Virulence-related characteristics of *Bordetella pertussis* mutants deficient in intracellular survival

Callie Effler[1], Kalyan Dewan[2], Israel Rivera[2], Randy Kim[2], Jonny Hancock[2], Monica Cartelle Gestal[2], Eric Harvill[2]

[1] Department of Natural Sciences and Mathematics, Lee University, Cleveland, TN

[2]University of Georgia, College of Veterinary Medicine, Athens, GA

Background

- Intracellular survival is a common trait among human pathogenic bacteria that has advantages for the bacteria's protection from the host immune response, persistence, and dissemination.
- *Bordetella pertussis*, the gram-negative bacteria that causes whooping cough in humans, is commonly regarded as an extracellular pathogen. However, it has been recovered from macrophages in *in vitro* experiments, and reported anecdotally in clinical samples. It is unknown what contribution to pathogenicity this has, if any, on the host.
- In this work, our broad objective was to evaluate the impact of *B. pertussis'* intracellular survival and its role in pathogenicity. To do so, we identify mutants similar to the wild type in all aspects of virulence, except for survival inside of macrophages.
- A transposon library of *B. pertussis* UT25 was screened, resulting in the identification of several putative mutants that are deficient in intracellular survival.

Objective

In vitro comparison of 6 putative mutant strains (E7, E8, E9, D5, G4, and B10) to the wild-type parent strain, BpUT25, to identify a mutant deficient in intracellular survival for use in *in vivo* experiments that has no differences in aspects of virulence, such as:

- Intracellular survival
- Cytotoxicity
- Hemolytic activity
- Resistance to serum complement
- Growth (as a measure of fitness)

Methods

Bacterial Culture: strains were plated on Bordet Gengou media supplemented with 10% defibrinated sheep blood. Liquid cultures were grown in Stainer-Scholte media supplemented with hepatkis and *B. pertussis* specific supplements.

Intracellular Survival Assay: RAW mouse macrophages were exposed to each strain at a target MOI of 100:1, and then extracellular bacteria were killed using gentamycin (300 µg/mL). Macrophages were lysed with Triton X 0.1%, and the contents plated on BG media.

Cytotoxicity Assay: Macrophages were exposed to each strain at a target MOI of 100:1. A Promega CytoTox Nonradioactive Cytotoxicity Assay kit was used according to the manufacturer protocol. LDH release levels were used as a measure of cytotoxicity.

Serum Complement Assay: Bacteria were incubated for 1 hour at 37ºC exposed to either PBS or mouse serum (20% in PBS), and then plated on BG agar. The amount of colony growth for bacteria exposed to each was used to calculate the percent survival.

OD_{600} Growth Curve: Bacteria were grown in SS media, beginning at an OD_{600} of 0.3. Measurements of OD_{600} were taken after 3 hours, and then every 2 hours thereafter, for 16 hours.

Results

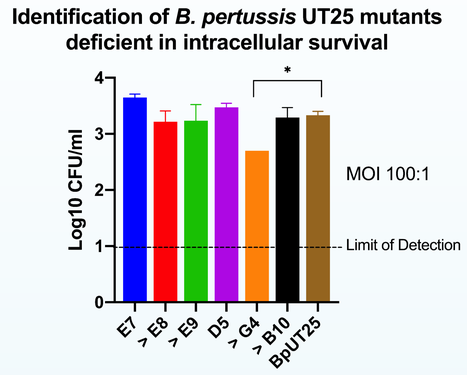


Figure 1: *B. pertussis* recovered from RAW 264.7 macrophages
G4 is seen to have a significant deficiency ($P < 0.05$) in intracellular survival in comparison to its parent strain, BpUT25. Some of the other strains, particularly those indicated, have been seen in other assays to have lower levels of intracellular survival at a MOI of 10:1.

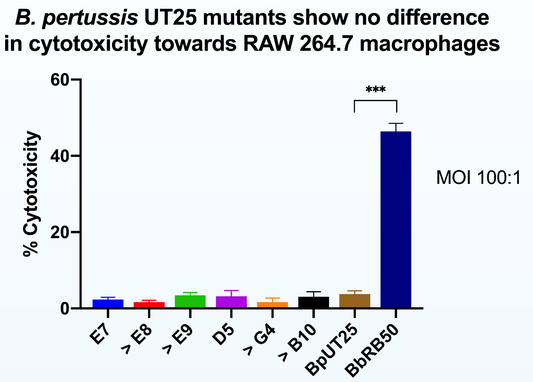
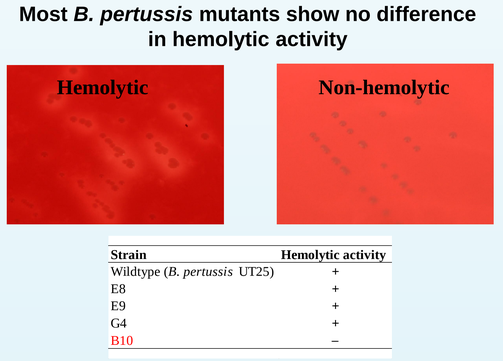


Figure 2: Cytotoxicity of *B. pertussis* UT25 strains and *B. bronchiseptica* RB50
All of the mutant strains show a significant deficiency in cytotoxicity ($P < 0.001$) compared to the known cytotoxic strain *B. bronchiseptica* RB50, and similar levels of cytotoxicity to the parent strain BpUT25, when tested at a MOI of 100:1.



