A data compendium of *Mycobacterium tuberculosis* antibiotic resistance

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Abstract

The Comprehensive Resistance Prediction for Tuberculosis: an International Consortium (CRyPTIC) presents here a global collection of 15,211 *Mycobacterium tuberculosis* clinical isolates, all of which have undergone whole genome sequencing and have had their minimum inhibitory concentrations to 13 antitubercular drugs measured. The isolates represent five major *M. tuberculosis* lineages originating from 23 countries across four continents. 6,814 isolates were found resistant to at least one drug, including 2,129 samples fully satisfy the clinical definitions of RR/MDR, pre-XDR or XDR. Resistance status to eight antitubercular drugs can be accurately predicted using a genetic mutation catalogue for over 90% of the isolates. Furthermore, we show the presence of suspected resistance conferring mutations for isolates resistant to the newly introduced drugs bedaquiline, clofazimine, delamanid and linezolid. Finally, a case study of rifampicin mono-resistance is presented to showcase how this compendium could be used to advance our genetic understanding of rare resistance

phenotypes and evaluate the likely performance of a widely used molecular diagnostic tool. It is hoped that this compendium, the largest *M. tuberculosis* matched phenotypic and genotypic dataset to date, will facilitate and inspire new research projects for years to come.

Introduction

Tuberculosis (TB) is a curable and preventable disease; 85% of those afflicted can be successfully treated with a six-month regimen. Despite this, TB is the world's top infectious disease killer with 10 million new cases and 1.2 million deaths estimated in 2019 alone [1]. Furthermore, drug resistant TB (DR-TB) is a continual threat; almost half a million people were estimated to have developed resistance to the first-line drug rifampicin (RR-TB), with three quarters of these cases estimated to be multidrug-resistant (MDR-TB, resistant to first-line drugs isoniazid and rifampicin) [1]. Worryingly, only 44% of DR-TB cases were officially notified and just over half of these cases were successfully treated (57%) [1].

To address these issues, the World Health Organisation (WHO) is encouraging the development of better, faster and more targeted diagnostic and treatment strategies through its EndTB campaign. Of particular interest is universal drug susceptibility testing (DST). Conventionally, DST relies on lengthy (4 weeks minimum) culture-based methods that require strict biosafety conditions for *Mycobacterium tuberculosis*. The development of rapid genetics-based assays has decreased diagnostic time to as little as 2 hours through the detection of specific resistance conferring mutations e.g. the Cepheid Xpert® MTB/RIF test [2, 3]. However, assay bias towards specific genic regions can result in misdiagnosis of resistance, the

prescription of ineffective treatment regimens and subsequent spread of multi-drug resistant disease, as seen during an outbreak in Eswatini of an MDR strain harbouring an *rpoB* I491F mutation not detected by the Xpert® MTB/RIF assay [4-6].

Furthermore, detection of rifampicin resistance with the Xpert® MTB/RIF line probe assay (LPA) assay is used to infer MDR-TB as epidemiologically, rifampicin resistance tends to coincide with resistance to isoniazid [7]. While this *modus operandi* is successful at pragmatically identifying potential MDR cases quickly and effectively, it is not generally true that a single path towards developing MDR or extensively drug resistant TB (XDR = MDR/RR + resistance to at least one fluoroquinolone and either bedaquiline or linezolid) exists. For example, *M. tuberculosis* strains that are sensitive to rifampicin but resistant to multiple other anti-tubercular drugs would be missed by current LPA diagnostic testing and remain undetected in the community. A better understanding of how various combinations of drug resistance in different backgrounds (nonMDR/MDR) arises is essential to not only prevent the spread of resistant strains and to promote the use of effective treatment regimens, but also to facilitate the development of suitable diagnostic assays unbiased in their approaches to detect *any* and *all* resistance where it occurs.

Whole-genome sequencing (WGS) has the potential to reveal the entirety of the *M. tuberculosis* genetic resistance landscape for any number of drugs simultaneously whilst enabling a more rapid turnaround time and affordable cost in comparison to DST culture methods [8]. The success of WGS as a diagnostic method wholly depends on a comprehensive and accurate catalogue of resistance-conferring mutations for each drug. Recent advances have shown that genotypic predictions of

resistance correlate well with DST results for first-line drugs [7]. However, the mechanisms of resistance to second-line, new and re-purposed drugs (NRDs) are less well understood despite their increased administration in clinics as MDR cases climb [9]. To align with the goals of the EndTB campaign, further research is vital to construct an extensive catalogue of genetic variants associated with resistance, and indeed susceptibility, if WGS-based diagnostics are to become routine practice for DR-TB management.

The Comprehensive Resistance Prediction for Tuberculosis: an International Consortium (CRyPTIC) has collected *M. tuberculosis* clinical isolates worldwide to survey the genetic variation associated with resistance to 13 antitubercular drugs, specifically the first-line drugs rifampicin, isoniazid, ethambutol, the second-line drugs amikacin, kanamycin, rifabutin, levofloxacin, moxifloxacin, ethionamide, and the new and re-purposed drugs bedaquiline, clofazimine, delamanid and linezolid. Here, we present a data compendium of 15,211 global clinical isolates, each of which has had its genomic sequence determined and DST profile measured [10]. This compendium is the largest drug screening effort to date for *M. tuberculosis* in a ‘one isolate – one microscale assay’ format across defined compound concentration ranges. Finally, this matched genotypic-phenotypic dataset will prove an invaluable resource to accelerate and improve AMR diagnostic development for TB, both by the enrichment of mutation catalogues for WGS resistance prediction and the identification of important diagnostic gaps and drug resistance patterns.

Methods

Ethics

Approval for CRyPTIC study was obtained by Taiwan Centers for Disease Control IRB No. 106209, University of KwaZulu Natal Biomedical Research Ethics Committee (UKZN BREC) (reference BE022/13) and University of Liverpool Central University Research Ethics Committees (reference 2286), Institutional Research Ethics Committee (IREC) of The Foundation for Medical Research, Mumbai (Ref nos. FMR/IEC/TB/01a/2015 and FMR/IEC/TB/01b/2015), Institutional Review Board of P.D. Hinduja Hospital and Medical Research Centre, Mumbai (Ref no. 915-15-CR [MRC]), scientific committee of the Adolfo Lutz Institute (CTC-IAL 47-J / 2017) and in the Ethics Committee (CAAE: 81452517.1.0000.0059) and Ethics Committee review by Universidad Peruana Cayetano Heredia (Lima, Peru) and LSHTM (London, UK).

Sample collection

Participating collection centres varied in their isolate collection approaches, but the consortium collectively aimed to oversample for *M. tuberculosis* isolates with drug resistance and multi-drug resistance.

Plate assay

The CRyPTIC consortium designed two variants of the Sensititre MYCOTB plate (Thermo Fisher Scientific Inc., USA) named the “UKMYC5” and “UKMYC6” microtitre plates [10, 11]. These plates contain five to ten doubling dilutions of 13 antibiotics (rifampicin (RIF), rifabutin (RFB), isoniazid (INH), ethambutol (EMB), levofloxacin (LEV), moxifloxacin (MXF), amikacin (AMI), kanamycin (KAN), ethionamide (ETH), clofazimine (CFZ), linezolid (LZD), delamanid (DLM), and bedaquiline (BDQ)). Delamanid and bedaquiline were provided by Otsuka Pharmaceutical Co., Ltd. and Janssen Pharmaceutica respectively. The UKMYC5

plate also contained para-aminosalicylic acid (PAS), however the MICs were not reproducible and hence it was excluded from the UKMYC6 plate design and is not included in any subsequent analysis [10].

A standard operating protocol for sample processing was defined by CRyPTIC as previously described [10, 11]. Clinical samples were sub-cultured using 7H10 agar plates, Lowenstein-Jensen tubes or MGIT tubes. Bacterial cell suspensions (0.5 McFarland standard, saline Tween) prepared from (no later than) 14-day old colonies were diluted 100X in 10 ml enriched 7H9 broth prior to plate inoculation. A semi-automated Sensititre Autoinoculator (Thermo Fisher, Scientific Inc., USA) was used to inoculate 100 µl prepared cell suspensions (1.5×10^5 CFU/ml [5×10^4 CFU/ml - 5×10^5 CFU/ml]) into each well of a UKMYC5/6 microdilution plate. The plate was sealed and incubated for 14 days at 37°C. Quality control runs were performed periodically using *M. tuberculosis* H37Rv ATCC 27294, which is sensitive to all drugs on the plates.

