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Effects of low incubation temperatures on the bactericidal activity of anti-tuberculosis drugs

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Abstract

Objectives: To explore the effect of low incubation temperatures and the consequent slowing of bacterial metabolism on the bactericidal action of anti-tuberculosis drugs against *Mycobacterium tuberculosis*.

Methods: Counting of surviving bacteria during exposure of static cultures to isoniazid 1 mg/mL, rifampicin 2 mg/mL, TMC207 0.5 or 2 mg/mL and pyrazinamide 40 or 160 mg/mL drugs, usually for periods of 21 days at temperatures of 37, 25, 22, 19. 16 or 8°C

Results: The bactericidal activities of isoniazid and rifampicin were progressively reduced at 25°C and 22°C, and were minimal at lower temperatures. TMC207 was immediately bactericidal at 37°C, in contrast to the early static phase reported with log phase cultures, and showed less change in activity as incubation temperatureswere reduced than did rifampicin or isoniazid. Pyrazinamide was more bactericidal when incubation temperatures were reduced below 37°C and when the static seed cultures were most dormant.

Conclusions: These results can be explained by the surmise that at low temperatures bacterial energy is at a low level with only just sufficient ATP to maintain homeostasis, making them more susceptible to the blocking of ATP synthesis by TMC207. Insufficient ATP at low temperature would also hinder the export of pyrazinoic acid, the toxic product of the pro-drug pyrazinamide, from the mycobacterial cell by an inefficient efflux pump which requires energy.

1. Introduction

Perhaps the most important issue in understanding the chemotherapy of tuberculosis is why it takes so long to kill *Mycobacterium tuberculosis* in the lesions of patients when axenic cultures can be sterilized in much shorter periods. The tolerance of these persistent bacilli to antibacterial drugs is characterized by the cessation of multiplication and a slowing of metabolism, so that inhibition of cellular biochemical machinery after drug exposure is less effective in killing the cell.[1,2] A variety of *in vitro* model systems have been proposed to mimic *in vivo M. tuberculosis* populations and interrogate drug action, [3-5] but none have identical transcriptional signatures to those found by global gene expression profiling of the "fat and lazy" bacilli encountered in the sputum of patients with pulmonary tuberculosis[6] or from bacilli isolated from human lung sections.[7] Furthermore, most model systems use culture media at the conventional pH of 6.6-6.8 whereas lesions must be more acid in the range of pH 5.5-6.0 to account for the known bactericidal activity of pyrazinamide whilst allowing multiplication of *M. tuberculosis.*[8,9] In the search for inexpensive model systems that would be more representative of persistent bacilli and yet easy to use in assays extending for several weeks, we decided to explore the effects of lowering the incubation temperature from 37 ^{o}C to 25^{o}C where multiplication ceases but metabolism continues, to lower temperatures that likely reduce metabolism further. We used a static stationary phase culture, with characteristic adaptations of persistence including anaerobic respiration and energy production from β-oxidation of fatty acids,[10,11] at pH 6.0 to allow the action of pyrazinamide to be explored. The other drugs tested were the first line drugs isoniazid and rifampicin together with the

diarylquinoline TMC207 (previously R207910) which inhibits mycobacterial ATP synthase, because it is the first of the new drugs being developed and has been shown to be promising both in its characteristics[12] and in the preliminary results of clinical trials.[13]

Methods

Bacterial Strain. *M. tuberculosis*, strain H37Rv.

Chemicals. TMC207 was gift from Tibotec Pharmaceuticals (Beerse, Belgium). Other antibacterial compounds were purchased from Sigma. All other chemicals were obtained from VWR International (Magna Park, Leics, UK) except where specified otherwise.

Media. Dubos broth buffered to pH 5.95 was prepared as follows: To 850 mL distilled water, 8.2g KH_2PO_4 and 3.2g K_2HPO_4 was added and mixed until dissolved. Then 50 mL glycerol and 6.5g Dubos broth base (Difco 238510, Becton-Dickinson, Sparks, MD) was added and mixed on a magnetic stirrer until dissolved. The pH was adjusted to 5.90 with 1M citric acid (approximately 5mL), to give a final pH of 5.95+/-0.05. The medium was sterilized through a 0.2 µm filter. Dubos Medium Albumin (Difco 230910, Becton-Dickinson, Sparks, MD) 100 mL was added to complete the medium.

