FEMS Pathogens and Disease, 73, 2015, ftv037

doi: 10.1093/femspd/ftv037
Advance Access Publication Date: 20 May 2015
Perspective

PERSPECTIVE

Pyrazinamide resistance in *Mycobacterium tuberculosis* fails to bite?

Alice L. den Hertog, Sarah Sengstake and Richard M. Anthony*

KIT Biomedical Research, Royal Tropical Institute (KIT), Amsterdam, the Netherlands

*Corresponding author: KIT Biomedical Research, Royal Tropical Institute (KIT), Meibergdreef 39, 1105 AZ Amsterdam, the Netherlands. Tel: +31205665450; Fax: +31206971841; E-mail: r.anthony@kit.nl

One sentence summary: The authors propose an intriguing concept, that PZA resistance typically imposes a fitness cost that impairs TB transmission.
Editor: Patrick Brennan

ABSTRACT

In contrast to most other antimycobacterial drugs where—particularly in multidrug-resistant (MDR) strains—a limited number of resistance mutations dominate, pyrazinamide (PZA) resistance associated mutations remain highly diverse with limited clustering. This apparent lack of evolutionary selection for successful PZA resistance mechanisms deserves attention. A clear understanding of the epidemiology of PZA resistance acquisition and spread would be expected to result in important insights into how PZA might be better exploited in treatment regimens to minimize the amplification of *Mycobacterium tuberculosis* (MTB) drug resistance. We propose that PZA resistance typically induces a fitness cost that impairs MTB transmission. This would explain the lack of extensive clustering for PZA-resistant mutants. Our hypothesis also leads to a series of testable predictions which we outline that could confirm or refute our ideas.

Keywords: *Mycobacterium tuberculosis*; pyrazinamide; resistance; multidrug resistance; transmission, fitness

INTRODUCTION

Just as Sherlock Holmes noted regarding the dog in the 'Silver Blaze' case, we suggest that the most curious aspect of PZA resistance is what hasn't happened:

> Gregory (detective): 'Is there any other point to which you would wish to draw my attention?'
>
> Holmes: 'To the curious incident of the dog in the night-time'.
>
> Gregory: 'The dog did nothing in the night-time'.
>
> Holmes: 'That was the curious incident'.
>
> *Arthur Conan Doyle* 1892

A limited number of mutations have emerged that are responsible for the vast majority of transmitted drug resistance in *Mycobacterium tuberculosis* (MTB). This fact is exploited by line probe assays which are surprisingly sensitive for the detection of drug resistance in MDR-TB despite targeting only one or two small regions and a few specific mutations (Morgan *et al.* 2005). It is also widely recognized that line probe and related assays become more sensitive as MTB strains become resistant to increasing numbers of drugs (Van Deun, Martin and Palomino 2010), demonstrating a strong purifying selection for specific mutations worldwide. This extremely low mutational diversity in multidrug-resistant isolates is true for most widely used antimycobacterial drugs, with one notable exception: pyrazinamide (PZA) (Brown, Tansel and French 2000; Louw *et al.* 2006; Singh *et al.* 2006).

WHY HAVE PZA RESISTANCE MUTATIONS REMAINED SO DIVERSE AND WHAT ARE THE IMPLICATIONS?

The list of mutations known to confer PZA resistance in clinical strains is long and growing, making the development of clinically applicable assays extremely challenging. The difficulty of

Received: 13 March 2015; **Accepted:** 11 May 2015

developing practical PZA resistance assays has received considerable attention (Daum *et al.* 2014; Miotto *et al.* 2014), but this practical issue—although valid—has in our opinion overshadowed the real importance of the observation, namely that despite the fact that PZA resistance acquisition occurs with high frequency it has for decades remained incompatible with epidemic spread. We believe that the significance of this may have been seriously overlooked.

PZA is a relatively old drug. Introduced in the year 1952 for the treatment of TB (Yeager, Munroe and Dessau 1952), PZA was subsequently used in combination with isoniazid, especially for the treatment of streptomycin-resistant forms of TB. PZA has been included in the first two months of standard first line treatment of TB since the late 1980s and is also almost universally given in the intensive phase of treatment of patients with MDR-TB irrespective of resistance profile. (WHO 2011, 2014). Thus, there has been ample time for the selection of successful resistant clones and for them to become established and spread. Although PZA resistance in clinical isolates is not particularly rare, resistant strains remain highly diverse in respect of PZA resistance mutations in MDR-TB and even XDR-TB (Wang *et al.* 2015) strains.

