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Improved Bactec MGIT 960 Pyrazinamide Test Decreases Detection of False *Mycobacterium tuberculosis* Pyrazinamide Resistance

Running title: A modified MGIT 960 test to decrease PZA resistance

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Pyrazinamide (PZA) is a key drug for the treatment of tuberculosis (TB). Resistance to PZA is mostly caused by mutations in the *pncA* gene encoding pyrazinamidase, which converts the prodrug PZA to the active form pyrazinoic acid [1-2]. For testing PZA susceptibility, the World Health Organization (WHO) recommends to perform the assay in liquid medium at pH 5.9 in the BACTEC MGIT 960 (M960) system (Becton Dickinson, Sparks, MD, USA) [3]. However, false resistances to PZA were reported for this phenotypic assay [4-7], due to high *Mycobacterium tuberculosis* inoculum that may impair pyrazinamidase activity by increasing the pH of the medium [8]. Indeed, a reduced M960 inoculum decreased detection of false resistances [9-10] when results were compared with the previous reference radiometric method BACTEC 460 (Becton Dickinson) [11] and with *pncA* sequencing, a method providing from 83% to 90% sensitivity [2, 12-13]. Since 2013, the WHO has been yearly offering to the global Supranational Reference Laboratory (SRL) network proficiency test panels of *M. tuberculosis* strains for PZA drug susceptibility testing (DST).

At the SRL in Rome, the PZA M960 assay is performed according to the manufacturer's instructions [14], with minor modifications. Briefly, a positive MGIT tube obtained 1 or 2 days after the positivity signal of the M960 instrument (seed tube) is vortexed for 30 seconds, and then allowed to settle for 20 to 30 minutes. Thereafter, 1 ml aliquot of the settled seed tube is taken with a 1-ml pipet from the top surface, instead of the lower down. Of this aliquot, 0.5 ml is transferred to the PZA test tube containing 100 µg/ml of PZA and 0.5 ml to a tube containing 4.5 ml of sterile saline. This 1:10 dilution tube is repeatedly mixed by a new pipet, and 0.5 ml is used to inoculate the tube without PZA (growth control tube). After inversion, both growth control and PZA test tubes are incubated in the M960 instrument. The seed tube is used firstly for PZA DST and then for other drugs.

In 2013-2016, the SRL tested PZA susceptibility of 106 WHO *M. tuberculosis* strains (41 resistant and 65 susceptible) with known *pncA* mutations. Using the modified M960 PZA assay (MMPA), 1/106 strain was false-resistant (0.9%) and no strain was false-susceptible. In Italy, the SRL coordinates a laboratory network (SMIRA: Italian Multicentre Study on Resistance to

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Antituberculosis Drugs) periodically examined by first- and second-line drug proficiency testing exercises [15-16]. In 2016, 17 SMIRA-laboratories performed in parallel the standard M960 PZA assay [14] and the MMPA on 10 *M. tuberculosis* strains from the 21st WHO round (4 resistant and 6 susceptible). Out of a total of 170 strains (68 resistant and 102 susceptible) examined by each of the two methods, 8/170 showed false-resistance by the standard assay (4.7%) and 2/170 false-resistance by MMPA (1.2%); no strain was false-susceptible. Overall, these observations suggest that the MMPA performed by withdrawing inoculum from the top surface of the settled MGIT 960 seed tubes may be useful to decrease false phenotypic PZA resistance.

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