays demonstrated that several methylobacterial and skin-derived isolates effectively inhibited the growth and biofilm formation of both environmental and clinical NTM strains. Clinical NTM isolates showed markedly higher antibiotic resistance and biofilm-forming capacity, aligning with their enhanced pathogenic potential. Whole-genome sequencing of active isolates revealed biosynthetic gene clusters (BGCs) potentially encoding diverse secondary metabolites, including candidates related to known antimycobacterial agents. Overall, antimicrobial activity was attributed to diffusible molecules, with some strains requiring competitive conditions to trigger bioactivity. These findings highlight the dynamic competitive ecology of NTM in both environmental and host-associated niches and emphasize the potential of microbial interactions and/or antagonism as a source of novel antimycobacterials. By integrating perspectives from waterborne and skin-associated microbiota, this work advances our understanding of NTM ecology and pathogenesis and suggests new strategies for targeted control of NTM infections.

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