Strain	Hemolytic activity
Wildtype (*B. pertussis* UT25)	+
E8	+
E9	+
G4	+
B10	–

Figure 3: Hemolytic Zones
Each of the strains exhibited hemolytic activity, indicating the presence of adenylate cyclase toxin, with the exception of B10.

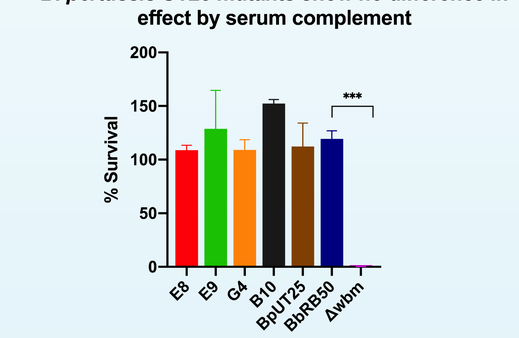


Figure 4: Percent survival of *B. pertussis* strains and *B. bronchiseptica* RB50 after exposure to blood serum
Each of the mutant strains are seen to survive exposure to blood serum at similar levels to the parent strain BpUT25.

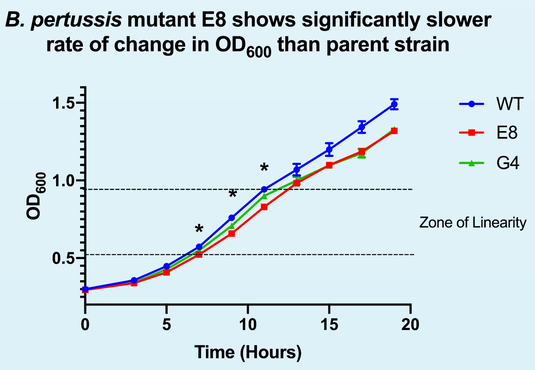


Figure 5: OD_{600} measurements of bacterial cultures of E8, G4, and BpUT25 over the course of 16 hours
While the OD of G4 increases at a similar rate to the parent strain BpUT25 throughout the entire time, E8 increases at a statistically slower amount ($P < 0.05$) during the exponential phase of growth.

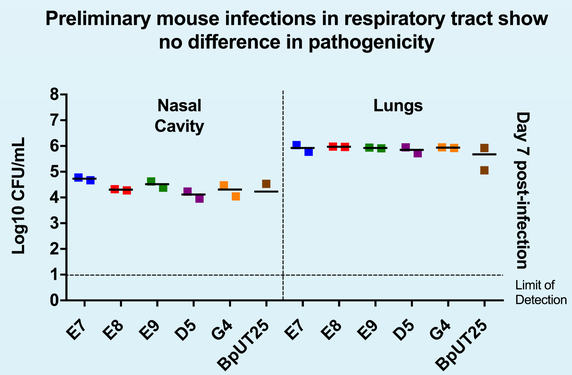


Figure 6: Amounts of *B. pertussis* strains recovered from upper and lower respiratory tract of mice 7 days post-infection
Based upon preliminary data, each of the mutants show similar levels of colonization in the nasal cavity and lungs to the parent strain BpUT25 at 7 days post-infection, indicating that there is likely no difference in *in vivo* pathogenicity.

Summary

- All of the mutants analyzed show similar levels of cytotoxicity, and G4, E8, E9 and B10 have the most pronounced deficiencies in intracellular survival
- These four mutants show similar levels of resistance to serum complementation, but B10 lacks hemolytic activity.
- An OD_{600} growth curve of the two most promising mutants reveals that E8 shows differences from the parent strain, BpUT25, that are not present with G4.

Discussion and Future Work

- G4 seems to be the best candidate among those analyzed for a mutant deficient in intracellular survival, but similar in other virulence-related characteristics.
- Preliminary mouse trials are continuing to see how the mutants behave *in vivo* in comparison to the parent strain.
- Screening of the *B. pertussis* UT25 transposon library will continue until 3x coverage of the approximately 3500 genes has been completed, and any further intracellularly-deficient mutants found must be tested for differences in virulence-related characteristics.
- Whole genome sequencing of G4 and other promising mutants must be completed to identify the mutation causing their deficiencies in intracellular survival, and to ensure that no other mutations are present.
- Complementation assays, which restore the mutation thought to cause intracellular deficiencies and then observe if function is restored, must be conducted for each mutant to ensure that no other mutations are present.

Acknowledgements and References

Many thanks to the National Science Foundation (NSF) for funding this REU program, and to the University of Georgia for organizing and hosting the program.

I would like to thank Dr. Harvill for opening his lab to me and letting me use their resources. Thank you especially to Dr. Dewan, Israel Rivera, and Dr. Gestal for their guidance, advice, and their time spent mentoring me throughout this process.

1) Lamberti, Y et al. (2013). Pathogens and Disease. Vol. 69, Issue 3: 194-204.

2) Hellwig, SM et al. (1999). FEMS Immunol Med Microbiol. Vol. 26, Issue 3-4: 203-7.

3) Taylor-Mulneix, D et al. (2017). Frontiers in Cellular and Infection Microbiology. Vol. 7: 510.