

Protocol for *in vivo* evaluation of growth rates and pathogenesis of *M. tuberculosis* strains found to have rapid or slow growth phenotypes in an *in vitro* model

Experiment #1
TARGET Mouse Model Group

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Background

An *in vitro* model using activated THP-1 cells has recently been proposed as an attractive model for assessing the intracellular growth rate of *M. tuberculosis* isolates as a measure of virulence (Theus et al, 2004). The model has been used to demonstrate that the clinical isolate TB282 representing strain 210 (a W Beijing strain) and associated with a large TB outbreak grew more rapidly in the model than did the clinical isolate TB284, which was considered to have lesser transmissibility. TB282 and the H37Rv control grew at similar rates in THP-1 cells. All 3 strains grew at indistinguishable rates in broth culture, suggesting that the growth defect of TB284 was specific to intracellular growth.

We have obtained 2 new isolates from the Eisenach group that have been assessed in the THP-1 model. Strain SA310 had a “rapid growth” phenotype, while strain SA294 had a “slow growth” phenotype (Theus et al, 2005). The senders have requested that we test the growth of these strains in the mouse model to validate the intracellular assay.

Rationale

Virulence can be assessed in mice by measuring growth rate, assessing the extent of histopathological disease, and determining the mortality rate after intravenous infection. We will assess the virulence of the submitted strains using both low-dose and high-dose intravenous infections. H37Rv and BCG will be used as virulent and attenuated controls, respectively. The submitter’s request is that the strains be used for infection “as is”, but we would like to compare virulence before and after passage.

Methods

The 2 new strains SA310 and SA294 were passaged twice by the IV route in Swiss mice before the initial comparative experiment.

Once the isolates were passaged, all strains to be used in the experiment were sub-cultured in 7H9 broth with OADC and Tween. Intravenous infection of mice took place when the OD was approximately 1 ($\sim 10^8$ CFU/ml). A 5-fold dilution of the broth culture was used for high-dose IV infection (to inoculate $\sim 4 \times 10^6$ CFU in 0.2 ml), while a 500-fold dilution was used for the low-dose infection (to inoculate $\sim 4 \times 10^4$).

182 4-5 week-old female BALB/c mice were infected according to the experimental scheme presented in Table 1 below. Three mice per group were sacrificed at 1, 7, 14, 21, and 28 days post-infection for mouse body weight, spleen weights, and lung and spleen CFU counts. An additional 8 mice/group in the high-dose IV infection groups were kept to determine the mortality rate. Mortality was assessed daily beginning at D14.

Table 1. Experimental scheme to test growth rate/lethality of Eisenach strains

Strain	Dose	Date and # of mice to be sacrificed					For mortality	Total
		D1	D7	D14	D21	D28		
H37Rv	Low	3	3	3	3	3		15
	High	3	3	3	3	3	8	23
BCG	Low	3	3	3	3	3		15
	High	3	3	3	3	3	8	23
SA310	Low	3	3	3	3	3		15
	High	3	3	3	3	3	8	23
SA294	Low	3	3	3	3	3		15
	High	3	3	3	3	3	8	23
SA310*	Low	3	3	3	3	3		15
SA294*	Low	3	3	3	3	3		15
Total		30	30	30	30	30	32	182

* Unpassaged isolates

Results

The body weight increased similarly for all groups except high-dose H37Rv (data not shown). After a modest weight gain in the first week after infection, mice in the latter group experienced a precipitous decline in body weight as the mice became severely ill. By D14, 2 of the 8 mice retained for survival analysis had died. The remainder of the mice died before D21.

The results of spleen weights are presented in Figure 1. After low-dose infection, the spleen weights increased more rapidly and to a larger extent in mice infected with H37Rv, SA310 and SA294 compared to the BCG group. The greatest increase occurred between D7 and D14, after which the weights generally reached a plateau. None of the differences between strains H37Rv, SA310 or SA294 were statistically significant. After high-dose infection, spleen weights increased more rapidly, especially in mice infected with SA310 and SA294. At D7, the spleen weight was significantly higher in the SA294 group compared to the SA310 group. Spleen weights in both these 2 groups were significantly higher than in the BCG group until D21, when statistical significance was lost. As noted above, mice in the high-dose H37Rv group died between D14 and D21. Due to overwhelming infection resulting in extreme illness and cachexia, the spleen weights never increased significantly from baseline.

