Optimizing the Macrophage Assay

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Introduction

Francisella tularensis is a highly infectious intracellular human pathogen. Due to its low infectious dose and its variety of hosts and transmission vectors, *F. tularensis* has been classified as a potential bioweapon by the CDC (Charity et al 2009). Once the bacterium enters a host it can replicate to very high numbers inside a multitude of different cell types with macrophage being a notable key niche. There is still much that is not understood about the factors controlling virulence in this pathogen. By creating mutants that delete or change potential essential genes and testing their ability to replicate in macrophage, we can begin to determine which genes are necessary for virulence. The macrophage assay is an important tool that we can use to test these mutants, but this assay needs to be optimized to guarantee meaningful results. One important factor to optimize is growth media. The bacteria need to grow on solid media before being used in the assay and it is important to consider any variability between suppliers of growth media. This report compares macrophage assay results using growth media from two different suppliers Alpha Biosciences and BD.

Methods

Three strains of *F. tularensis* were tested in this assay. These strains were LVS, the wildtype strain, dPigR, a strain where the gene producing the transcription factor PigR has been deleted, and dPmrA where the transcription factor PmrA has been deleted. Both dPigR and dPmrA have been shown to not be able to replicate in macrophage (Ramsey & Dove 2016). In this assay the dPmrA strain was grown on media from Alpha Biosciences and on media from BD to compare any variability in results.

To perform this assay murine macrophage cells are seeded in two 96 well plates with 3 wells devoted to each strain being tested in each plate. A fixed amount of about 200 bacterial cells are added to the wells with the macrophage. The bacteria are allowed to incubate with the macrophage for two hours to allow the bacteria time to enter the macrophage and then the wells are treated with gentamicin to kill any bacteria that were not able to enter the macrophage. Two hours after treating with gentamicin, one of the 96 well plates is treated with saponin to break open the macrophage and the culture in the wells are plated on agar plates. The bacterial colonies that grow on these plates represent the number of bacteria that were able to enter the macrophage for each strain tested.

Twenty-four hours after the 96 well plates were treated with gentamicin, the other 96 well plate is removed from the incubator and the saponin is added to the wells. The culture from these wells is plated on agar plates. The bacterial colonies that grow on these plates represent the number of bacteria that replicated inside the macrophage during the 24-hour period.

Results

R Studio was used to interpret results from the plate counts. Each strain tested had a total of 6 plates to be counted from the 24-hour time point. Using R Studio the plate counts for each strain were averaged and the CFU/well (colony forming units per well) was determined for each of the three wells for each strain. Next the average CFU/well was determined for each strain and this number was plotted using R Studio for each strain (Figure 1). Please note that the y-axis is in log form. To further examine the relationships between strains a t-test was performed using R Studio and compares the mutant strains to the wildtype strain (LVS). An additional t-test was performed to compare the dPmrA strain grown on the Alpha Biosciences media and the dPmrA strain grown on the BD media (Table 1).

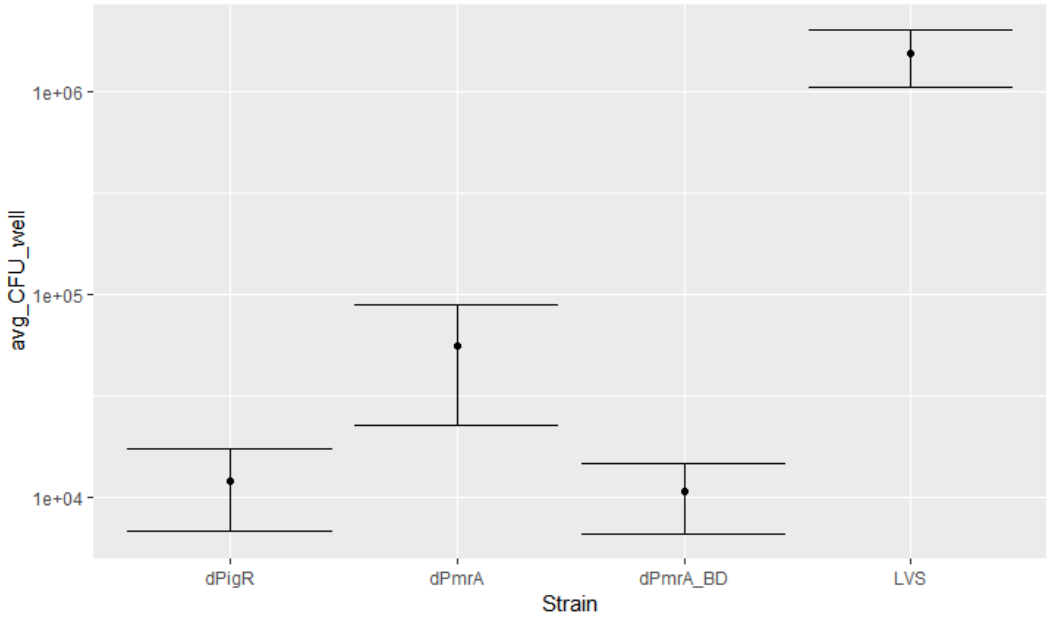

Figure 1

The average CFU/mL for all strains at the 24-hour time point. The y-axis is displayed in log form. Error bars are based on the standard deviation for each strain.

Table 1

Summary of statistical t-tests preform in R Studio, assuming that a p-value of below 0.05 indicates statistical significance between strains.

Strains Compared	p-value
LVS, dPigR	0.03190216
LVS, dPmrA	0.03320627
LVS, dPmrA_BD	0.03185131
dPmrA, dPmrA_BD	0.1395975

Discussion

Going through the different variables used in the macrophage assay is essential if it will be used to support further research. The results of this assay was able to tell us something about how using growth media from two different suppliers could have an effect on the results. Graphically, the results of this assay do seem to show a difference between growing the dPmrA strain on the growth medium from the two suppliers. When comparing the ability of the different strains to replicate in macrophage a t-test was used to determine statistical significance. In this comparison all of the deletion strains did show a statistically significant difference from the wildtype strain (LVS). This can be supported by p-values of 0.03190216 for dPigR, 0.03320627 for dPmrA and 0.03185131 for the dPmrA strain that was grown in the BD media (Table 1). The comparison between the dPmrA strain grown on the Alpha Biosciences media and the dPmrA strain grown on the BD media was not statistically significant with a p-value of 0.1395975 (Table 1). While visually there seemed to be a difference in the replication ability of the dPmrA strain on the two types of media, statistically the difference was not significant. This information will be important in performing future assays because if the ability to obtain media from supplier is impeded then we have an alternate supplier we can use from without corrupting assay results.

References

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