Serial 10-fold dilutions of Dubos broth cultures in 100 µL volumes were plated onto Mycobacteria 7H11 agar (Difco 283810, Becton-Dickinson, Sparks, MD) supplemented with 100 mg/L oleic acid, albumin, dextrose enrichment (BBL212260, Becton-Dickinson, Sparks, MD) and 50 mg/L of the broad spectrum antifungal carbendazim (Aldrich). Plates were incubated in the dark at 37°C for 28 days, when colonies were counted.

Anti-bacterial compounds. TMC207 5 mg/mL stock solution in sterile dimethyl sulphoxide was added at final concentrations of 0.5 and 2.0 mg/L. Pyrazinamide 10 mg/

stock solution in sterile, distilled water; final concentrations of 30, 40 and 160 mg/L. Rifampicin 10 mg/mL stock solution in sterile, absolute methanol; final concentration 2 mg/L. Isoniazid 10 mg/mL stock solution in sterile, distilled water; final concentration 1 mg/L. The drug concentrations used correspond with concentrations near peak and near trough obtained in patients under treatment[14] except that 160 mg/L pyrazinamide is well above peak concentration to see what effect higher concentrations would have.

Culture systems. The seed inoculum was prepared by growing *M. tuberculosis* in Dubos broth to an opacity of 0.4-0.6 at 580 nm and storing aliquots frozen in liquid nitrogen. Aliquots were pre-tested for the presence of contaminants. In each experiment, a series of 10 mL volumes of buffered Dubos broth at pH 5.95 were dispensed into 28 mL glass screw-capped tubes, which were inoculated with 100 µL seed culture and either, in procedure 1, incubated undisturbed for 30 days or, in procedure 2, incubated until they reached standard opacity (McFarland 2) and then inoculated at a 1:4 by volume dilution into fresh buffered Dubos broth at pH 5.95 and incubated undisturbed for 30 days. The cultures from either procedure were then vortex-mixed to create an even suspension, antibacterial compounds were added, and serial samples taken for colony counting at 3, 7, 14 and 21 days post exposure after incubation at various temperatures. At least two replicates of each antibacterial compound concentration were tested.

Incubation temperatures. The 28 mL screw-capped tubes were incubated either in a small conventional incubator with an adjustable temperature or in a Boekel PCB2 cooling incubator (Grant Instruments, Cambridge) at temperatures of 8, 16, 19, 22, 25 and 37 ^{o}C.

Statistics. Counts and linear regression coefficients were calculated in Excel and were further examined by ANOVA in Stata 8 (Stata Corp., College Station, TX)

Results

Drug-free cultures. Control cultures (no drug) grown at 37°C increased their cfu count by about 1 log unit over 21 days, whereas cultures grown at lower temperatures including 25°C showed no change in their counts over this incubation period.

Inoculum of 10-day static cultures. We first exposed static 10-day cultures to 2 mg/L rifampicin, 1 mg/L TMC207 or 30 or 160 mg/L pyrazinamide at 37, 25, 19 and 8°C. Linear regression coefficients were calculated from cfu counts over 7 days of incubation (Fig 1). None of the drugs had bactericidal activity at 8°C. The bactericidal activity of rifampicin was the greatest at 37°C, but declined sharply at 25°C and 19°C. TMC207 was less bactericidal than rifampicin at 37°C but its activity dropped more slowly at 25 and 19°C. The difference in behaviour between rifampicin and TMC207 was highly significant ($p<0.001$). Pyrazinamide at both concentrations allowed slight growth at 37°C but were marginally bactericidal at 25°C and 19°C.