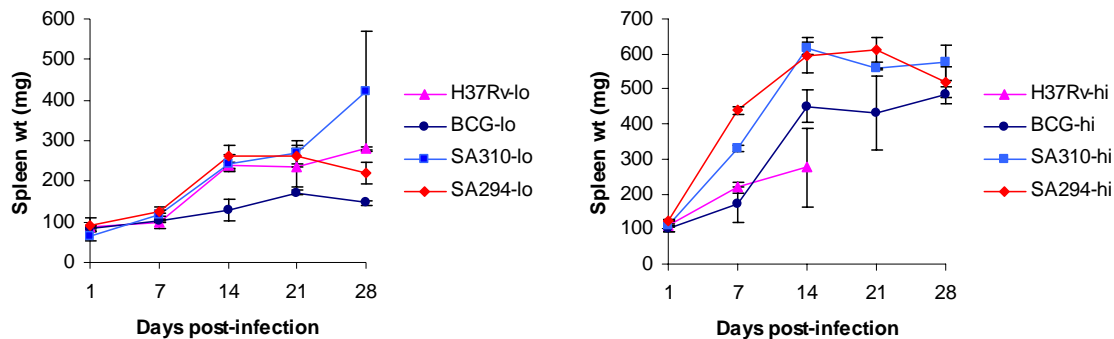


Figure 1. Mean spleen weights after IV infection with a low (left) or high (right) infectious dose

The results of spleen CFU counts are presented in Figure 2. Whether following low-dose or high-dose infection, the SA310 and SA294 strains behaved similarly to one another and to the H37Rv control. All 3 strains proliferated to higher CFU counts than did BCG.

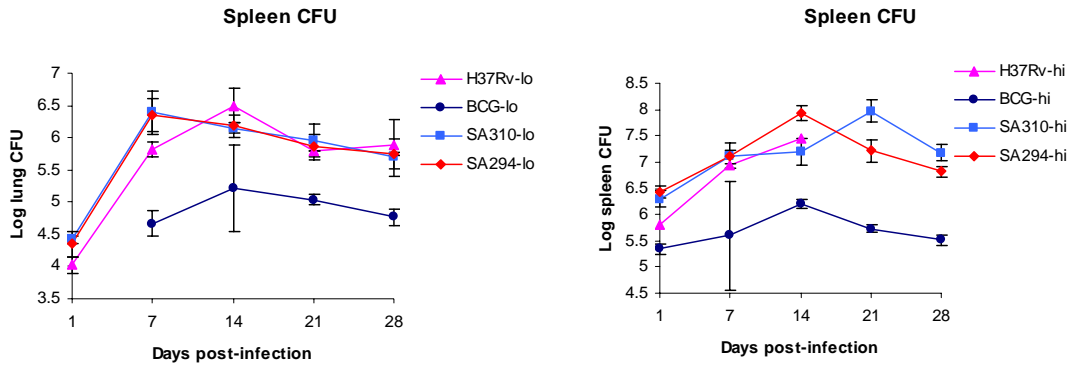


Figure 2. Mean log₁₀ spleen CFU counts after IV infection with a low (left) or high (right) infectious dose

When comparing the non-passaged (np) and the mouse-passaged (lo) isolates after low-dose infection, there were no significant differences in spleen weights or CFU counts (Figure 3).

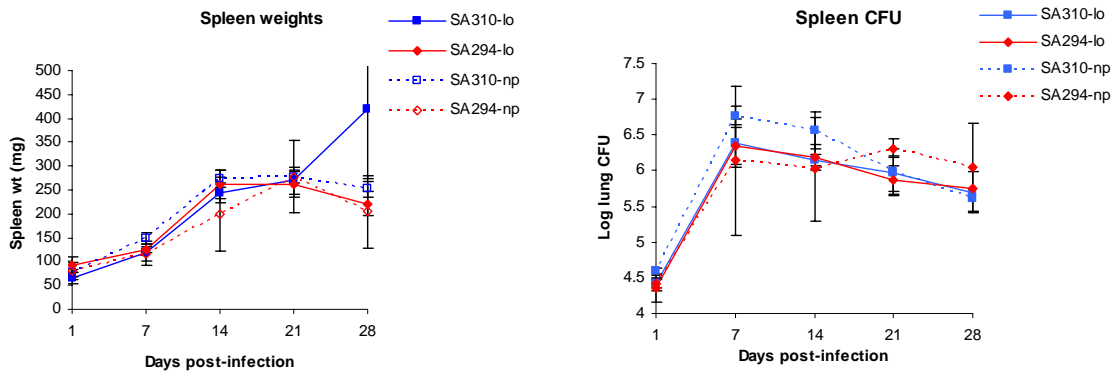


Figure 3. Mean spleen weight (left) and log₁₀ spleen CFU counts (right) after low-dose IV infection with a mouse-passaged (lo) or non-passaged (np) isolate

The results of lung CFU counts are presented in Figure 4. Curiously, despite similar starting CFU counts in the spleen between H37Rv, SA310 and SA294, the lung CFU counts were much higher for H37Rv, implying that a greater proportion of injected bacilli were deposited in the lungs for H37Rv compared to the other strains. Because D1 lung CFU counts should be approximately 2% of infectious dose, the D1 lung CFU counts for the H37Rv strain seem inappropriately high.