Phenotypic and genotypic resistance testing for PZA is more complex than for other TB drugs (Hoffner *et al.* 2013). Phenotypic screening must be carried out at low pH which is suboptimal for growth of TB. Genotypic analysis requires sequencing of at least the entire *pncA* gene followed by interpretation of the significance of any, often previously unreported, mutations. In fact, the diagnostic accuracy of screening for identified high confidence markers of PZA resistance, by sequencing the *pncA* gene, has been predicted to have an accuracy in the range of 89.5–98.8% (Miotto *et al.* 2014), but routine collection of these data in the pre-whole genome sequencing era has been uncommon. Thus, *pncA* mutations appear to account for the majority of PZA resistance although other mechanisms and targets have been proposed (Shi *et al.* 2011; Zhang *et al.* 2013; Yang *et al.* 2015). However, multiple studies independently demonstrate around 40–50% PZA resistance in primary MDR-TB cases with little clustering of strains with particular PZA resistance associated mutations (Chang, Yew and Zhang 2011; Stoffels *et al.* 2012; Casali *et al.* 2014). Most often only a few small clusters (typically 2–10 strains) of PZA-resistant isolates that share identical resistance mutations and are geographically and temporally localized are seen. Thus, in contrast to other TB drugs, there is no evidence that a particular PZA resistance mutation is becoming increasingly common in different bacterial genotypes and geographical settings. Rather, the wide diversity and limited clustering indicate that resistant strains are both appearing and disappearing continuously, which we believe suggests that the transmission of PZA-resistant isolates is in one way or another impaired.

PZA resistance clearly confers an advantage during therapy that includes PZA and can be selected under those circumstances; indeed, patients infected with PZA-resistant strains respond less well when treated with this drug (Franke *et al.* 2015). However, the lack of large-scale clustering in panels of MDR-TB strains that are PZA resistant demonstrates that for some reason these strains don't become established at the level of the epidemic. It could be claimed that this is simply due to the fact that PZA resistance is rapidly generated and all mutations have a similar (low?) fitness cost, but we believe that this curious lack 'of the dog's bark' reveals a significant cost to PZA resistance which is difficult for the bacterium to circumvent. Even though strains with PZA mutations can be isolated from patients and occasionally undergo limited local spread, available data shows that they have a considerably reduced epidemic potential and probably represent evolutionary dead-end mutations.

The primary mechanism of PZA resistance closely resembles that of INH resistance. Both PZA and INH are prodrugs activated by MTB enzymes [pyrazinamidase (PZase, encoded by *pncA*) and catalase, respectively] and loss of these enzymes results in resistance. Resistance to both PZA and INH is acquired *in vitro* at a high rate (David *et al.* 1970; Stoffels *et al.* 2012), probably because of the large range of potential mutations available that impair the function of the enzyme. Indeed, a wide variety of mutations is seen after *in vitro* selection with either PZA or INH (Scorpio *et al.* 1997; Bergval *et al.* 2009; Stoffels *et al.* 2012;). However, in the case of INH, a decreased virulence of almost all INH-resistant mutants has been reported (Barnett, Busby and Mitchison 1953), probably due to the loss of functional catalase. Probably as a consequence of this, the vast majority of clinical katG mutants contain the same mutation, katG-S315T a mutation which prevents INH activation while preserving the normal function of the enzyme (Rouse *et al.* 1996; Soolingen *et al.* 2000). Alternative katG mutations represent only a small proportion of clinically isolated isoniazid-resistant strains and are very rare in MDR-TB strains. This situation is in strong contrast to clinically isolated PZA-resistant mutants that show a diversity comparable to those generated *in vitro* (Stoffels *et al.* 2012).