Inoculum of 30-day static cultures using procedure 1. To extend these findings of a drug-specific effect of incubation temperature, we exposed static 30-day cultures for 21 days to 2 mg/L rifampicin, 1 mg/L isoniazid, 0.5 and 2 mg/L TMC207 or 40 or 160 mg/L pyrazinamide at 37, 25, and 19°C. Cultures were also exposed to the same drugs at 22°C and at 16°C in additional follow up experiments. The counts on RIFAMPICIN (Fig 2) show an almost exponential decline, most rapid at 37°C, less at 25°C and at similar slower rates of killing at 22, 19 and 16°C. The counts on isoniazid (Fig 3) at 37°C showed a rapid decline of 3 log units during the first 7 days of exposure followed by no

loss of viability to 21 days. At 25°C, the initial fall was about 2 log units, while at lower temperature only a very slight decline occurred throughout The counts on 2 mg/L TMC207 showed an exponential fall at 37°C (Fig 4), starting with an initial substantial fall during the first 3 days, and with a slight reduction, much less than with either rifampicin or isoniazid, in the rate of fall at the lower temperatures. The counts on 160 mg/L pyrazinamide showed little change over the 21 days at 37°C, but increased bactericidal activity with similar steady falls at 19 and 25°C (Fig 5). Linear regressions of the viable counts, which estimate overall bactericidal activity, were calculated for isoniazid over 0-7 days during which period all bactericidal activity occurred and for the remaining drugs over 0-21 days since their bactericidal activity was more prolonged (Fig 6). The loss of bactericidal activity as temperature was lowered was most evident with rifampicin and isoniazid. The corresponding fall in activity for TMC207 at both concentrations was smaller than with rifampicin or isoniazid. In contrast to the other drugs, the bactericidal activity of pyrazinamide at both concentrations increased with temperatures below 37°C.

Inoculum of 30-day static cultures using procedure 2. To confirm the enhanced efficacy of pyrazinamide at low temperature in a model where isoniazid-mediated killing was not so prominent, we adopted a second culture system (procedure 2) and re-tested pyrazinamide and isoniazid killing. In procedure 1 for preparation of the test inoculum, isoniazid at 37°C caused a drop of approximately 3 log units during the first 7 days (Fig 3A), whereas in procedure 2 cultures only a 1 log unit reduction in cfu was detected after isoniazid exposure (Fig 3B). Drug-free control cultures grew successfully at 37 °C, and there was no change in viable count over time at 25 or 22 °C, as expected. The viable

counts during exposure to 40 and 160 mg/L pyrazinamide demonstrate unequivocally that reductions in incubation temperature from 37°C to 25°C resulted in a striking increase in bactericidal activity which was even greater at 22°C (Fig 7). At 37°C, pyrazinamide at either concentration had negligible bactericidal activity, but at 22°C the counts on cultures exposed to 40 µmg/L pyrazinamide dropped from log 7.7 cfu/mL to log 5.4 cfu/mL (2 log kill) over 21 days, while those exposed to 160 µmg/L pyrazinamide dropped even further to log 4.3 cfu/mL (3 log kill).

Discussion

The most striking finding of this study was the increase in bactericidal activity of pyrazinamide at temperatures below 37°C, particularly evident when the test cultures had been prepared by procedure 2 (Fig7). This preparation procedure was hypothesized to increase the resemblance of the test cultures to persistent *M. tuberculosis* in sputum and presumably also in lesions because isoniazid exposure caused an initial drop of about 1 log unit in counts Fig 3B). This is similar to the drop in viability demonstrated during the first week of early bactericidal studies on patients given isoniazid monotherapy, [15] and is consistent with evidence suggesting that isoniazid-tolerance may be a feature of persistent bacilli.[5,16] This drop of 1 log unit using procedure 2 is appreciably smaller than the reduction of 3 log units using bacilli prepared by procedure 1 (Fig 3A), and is, as predicted, inversely correlated with pyrazinamide efficacy in these models. An explanation for the increase in bactericidal activity of pyrazinamide at low temperatures is provided by the Zhang hypothesis for the mode of action of pyrazinamide.[8] According