Minimum Inhibitory Concentration (MIC) measurement

As stated in reference [10], minimum inhibitory concentrations (MICs) for each drug were read after incubation for 14 days by a laboratory scientist using a Thermo Fisher Sensititre™ Vizion™ digital MIC viewing system. The Vizion apparatus was also used to take a high contrast photograph of the plate with a white background, from which the MIC was measured again using the Automated Mycobacterial Growth Detection Algorithm (AMyGDA) software [12]. The AMyGDA software detects the boundaries of each well using a Hough transform for circles and measures growth as the number of dark pixels within the area contained by this boundary. All images where the MICs measured by Vizion and AMyGDA were different were uploaded to a citizen

science project, BashTheBug, on the Zooniverse platform [13]. Each image was then classified by ≥ 11 volunteers and the median classification taken.

MICs were then classified as high (at least two methods concur on the MIC), medium (either there is no plate image, or Vizion and AMyGDA disagree and there is no BashTheBug measurement), or low (all three methods disagree) quality. When considering the pattern of resistance in a sample we took the MIC from the Vizion reading, if it was annotated as having medium or low quality, to ensure adequate data coverage.

Genomic data processing and variant calling

Sequencing arrangements differed slightly between each CRyPTIC participating laboratory. Short-read, paired end libraries were sequenced on Illumina machines and the resulting FASTQ files were processed using the bespoke pipeline Clockwork (v0.8.3, <https://github.com/iqbal-lab-org/clockwork>). Briefly, all raw sequencing files are indexed into a relational database with which Clockwork proceeds. Human, nasopharyngeal flora and human immunodeficiency virus related reads are removed and remaining reads are trimmed (adapters and low quality ends) using Trimmomatic and mapped with BWA-MEM to the *M. tuberculosis* H37Rv reference genome (NC000962.3) [14, 15]. Read duplicates are removed. Genetic variants are called independently using Cortex and SAMtools, two variant callers with orthogonal strengths (SAMtools a high sensitivity SNP caller, and Cortex a high specificity SNP and indel caller) [16, 17]. The two call sets are merged to produce a final call set, using the Minos adjudication tool to resolving loci where the two callers disagreed, by remapping reads to an augmented genome containing each alternative allele [18]. Default filters of a minimum depth of 5x and a Fraction of Supporting Reads

of 0.9 (minos) and a Genotype confidence percentile (GCP) filter of 0.5 were applied. The GCP filter is, roughly speaking, a normalised likelihood ratio test, giving a measure of confidence in the called allele compared with the other alternatives, and is described in [18]. This produced one VCF file per sample, each only describing positions where that sample differed from the reference.

These filtered VCFs were then combined, to produce a single non-redundant list of all variants seen in the cohort. All samples were then processed a second time with Minos, remapping reads to a graphical representation of all the segregating variation within the cohort, generating VCF files which had an entry at all variable positions (thus for all samples, most positions would be genotyped as having the reference allele).

In order to remove untrustworthy loci, a genome mask was applied to the resulting VCF files (regions identified with self-blast matches in [19] comprising of 324,971 bp of the reference genome. Furthermore, positions with less than 90% of total samples passing default Clockwork/Minos variant call filters (described above) were filtered out, comprising 95,703 bp of the genome, of which 55,980 bp intersect with the genome mask.

Phylogenetic tree construction

A pairwise genetic distance matrix was constructed for 15,211 isolates by comparing each two variant call format (VCF) files (relative to the reference *M. tuberculosis* H37Rv genome, see [11] and the above methods section for more details) under consideration position-wise. A neighbourhood-joining tree was constructed from

the distance matrix using *quikctree* [20]. Tree visualisation and annotation was performed using the R library *ggtree* [21]. *M. tuberculosis* lineages were assigned using Mykrobe and are represented by the coloured dots at the branch termini of the tree [18]. For isolates that had ‘mixed’ lineage classification (*i.e.*, 2 lineages were found present in the sample by Mykrobe, $n = 225$, 1.5%), the first of the two lineages was assigned to the isolate. *ggtree* was also used to construct the trees depicting bedaquiline, clofazimine and delamanid resistant isolates.

Data analysis and visualisation

All data can be found in <ftp address: tbc> and were analysed and visualised using either R or python3 libraries and packages. See <github: tbc> for codebase.

Results

15,211 M. tuberculosis clinical isolates

The CRyPTIC dataset comprises isolates from 27 countries collected by 14 partner sites worldwide. The compendium presented here contains 15,211 isolates for which both genomic and phenotypic data was collected by 23 of the partner countries across the continents of Asia, Africa, South America and Europe (Fig. 1). Where the origin of an isolate was not known, the isolate was assumed to originate in the same country as the contributing laboratory (269 isolates in Germany, 17 isolates in India, 6 isolates in Peru, 885 isolates in Italy, 510 isolates in South Africa, 357 isolates in Sweden, 208 isolates in Taiwan, 1 isolate in Brazil and 4 isolates in the UK). The largest number of isolates were contributed by India ($n = 4,004$), Peru ($n = 2,691$), South Africa ($n = 2,155$), Vietnam ($n = 1,288$) and China ($n = 1,121$).

Lineage assignment revealed that all isolates (except 41, 0.3%) belong to the four main *M. tuberculosis* lineages (L1-L4). Like previous studies, we see a strong association between geolocation and lineage (Pearson's chi-squared test, $p < 2.2 \times 10^{-16}$, Fig. S3)[22, 23]. The pie-charts in Fig. 1 and supplemental tables show the breakdown of isolate number -v- lineages (Table S2) and sub-lineages (Table S3) for each location. The phylogenetic tree in Fig. 2 further highlights the strong population structure of this collection of isolates, with isolates clustering according to lineage.

Isolates of the ancient Indo-oceanic lineage/L1 ($n = 1,150$) were mostly contributed by India ($n = 676$ isolates) and Vietnam ($n = 283$ isolates). 85% of the L1 Indian isolates belong to sub-lineages 1.1.2 and 1.2.2 while 66% of the Vietnamese isolates are sub-lineage 1.1.1.1. No L1 isolates were contributed by 10 of the 23 countries in this study with only 2 isolates collected in South America (one in each of Brazil and Peru).

There are 5,598 L2 (East Asian) isolates, making it the second largest group in the dataset. L2 was found most prevalent in Asia and Europe with the largest proportion found in amongst isolates contributed by China ($n = 722$, 64% of isolates) and India ($n = 1,573$, 39% of isolates). Sub-lineages 2.2 and 2.2.7 dominate the L2 isolates ($n = 1,421$ and 1,249 respectively); 2.2 was found mostly amongst Peruvian and South African isolates ($n = 231$ and 161 respectively) apart from those contributed by the Asian countries of Vietnam ($n = 271$), China ($n = 284$) and India ($n = 272$), while 85% of sub-lineage 2.2.7 isolates were contributed by South Africa ($n = 206$), Vietnam ($n = 164$) and India ($n = 691$). 70% of sub-lineage 2.2.1 originated from South Africa (10% of isolates found here) and has recently been associated with more favourable

transmission rates [24]. Lastly, 86% and 72% of isolates contributed by Kyrgyzstan and Turkmenistan respectively belong to L2 with sub-lineage 2.2.10 dominating (16/24 and 75/86 isolates for both countries respectively). 2.2.10 has been previously described as restricted to Central Asia and this is reflected in the compendium [25].

The majority (1184/1850, 65%) of L3 (East African/Indian) isolates were contributed by India, followed by 19.6% (363/1850) isolates from Pakistan. L3 is typically under-sampled and under-studied in current databases and biobanks in comparison to L2 and L4; the L3 isolates in this study are the largest collected to date in a single study [26].

L4 (Euro-American) is the largest lineage group in the compendium ($n = 6,572$). Isolates donated from Peru dominate; 87% of all L4 isolates are Peruvian with 4.1.2.1 and 4.3.3 being the most prevalent sub-lineages (24% and 22% respectively). There are 34 different L4 sub-lineages in the dataset, making L4 the most diverse in comparison to the other lineage groupings.

There were no L5 isolates found in the compendium and only 6 L6 isolates were identified. Animal-restricted pathogenic mycobacterial isolates are also rare in the compendium; only 16 cases were identified ($n = 15$ *M. bovis* and $n = 1$ *M. caprae*).

Figure 1: Geographical distribution of CRyPTIC *M. tuberculosis* clinical isolates.

