A New Approach for Pyrazinamide Susceptibility Testing in *Mycobacterium tuberculosis*

Mirko Zimic,¹ Sebastian Loli,¹ Robert H. Gilman,^{1,2} Andrés Gutierrez,¹
Patricia Fuentes,¹ Milagros Cotrina,¹ Daniela Kirwan,³ and Patricia Sheen¹

Background: Pyrazinamide (PZA) is an important drug in the treatment of tuberculosis. Microbiological methods of PZA susceptibility testing are controversial and have low reproducibility. After conversion of PZA into pyrazinoic acid (POA) by the bacterial pyrazinamidase enzyme, the drug is expelled from the bacteria by an efflux pump. **Objective:** To evaluate the rate of POA extrusion from *Mycobacterium tuberculosis* as a parameter to detect PZA resistance. **Methods:** The rate of POA extrusion and PZA susceptibility determined by BACTEC 460 were measured for 34 strains in a previous study. PZA resistance was modeled in a logistic regression with the pyrazinoic efflux rate. **Result:** POA efflux rate predicted PZA resistance with 70.83%–92.85% sensitivity and 100% specificity compared with BACTEC 460. **Conclusion:** POA efflux rate could be a useful tool for predicting PZA resistance in *M. tuberculosis*. Further exploration of this approach may lead to the development of new tools for diagnosing PZA resistance, which may be of public health importance.

Introduction

TUBERCULOSIS (TB) kills 1.7 million people annually,⁴ and TB control efforts are increasingly hindered by antibiotic resistance.¹¹ Pyrazinamide (PZA) is an important drug in TB management: It is the most effective drug against semi-dormant *Mycobacterium tuberculosis* bacilli residing in acidic regions of acute inflammation that are inaccessible to other anti-TB drugs. Its incorporation in drug regimens permits faster elimination, and, thus, reduction of treatment duration.^{5,6,8,14} Although PZA resistance is common in endemic countries,¹⁶ the lack of reliable culture-based resistance tests and the high cost and modest accuracy of molecular methods means that testing is not generally performed. Consequently, patients with PZA-resistant TB are frequently treated inappropriately with PZA. The frequency and significance of undetected PZA resistance to the success of treatment regimens is unknown. The emergence of PZA-resistant strains could present a significant public health problem, as both first- and second-line treatment regimens include PZA.

PZA requires an acidic environment to function, making resistance testing difficult.⁵ As such, it has often been excluded from efforts to develop tools to detect drug resistance.² Since testing is not routinely performed, the prevalence of PZA resistance is not well known; however,

surveillance studies have reported prevalence of 52%–78% in treatment failure or MDR-TB cases, and 2%–10% in non-MDR-TB cases.^{1,7,9,10}

PZA is a pro-drug that enters bacteria by passive diffusion and is converted to its active form, pyrazinoic acid (POA), by bacterial pyrazinamidase (PZAse).^{6,12,19} POA is expelled from the mycobacterium by an efflux pump, it is protonated in the acidic extracellular space, and the protonated form is then reabsorbed into the bacilli, where the proton is released. This cycle is repeated, resulting in a lethal combination of POA accumulation and intracellular acidification, causing disruption of membrane permeability and cellular damage.^{3,17,19}

The major mechanism of PZA resistance in *M. tuberculosis* arises from mutations in the PZAse, resulting in a loss of enzymatic activity, preventing conversion of PZA to POA.¹⁷ The same mechanism is responsible for the natural resistance to PZA of *Mycobacterium bovis* and *Mycobacterium kansasii*.^{6,12,15,18} However, these mutations cannot fully account for *M. tuberculosis* PZA resistance. Variations in the rate of bacterial POA expulsion (POA efflux) are implicated in PZA resistance^{15,18,20}; these variations may depend on PZAse activity levels, intracellular PZAse concentrations, and POA efflux pump efficiency. *Mycobacterium smegmatis*, naturally PZA resistant, has a much more active POA efflux

¹Unidad de Bioinformática y Biología Molecular, Laboratorios de Investigación y Desarrollo, Facultad de Ciencias y Filosofía, Universidad Peruana Cayetano Heredia, Lima, Peru.

²Department of International Health, Johns Hopkins Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland.

