Pyrazinamide is active against *Mycobacterium tuberculosis* at low temperature at neutral pH

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Abstract:
At 28ºC, Pyrazinamide (PZA) is active against *Mycobacterium tuberculosis* (Mtb) at neutral pH at the standard breakpoint concentration. This finding should help to unravel the mechanism of action of PZA and allow drug susceptibility testing (DST) testing methods to be optimized.

Introduction:
The mechanism of antimicrobial activity of PZA against Mtb is unclear. The dogma is that acidic conditions are required for PZA activity, to protonate pyrazinoic acid (POA, the enzymatically activated form of PZA) to allow internalization. But low pH also triggers transcriptional remodeling of the bacteria towards phenotypes resembling *in vivo* bacteria.
PZA drug susceptibility testing at low pH is difficult to perform; there is a need for better methods at neutral pH.

We decided to look at the effects of temperature on antimicrobial PZA activity, as
- Changing the culture temperature is a simple and non-chemical metabolic stress,

and
- it was previously demonstrated that PZA activity increased at reduced culture temperatures at low pH (Coleman *et al.* 2011).

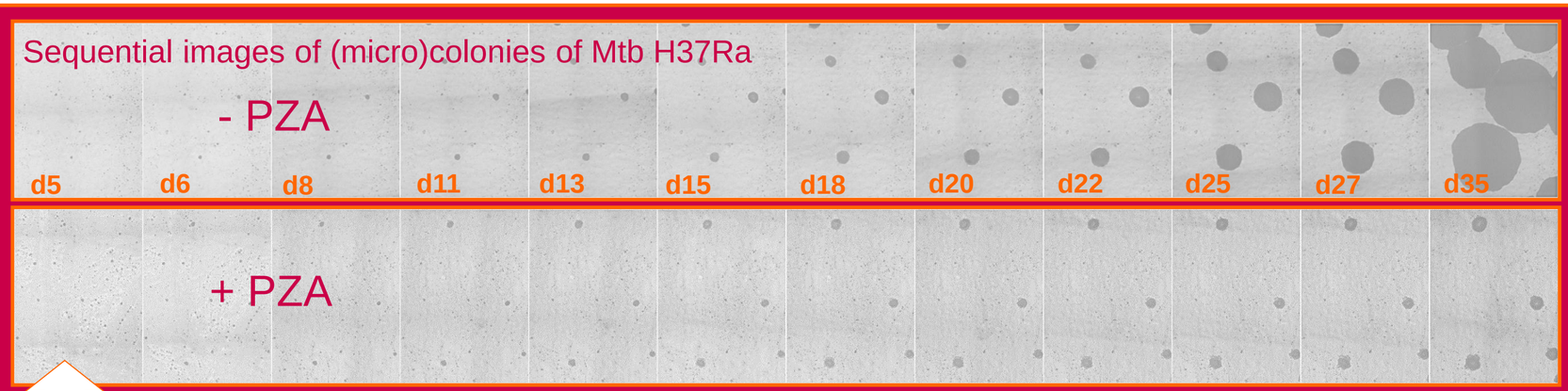
Methods:
PZA activity against Mtb was tested on solid media (microcolony growth on MB7H11) and in liquid broth (MGIT method).

MGIT assay
Mtb suspensions were adjusted to 0.1 McFarland standard to inoculate standard MGIT tubes (neutral medium) and MGIT PZA test tubes (medium at pH5.9) supplemented with MGIT culture supplement (BD) with 100mg/L PZA in duplicate. Control tubes without PZA were inoculated at a 10-fold lower density. Tubes were incubated at 28 or 37ºC and manually measured 3 times per week with a manual MGIT reader.

Susceptibility of Mtb strains to PZA at 37°C and 28°C at neutral and acidic pH by MGIT method.

Strain	Phenotype	Inoculation density GC (bacteria/ml)	PZA DST testing results					
			37°C				28°C	
			neutral pH		low pH		neutral pH	low pH
			Result	TtP[1] (control/+PZA)	Result	TtP (control/+PZA)	Result	TtP (control/+PZA)	Result	TtP (control/+PZA)
Mtb ATCC 27294 (H37Rv)	S	$1.0x10^4$	R	(7 / 5)	S	(8,5 / 22,5)	S	(14 / N[3])	S	(12 / N)
Mtb ATCC 25177 (H37Ra)	S	$1.1x10^4$	R	(7 / 3)	S	(7 / 10)	S	(10 / 12)	S	(13 / N)
Mtb ATCC 35801	S	$1.0x10^3$	R	(5 / 3)	S	(5 / 18)	S	(10 / N)	S	(10 / N)
Mtb BD-201	moderate PZA-R[2]	$3.3x10^2$	R	(6 / 4)	S	(7 / 15,5)	S	(10 / 25)	S	(10 / N)
Mtb ATCC 35828	R	$4.3x10^3$	R	(5 / 3)	R	(5 / 3)	R	(8,5 / 5)	R	(8,5 / 5)
Mtb DIS529	R	$4.7x10^3$	R	(7 / 5)	R	(8,5 / 6)	R	(10 / 7)	R	(11 / 8,5)