to this hypothesis, pyrazinmide is deaminated in the cytoplasm of bacilli and the resulting pyrazinoic acid is pumped out to the micro-environment. It is then passively reabsorbed in a pH-dependent manner into the bacilli. Once inside, bacilli are required to actively pump pyrazinoic acid out again. If the mycobacterial energy supply is diminished, pyrazinoic acid accumulates within the cell, eventually killing it.[17] Thus, the elimination of pyrazinoic acid is energy-dependent. If ATP levels are diminished by sustained incubation at low temperature, pyrazinoic acid will accumulate more rapidly, and killing will occur more readily. The finding that the bactericidal activity was greater in procedure 2 than in procedure 1 test cultures indicates that less energy, perhaps in the form of ATP, was available in the non-replicating procedure 2 cultures. This hypothesis is consistent with ATP measurements, and transcriptional signatures of energy production and ribosomal biosynthesis, showing a reduction in ATP levels in hypoxic non-replicating *in vitro* models of persistence.[6,10, 18-20] The bactericidal activity of pyrazinamide is therefore at a maximum when the energy sources available to the bacilli are at their lowest, either because metabolism is limited by adaptation to the *in vivo* environments encountered or by low incubation temperatures.

The second finding of interest was the rapid onset of the bactericidal activity of TMC207 at 37^{o}C evident during the first 3 days of exposure. This rapid onset is in contrast to the existence of an initial phase lasting 7-14 days when no change in the cfu count occurs after exposure of log phase bacilli to TMC207.[12,21] The results from log phase organisms gave rise to the view that TMC207 has time dependent but not dose dependent activity.[12] We suggest that the gradual use of the energy store available in log phase bacilli is responsible for the static phase, which is eventually followed by rapid

dose-dependent killing when ATP levels are low and the effects of the ATP synthase isoniazidibitor become cidal. A further feature of interest was the small decrease in the bactericidal activity of TMC207 as the incubation temperature was reduced compared to the larger effects on the bactericidal activities of rifampicin and isoniazid (Fig 6). Since bacterial metabolism is likely to progressively decrease as the temperature goes down, TMC seems to retain its activity even when metabolism is low. This indicates its potential value as a drug capable of sterilizing the more persistent bacterial populations, and identifies energy generation as important for maintenance of cell viability in bacilli isolated from *in vitro* models of persistence.[18-20] This effect may be noticeable because TMC207 was considerably less bactericidal at 37^{o}C than rifampicin or isoniazid, but this greater kill caused by isoniazid and rifampicin may not be reflected by a similar greater activity in treating patients because their half-life and therefore the period of exposure to the drugs in lesions is considerably shorter than that of TMC207.[12,14]

In brief, the effects of reducing the incubation temperature are particularly illuminating in exploring the action of drugs, such as pyrazinamide and TMC207 whose activity is greatly dependent on the energy resource status of bacilli.

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Transparency Declaration

None to declare.

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Fig 1. The speed of bactericidal activity of rifampicin (RIF) 2 mg/L, TMC207 (TMC) 1 mg/L or pyrazinamide (PZA) 30 or 160 mg/L against 10-day static cultures of *M. tuberculosis* at incubation temperatures of 37, 25, 19 or 8°C. Speed is represented by the linear regression coefficients for log cfu/mL/day.

Fig 2. Survival curves for 30-day static cultures of *M. tuberculosis* exposed to rifampicin (RIF) 2 mg/L for 21 days at temperatures ranging from 37°C to 16°C.

Fig 3. A. Survival curves for 30-day static procedure 1 cultures of *M. tuberculosis* exposed to isoniazid 1 mg/L for 21 days at temperatures ranging from 37°C to 16°C
B. Survivor curves for 30-day static procedure 2 cultures exposed to isoniazid (INH) 1 mg/L at 37°C.

Fig 4. Survival curves for 30-day static cultures of *M. tuberculosis* exposed to TMC207 2 mg/L for 21 days at temperatures ranging from 37°C to 16°C

Fig 5. Survival curves for 30-day static cultures (procedure 1) of *M. tuberculosis* exposed to pyrazinamde 160 mg/L for 21 days at temperatures ranging from 37°C to 16°C

Fig 6. The speed of bactericidal activity of rifampicin (RIF) 2 mg/L, TMC207 (TMC) 2 mg/L or pyrazinamide (PZA) 40 or 160 mg/L against 10-day static cultures of *M.*

tuberculosis at incubation temperatures of 37, 25, 19 or 16°C. Speed is represented by the linear regression coefficients for log cfu/mL/day.

Fig 7. Experiments 4. Survival curves for 30-day static cultures (procedure 2) of *M. tuberculosis* exposed to pyrazinamde 40 or 160 mg/L for 21 days at temperatures of A. 37°C or B. at 25°C or 22°C.