³Department of Infectious Diseases and Immunity, Imperial College London, London, United Kingdom.

mechanism than *M. tuberculosis*, which prevents intracellular POA accumulation.^{15,18,19}

M. tuberculosis strains exhibit a range of POA efflux rates, associated with different PZA-susceptibility levels. Recently, POA efflux rates from susceptible *M. tuberculosis* strains were found to fall within a critical range, with strains with rates below this range showing resistance.²⁰ The POA efflux rate accounted for 61% of the variability in BACTEC460 PZA susceptibility and 51% of the BACTEC460 growth index. POA efflux rates were able to account for differences in BACTEC460 susceptibility to a greater extent than the detection of PZAse mutations.

Based on these results, it was hypothesized that measurement of the POA efflux rate could lead to the development of a novel test for PZA resistance. The present study aims at evaluating the accuracy of POA efflux rate as a predictor of PZA resistance as determined by BACTEC460.

Materials and Methods

Thirty-four *M. tuberculosis* clinical isolates, including the PZA-susceptible reference strain H37Rv, were analyzed in a previous study¹³ from which data concerning PZA susceptibility based on BACTEC460 were available.

BACTEC460 was performed at pH 6.0 using 100 µg/ml PZA. The resistance level was estimated using the ratio of the radioactive growth index of the culture media containing bacteria and PZA to the radioactive growth index of the control (media without PZA), expressed as a percentage (Table 1). PZA-resistant strains were classified according to the BACTEC growth index. Two classifications were used for considering PZA resistance: a growth index greater than 11% (Bactec11) and greater than 20% (Bactec20).

In a previous study,²⁰ the POA efflux rate was measured for each strain. Briefly, bacteria were incubated at 37°C for 12, 24, 36, and 48 hr with 1 mM of PZA. Samples were centrifuged, and the supernatants were recovered. POA in the extracellular environment was quantified by Wayne reaction and fitted to a linear regression in time. The POA efflux rate was estimated using the readings until 48 hr [$V_{\text{POA}}(48\text{h})$]. We extended the previous study by estimating the POA efflux rate using only the reading at 12 hr (Table 1).

BACTEC460 susceptibility (BACTEC11 and BACTEC20) was modeled in a logistic regression with the POA efflux rate [$V_{\text{POA}}(48\text{h})$ and $V_{\text{POA}}(12\text{h})$] as the predictor covariate. Sensitivity and specificity were estimated for different probability cutoffs.

Results

The POA-efflux rate estimated with a single reading at 12 hr [$V_{\text{POA}}(12\text{h})$] was highly correlated with the corresponding POA-efflux rate estimated with readings at 12, 24, 36, and 48 hr [$V_{\text{POA}}(48\text{h})$], with a Pearson correlation coefficient of 0.94 ($p < 0.0001$) (Fig. 1).

The POA efflux rate at 12 hr was significantly associated with BACTEC460 susceptibility (OR = 0.80, $p = 0.024$). For the growth index $> 11\%$, the highest specificity, 100%, was associated with 89.7% sensitivity for $V_{\text{POA}}(48\text{h})$ and 92.9% for $V_{\text{POA}}(12\text{h})$. For the growth index $> 20\%$, the highest specificity, 100%, was associated with 70.8% sensitivity for $V_{\text{POA}}(48\text{h})$ and 78.3% for $V_{\text{POA}}(12\text{h})$ (Table 2). The area under the receiver-operating curve (ROC) ranged in all cases from

