Tuberculosis 99 (2016) 41–46

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube

DIAGNOSTICS

A quantitative adaptation of the Wayne test for pyrazinamide resistance

Christopher Meinzen [a], Alvaro Proaño [b], Robert H. Gilman [a, c, d], Luz Caviedes [d, †], Jorge Coronel [d], Mirko Zimic [d, e], Patricia Sheen [d, e, *]

[a] Asociación Benéfica PRISMA, Peru
[b] Faculty of Medicine 'Alberto Hurtado', Universidad Peruana Cayetano Heredia, Peru
[c] Department of International Health, Johns Hopkins University, School of Public Health, USA
[d] Laboratory of Tuberculosis – Infectious Diseases, Laboratories of Research and Development, Faculty of Science, Universidad Peruana Cayetano Heredia, Peru
[e] Laboratory of Bioinformatics and Molecular Biology, Laboratories of Research and Development, Faculty of Science, Universidad Peruana Cayetano Heredia, Peru

ARTICLE INFO

Article history:
Received 26 July 2015
Received in revised form
3 March 2016
Accepted 30 March 2016

Keywords:
Tuberculosis
Pyrazinamide
Pyrazinoic acid
Resistance
Wayne
Bactec
TEMA
Optic density
pncA
Peru

SUMMARY

Background: Pyrazinamide (PZA) is the most important drug against the latent stage of tuberculosis (TB) and is used in both first and second line treatment regimens. The continued increase in multi-drug resistant TB and the prevalence of PZA resistance makes the development of alternative assays for prompt identification of PZA resistance all the more important.
Methods: We standardized and evaluated a quantitative variant of the Wayne assay (QW) for determining PZA resistance in *Mycobacterium tuberculosis* strains. This assay quantifies *M. tuberculosis* metabolism of PZA and production of pyrazinoic acid (POA) using visible spectrophotometry. We evaluated this method using PZA concentrations of 400 μg/ml and 800 μg/ml at incubation periods of 3, 5 and 7 days. *M. tuberculosis* strains from 68 sputum samples were also tested with the standard Wayne assay, Tetrazolium Microplate Assay (TEMA), Bactec 460TB and *pncA* sequencing. We compared QW and standard Wayne assay against a dichotomous reference classification using concordant Bactec 460TB and *pncA* sequencing. Secondarily, we determined the quantitative correlation between both QW values and TEMA's minimum inhibitory concentration (MIC) against Bactec 460TB percentage growth.
Results: The standard Wayne showed sensitivity of 88% and specificity of 97.5%, giving a Youden Index (YI) of 0.855 against reference tests. The QW showed maximum YI of 0.934 on day 7 at 400 μg/ml PZA with 96% sensitivity and 97.4% specificity. Absorbance OD values for 400 μg/ml PZA were more accurate than 800 μg/ml PZA. Although QW showed high accuracy for PZA susceptibility, it did not correlate quantitatively with Bactec percentage growth. TEMA testing was unreliable and did not correlate with Bactec results.
Conclusions: The proposed QW assay is an inexpensive method capable of providing standardization and automation of colorimetric PZA resistance testing, with better discriminatory than the standard Wayne assay.

1. Introduction

Tuberculosis (TB) is a disease affecting 9.0 million people globally [1], with drug resistance becoming a growing threat [2]. Among first-line anti-tuberculosis drugs, pyrazinamide (PZA) is the most important drug against latent stage infection. Although incompletely understood [3], its mechanism of action requires an acid pH that makes difficult standard cultures for resistance [4]. With an estimated 50% resistance to PZA in the concerning multidrug-

* Corresponding author. Universidad Peruana Cayetano Heredia, Facultad de Ciencias y Filosofía, Laboratorios de Investigación y Desarrollo, Laboratorio de Bioinformática y Biología Molecular, Av. Honorio Delgado 430, SMP, Lima, Peru. Tel.: +51 1 3190000x2604.
E-mail address: patricia.sheen@upch.pe (P. Sheen).
† *in memoriam* Luz Caviedes who passed away in November 2012.

http://dx.doi.org/10.1016/j.tube.2016.03.011
1472-9792/

resistant tuberculosis (MDR-TB) strains [5], identifying these cases becomes critical.

There are three commonly used tests for PZA resistance, none of which have ideal accuracy or reproducibility [5]. The prior test of choice, the Bactec 460-TB radiometric method [6,7], has been pulled from the market due to concern of contaminating byproducts and the replacement tool, Bactec 960-TB, has not shown the same diagnostic accuracy [8]. A newer method with good results is genetic testing of the TB strain, particularly focused at identifying mutations in the *pncA* gene, though this remains relatively expensive and does not identify all resistant strains [9]. The Wayne test is an inexpensive assay based on a color change proportional to the extracellular concentration of pyrazinoic acid (POA), a metabolite of PZA in susceptible strains [10]. The primary drawbacks are inferior accuracy compared to the other two methods, the subjective interpretation of color-change and the concern for over reporting of positive results [5].