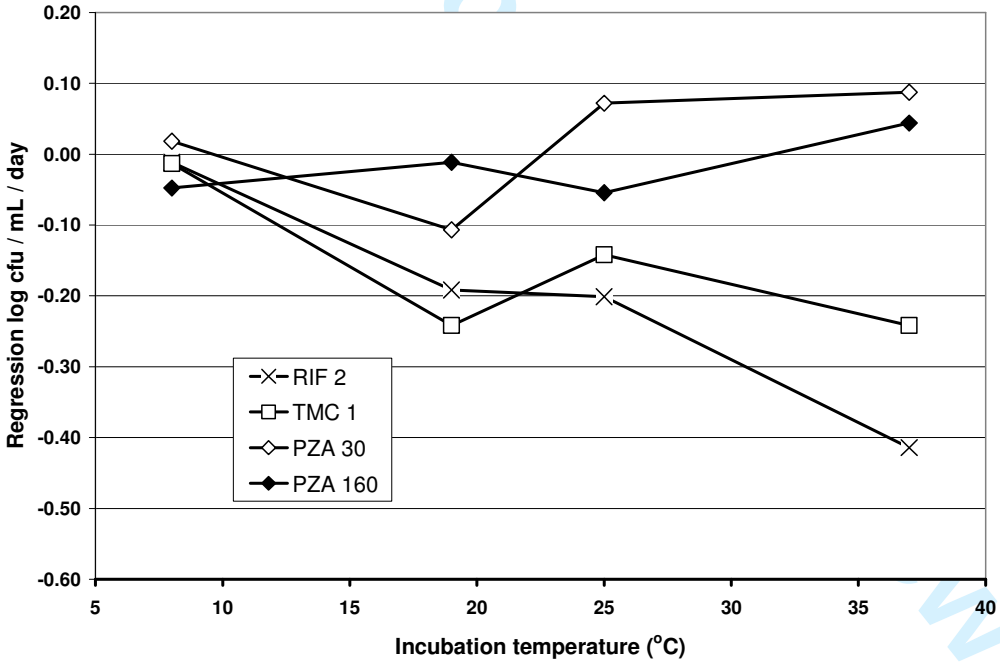

Fig 1

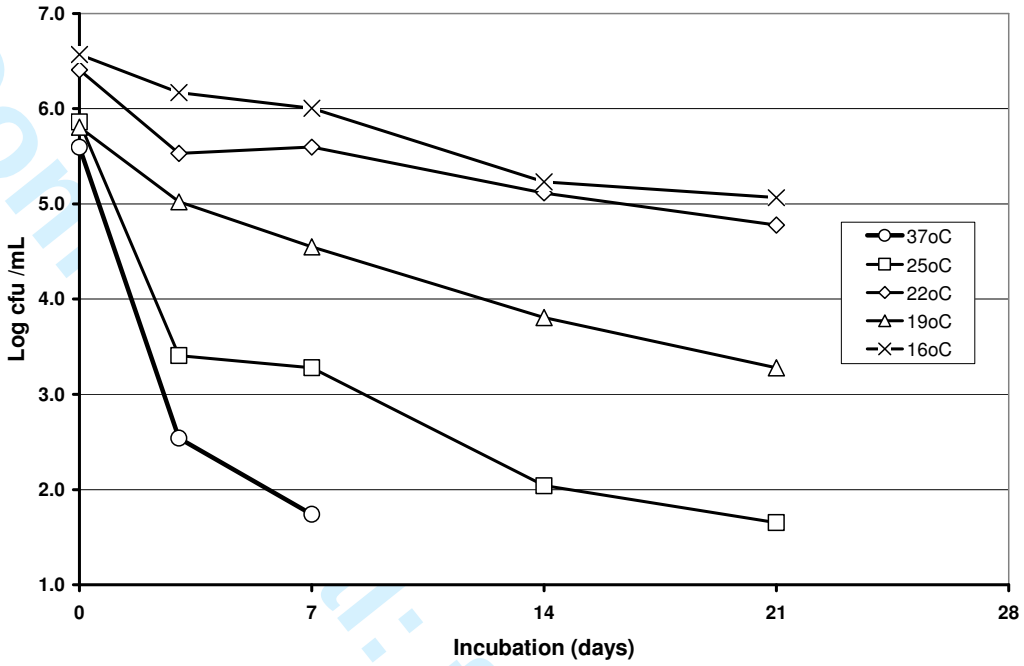

Fig 2

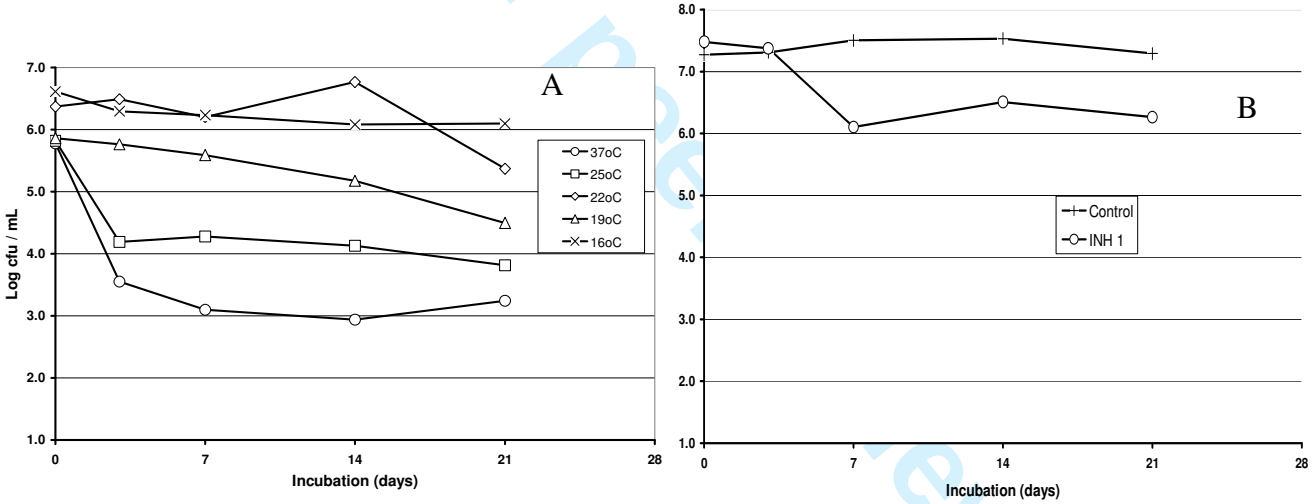

Fig 3

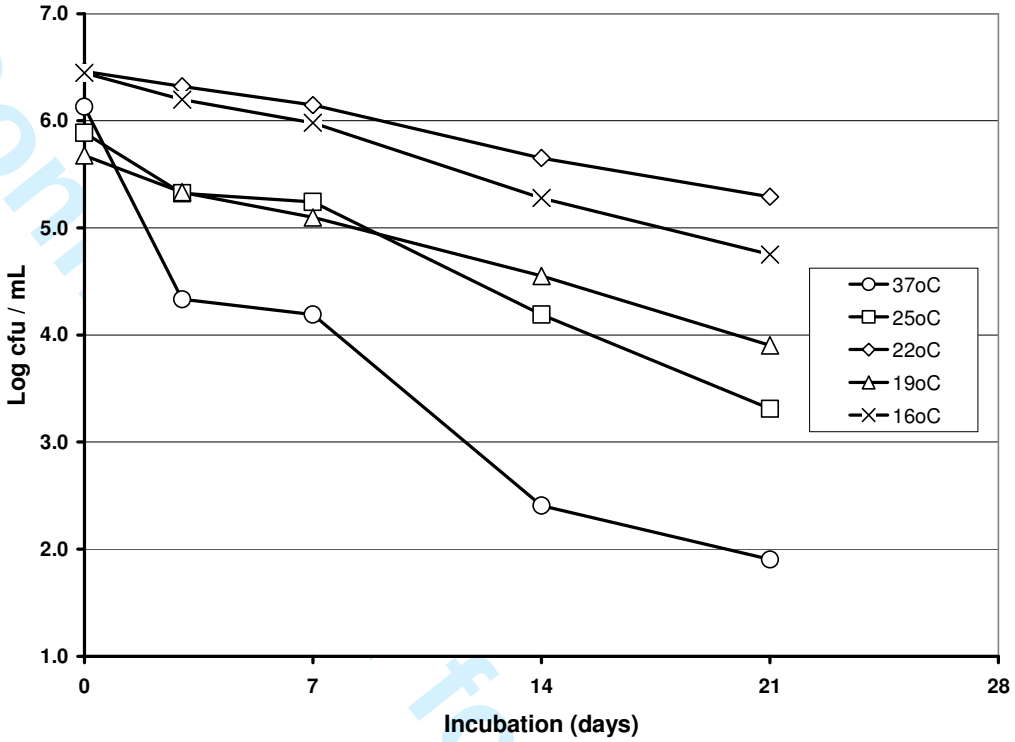