The continuous generation of PZA mutants rather than their epidemic spread is supported by available epidemiological data. A high diversity of *pncA* mutations among all collections of isolates studied to date has been observed (Stoffels *et al.* 2012; Casali *et al.* 2014; Miotto *et al.* 2014). In one of the larger studies, Miotto *et al. et al.* (2014) found 280 genetic variants among 888 phenotypically PZA-resistant clinical isolates from various geographical regions with the largest cluster of isolates with identical *pncA* mutations consisting of only14 isolates. Although examples of the transmission of PZA-resistant isolates have occasionally been reported (Thomas *et al.* 2014). One of the few examples for a large *pncA* cluster of isolates sharing an identical mutation in *pncA* reported was a cluster of 157 isolates carrying a non-synonymous SNP in *pncA* (I6L) in a collection of 1000 Russian isolates analyzed by WGS from the Samara region of Russia. However, strains carrying this mutation did not show *in vitro* phenotypic resistance and all MTB isolates with this mutation belonged to the same genotypic lineage, thus the significance of this finding remains unclear (Casali *et al.* 2014). Apart from this I6L mutation clustering of strains sharing the same *pncA* mutation was minimal, with no other clusters of more than seven isolates identified (Casali *et al.* 2014).

What might explain this situation? The ability of MTB to cause a chronic infection and transmit depends on its capacity to switch between different (active and latent) phenotypes. Although most PZA resistance mutations in *pncA* are reported to have a low fitness cost in actively growing MTB (Stoffels *et al.* 2012) and frequently emerge in the host, there is evidence that these mutations disrupt the formation or survival of latent bacterial cells, which could impair transmissibility. As mentioned above PZase is required to convert the prodrug PZA to its active form. The biological function of PZase is related to the recycling of NAD (Boshoff *et al.* 2008; Vilchèze *et al.* 2010), which is upregulated under oxygen/nutrient restricted conditions (Betts *et al.* 2002). There is little data on the role of PZase in infection disease progression or transmission in humans, and infection with

pncA mutant MTB does not appear to have been studied using animal models. Clearly subpopulations of MTB encounter hypoxic and/or nutrient limited environments at specific stages of the infection and disease locations, thus it is likely that PZase plays a role in NAD recycling under those circumstances, loss of PZase activity may disrupt the disease process. Tantalizingly, there are some indications from other (intracellular) pathogens that a functional nicotinamidase/ PZase is of importance for establishing a new infection: mutants of *Brucella* spp. and *Borrellia* spp. lacking nicotinamidase or PZase, show reduced intracellular replication and infectivity in a mammalian host, respectively (Purser *et al.* 2003; Kim *et al.* 2004).

Of interest (but also of unknown significance) is the observation that PZA-monoresistant infections are associated with extrapulmonary disease (Budzik *et al.* 2014). Association with extrapulmonary disease and lack of transmission have been reported for human infections with *M. bovis* and *M. canettii* both of which are phylogenetically related to MTB and capable of causing TB in humans, but intrinsically resistant to PZA (Koeck *et al.* 2011; LoBue *et al.* 2003). Another observation that deserves mentioning in this context are the so far excellent outcomes, with respect to cure and relapse, of the so-called 'Bangladesh regimen' a regimen that includes PZA for 15 months (van Deun *et al.* 2014) although no statistical significant difference in outcome between patients infected with PZA-resistant isolates could be identified on this regimen except when PZA resistance was also associated with quinolone resistance (Aung *et al.* 2014).

There may be alternative explanations for the observations above and of the failure of specific PZA resistance mutations to co-select with an increasingly MDR-TB genotype. For example, PZA is unequally or at least primarily active against a population of bacteria in the host not affected by other antibiotics. This could mean that even with multiple active drugs a subpopulation of mycobacteria would effectively receive monotherapy with PZA, and this proportion could become resistant. As this PZA-resistant population may have limited or no advantage in the growth phases where PZA is not active, one could argue that accumulation of additional resistance would be no more likely than for a PZA-sensitive variant. Nonetheless, unless acquisition of PZA resistance typically induces a strong fitness cost as we propose, an MDR-TB strain (for instance during empirical treatment with first line drugs) acquiring PZA resistance would be expected to have an advantage and spread.

