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SHORT COMMUNICATION

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Minimum inhibitory concentration distributions for first- and second-line antimicrobials against *Mycobacterium tuberculosis*

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Abstract

We report the range of minimum inhibitory concentrations for six antimicrobial drugs in 228 clinical *Mycobacterium tuberculosis* (MTB) isolates from three distinct groups of patients (unselected patients, patients at high risk of drug-resistant TB and HIV-positive patients) in Lima, Peru. These data highlight the challenges of and discriminatory characteristics required for MTB drug susceptibility testing.

Development of accurate, timely and straightforward methods for drug susceptibility testing (DST) of *Mycobacterium tuberculosis* (MTB) is crucial in order to detect, appropriately treat, and tackle the spread of drug-resistant tuberculosis. Clearly distinguishing susceptible from resistant isolates can be fraught with difficulty. The World Health Organization (WHO) has provided guidance on 'critical concentrations' of various anti-tuberculous drugs, but much of this is based on work from the 1960s and may not accurately represent the most clinically useful or microbiologically logical breakpoints [1–5].

Understanding the distribution of minimum inhibitory concentrations (MICs) amongst MTB isolates circulating in a cross-section of patient groups is important both to be able to interpret traditional DST – where this is available – and also to inform the development of rapid DST assays. In this short communication, we present MIC distribution data for six drugs (first and second line) against MTB isolates from three groups of patients in Lima, Peru and illustrate where the WHO critical concentrations lie in the population distribution. MICs were calculated using the microplate alamarBlue assay (MABA), an indirect colorimetric method using Middlebrook 7H9 broth.

This analysis uses isolates from a previous study evaluating the microscopic observation drug susceptibility (MODS) assay for rapid detection of MTB and multidrug-resistant TB (MDR-TB); that previous study is more fully described elsewhere [6]. MTB isolates were harvested from sputum cultures positive for *M. tuberculosis* by the MODS assay.

The MABA DST method is more fully described elsewhere [7]. Briefly, 100 µl of serial 1 : 2 dilutions of the six drugs tested mixed in Middlebrook 7H9-oleic acid-albumin-dextrose-catalase broth were prepared in a 96-well plate. MTB suspensions at a McFarland standard of 1 were diluted 1 : 25 in Middlebrook 7H9-oleic acid-albumin-dextrose-catalase broth and 100 µl of the MTB containing broth was added to the drug-containing broth. A drug-free (inoculum only) control well was also prepared. The final drug concentration ranges were as follows: isoniazid, 0.125 to 32.0 µg ml^{-1}; rifampacin, 0.063 to 16 µg ml^{-1}; streptomycin, 0.125 to 32.0 µg ml^{-1}; ethambutol, 0.5 to 128 µg ml^{-1}; capreomycin, 0.031 to 8 µg ml^{-1}; and ciprofloxacin, 0.063 to 16 µg ml^{-1}. Plates were sealed in individual ziplock bags and incubated at 37 °C for five days; after five days control wells were examined under an inverted light microscope daily for evidence of growth. If growth was observed in a control well, a freshly prepared 50 µl 1 : 1 mixture of alamarBlue (Trek Diagnostic Systems, OH) and 10 % Tween 80 was added to this well. Plates were reincubated for 24 h, and if a control well turned pink, the reagent mixture was added to all wells. The plate was resealed and incubated for an additional 24 h at 37 °C, after which all well colours were recorded. Blue was interpreted to indicate no growth, and pink was interpreted to indicate growth. The MIC was defined as the lowest drug concentration that prevented a blue-to-pink colour change (indicating inhibition of growth). There was one replicate only per isolate. Data were analysed in Stata 11 (Statacorp) and histograms plotted to graphically display the range of MICs. χ^2 tests were used to compare the

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Abbreviations: DST, drug sensitivity testing; EUCAST, European committee on antimicrobial sensitivity testing; MABA, microplate alamar Blue assay; MDR-TB, multi-drug resistant tuberculosis; MIC, minimum inhibitory concentration; MODS, microscopic observation drug susceptibility; MTB, *Mycobacterium tuberculosis*; WHO, World Health Organization.