After low-dose infection, the H37Rv, SA310 and SA294 strains increased by over 2 logs in the first 2 weeks. The SA310 strain had a higher CFU count than the SA294 strain at D7, but the difference did not reach statistical significance ($p=0.08$) and may have occurred by chance if one takes into account the overall appearance of the two curves. BCG proliferated poorly after low-dose infection and was below the limit of detection at D28. After high dose infection,

SA310, SA294 and BCG all behaved similarly to each other and to H37Rv after low-dose infection.

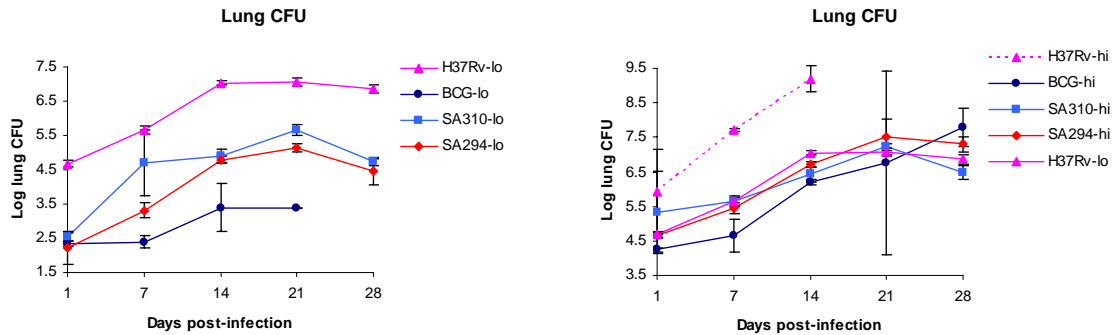


Figure 4. Mean log₁₀ lung CFU counts after IV infection with a low (left) or high (right) infectious dose

Comparison of the non-passaged (np) with the mouse-passaged (lo) isolates of SA310 and SA294 in the lungs after low-dose infection also showed no differences between the isolates based on mouse passage (Figure 5). As seen with the mouse-passaged isolates above, the non-passaged SA310 isolate had higher CFU counts than the SA294 isolate at D7, although the difference was not statistically significant.

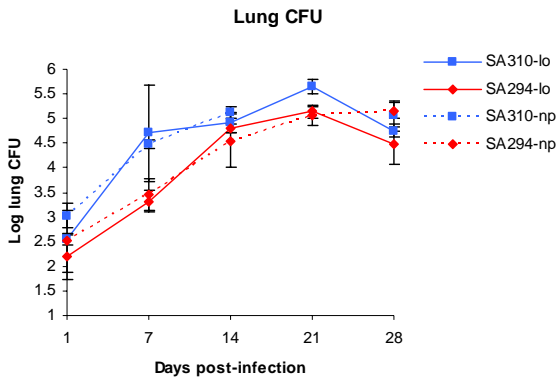


Figure 5. Mean log₁₀ lung CFU counts after low-dose IV infection with a mouse-passaged (lo) or non-passaged (np) isolate

There was no mortality after 12 weeks in any group other than high-dose H37Rv

Conclusions

Overall, both the SA310 and the SA294 strains behaved similarly to each other and to the virulent H37Rv control after IV infection in this short-term experiment. However, we cannot exclude the possibility that the early proliferation (i.e., from D1 to D7) is more rapid for the SA310 strain in the lungs of mice after low-dose intravenous infection. This was assessed next in a low-dose aerosol infection model (see Experiment #2 below).

The virulence of the SA310 and SA294 isolates did not vary by mouse passage status. This may not be surprising, though, because these were clinical isolates that had not been repeatedly passed *in vitro* prior to use.

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Experiment #2
TARGET Mouse Model Group

Rationale

A low-dose aerosol model was used to re-examine the possibility that the SA310 strain was associated with more rapid proliferation in initial 1-2 weeks after infection. Only the previously passaged isolates of the indicated strains were tested.

Experimental Plan

Table 2.

Strain	Time point after infection & No. of mice to be sacrificed					Total
	D1	D7	D14	D28	Survival	
H37Rv	6	6	6	6	12	36
SA310	6	6	6	6	12	36
SA294	6	6	6	6	12	36
Total	18	18	18	18	36	108

Methods

Aerosol infection of 108 5-week-old female BALB/c mice was performed using late log-phase cultures of the indicated strains. Six mice per group were sacrificed at 1, 7, 14, and 28 days post-infection for mouse body weight, spleen weights, and lung and spleen CFU counts. An additional 12 mice/group were kept to determine the mortality rate. Mortality was assessed daily beginning at D14.