Resistance to most antibacterials can be characterized with proportion susceptibility methods, but none have been implemented with PZA. Tetrazolium Microplate Assay (TEMA) has been successfully used to test resistance with proportion susceptibility against isoniazid and rifampin [11], however it has not undergone testing with PZA. Prior studies have confirmed that the rate of POA efflux and thus the amount of extracellular POA accumulated in a certain period of time is a highly sensitive and specific indicator of PZA resistance [12]. Unfortunately, direct measurement of POA proves to be too expensive and cumbersome for routine use.

In this study, we seek to use the strengths of the Wayne test's indication of extracellular POA, while addressing the subjectivity in interpretation of "color change" by quantifying that measure. We propose to measure optical density (OD) absorbance to quantify the extracellular POA in the Wayne test and thus provide an objective cut-off for quantitative positive results. To judge the success of our method, we compare both the standard Wayne test and this quantitative variant of the Wayne test (QW) against concordant testing by Bactec radiometric method and *pncA* sequencing. Furthermore, we attempt to correlate Bactec radiometric percentage growth with both TEMA concentrations and OD absorbance by QW in order to measure the degree of PZA resistance.

2. Methods

2.1. Setting and samples

Mycobacterium tuberculosis samples came from cultured sputum samples maintained in the Mycobacterial Laboratory in Universidad Peruana Cayetano Heredia (UPCH). We used random sampling of our team's preexisting culture bank from sputum from two studies with two sites each [13]. In brief, these were an unselected community-based cohort from south Lima and a hospital HIV unit (1999–2005) [14]; and an unselected community-based cohort from north Lima (2003–2004) with a selected community-based cohort from east Lima of TB patients with risk factors for drug resistance (2003–2005) [15]. From the former study, 18% of donors had MDR-TB and 61% of them had concomitant HIV [14]; in the latter study, 33% of the donors had MDR-TB [15]. Additionally, drug resistant TB has been noted to be prevalent in Peru [13].

All strains had been culture-confirmed tuberculosis prior to being stored in the culture bank. These initial strains have been maintained at −70 °C since collection. The age, gender, HIV status, and MDR-TB status of all participants reported here came from data collected in those studies. All strains and data were de-identified and stored as sequential codes with clinical site prefixes. All procedures in this study received the approval of the IRB ethics committee from UPCH for the original studies in which the samples were collected and tested.

A total of 68 strains were randomly selected and cultured in 7H10 media. Upon growth, these strains were separated into samples which were tested according to the protocols for Wayne, QW, Bactec, *pncA* sequencing and TEMA.

2.2. Wayne test

The standard Wayne test for PZAse activity was performed independently of our proposed quantitative variant, according to the original protocol and described briefly here [10]. A heavy loopful of several actively growing colonies was transferred into a screw-cap tube with 5 ml of Dubos medium containing 100 μg/ml PZA and 2 mg/ml sodium pyruvate. These were then incubated at 37 °C for seven days. On the seventh day, 1 ml of freshly prepared 10% ammonium iron sulfate was added to the culture tube and allowed it to sit at room temperature for 30 min. After that time, we observed the upper layer for color change indicative of extracellular POA. If the sample initially showed no color change, we checked again for color change after 4 h additional refrigeration. A pink-purple tint or ring on either inspection was considered a positive test, indicative of pyrazinamide susceptibility. If the color change was ambiguous, the result was indeterminate.

2.3. Quantitative variant of Wayne test (QW)

The QW method proposed here proceeds as follows: 3 ml-suspensions were prepared in citrate buffer pH 7.0 so they matched a McFarland 4 turbidity standard. PZA was added to reach 400 μg/ml and 800 μg/ml final concentrations. The suspensions were incubated at 37 °C until days 3, 5, and 7 in which the tests were performed. At each testing day, 1 ml of suspension was removed and centrifuged at 5000 rpm for 10 min. The supernatant was taken and boiled for 30 min to remove any remaining mycobacteria. The resulting solution was removed from the biosecurity lab and centrifuged at 12,000 rpm for 10 min to remove particulate debris. Then aliquots of 100 μl was placed in 3 separate wells of Microtest wells (Falcon, No.35-3072) for each PZA concentration that had each had optic density measurements before reagent was added. 20 μl 10% ammonium iron sulfate was added to each well to initiate color change. The optic density was then again measured at 450 nm using a spectrophotometer (VersaMax ELISA Microplate ReadeR), Prior to the present experiment, we had conducted a short series of OD absorbance tests using this proposed method at 100, 200, 400, and 800 μg/ml PZA in known strains. The 400 and 800 μg/ml PZA tests showed the greatest difference between values for susceptible and resistant strains and so we used both in the present experiment (data not shown).

2.4. Bactec

The strains were tested for PZA susceptibility using Bactec 460-TB method following the manufacturer's instructions (Becton Dickinson, Sparks, MD), described briefly here. In two vials with 4 ml test medium at pH 6.0, we added McFarland 1 of the test strain. One vial received 100 μg/ml PZA and the other control vial received no drug. The vials were incubated at 37 °C and tested each day. The Bactec 460TB test was repeated twice for each strain, and the mean percentage of growth was determined. A growth index (GI) of the test vial compared to control vial was recorded numerically and further interpreted according to the standard: <9% GI is susceptible, 9–11% is borderline, and >11% is resistant. If the test vial failed to achieve a raw GI of 200, the test was considered invalid and repeated.