The total number of isolates contributed by each country is depicted, with pie charts representing the proportion of *M. tuberculosis* lineages. Where the origin of an isolate was not known, the collection site identity was assigned (269 isolates in Germany, 17 isolates in India, 6 isolates in Peru, 885 isolates in Italy, 510 isolates in South Africa, 357 isolates in Sweden, 208 isolates in Taiwan, 1 isolate in Brazil and 4 isolates in the UK).

Figure 2: Phylogenetic tree of CRYPTIC *M. tuberculosis* clinical isolates. A phylogenetic cladogram of 15,211 *M. tuberculosis* clinical isolates. A neighbour-joining tree was constructed from a pairwise distance matrix using *quikctree* [20]. Coloured dots at the branch termini represent the lineage assigned to each isolate. “Animal/Other” includes 16 isolates that were assigned the following lineages: *M. caprae*, *M. bovis*, *unknown* along with 17 quality control isolates representing specific sub-lineages [27].

Quality assurance of the drug minimum inhibitory concentrations

Regular quality assurance checks detected problems with plate inoculation and reading in two laboratories during collection of the 15,211 isolates. In addition, some plates were not able to be read, usually because of inadequate growth in the positive control wells or extensive contamination. These anomalies were investigated, and samples were removed from the dataset where appropriate, resulting in 2,922 isolates being held out of the final dataset. Further, due to skip wells and other phenomena that prevent an MIC being measured, 11.9% of isolates did not return a phenotype for all 13 drugs on the plate. The compendium therefore has a total of 157,401 MIC measurements for 12,289 isolates (Table 1).

	MIC MEASUREMENTS	HIGH QUALITY	MEDIUM QUALITY	LOW QUALITY
INH	12070	9519	1351	1200
RIF	12099	8955	1356	1788
EMB	12158	7506	1355	3297
LEV	12163	7774	1354	3035
MXF	12194	6785	1353	4056
AMI	12072	8973	1350	1749
KAN	12130	9333	1355	1442
BDQ	12068	8536	1355	2177
CFZ	12049	7763	1352	2934
DLM	11927	8095	1349	2483
LZD	12189	7141	1355	3693
ETH	12132	8821	1355	1956
RFB	12150	10042	1352	756
TOTAL	157401	109243	17592	30566

Table 1: Quality metrics for phenotype data Stated for each drug is the total number of MIC readings (“PHENOTYPED”) stratified into “high” quality (at least two MIC measurement methods agree), “medium” quality (either Vizion and AMyGDA disagree, or there is no plate picture) or “low” quality (all three MIC measurements methods disagree) phenotype classifications as described in Methods. Drug acronyms: INH = isoniazid, RIF = rifampicin, EMB = ethambutol, LEV = levofloxacin, MXF = moxifloxacin, AMI = amikacin, KAN = kanamycin, BDQ = bedaquiline, CFZ = clofazimine, DLM = delamanid, LZD = linezolid, ETH = ethionamide, RFB = rifabutin.

Resistance classification and distribution

Binary drug resistant/susceptible phenotypes were calculated from the MICs by applying an epidemiological cut-off value (ECOFF); samples with MICs at or below the ECOFF are, by definition, wild-type and hence assumed to be susceptible to the drug in question [11] (Fig. S1). Samples with MICs above the ECOFF are therefore assumed to be resistant.

Unsurprisingly given its size, resistance to each of the 13 drugs is represented within the CRyPTIC compendium (Fig. 3a). The drugs with the highest percentage of resistance are the first line drugs isoniazid and rifampicin (49.0% and 38.7% respectively). Of the second line drugs, levofloxacin had the highest proportion of

resistant isolates in the dataset (17.6%) and amikacin the lowest (7.3%). Reassuringly, a low proportion of isolates were resistant to the NRDs, bedaquiline (0.9%), clofazimine (4.4%), delamanid (1.6%) and linezolid (1.3%).

Of the 12,289 isolates which returned a binary phenotype for at least one drug, 6,814 (55.4%) were 'resistant' to at least one drug (Fig. 3b). For the purpose of describing the broader resistance categories present in the dataset, we assumed that all MICs that could not be read had susceptible phenotypes in this section. Consequently, the calculated prevalence of MDR, XDR etc in the dataset (Fig. 3) are likely under-estimates.

At least 68.8% ($n = 4,685$) of drug resistant isolates were either MDR or RR (Fig. 3b) and of these 38.8% ($n = 1,819$) were pre-XDR (MDR/RR + fluoroquinolone resistance) and 3.0% were XDR ($n = 142$). Two of the XDR isolates returned a resistant phenotype to all 13 of the drugs assayed (Table S1) and therefore could be reasonably described as totally drug resistant (TDR). One such isolate belonged to L4 and was contributed by South Africa, and the other belonged to L2 with an unknown country of origin.

Of the 2,129 resistant isolates that did not fit into either of the MDR or RR categories, more were categorised as isoniazid resistant (73.1%, $n = 1,556$) than were categorised as susceptible to both isoniazid and rifampicin and resistant to at least one other drug (26.9%, $n = 573$).

Whilst many partner laboratories oversampled for resistance, others used a less biased collection protocol (Fig. 3c). Therefore, the proportions of resistant phenotypes are not necessarily representative of the overall distribution of resistance in the country and are not comparable between countries. Isolates with unknown country of origin were excluded from this analysis. In all countries that contributed more than 100 resistant isolates, each of the five phenotypic resistance categories in Fig. 3b were identified, except for Peru, Vietnam and Nepal which did not contribute any XDR isolates. Vietnam and Brazil sampled a particularly high proportion of non-MDR/RR resistant phenotypes; 73.9% and 55.1% of resistant isolates contributed by these countries, respectively, were neither MDR nor RR.

For Nepal and India, an especially high proportion of the MDR/RR isolates contributed were fluoroquinolone resistant (92.9% ($n = 132/142$ isolates) and 69.8% ($n = 538/770$ isolates) respectively), as has been previously observed for this geographical region [28, 29].

All five categories of resistance were represented in the four major *M. tuberculosis* lineages. We note that these relative proportions will have been influenced by the different sampling approaches at each partner laboratory since lineage distributions are typically geographically distinct (Fig. 1). Bearing this in mind, we observe that in the compendium, L3 isolates contained the most MDR/RR isolates as a proportion of resistant isolates (77.6%), followed by L2 (74.3%), L4 (63.4%), and L1 (50.0%). L2 is known to be associated with MDR/RR and XDR and although it did not contain the highest proportion of MDR/RR isolates, it did have the most pre-XDR isolates as a proportion of MDR/RR isolates (54.2%) [30, 31]. L1 and L2 contained the

most XDR isolates as a proportion of MDR/RR isolates (4.7% and 4.2% of isolates respectively).

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443 **Figure 3: Drug phenotype data for the CRyPTIC compendium** a) Prevalence of
 444 resistance to each of 13 drugs in the CRyPTIC dataset. The total number of isolates with a binary
 445 phenotype (of any quality) for the corresponding drug is presented in Table 1. b) Phenotypes of
 446 12, 289 CRyPTIC isolates with a binary phenotype for at least one drug. c) Geographical
 447 distribution of phenotypes of 12, 289 CRyPTIC isolates. Intensity of blue shows the percentage of
 448 isolates contributed that were categorised as susceptible to all 13 drugs. Donut plots show the

proportions of resistant phenotypes identified in (b) for countries contributing ≥ 100 isolates with drug resistance. **d)** Proportions of resistance phenotypes in the 4 major *M. tuberculosis* lineages. N is the number of isolates of the lineage called resistant to at least one of the 13 drugs. Drug acronyms: INH = isoniazid, RIF = rifampicin, EMB = ethambutol, LEV = levofloxacin, MXF = moxifloxacin, AML = amikacin, KAN = kanamycin, BDQ = bedaquiline, CFZ = clofazimine, DLM = delamanid, LZD = linezolid, ETH = ethionamide, RFB = rifabutin.

Co-occurrence of drug resistance amongst the CRyPTIC isolates

Isolates with all possible two-drug resistant combinations were present in this dataset (Fig. 4a). Resistance to any of the remaining 11 drugs was associated with resistance to both isoniazid and rifampicin. Isoniazid resistance was the most strongly associated bar the following two exceptions, both of which arise because drugs from the same class are being compared: a greater proportion of isolates resistant to rifabutin were also resistant to rifampicin (96.8% had rifabutin - rifampicin co-resistance versus 93.3% rifabutin - isoniazid co-resistance) and a greater proportion of isolates resistant to moxifloxacin were associated with levofloxacin resistance (97.6% had moxifloxacin – levofloxacin co-resistance versus 95.0% moxifloxacin – isoniazid co-resistance). Of the second line drugs, levofloxacin and moxifloxacin were more commonly seen as a second resistant phenotype than the injectable drugs kanamycin and amikacin.