Fig 4

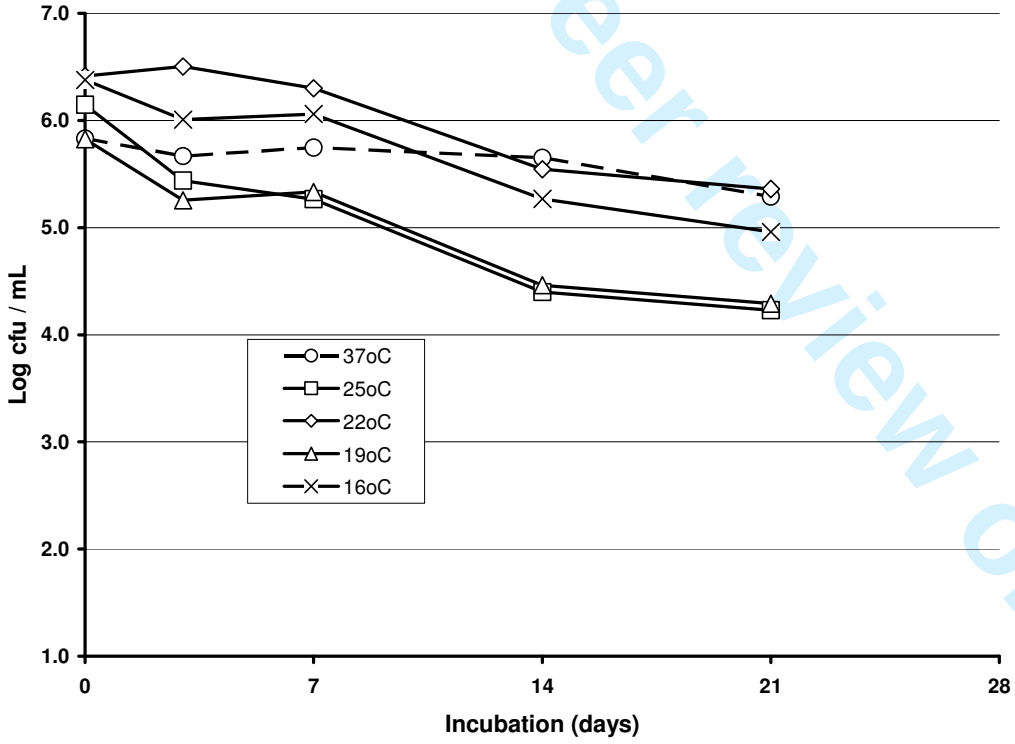

Fig 5

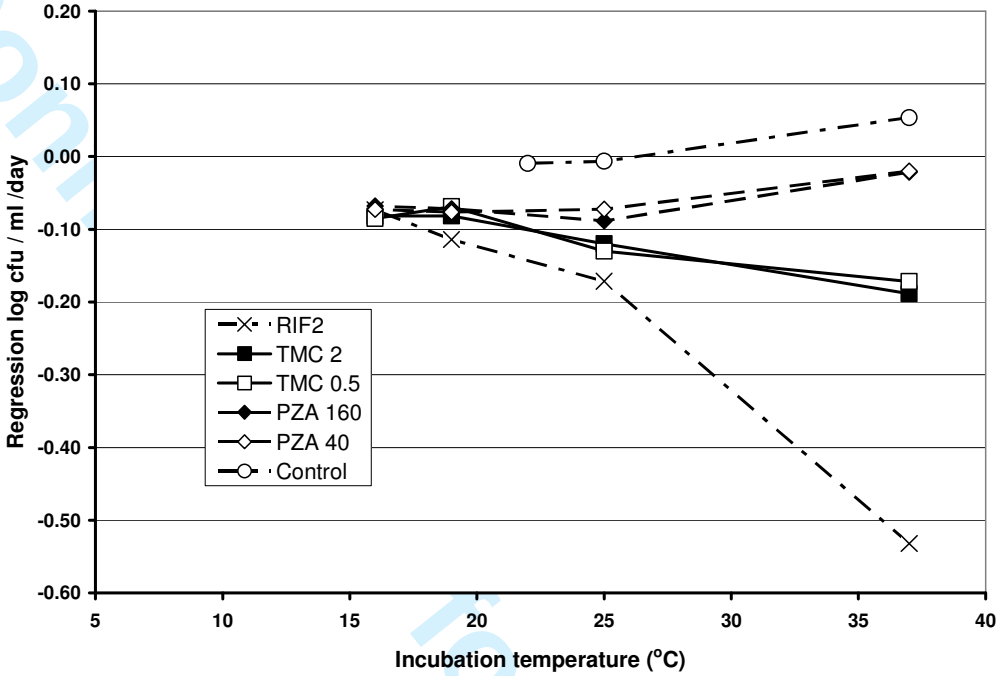
0.20
0.10
0.00
-0.10
-0.20
-0.30
-0.40
-0.50
-0.60
Regression log cfu / ml /day
5
10
15
20
25
30
35