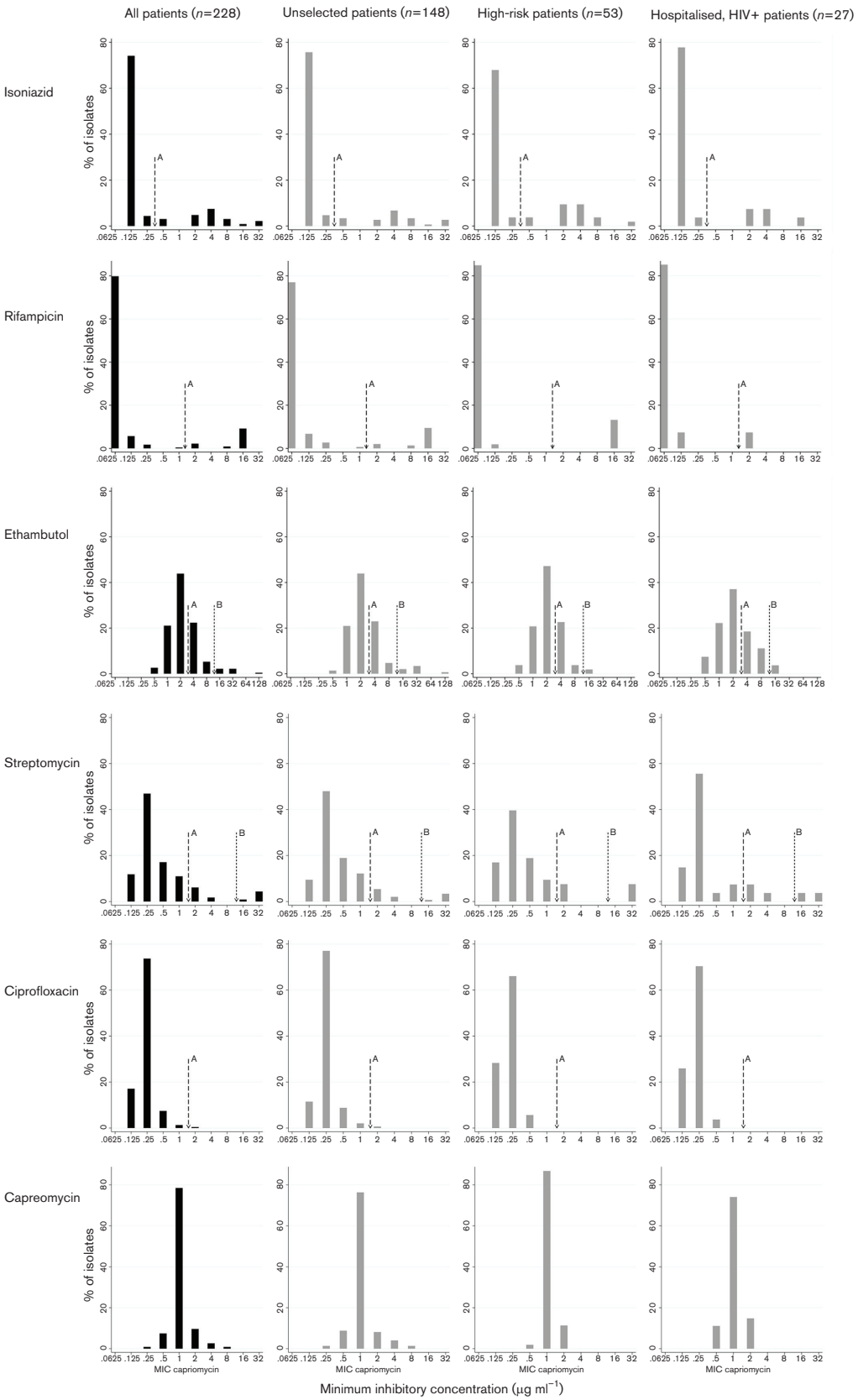

Fig. 1. Percentage distribution plot of MICs of isoniazid, rifampicin, ethambutol, streptomycin, ciprofloxacin and capreomycin against MTB isolates. The first column (black bars) shows MICs for all isolates. Subsequent columns (dark grey bars) show MICs from an

unselected population, a population of perceived higher risk for MDR-TB, and a population of HIV-positive hospitalized patients. Arrows 'A' represent suggested breakpoints for resistance, and arrows 'B' for higher-level resistance (where applicable).

proportions of isolates that were resistant, with the unselected patient group used as the baseline for comparison.