Despite this, resistance to both drugs in the aminoglycoside class was common the dataset; 90.4% of amikacin resistant isolates were also resistant to kanamycin although significantly fewer kanamycin resistant isolates were resistant to amikacin (72.0%, $p < 0.00001$). In a similar fashion, a smaller proportion of rifampicin resistant isolates were resistant to rifabutin than rifabutin resistant isolates that were resistant to rifampicin (91.3%, 96.8%, $p < 0.00001$) while a smaller proportion of levofloxacin

resistant isolates were resistant to moxifloxacin than moxifloxacin resistant isolates that were resistant to levofloxacin (78.5%, 97.6%, $p < 0.00001$). One possible explanation is that the cut off, in this case the ECOFFs, have been inconsistently defined for the different compounds, however here all ECOFFs have been determined in the same way using a much larger dataset than is typically the case [11]. Differences in drugs of the same class are also well documented by *in vitro* studies [32-34].

Bedaquiline, clofazimine, delamanid and linezolid were less commonly seen as a second resistance phenotype and the smallest proportional resistance combinations involved the NRDs (e.g. 1.5% of isoniazid resistant isolates were bedaquiline resistant and 1.7% were delamanid resistant). Within the NRDs however, co-occurrence of resistance was proportionally higher; bedaquiline resistance was most seen with clofazimine resistance (52.4%), clofazimine resistance was most seen with bedaquiline resistance (10.6%), delamanid resistance was most seen with clofazimine resistance (26.3%) and linezolid resistance was most seen with clofazimine resistance (34.2%).

Additional antibiotic resistance in isolates with MDR or non-MDR phenotypic backgrounds

We next examined in more detail the correlation structure of phenotypes by conditioning on different phenotypic “backgrounds”. (Fig. 4b-f). We found that a greater proportion of isolates that were susceptible to isoniazid and rifampicin were resistant to second-line drugs than the first line drug ethambutol (15.9%) (Fig. 4b). The proportion of isolates resistant to clofazimine or levofloxacin was particularly high

(32.9% and 24.1%, respectively), and more isolates were resistant to these two drugs than ethambutol in an isoniazid resistant and rifampicin susceptible background but not in MDR/RR isolates (Fig. 4c-f).

MDR/RR isolates (asides from rifabutin) were most commonly resistant to the first line drug ethambutol (46.3%), closely followed by levofloxacin (41.4%), then moxifloxacin (34.1%). A greater proportion of isolates resistant to levofloxacin than moxifloxacin was also seen in all other backgrounds and as expected, the proportion of fluoroquinolone resistance was higher in MDR/RR isolates than non-MDR isolates [35]. For the aminoglycosides, a greater percentage of MDR/RR isolates were kanamycin resistant (21.8%) than amikacin resistant (18.1%), a trend seen in all other backgrounds.

For isolates with an XDR phenotype, a higher proportion were also resistant to linezolid than bedaquiline (66.7% compared to 44.6%) and 11.3% of XDR isolates were resistant to both bedaquiline and linezolid (Fig. 4f). XDR isolates were also resistant to the other NRDs, clofazimine (41.3%) and delamanid (18.8%). For isolates with an MDR/RR or pre-XDR background, the most common NRD resistance seen was clofazimine (6.0% and 9.3%, respectively) followed by linezolid (2.3% and 4.7% respectively), bedaquiline (1.8% and 3.2%, respectively), and delamanid (1.7% and 2.3%, respectively). In non-MDR/RR backgrounds: clofazimine resistance remained the most common followed by delamanid resistance, linezolid resistance and bedaquiline resistance (Fig. 4b-c).

Figure 4: Co-occurrence of antibiotic resistance in CRyPTIC *M. tuberculosis* isolates **a)** The heatmap shows the probability of an isolate being resistant to Drug 2 if it is resistant to Drug 1, percentages are given in Table S4. **(b-g)** Percentage of isolates that are resistant to another of the 13 drugs in a background of **(b)** isoniazid susceptible + rifampicin susceptible, **(c)** isoniazid resistant + rifampicin susceptible, **(d)** MDR/RR **(e)** Pre-XDR, **(f)** XDR. Only samples with definite phenotypes for RIF in MDR backgrounds and RIF and INH in non-MDR backgrounds and the additional drug are included. Drug acronyms: INH = isoniazid, RIF = rifampicin, EMB = ethambutol, LEV = levofloxacin, MXF = moxifloxacin, AMI = amikacin, KAN = kanamycin, BDQ = bedaquiline, CFZ = clofazimine, DLM = delamanid, LZD = linezolid, ETH = ethionamide, RFB = rifabutin.

Genetic determinants of resistance

In order to establish a baseline measure of how well resistance and susceptibility could be predicted based on the state of the art prior to the CRyPTIC project, we constructed a hybrid catalogue of genetic variants associated with resistance to the first-line compounds isoniazid, rifampicin & ethambutol and the

second-line compounds levofloxacin, moxifloxacin, amikacin, kanamycin, ethionamide based on existing catalogues [7, 36]. (We note in passing that we specifically did not use the recent WHO catalogue in order to avoid circularity and over-training, as that catalogue was developed (via prior literature, expert rules and a heuristic algorithm) based partially on these isolates [37]. We applied our hybrid catalogue to predict the susceptibility and resistance of all isolates in this compendium to these compounds, and the resulting genetic-based predictions were then compared with the binary phenotypes derived from the MICs (Table 2). Since these data were not collected prospectively or randomly, but indeed are enriched for resistance, the calculated error rates are not representative of how well such a method would perform in routine clinical use. We also did not apply the approach used in [7] of refusing to make a prediction if a novel mutation was detected in a known resistance gene, as we simply wanted to measure how well a pre-CRyPTIC catalogue could predict resistance in the compendium. The results were broadly in line with prior measurements on a smaller (independent) set [18]. The hybrid catalogue does not make predictions for rifabutin, linezolid, bedaquiline, delamanid or clofazimine; indeed, this is one of the main aims of the consortium and new catalogues published by CRyPTIC and the WHO will begin to address this shortcoming (Fig. 5) [19].

Table 3 shows the top mutations found amongst isolates phenotypically resistant to first- and second- line drugs. As expected, *rpoB* S450L is the most prevalent mutation associated with rifampicin resistance, with 62.2% of all phenotypically rifampicin-resistant isolates carrying it, while 70% of isoniazid resistant isolates have *katG* S315T mutations. Mutations in *gyrA* dominate amongst fluoroquinolone resistant isolates; D94G and A90V are the two most frequently

occurring mutations for levofloxacin and moxifloxacin, with just under 77% and just over 94% of phenotypically resistant isolates carrying at least one mutation in *gyrA* respectively. Mutations in *rpoC* are amongst those most prevalent for rifabutin resistant isolates; these mutations are typically described as compensatory, while G594E was subsequently defined as a lineage-specific marker for L4 LAM and Haarlem lineages [38].

	TP	FP	TN	FN	VME	ME	PPV	NPV
INH	5493	142	5622	224	0.039	0.025	0.961	0.975
RIF	4535	435	6669	107	0.023	0.061	0.977	0.939
EMB	1919	513	6702	111	0.055	0.071	0.945	0.929
LEV	1689	255	8104	184	0.098	0.031	0.902	0.969
MXF	1358	504	9022	160	0.105	0.053	0.895	0.947
AMI	632	84	10117	163	0.205	0.008	0.795	0.992
KAN	735	124	9043	197	0.211	0.014	0.789	0.986
ETH	971	114	9183	511	0.345	0.012	0.655	0.988

Table 2: Predicting phenotypic resistance using genetics

TP: the number of phenotypically resistant samples are that correctly identified as resistant ("true positives"); FP, the number of phenotypically susceptible samples that are falsely identified as resistant ("false positives"); TN, the number of phenotypically susceptible samples that are correctly identified as susceptible ("true negatives"); FN, the number of phenotypically resistant samples that are incorrectly identified as susceptible ("false negative"); VME, very major error rate (false-negative rate), 0-1; ME, major error rate (false-positive rate), 0-1; PPV, positive predictive value, 0-1; NPV, negative predictive value, 0-1.