Results

Although the SA294 strain, which has a less virulent phenotype in macrophages is also a slow grower *in vitro* and had approximately 1 log₁₀ fewer bacilli implanted by the aerosol infection, the strain showed no growth rate deficits in the mouse over the 28-day test period when compared to H37Rv and the intramacrophage virulent strain, SA310 (Figure 6). Between D14 and D28, there was a slight decline in the growth of SA310, such that the two test strains had nearly identical values on D28. The growth curves for bacteria that disseminated to the spleen were superimposable. Figure 7 shows that infection with H37Rv caused a greater increase in spleen weight and decline in body weight than did either of the other strains. These differences were not statistically significant. Although there were deaths in two mice infected with H37Rv at day 31, there were no other deaths in any of the groups up to 12 weeks after infection.

Conclusions

There were no significant differences in growth rate or mortality between the SA310 and SA294 strains in these short-term infection models. Neither strain was more virulent than the H37Rv control strain.

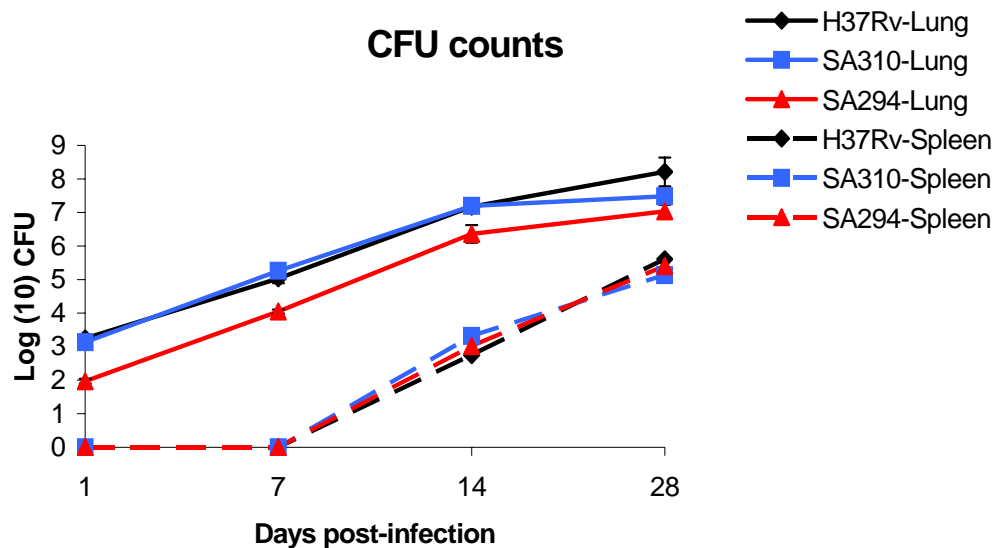
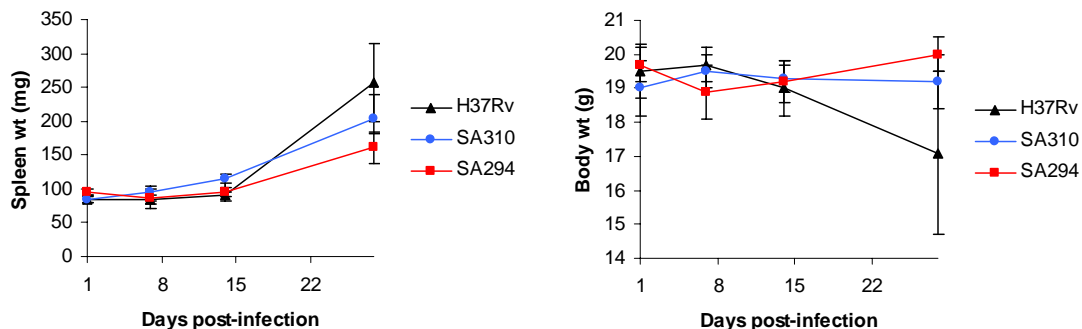


Figure 6. Growth of *M. tuberculosis* H37Rv, SA310, and SA294 in mice after aerosol infection.

Figure 6. Changes in spleen and body weight after aerosol infection with *Mtb* H37Rv, SA310, or SA294



References

Theus SA, et al. Activated THP-1 cells: an attractive model for the assessment of intracellular growth rates of *Mycobacterium tuberculosis* isolates. *Infect Immun* 2004;72:1169.

Theus SA, Cave MD, Eisenach KD. Intracellular macrophage growth rates and cytokine profiles of *Mycobacterium tuberculosis* strains with different transmission dynamics. *J Infect Dis*. 2005;191:453-60.