The hypothesis, that PZA resistance typically induces a fitness cost for MTB transmission, would explain the presence of culture positive PZA-resistant cases and the lack of extensive clustering. However, it is inconsistent with the observation that frequently around 40–50% of primary MDR-TB is reported to have resistance to PZA (Günther *et al.* 2015). Can this apparent contradiction be explained? One possible solution to this puzzle could relate to how TB patients are diagnosed and treated. Typically, even where primary MDR-TB is relatively common standard therapy remains based on rifampicin, INH, ethambutol and PZA. Due to the unavailability of simple rapid diagnostics for TB and TB drug resistance, TB patients are usually started on first line therapy upon diagnosis. MDR-TB infection is frequently only identified when DST results become available and/or patients fail to respond, after which time therapy is changed to second line therapy. For these reasons, primary MDR-TB cases typically have received more than two weeks of first line therapy before a MDR-TB diagnosis is made, and possibly even two or three months of first line therapy if MDR-TB is detected as a result of treatment monitoring (Naidoo *et al.* 2014). Thus, at least a proportion of the PZA resistance detected in primary MDR-TB patients is likely to be selected *de novo* as a result of effectively two or more weeks of more or less mono-PZA therapy (in addition to at least two inactive drugs). Repeated *de novo* selection of PZA resistance in MDR-TB patients early in treatment is an appealing hypothesis as it would both explain the seemingly contradictory but common finding of PZA resistance in an MDR-TB strain due to novel mutations. If this is true, we expect that the implementation of rapid molecular testing eventually allowing patients to start immediately on second line drugs, will cause a reduction in PZA resistance in primary MDR-TB strains.

DIRECTIONS FOR FUTURE RESEARCH

It is our opinion that this curious and poorly explained lack of clustering and evolutionary selection of successful PZA resistance mechanisms deserves attention. Not least because a clear understanding of the epidemiology of PZA resistance acquisition and spread would be expected to result in important insights into how this drug could be better exploited in treatment regimens to minimize the amplification of resistance. Importantly our line of reasoning, outlined above, leads to a series of testable predictions (Text box 1) that could confirm or refute our ideas as well as narrow down the possible explanations for this curious case.

Text box 1.

Hypotheses and testable predictions that could support or refute our 'elementary' speculation:
Primary hypothesis: *pncA* mutations conferring PZA resistance have a reduced ability to spread and are often dead end mutations.
Predictions are as follows.

i. Contacts of patients infected with PZA-resistant strains will be less likely to develop active TB disease compared to contacts of PZA susceptible strains.
ii. PZA resistance will rapidly emerge if MDR-TB patients receive standard therapy before a diagnosis of MDR-TB.
iii. The rate of PZA resistance in primary MDR-TB patients who have never received PZA in either first or second line (empirical) will be lower than in primary MDR-TB patients who have received a few weeks of empirical first line therapy prior to (or after) diagnosis as an MDR-TB patient. Thus, MDR-TB patients diagnosed on the basis of a molecular test on the day of presentation will be less likely to harbor PZA-resistant strains.

ACKNOWLEDGEMENTS

We thank Indra Bergval and Paul Klatser for their critical input during the preparation of this manuscript.

CONTRIBUTORS

Based on discussions with all authors RMA wrote the first draft, all authors provided critical input on subsequent drafts.

Conflict of interest. None declared.

REFERENCES

Aung KJM, Van Deun A, Declercq E, *et al.* Successful '9-month Bangladesh regimen'for multidrug-resistant tuberculosis among over 500 consecutive patients. *Int J Tuberc Lung D* 2014;**18**:1180–87.

Barnett M, Busby SR, Mitchison DA. Tubercle bacilli resistant to isoniazid: virulence and response to treatment with isoniazid in guinea-pigs and mice. *Brit J Exp Pathol* 1953;**34**:568–81.

Bergval IL, Schuitema ARJ, Klatser PR, *et al.* Resistant mutants of *Mycobacterium* tuberculosis selected in vitro do not reflect the in vivo mechanism of isoniazid resistance. *J Antimicrob Chemoth* 2009;**64**:515–23.