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	GENE	VARIANT	<i>n</i>	%
RIF	<i>rpoB</i>	S450L	2914	62.2
	<i>rpoB</i>	D435V	506	10.8
	<i>rpoB</i>	H445D	202	4.3
	<i>rpoB</i>	H445Y	147	3.1
	<i>rpoB</i>	D435Y	112	2.4
INH	<i>katG</i>	S315T	3668	70.
	<i>fabG1</i>	c-15t	829	15.8
	<i>fabG1</i>	g-17t	176	3.4
	<i>fabG1</i>	t-8c	154	2.9
	<i>inhA</i>	I194T	56	1.1
EMB	<i>embB</i>	M306V	1131	50
	<i>embB</i>	M306I	1001	44.3
	<i>embB</i>	Q497R	449	19.9
	<i>embB</i>	G406A	164	7.2
	<i>embB</i>	G406D	105	4.6
KAN	<i>rrs</i>	a1401g	660	58.9
	<i>eis</i>	c-14t	70	6.2
	<i>eis</i>	g-10a	53	4.7
AMI	<i>rrs</i>	a1401g	660	74.7
	<i>rrs</i>	g1484t	7	0.8
LEV	<i>gyrA</i>	D94G	783	36.5
	<i>gyrA</i>	A90V	487	22.7
	<i>gyrA</i>	D94N	157	7.3
	<i>gyrA</i>	D94A	133	6.2
	<i>gyrA</i>	S91P	92	4.3
MXF	<i>gyrA</i>	A90V	487	28.2
	<i>gyrA</i>	D94G	783	45.4
	<i>gyrA</i>	D94N	157	9.1
	<i>gyrA</i>	D94A	133	7.7
	<i>gyrA</i>	D94Y	70	4.1
ETH	<i>fabG1</i>	c-15t	829	48
	<i>fabG1</i>	L203L	124	7.2

598 **Table 3: The top mutations associated with phenotypic drug resistance**

599 A genetic resistance catalogue was used for genotypic prediction of resistance for each drug in
600 column 1 [7, 36]. "VARIANT": non-synonymous amino acid mutations are denoted by upper case
601 letters while nucleotide substitutions for non-coding sequences are denoted by lower case letters.
602 Negative numbers denote substitutions in promoter regions; "GENE": genic region of interest in
603 which "Variant" can be found; "*n*": number of phenotypically resistant isolates with "VARIANT"; "%":
604 percentage of total phenotypically resistant isolates with "VARIANT". Drug acronyms: INH =
605 isoniazid, RIF = rifampicin, EMB = ethambutol, AMI = amikacin, KAN = kanamycin, LEV =
606 levofloxacin, MXF = moxifloxacin, ETH = ethionamide.

Resistance to new and re-purposed drugs

As previously stated, relatively few isolates are resistant to the NRDs, bedaquiline ($n = 109$), clofazimine ($n = 525$), delamanid ($n = 186$) and linezolid ($n = 156$). South Africa contributed the greatest number of isolates resistant to bedaquiline, clofazimine and linezolid (Fig. 5a), while China and India contributed the most isolates resistant to delamanid. Since the collection protocol differed between laboratories it is not possible to infer any differences in the relative prevalence of resistance to the NRDs in these countries. The results of a survey of all non-synonymous mutations in genes known or suspected to be involved in resistance to these four drugs (e.g. *rv0678*, *mmpL5*, *pepQ*, *ddn*, *rplC*, *rrl* etc.) are depicted in Fig. 5b-e [39-43]. Mutations known to be associated with sensitivity were ignored, along with mutations that occurred at a frequency of $\geq 5\%$ amongst all isolates. In contrast to first- and second-line drugs, there are no mutations within a single gene/small group of genes that can fully explain resistance to any NRD. Note that the role of most of these mutations in resistance remains undetermined. Work is ongoing within the consortium to unravel the association of these (and novel) mutations with resistance to the NRDs along with the relevant mechanisms of resistance.

Figure 5: Resistance to bedaquiline, clofazimine, delamanid and linezolid amongst *M. tuberculosis* CRYPTIC isolates a) The prevalence of resistance to bedaquiline (BDQ), clofazimine (CFZ), delamanid (DLM) and linezolid (LZD) per country or origin or collection site. Phylogenies are shown for isolates phenotypically resistant to b) BDQ, c) CFZ, d) DLM and e) LZD. Tip point colours denote lineage. Each outer track represents a gene thought to

be associated with resistance and coloured blocks denote the presence of a non-synonymous mutation in the relevant gene for a given isolate. Mutations in these genes that are either associated with sensitivity or present in >5% of the collection of isolates as a whole were ignored.

Case study on rifampicin mono-resistance

Around 1% of TB cases are rifampicin mono-resistant (RMR) and the frequency is increasing [1, 44]. The WHO does not recommend isoniazid for RMR treatment, despite it being effective; this is likely due to the reliance on the Xpert® MTB/RIF assay which cannot distinguish between RMR and MDR. Use of isoniazid could improve treatment outcomes for RMR patients which are currently similar to that of MDR TB, including a higher risk of death compared to drug susceptible infections [45, 46]. Due to its low natural prevalence, RMR has been poorly studied to date but increasingly large clinical TB datasets, such as the one presented here, make its study now feasible.

For this case study, we defined RMR as any isolate that was rifampicin resistant and isoniazid susceptible, discounting isolates with no definite phenotype for either isoniazid and/or rifampicin. Of the 4,655 rifampicin resistant isolates in the CRyPTIC database that also had a phenotype for isoniazid, 302 (6.5%) were RMR. These isolates were contributed by South Africa, Peru, India, Italy, China, Pakistan, Brazil, Vietnam, Germany, Nigeria, Turkmenistan and Tajikistan (Fig. 6a). South Africa and Nigeria contributed a significantly higher number of RMR isolates, as a proportion of rifampicin resistant isolates, than that of the total dataset at 17.5% ($p < 0.00001$) and 27.3% ($p = 0.00534$) respectively. We note that countries may have oversampled for solo resistance, but the higher contribution of RMR isolates from South Africa is consistent with previous studies [44].

RMR has evolved in all four major lineages, with L4 having a significantly higher RMR prevalence than that of the total rifampicin resistant dataset at 9.4% ($p = 0.00031$) and L2 significantly lower RMR prevalence at 4.2% ($p = 0.00026$). Although these numbers will have been somewhat influenced by differences in sample collection bias at different geographical sites, we would expect L2 to have a lower proportion of RMR isolates due to its association with MDR phenotypes [31, 47] (Fig. 6b).

Rifampicin mono-resistance is incorrectly predicted by current diagnostics

A widely used, WHO-endorsed diagnostic tool, the Xpert® MTB/RIF assay, uses a proxy whereby any SNP detected in the “rifampicin-resistance determining region” (RRDR) of *rpoB* results in a prediction of MDR. However, the suitability of the proxy is dependent upon prevalence of RMR in the population [44]. We tested the reliability of this on the 4,655 rifampicin resistant isolates in our dataset that had a phenotype for isoniazid (Fig. 6c).

Of these isolates, 4,353 (93.5%) were MDR and 302 (6.5%) were RMR. 187 of the MDR isolates had no RRDR mutation and therefore 4.0% of isolates in this study would be predicted as false negative MDR by the Xpert® MTB/RIF assay. 276 of the RMR isolates had a mutation in the RRDR of *rpoB* and so the Xpert® MTB/RIF assay proxy would incorrectly predict 5.9% of the rifampicin resistant isolates as false positive MDR cases. However, overall, the Xpert® MTB/RIF assay proxy correctly predicts 89.5% of the rifampicin resistant isolates as MDR and 0.6% of the isolates as non-MDR in this dataset, which suggests it is a reasonably successful diagnostic tool

with >90% accuracy for MDR classification of rifampicin resistant isolates. As most sites oversampled for resistance, our dataset likely contains a higher prevalence of RMR than the global average and hence the Xpert® MTB/RIF assay is likely to perform better on more representative data. However, the analysis shows how the increasing global levels of RMR TB cases could increase the number of false positive MDR diagnoses by the Xpert® MTB/RIF assay, denying isoniazid treatment to a greater number of patients who would then be moved on to less effective drugs.

There are genetic differences between rifampicin mono-resistant and multidrug resistant isolates

We have analysed our matched phenotypic and genotypic data to examine whether there were any differences in the genetic determinants of rifampicin resistance between RMR and MDR isolates. The proportion of RMR isolates with no *rpoB* mutation (5.3%, Fig. 6c) was significantly higher than that of MDR isolates (1.8%, $p < 0.00001$). This suggests that non-target-mediated resistance mechanisms, such as upregulation of rifampicin specific efflux pumps, could be more influential in providing protection against rifampicin in RMR isolates than in MDR isolates.