40
Incubation temperature (°C)
RIF2
TMC 2
TMC 0.5
PZA 160
PZA 40
Control

Fig 6

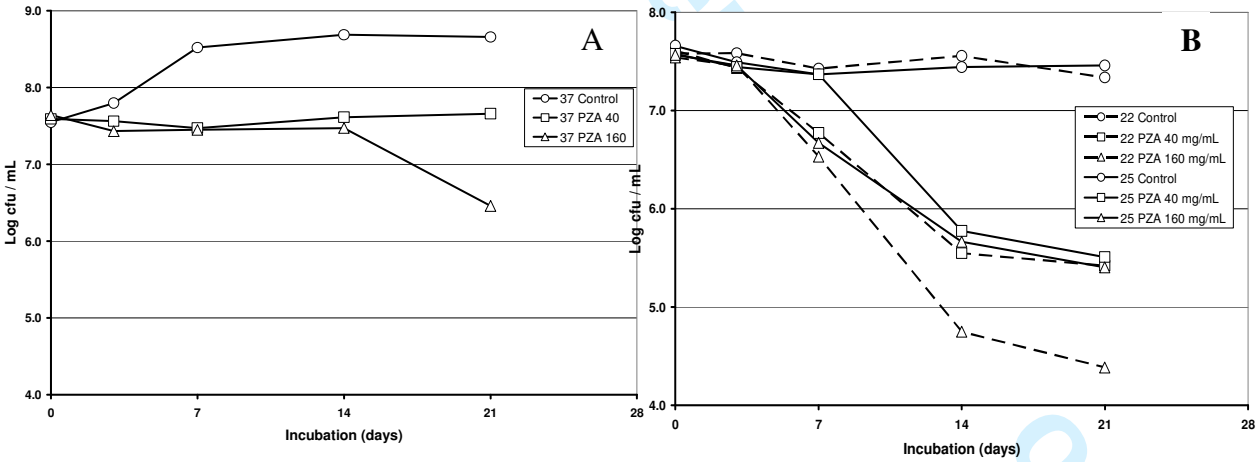
A
9.0
8.0
7.0
6.0
5.0
4.0
Log cfu / mL
0
7
14
21
28
Incubation (days)
37 Control
37 PZA 40
37 PZA 160
B
8.0
7.0
6.0
5.0
4.0
Log cfu / mL
0
7
14
21
28
Incubation (days)
22 Control
22 PZA 40 mg/mL
22 PZA 160 mg/mL
25 Control
25 PZA 40 mg/mL
25 PZA 160 mg/mL

Fig 7