Betts JC, Lukey PT, Robb LC, *et al.* Evaluation of a nutrient starvation model of *Mycobacterium* tuberculosis persistence by gene and protein expression profiling. *Mol Microbiol* 2002;**43**: 717–31.

Boshoff HIM, Xu X, Tahlan K, Dowd CS, *et al.* Biosynthesis and recycling of nicotinamide cofactors in *Mycobacterium* tuberculosis. An essential role for NAD in nonreplicating bacilli. *J Biol Chem* 2008;**283**:19329–41.

Brown TJ, Tansel O, French GL. Simultaneous identification and typing of multi-drug-resistant *Mycobacterium* tuberculosis isolates by analysis of *pncA* and rpoB. *J Med Microbiol* 2000;**49**:651–6.

Budzik JM, Jarlsberg LG, Higashi J, *et al.* Pyrazinamide resistance, *Mycobacterium* tuberculosis lineage and treatment outcomes in San Francisco, California. *PLoS ONE* 2014;**9**: e95645.

Casali N, Nikolayevskyy V, Balabanova Y, *et al.* Evolution and transmission of drug-resistant tuberculosis in a Russian population. *Nat Genet* 2014;**46**:279–86.

Chang KC, Yew WW, Zhang Y. Pyrazinamide susceptibility testing in *Mycobacterium* tuberculosis: a systematic review with meta-analyses. *Antimicrob Agents Ch* 2011;**55**: 4499–505.

Daum LT, Fourie PB, Bhattacharyya S, *et al.* Next-generation sequencing for identifying pyrazinamide resistance in *Mycobacterium* tuberculosis. *Clin Infect Dis* 2014;**58**:903–4.

David HL. Probability distribution of drug-resistant mutants in unselected populations of *Mycobacterium* tuberculosis. *Appl Microbiol* 1970;**20**:810–4.

Doyle AC. *The Memoirs of Sherlock Holmes, Silver Blaze*. Great Britain: George Newnes, 1892,**1**:1–31

Franke MF, Becerra MC, Tierney DB, *et al.* Counting pyrazinamide in regimens for multidrug-resistant tuberculosis. *Ann Am Thorac Soc* 2015;**12**:674–9.

Günther G, van Leth F, Alexandru S, *et al.* Multidrug-resistant tuberculosis in Europe, 2010–2011. *Emerg Infect Dis* 2015;**21**: 409–16.

Hoffner S, Angeby K, Sturegård E, *et al.* Proficiency of drug susceptibility testing of *Mycobacterium* tuberculosis against pyrazinamide: the Swedish experience. *Int J Tuberc Lung Dis* 2013;**17**:1486–90.

Kim S, Kurokawa D, Watanabe K, *et al. Brucella abortus* nicotinamidase (*PncA*) contributes to its intracellular replication and infectivity in mice. *FEMS Microbiol Lett* 2004;**234**:289–95.

Koeck J-L, Fabre M, Simon F, *et al.* Clinical characteristics of the smooth tubercle bacilli '*Mycobacterium canettii*' infection suggest the existence of an environmental reservoir. *Clin Microbiol Infect* 2011;**17**:1013–9.

LoBue PA, Betacourt W, Peter C, *et al.* Epidemiology of *Mycobacterium bovis* disease in San Diego County, 1994–2000. *Int J Tuberc Lung Dis* 2003;**7**:180–5.

Louw GE, Warren RM, Donald PR, *et al.* Frequency and implications of pyrazinamide resistance in managing previously treated tuberculosis patients. *Int J Tuberc Lung Dis* 2006;**10**:802–7.

Miotto P, Cabibbe AM, Feuerriegel S, *et al. Mycobacterium* tuberculosis pyrazinamide resistance determinants: a multicenter study. *MBio* 2014;**5**:e01819–4.

Morgan M, Kalantri S, Flores L, *et al.* A commercial line probe assay for the rapid detection of rifampicin resistance in *Mycobacterium* tuberculosis: a systematic review and meta-analysis. *BMC Infect Dis* 2005;**28**:62.

Naidoo P, du Toit E, Dunbar R, *et al.* A comparison of multidrug-resistant tuberculosis treatment commencement times in MDRTBPlus line probe assay and Xpert® MTB/RIF-based algorithms in a routine operational setting in Cape Town. *PLoS ONE*. 2014;**9**:e103328.