The majority of RMR and MDR isolates contained one or more SNPs in *rpoB*, with the majority having at least one mutation in the RRDR. To date, several non-synonymous RRDR mutations have been found in RMR *M. tuberculosis* isolates, including the resistance conferring mutations S450L, H445D and D435Y, which are also seen in MDR isolates [48, 49]. For both RMR and MDR isolates in this dataset, the most common *rpoB* RRDR mutation seen was S450L (63.6% and 41.1% of isolates respectively, Fig. 6d). Five mutations were present in RMR isolates that were

not seen in MDR isolates: S428G, S441A, S441V, S450M and S450Q, however these were seen at low prevalence (less than 2%) of RMR isolates. We found more RMR isolates had His445 mutated than MDR isolates (27.8% of RMR and 9.5% of MDR, $p < 0.00001$), and mutations to amino acids Ser450 and Asp435 were more prevalent in MDR isolates than RMR isolates (43.7% of RMR and 65.8% of MDR ($p < 0.00001$), and 9.3% of RMR and 15.5% of MDR ($p = 0.00328$) respectively).

Relatively little difference in the mutational profiles for *rpoB* mutations falling outside the RRDR was found; many RMR mutations were also seen in MDR isolates and those found exclusively in RMR were at <2% prevalence (Fig. S2). The most common non-RRDR mutation in both MDR and RMR isolates was a cytosine to thymine mutation 61 bases upstream of the *rpoB* start codon (10.1% and 8.6% of isolates respectively). The resistance conferring mutations, V695L and V170F, were seen at low proportions with no significant difference between MDR and RMR isolates (V695L was seen in 0.5% MDR isolates and 1.3% of RMR isolates, and V170F was seen in 0.8% of MDR isolates and 1.7% of RMR isolates).

Figure 6: Rifampicin mono resistance **a)** Percentage of rifampicin resistant isolates that are rifampicin mono-resistant (RMR) by country of isolate origin. * indicates RMR proportions that were significantly different from that of the total dataset using a two tailed z-test with 95% confidence. **b)** RMR prevalence by lineage. * indicates RMR proportions that were significantly different from that of the total dataset using a two tailed z-test with 95% confidence. **c)** MDR predictions for rifampicin resistant isolates made using the Xpert® MTB/RIF assay proxy. N is the total number of rifampicin resistant isolates. The inner ring shows the proportion of rifampicin resistant isolates that are MDR and RMR. The middle ring represents the proportions of MDR and RMR isolates that have a SNP (synonymous or non-synonymous) in the RRDR of *rpoB* (RRDR), no RRDR SNP but a SNP elsewhere in the *rpoB* gene (*rpoB*) and no *rpoB* mutations (none). The outer ring shows the expected true positive (TP), true negative (TN), false positive (FP) and false negative (FN) MDR predictions of Xpert® MTB/RIF assay, based on the SNPs present in the rifampicin resistant isolates. **d)** Non-synonymous mutations found in the RRDR of *rpoB* in RMR isolates and MDR isolates. Presence of a coloured spot indicates that the mutation was found in RMR/MDR isolates and spot size corresponds to the proportion of RMR or MDR isolates carrying that mutation.

Discussion

This compendium of *M. tuberculosis* clinical isolates is the result of an extensive global effort by the CRyPTIC consortium to better map the genetic variation associated with drug resistance. Through its sheer size and by oversampling for resistance, the compendium gives an unparalleled view of resistance patterns in the panel of 13 antitubercular compounds studied, providing more nuance to the traditional classification (nonMDR/MDR/XDR) nomenclature. These insights could be used to inform future treatment guidelines and steer the development of improved diagnostics.

For first-line drugs, molecular based diagnostic assays have vastly improved the detection of and the speed at which we find drug resistant TB cases, resulting in improved quality of care for patients. However, relying solely on these diagnostic methods has several drawbacks. Aside from the Xpert® MTB/RIF assay potentially increasing false positive MDR diagnoses as discussed earlier in the RMR case study, the assay assumes isoniazid resistance upon detection of rifampicin resistance. Thus, less is known about the prevalence of mono isoniazid resistance or 'true' cases of MDR (confirmed rifampicin *and* isoniazid resistance) (REFWHO). A greater amount of isoniazid-resistant and rifampicin-susceptible (Hr-TB) isolates were contributed by CRyPTIC countries than RMR isolates ($n = 1470$ versus $n = 302$), a pattern also recently observed in a global prevalence study [50]. A modified 6-month treatment regimen is now recommended for Hr-TB (rifampicin, ethambutol, levofloxacin and pyrazinamide), and as a result of inadequate diagnosis many of the 1.4 million global Hr-TB estimated cases would have received inadequate and unnecessarily longer treatment regimens [1, 50]. Encouragingly, CRyPTIC isolates with a Hr-TB background exhibited relatively low levels of resistance to other antitubercular drugs,

including those in the augmented regimen (Fig. 4c). However, without the tools appropriate to assess and survey this, we will continue to misdiagnose and infectively treat these clinical cases.

In 2018, CRyPTIC and the 100,000 Genomes project demonstrated using WGS that genotypic prediction correlates well with culture-based phenotype for first-line drugs, which is reflected in our summary of the genetic catalogue applied to this dataset (Table 3) [7]. While predictions can be made to a high level of sensitivity and specificity, there is still more to learn, as exemplified by the isolates in the compendium that despite being resistant to rifampicin and isoniazid could not be described genetically (Table 2). This shortfall, along with the limitations of molecular based diagnostic assays, highlights the need for continual genetic surveillance as drug resistant cases rise and shines a favourable light on a WGS-led approach.

One of the particular strengths of this compendium lies with the data collated for second-line drugs. A greater proportion of drug resistant isolates had additional resistance to fluoroquinolones than second line injectable drugs (Fig. 4a). This could be because of more widespread use of fluoroquinolones to treat bacterial infections in general as well as their ease of administration, hence being recommended over injectables for longer MDR treatment regimens [1]. Concerningly, we found that resistance to levofloxacin and moxifloxacin, and kanamycin and amikacin, were more common than resistance to the mycobacterial specific drug ethambutol in an isoniazid and rifampicin susceptible background (Fig. 4b) which suggests a level of pre-existing second line resistance. This concurs with a systematic review that found patients previously prescribed fluoroquinolones were 3 times more likely to have

fluoroquinolone resistant TB [51]. Careful stewardship of fluoroquinolones both in TB and other infectious diseases will be paramount for the success of treatment regimens. Despite the variability in sample collection, the observed high proportions of fluoroquinolone resistant MDR/RR isolates from some countries also suggests that MDR treatment regimens could be improved by optimisation on a geographic basis.

Further improvement could also be made by the selection of appropriate drugs from each class; for example, the WHO recommends switching from kanamycin to amikacin when treating MDR TB patients [52]. The compendium supports such recommendations as we saw more resistance to kanamycin than amikacin in not just MDR/RR but also all other phenotypic backgrounds. For fluoroquinolones, more isolates were resistant to levofloxacin than moxifloxacin in all phenotypic backgrounds suggesting moxifloxacin may be the most appropriate fluoroquinolone to recommend. However, the amenability of drugs to catalogue-based genetic diagnostics is also an important consideration, and our data suggest levofloxacin resistance could be predicted more reliably than moxifloxacin with fewer false positives predicted (Table 2).

Testing for fluoroquinolone resistance using molecular diagnostic tests remains limited. However, the limited global data from the past 15 years suggests that the proportion of MDR/RR TB cases resistant to fluoroquinolones sits at around 20%, with these cases primarily found in regions of high MDR-TB burden [1]. While tools such as the Cepheid Xpert® MTB/XDR cartridge, which has just been approved for use by the WHO, will permit both isoniazid and fluoroquinolone testing to be increased, the same pitfalls are to be encountered regarding targeted diagnostic assays [53]. In

contrast, the genetic survey in this study demonstrates the potential of WGS for genetic prediction of resistance to second-line drugs and studies within the consortium to investigate this are underway.

The CRyPTIC compendium has facilitated the first global survey of resistance to NRDs. Reassuringly, prevalence of resistance to the NRDs was lower than for first- and second-line agents in the dataset as a whole (Fig. 3a), and resistance to the new drugs bedaquiline and delamanid was less common than the repurposed drugs clofazimine and linezolid in an MDR/RR background (Fig. 4c). However, the presence of higher levels of delamanid and clofazimine resistance than ethambutol resistance in the isoniazid and rifampicin susceptible background does suggest some pre-existing propensity towards NRD resistance (Fig. 4b).