Sputum samples from 1975 different patients were collected between April 2003 and March 2005. Overall, 235/1975 (12 %) were positive for MTB by one or more of Lowenstien–Jensen culture, MBBact automated system (bioMérieux) or MODS. Altogether, 228/235 (97 %) yielded a suitable isolate from MODS for MABA DST. A total of 148 patients were from an unselected population presenting to the National TB Control Programme in 10 clinics in North Lima for investigation of possible TB. There were 53 patients presenting to five clinics in East Lima who had one or more risk factors for drug-resistant TB (previous TB treatment, HIV positive, previous incarceration, contact with TB patient, healthcare or prison worker, hospitalization in the past year). Finally, 27 isolates were from hospitalized HIV-positive patients.

Fig. 1 shows the distribution of MICs of isoniazid, rifampicin, streptomycin, ethambutol, ciprofloxacin and capreomycin by MABA and breakpoints. The MABA breakpoints are from earlier work, maximizing agreement with a BACTEC 460 assay and using WHO-suggested critical concentrations [7–9]. For capreomycin there is no accepted consensus breakpoint [10]. Ciprofloxacin is no longer recommended as a treatment for TB, although sensitivity to ciprofloxacin implies sensitivity to second-generation fluoroquinolones.

Overall, 29/148 (20 %), 15/53 (28 %) and 6527 (19 %) of isolates in unselected, high-risk and HIV-positive hospitalized groups, respectively, had MICs $\geq 0.5\,\mu g\,ml^{-1}$ for isoniazid; these differences did not reach statistical significance (P=0.19 comparing high-risk vs unselected and 0.9 comparing HIV positive vs unselected). Altogether, 19/148 (13 %), 7/53 (13 %) and 2/27 (7 %) of patients in unselected, high-risk and hospitalized groups had MICs $\geq 2\,\mu g\,ml^{-1}$ for rifampicin; again this was not statistically significant (P=0.95 comparing high-risk vs unselected and 0.42 comparing HIV-positive vs unselected). All but one rifampicin-resistant isolates were also isoniazid resistant (i.e. there were 27 MDR-TB isolates). For ethambutol and streptomycin, critical concentrations lie in the middle of the frequency distribution in all groups in this analysis, highlighting why DST for these agents is regarded as less reliable and is associated with significant inter-laboratory variability [11, 12]. One isolate (unselected group) was resistant to ciprofloxacin.

There is a paucity of published information on the range of MICs to first- and second-line anti-TB drugs, and most studies report small sample numbers [12–14], which contributes to the struggle for consensus about breakpoints for DST. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) does not publish recommendations for mycobacteria breakpoints for most drugs [15]. WHO has suggested 'critical concentrations' of various TB drugs, but this relies on work on small samples dating back to 1963 and it is unclear whether these critical concentrations are clinically representative of drug concentrations likely to be found at respiratory epithelial linings *in vivo* based on pharmacokinetic data [1, 2]. Our data from 228 isolates provide valuable information about the range of MICs in circulating strains of MTB and will help elucidate rational DST cut-off points for existing and newer DST methods.

This study used phenotypic resistance profiles. This is the gold standard for DST, and the only feasible method for many second-line drugs for which knowledge of resistance-conferring genomic mutations is incomplete. A limitation of this analysis is the lack of genetic mutation data, so it was not possible to map MICs to strains with or without known resistance genes. Each MTB isolate was from a different patient, but we do not have genetic proof that all isolates are unique. A further limitation is that all strains were from a single city in Peru, and though the phylogenetic diversity is known [16], there is not global representation of all MTB families. To put this study into context, Peru in 2003–2005 was considered to have a growing MDR-TB problem. Since 2005, MDR-TB case notifications in Peru have remained high but there is now increased coverage of DST and increased treatment success for MDR-TB cases [17].

Distinguishing highly drug-susceptible and highly drug-resistant strains is the low-hanging fruit of DST; the challenge is in correctly separating the more borderline strains. Rational development and validation of novel phenotypic DSTs requires understanding of the distribution of MICs within the circulating population of MTB isolates. Clinicians, researchers and laboratory scientists diagnosing and treating people with possible drug-resistant TB should be aware of the inherent difficulties of using cut-offs to create binary categories from continuous and overlapping distributions. Where resources permit, clinicians should seek expert advice in clinical interpretation of DST results to guide treatment decisions. We believe these data will be of use to researchers and clinicians in order to better understand what they are trying to distinguish between when dividing DST results into binary categories of susceptible versus resistant [1].

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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