TABLE 1. PYRAZINAMIDE SUSCEPTIBILITY OF SELECTED *MYCOBACTERIUM TUBERCULOSIS* STRAINS AND PYRAZINOIC ACID EFFLUX RATE

Mutation	Bactec % growth	$V_{\text{POA}}(48\text{h})$ (nmol POA/mg protein/min)	$V_{\text{POA}}(12\text{h})$ (nmol POA/mg protein/min)
H57R	100	4.32	1.76
V139A	100	29.22	27.33
T135P	98	0.67	0.39
H51R	90	0.45	0.75
F58L	88	5.17	7.47
Y34D	78	1.42	1.07
D12A	77	3.61	2.97
D49N	74	0.65	0.59
D12G	70	1.53	3.6
F94L	70	4.18	4.33
G24D	68	0.72	1.6
L116P	46	4.39	ND
G78C	27	6.39	4.01
K48T	20	1.33	0.57
C14G	13	7.24	6.18
C14G	8	12.59	14.68
D136G	3	7.3	8.71
WT	97	0.67	0.77
WT	91	0.52	0.15
WT	88	0.25	0.31
WT	69	1.45	1.45
WT	68	2.04	2.73
WT	63	3.07	2.83
WT	53	1.76	1.05
WT	53	5.92	1.25
WT	32	2.58	1.08
WT	22	3.89	4.14
WT	19	9.19	4.64
WT	19	4.73	10.45
WT	16	3.97	4.01
WT	12	5.94	7.67
WT	1	6.91	10.38
WT	1	14.42	17.88
WT	1	15.98	20.00

ND, nondetermine; POA, pyrazinoic acid.

FIG. 1. Correlation between the pyrazinoic acid (POA) efflux rate estimated from a 48-hr culture, $V_{\text{POA}}(48\text{h})$ (0, 12, 24, 36, and 48 hr), and estimated from a 12-hr culture, $V_{\text{POA}}(12\text{h})$ (0, 12 hr).

TABLE 2. SENSITIVITY AND SPECIFICITY OF PYRAZINOIC ACID EFFLUX RATE IN DIFFERENT SCENARIOS

	Specificity	Sensitivity	Probability cutoff	Area under ROC
Bactec11 vs. $V_{\text{POA}}(48\text{h})$	100	89.65	0.86	0.94
Bactec11 vs. $V_{\text{POA}}(12\text{h})$	100	92.85	0.86	0.95
Bactec20 vs. $V_{\text{POA}}(48\text{h})$	100	70.83	0.76	0.92
Bactec20 vs. $V_{\text{POA}}(12\text{h})$	100	78.26	0.79	0.93

ROC, receiver-operating curve.

0.92 to 0.95. Sensitivity and specificity to determine PZA resistance based on the POA efflux rate for different cutoffs of the predicted probability are shown in the nonparametric sensitivity-specificity curves in Fig. 2.

Discussion

The analysis performed in this study showed that the POA efflux rate was able to predict PZA resistance with high specificity and sensitivity. A 7% of true resistant strains are not detected when using the growth index cutoff of 11%, while a 22% is missing if using a 20% cutoff.

PZA plays a key role in TB control. However, although PZA resistance is common in some countries,¹⁶ the lack of

reliable culture-based resistance tests and the high cost and modest accuracy of molecular methods means that testing is not generally performed. Consequently, patients with PZA-resistant TB are usually treated inappropriately with this hepatotoxic drug. The frequency and significance of undetected PZA resistance to the success of treatment regimens is unknown.

This study confirms the “proof of concept” that the quantitative measurement of POA accumulation is an important predictor of PZA resistance, although the direct measurement of extracellular POA accumulation with a protocol similar to the one described in this article may not be practical for routine testing given the demanding amount of the cultured strain required.

FIG. 2. Sensitivity and specificity that determine pyrazinamide resistance based on the POA efflux rate for different cutoffs of the predicted probability. (A) Prediction of Bactec resistance (% growth > 11%) based on $V_{\text{POA}}(12\text{h})$; (B) prediction of Bactec resistance (% growth > 11%) based on $V_{\text{POA}}(48\text{h})$; (C) prediction of Bactec resistance (% growth > 20%) based on $V_{\text{POA}}(12\text{h})$; (D) prediction of Bactec resistance (% growth > 20%) based on $V_{\text{POA}}(48\text{h})$.

However, the evidence found in this study suggests that it could be feasible to develop a novel approach and a simple assay for testing PZA resistance. We are currently in the process of developing rapid immunoassays for measuring POA levels, and this will be the subject of a future article.

The development of a reliable tool for detecting PZA resistance is likely to have an impact on the clinical outcomes of individuals and populations, and the cost of providing TB control services. If widely implemented, this could have significant public health implications.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Dr. Mirko Zimic
Unidad de Bioinformática y Biología Molecular
Laboratorios de Investigación y Desarrollo
Facultad de Ciencias y Filosofía
Universidad Peruana Cayetano Heredia
Av. Honorio Delgado 430 SMP
Lima 31
Peru

E-mail: mirko.zimic@upch.pe