Co-resistance between all two-drug combinations of NRDs was seen in isolates in the compendium, the most common being isolates resistant to both bedaquiline and clofazimine. This link is well documented and has been attributed to shared resistance mechanisms such as non-synonymous mutations in *rv0678* which were found in both clofazimine and bedaquiline resistant isolates in the compendium [42] (Fig. 5b,c). Increased clofazimine use could further increase the prevalence of *M. tuberculosis* isolates with clofazimine and bedaquiline co-resistance, limiting MDR treatment options such as using bedaquiline as the backbone of a shorter MDR regimen [54]. Therefore, proposed usage of clofazimine for other infectious diseases, including COVID-19, should be carefully considered [55].

The WHO recommends not using bedaquiline and delamanid in combination to prevent the development of co-resistance, which could occur relatively quickly [56]; the rates of spontaneous evolution of delamanid resistance *in vitro* has been shown to be comparable to that of isoniazid, and likewise bedaquiline resistance arises at a comparable rate to rifampicin resistance [57]. In this compendium, 12.9% bedaquiline resistant isolates were resistant to delamanid and 7.1% delamanid resistant isolates were resistant to bedaquiline. Several scenarios could account for this, including the presence of shared resistance mechanisms. For example, as bedaquiline targets energy metabolism within the cell, changes to cope with energy/nutrient imbalances upon the acquisition of resistance-associated ATPase pump mutations may result in cross resistance to delamanid in a yet unknown or unexplored mechanism [58]. Additionally, off-target mutations in the *mmpI3* gene (involved in export), are associated with resistance to delamanid, bedaquiline, clofazimine and linezolid, and were seen in isolates resistant to each of these drugs, although we note this could be artefactual (Fig. 5b-e). In any case, it is imperative that genetic determinants of resistance are fully explored for our last resort NRDs, with special attention given to those mechanisms that could be shared with other agents. In the meantime, careful stewardship and phenotypic and genotypic surveillance of the NRDs should be implemented, including linezolid and clofazimine which are now group A and B drugs respectively for MDR treatment [1].

Several research avenues are being actively explored by the CRyPTIC consortium that make use of this compendium, including: *i*) relating genetic mutations to quantitative changes in the minimum inhibitory concentrations of different drugs [58]; *ii*) genome-wide association studies [59]; *iii*) training machine learning models

that can predict resistance [60]; *iv*) exploration of the genetic determinants of resistance to second line and NRDs [61]. Collectively these studies share the same aim of facilitating the implementation of WGS-directed resistance prediction in the clinic. Finally, we urge other researchers to explore this large dataset of *M. tuberculosis* clinical isolates and hope it will lead to a wave of new and inciteful studies that will positively serve the TB community for years to come.

Author contributions

D.W.C, T.E.A.P, S.H, A.L.G.C, A.W.S, T.M.W, P.W.F, D.M.C designed the CRyPTIC study and all contributing laboratories collected samples and provided data. MIC data and genetic information was retrieved and processed by P.W.F, S.H, A.L.G.C, Z.I, M.H and J.K. A.B and K.M.M designed and performed all analyses for this manuscript. A.B and K.M.M wrote the manuscript with feedback from CRyPTIC partners.

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Competing Interest

E.R. is employed by Public Health England and holds an honorary contract with Imperial College London. I.F.L. is Director of the Scottish Mycobacteria Reference Laboratory. S.N. receives funding from German Center for Infection Research, Excellenz Cluster Precision Medicine in Chronic Inflammation, Leibniz Science Campus Evolutionary Medicine of the LUNG (EvoLUNG)tion EXC 2167. P.S. is a consultant at Genoscreen. T.R. is funded by NIH and DoD and receives salary support from the non-profit organization FIND. T.R. is a co-founder, board member and shareholder of Verus Diagnostics Inc, a company that was founded with the intent of developing diagnostic assays. Verus Diagnostics was not involved in any way with data collection, analysis or publication of the results. T.R. has not received any financial support from Verus Diagnostics. UCSD Conflict of Interest office has reviewed and approved T.R.'s role in Verus Diagnostics Inc. T.R. is a co-inventor of a provisional patent for a TB diagnostic assay (provisional patent #: 63/048.989). T.R. is a co-inventor on a patent associated with the processing of TB sequencing data (European Patent Application No. 14840432.0 & USSN 14/912,918). T.R. has agreed to "donate all present and future interest in and rights to royalties from this patent" to UCSD to ensure that he does not receive any financial benefits from this patent. S.S. is working and holding ESOPs at HaystackAnalytics Pvt. Ltd. (Product: Using whole genome sequencing for drug susceptibility testing for *Mycobacterium tuberculosis*). G.F.G. is listed as an inventor on patent applications for RBD-dimer-based CoV vaccines. The patents for RBD-dimers as protein subunit vaccines for SARS-CoV-2 have been licensed to Anhui Zhifei Longcom Biopharmaceutical Co. Ltd, China.

1002 Supplemental

Figure S1: Per drug MIC distributions of isolates plated on CRYPTIC designed variations on the Thermo Fischer Sensititre MYCOTB MIC plate; UKMYC5 (a) and UKMYC6 (b). The solid black line represents the ecological resistance cut-off (ECOFF) for each drug as determined by [11]. Isolates with an MIC above the cut-off are considered resistant. N denotes the total number of isolates tested on each plate that returned a phenotype for each drug.

UNIQUEID	COUNTRY OF ORIGIN	LINEAGE
site.11.subj.XTB-18-224.lab.XTB-18-224.iso.1	UNKNOWN	Lineage 2
site.10.subj.YA00026182.lab.YA00026182.iso.1	ZAF	Lineage 4

Table S1: Sample information for isolates classified as resistant to all 13 CRYPTIC drugs tested. The country of origin is specified using the 3-letter country codes (alpha-3) defined by ISO 3166-1.

Figure S2: Non-synonymous mutations found outside the RRDR of *rpoB* in RMR isolates and MDR isolates. Presence of a coloured spot indicates that the mutation was found in RMR/MDR isolates and spot size corresponds to the proportion of RMR or MDR isolates carrying that mutation.

	Animal/ other	L1	L2	L3	L4	L6	Total
Albania	0	0	0	0	7	0	7
Algeria	0	0	0	0	25	0	25
Brazil	1	1	8	0	335	0	345
Burkina Faso	0	3	1	8	65	1	78
China	7	2	722	16	374	0	1121
Germany	1	25	235	61	403	1	726
India	11	676	1573	1184	560	0	4004
Italy	11	38	192	113	866	4	1224
Japan	0	0	0	0	1	0	1
Kyrgyzstan	0	0	25	0	3	0	28
Nepal	0	5	137	34	21	0	197
Nigeria	0	0	0	0	15	0	15
Pakistan	1	30	23	363	74	0	491
Peru	2	1	360	0	2328	0	2691
South Africa	1	61	874	57	1162	0	2155
Sweden	0	7	280	6	134	0	427
Taiwan	0	18	149	0	44	0	211
Tajikistan	0	0	15	0	4	0	19
Tunisia	0	0	0	0	2	0	2
Turkmenistan	0	0	88	1	30	0	119
Ukraine	0	0	21	0	9	0	30
UK	0	0	3	3	1	0	7
Vietnam	0	283	892	4	109	0	1288
Total	35	1150	5598	1850	6572	6	15211

Table S2: Lineages –v- geographical location of origin/contribution for CRyPTIC isolates