Purser JE, Lawrenz MB, Caimano MJ, *et al.* A plasmid-encoded nicotinamidase (*PncA*) is essential for infectivity of *Borrelia burgdorferi* in a mammalian host. *Mol Microbiol* 2003;**48**: 753–64.

Rouse DA, DeVito JA, Li Z, *et al.* Site-directed mutagenesis of the *katG* gene of *Mycobacterium* tuberculosis: effects on catalase-peroxidase activities and isoniazid resistance. *Mol Microbiol* 1996;**22**:583–92.

Scorpio A, Lindholm-Levy P, Heifets L, *et al.* Characterization of *pncA* mutations in pyrazinamide-resistant *Mycobacterium* tuberculosis. *Antimicrob Agents Ch* 1997;**41**:540–3.

Shi W, Zhang X, Jiang X, *et al.* Pyrazinamide inhibits trans-translation in *Mycobacterium* tuberculosis. *Science* 2011;**333**:1630–2.

Singh P, Mishra AK, Malonia SK, *et al.* The paradox of pyrazinamide: an update on the molecular mechanisms of pyrazinamide resistance in Mycobacteria. *J Commun Dis* 2006;**38**:288–98.

Stoffels K, Mathys V, Fauville-Dufaux M, *et al.* Systematic analysis of pyrazinamide-resistant spontaneous mutants and clinical isolates of *Mycobacterium* tuberculosis. *Antimicrob Agents Ch* 2012;**56**:5186–93.

Thomas TA, Heysell SK, Houpt ER, *et al.* Outbreak of pyrazinamide-monoresistant tuberculosis identified using genotype cluster and social media analysis. *Int J Tuberc Lung Dis* 2014;**18**:552–8.

Van Deun A, Martin A, Palomino JC. Diagnosis of drug-resistant tuberculosis: reliability and rapidity of detection. *Int J Tuberc Lung Dis* 2010;**14**:131–40.

Van Deun A, Salim MAH, Das APK, *et al.* Results of a standardised regimen for multidrug-resistant tuberculosis in Bangladesh. *Int J Tuberc Lung Dis* 2004;**8**:560–7.

van Soolingen D, de Haas PE, van Doorn HR, *et al.* Mutations at amino acid position 315 of the *katG* gene are associated with high-level resistance to isoniazid, other drug resistance, and successful transmission of *Mycobacterium* tuberculosis in the Netherlands. *J Infect Dis* 2000;**182**:1788–90.

Vilchèze C, Weinrick B, Wong K-W, *et al.* NAD+ auxotrophy is bacteriocidal for the tubercle bacilli. *Mol Microbiol* 2010;**76**:365–77.

Wang F, Shao L, Fan X, *et al.* Evolution and transmission patterns of extensively drug-resistant tuberculosis in china. *Antimicrob Agents Ch* 2015;**59**:818–25.

WHO. *Guidelines for the Programmatic Management of Drug-resistant Tuberculosis*. 2011, http://whqlibdoc.who.int/publications/2011/9789241501583˙eng.pdf?ua=1 (27 February 2015, date last accessed).

WHO. *Companion Handbook to the WHO Guidelines for the Programmatic Management of Drug-resistant Tuberculosis*. 2014 http://apps.who.int/iris/bitstream/10665/130918/1/9789241548809_eng.pdf?ua=1&ua=1 (27 February 2015, date last accessed)).

Yang J, Liu Y, Bi J, *et al*. Structural basis for targeting the ribosomal protein S1 of *Mycobacterium* tuberculosis by pyrazinamide. *Mol Microbiol* 2015;**95**:791–803.

Yeager RL, Munroe WGC, Dessau FI. Pyrazinamide (aldinamide) in the treatment of pulmonary tuberculosis. *Am Rev Tuberc* 1952;**65**:523–46.

Zhang S, Chen J, Shi W, *et al*. Mutations in panD encoding aspartate decarboxylase are associated with pyrazinamide resistance in *Mycobacterium* tuberculosis. *Emerg Microbes Infect* 2013;**2**:e34.