[1] Time to positivity in days (growth control/+PZA)
[2] Normally classifies as susceptible in MGIT PZA testing
[3] N indicates both duplicates remained negative at day 26



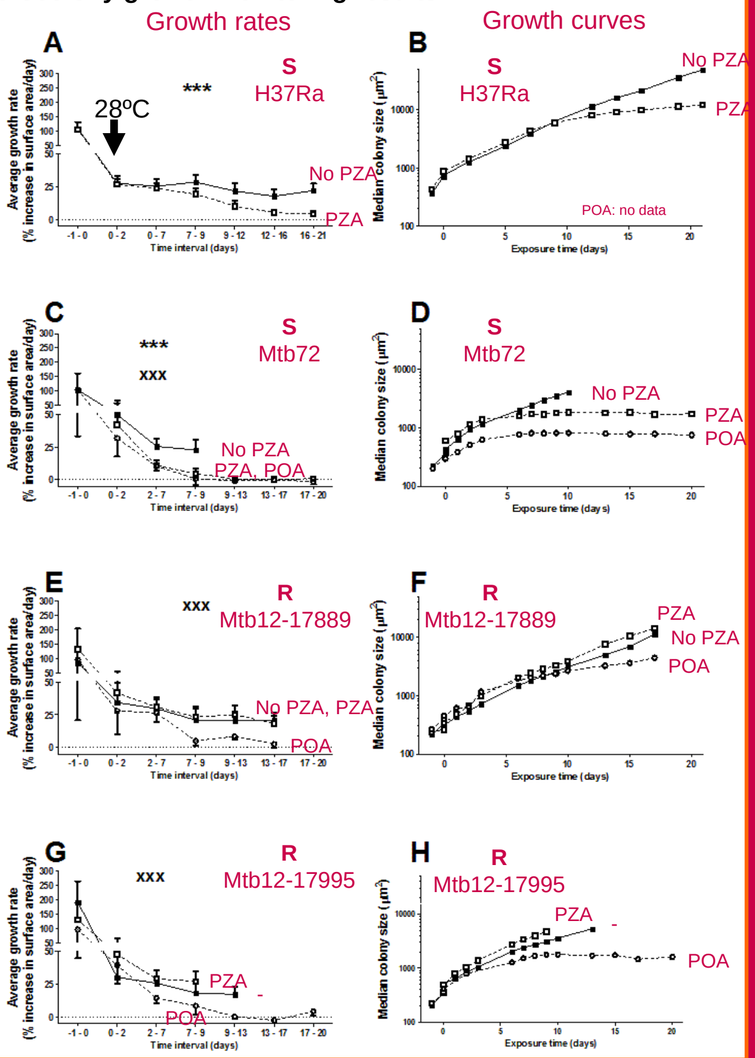
Microcolony growth monitoring
The assay was performed essentially as described by den Hertog *et al*. 2014.
Strains were inoculated ~ 10^4CFU/cm^2 on porous aluminum oxide filters on non selective MB7H11 agar supplemented with OADC + glycerol and cultured for 6 days at 37ºC. Then, the filters containing colonies of approx. 10^2 cells were transferred to MB7H11 agar with or without 100mg/L PZA or POA and culture continued at 28ºC.

Microcolonies were monitored before and during exposure by repetitive imaging in an automated microscope system (Lumibyte BV, www.lumibyte.eu). Based on the collected images, the size of each colony over time was measured, from which the average daily growth rates were extracted as previously described.

Strains: two PZA susceptible strains (Figure A-D, *pnc*A wt) and two clinically PZA resistant isolates (Figure E, F: *pnc*A A16C (I6L); G,H: *pnc*A A212G (H71R).
Statistics: 2-sided t-test; *** : p<0.0001, PZA vs control. ^{xxx} : p<0.0001, POA vs control.

Results:
Microcolony growth experiments demonstrate that at 28ºC, PZA inhibits susceptible Mtb strains. The resistant strains tested here are inhibited by POA, but not PZA.
In the MGIT assay, inhibition of the susceptible strains can also be detected, and the effects of PZA on Mtb at low pH, low temperature and combined are highly similar, although the time to positivity of the controls is longer at low temperature than under the standard (low pH) conditions.

Microcolony growth monitoring results



Conclusions:
PZA and POA are active at neutral pH at reduced temperature.
The finding that a low pH is not required for antimicrobial PZA activity is supported by recent work by Peterson *et al.* 2015 and historical data from McDermott *et al.* 1954. Combined, these data disprove the requirement for an acidic pH to protonate POA and thus should help elucidate the mechanism of action of PZA.
Despite the lack of activity of PZA under standard culture conditions, PZA is an essential drug for sterilizing Mtb infections due to its activity against slowly metabolizing *Mtb*. The finding that a simple adaptation of the temperature stimulates PZA activity, opens up new strategies for drug susceptibility testing and drug development.

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