ALB ALG BRZ BFA CHN GER IND ITL JPN KGZ NPL NGA PAK PER ZAF SWE TWN TJK TUN TKM UKR UK VNM

1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1.1.1	0	0	0	2	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	38
1.1.1.1	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	188
1.1.2	0	0	0	1	1	12	316	16	0	0	2	0	23	0	0	1	0	0	0	0	0	0
1.1.3	0	0	1	0	0	1	52	4	0	0	1	0	1	0	6	0	0	0	0	0	0	2
1.2.1	0	0	0	0	0	8	12	11	0	0	0	0	0	1	0	3	16	0	0	0	0	24
1.2.2	0	0	0	0	1	2	259	5	0	0	2	0	4	0	54	3	1	0	0	0	0	1
2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2.1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	20	0	0	0	0	34
2.2	0	0	5	1	284	19	272	14	0	0	68	0	6	231	181	13	56	0	0	0	0	271
2.2.1	0	0	0	0	37	4	2	2	0	0	0	0	0	3	224	1	21	0	0	0	0	26
2.2.10	0	0	0	0	1	117	1	84	0	16	0	0	3	0	0	70	0	5	0	75	9	1
2.2.2	0	0	0	0	32	8	5	11	0	1	0	0	1	0	0	1	5	0	0	0	1	8
2.2.3	0	0	2	0	172	3	290	4	0	0	21	0	2	1	3	1	10	0	0	0	0	100
2.2.4	0	0	0	0	3	3	4	8	0	0	0	0	0	0	37	1	1	0	0	0	0	70
2.2.5	0	0	0	0	59	1	12	5	0	0	0	0	2	84	1	1	13	0	0	0	0	45
2.2.6	0	0	1	0	22	8	226	2	0	0	9	0	7	4	204	1	4	0	0	0	0	154
2.2.7	0	0	0	0	97	4	691	5	0	1	38	0	0	20	206	5	18	0	0	0	0	164
2.2.8	0	0	0	0	4	0	42	0	0	0	1	0	2	0	0	0	0	0	0	0	0	2
2.2.9	0	0	0	0	0	64	0	51	0	6	0	0	0	2	0	174	0	10	0	11	11	2
3	0	0	0	8	15	37	828	81	0	0	28	0	346	0	34	5	0	0	0	1	0	2
3.1.1	0	0	0	0	0	17	17	25	0	0	1	0	0	0	19	1	0	0	0	0	0	1
3.1.2	0	0	0	0	1	5	201	3	0	0	1	0	7	0	2	0	0	0	0	0	0	0
3.1.2.1	0	0	0	0	0	2	119	3	0	0	4	0	6	0	0	0	0	0	0	0	0	0
4	0	0	1	0	6	4	0	6	0	0	0	0	0	100	1	1	0	0	0	1	0	0
4.1	0	0	0	1	0	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4.1.1	0	0	0	0	0	6	5	10	0	0	1	0	0	154	13	1	0	0	0	0	0	0
4.1.1.1	0	0	5	0	1	3	20	5	0	0	1	0	2	5	54	1	0	0	0	0	0	1
4.1.1.2	0	0	0	0	0	0	4	1	0	0	0	0	0	0	6	0	0	0	0	0	0	0
4.1.1.3	0	0	1	6	1	0	30	7	1	0	3	0	2	68	138	0	0	0	0	0	0	1
4.1.2	0	0	13	0	0	15	3	26	0	1	0	0	0	31	43	0	0	0	0	0	0	10
4.1.2.1	1	8	87	10	0	111	56	183	0	0	3	2	4	653	54	5	0	1	0	0	1	17
4.1.3	0	0	0	5	0	0	0	6	0	0	0	1	0	0	0	0	0	0	0	0	0	0
4.10	4	6	35	5	1	110	166	207	0	1	3	0	28	128	170	4	5	2	1	6	2	12
4.2	0	0	0	0	3	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4.2.1	0	0	0	0	0	9	3	46	0	0	1	0	2	0	2	4	0	0	0	8	3	1
4.2.2	0	1	0	0	53	31	76	51	0	0	5	0	12	2	10	10	1	0	0	0	0	11
4.2.2.1	0	0	0	0	0	5	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4.3	0	0	17	0	0	0	0	1	0	0	0	0	0	96	1	0	0	0	0	0	0	0
4.3.1	0	3	0	1	0	1	2	21	0	0	0	0	0	8	0	1	0	0	0	0	0	1
4.3.2	0	1	10	0	0	3	1	8	0	0	0	0	0	245	21	0	0	0	0	0	0	0
4.3.2.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	234	0	0	0	0	0	0	0
4.3.3	1	4	34	2	0	33	99	81	0	1	1	0	0	576	120	102	2	1	1	11	2	1
4.3.4	0	0	1	0	0	0	8	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
4.3.4.1	0	0	70	0	0	2	6	3	0	0	0	0	0	60	22	2	1	0	0	0	0	0
4.3.4.2	0	0	38	0	0	6	7	27	0	0	0	0	0	132	12	0	0	0	0	0	1	0
4.3.4.2.1	0	0	0	0	0	2	1	1	0	0	0	0	0	0	75	0	0	0	0	0	0	0
4.4	0	0	0	0	0	0	13	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
4.4.1	0	0	10	0	0	0	0	2	0	0	0	0	0	11	0	0	0	0	0	0	0	0
4.4.1.1	0	0	4	0	0	16	4	66	0	0	1	0	0	22	180	2	0	0	0	0	0	4
4.4.1.2	0	0	0	0	0	1	25	3	0	0	0	0	0	2	0	0	0	0	0	0	0	3
4.4.2	0	0	0	0	124	1	4	1	0	0	0	0	0	4	0	0	5	0	0	0	0	16
4.5	0	0	0	0	179	28	15	14	0	0	1	0	18	5	1	0	30	0	0	3	0	29
4.6	0	1	0	1	0	1	1	7	0	0	0	2	0	0	0	0	0	0	0	0	0	0
4.6.1.1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4.6.1.2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
4.6.2	0	0	0	0	0	2	2	14	0	0	0	0	4	0	0	0	0	0	0	0	0	0
4.6.2.1	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0
4.6.2.2	1	0	0	34	0	8	1	55	0	0	0	10	1	0	1	0	0	0	0	0	0	3
6	0	0	0	1	0	1	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Bovis	0	0	0	0	0	1	1	10	0	0	0	0	0	2	1	0	0	0	0	0	0	0
Caprae	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mixed	0	1	9	0	16	3	89	9	0	1	0	0	6	38	25	12	2	0	0	3	0	48
Unknown	0	0	1	0	7	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0

Table S3: Sub-lineages –v- geographical location of origin/contribution for CRYPTIC isolates

Figure S3: A significant association between country and lineage can be seen in the CRYPTIC data. Pearson's chi-squared test, X-squared = 7935.2, df = 110, $p < 2.2e-16$. The correlation plot indicates the relative contribution of each row-column pairing to the chi-square test score (%).

1047

	INH	RIF	EMB	LEV	MXF	AMI	KAN	BDQ	CFZ	DLM	LZD	ETH	RFB
INH	100.0	74.8	38.0	34.0	27.8	14.0	17.0	1.5	5.5	1.7	2.0	28.2	70.4
RIF	93.5	100.0	46.3	41.4	34.1	17.2	20.8	1.8	6.0	1.7	2.3	29.4	91.3
EMB	98.5	95.9	100.0	53.9	47.1	23.3	26.4	2.4	8.2	2.3	3.7	35.8	87.3
LEV	93.3	90.2	56.9	100.0	78.5	27.3	31.3	3.1	9.4	2.9	4.7	39.2	87.1
MXF	95.0	92.3	61.9	97.6	100.0	29.9	34.6	3.2	9.9	2.7	5.3	41.9	88.8
AMI	93.0	90.4	58.9	65.3	57.8	100.0	90.4	2.4	13.0	5.2	8.1	40.7	82.8
KAN	89.4	86.7	53.0	59.5	53.2	72.0	100.0	1.8	10.6	3.2	5.7	40.4	80.0
BDQ	79.4	77.8	49.5	60.7	49.5	20.0	18.9	100.0	52.4	12.9	14.0	34.9	76.9
CFZ	61.9	52.8	34.9	38.2	32.1	21.8	22.5	10.6	100.0	9.4	10.2	27.0	53.7
DLM	55.2	44.4	28.2	33.2	23.9	24.4	19.1	7.1	26.3	100.0	19.3	22.1	45.4
LZD	77.6	69.9	53.2	64.7	58.7	46.7	41.2	9.8	34.2	24.1	100.0	42.6	66.7
ETH	96.5	79.4	47.0	48.7	41.9	21.1	26.2	2.2	8.3	2.4	3.9	100.0	77.1
RFB	93.3	96.8	44.5	42.1	34.4	16.6	20.3	1.9	6.4	1.9	2.3	29.9	100.0

1048 **Table S4: Co-occurrence of antibiotic resistance in CRyPTIC *M. tuberculosis***
1049 **isolates.** The probability (%) of an isolate being resistant to Drug 2 (top) if it is resistant to Drug
1050 1 (left). Drug acronyms: INH = isoniazid, RIF = rifampicin, EMB = ethambutol, LEV = levofloxacin,
1051 MXF = moxifloxacin, AMI = amikacin, KAN = kanamycin, BDQ = bedaquiline, CFZ = clofazimine,
1052 DLM = delamanid, LZD = linezolid, ETH = ethionamide, RFB = rifabutin.

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