

Comparison of two adjuvants in an experimental *Actinobacillus equuli* vaccine in horses

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Abstract

Actinobacillus equuli is a Gram-negative bacterium that causes sleepy foal disease in neonatal foals. Mortality rates for foals infected with *A. equuli* tend to be higher than foals infected with other bacteria. The *A. equuli* bacterium is an oral commensal of adult horses and dams may produce detectable amounts of serum antibodies to *A. equuli*. Mare antibodies may pass to foals through colostrum, providing passive protection against *A. equuli*. Vaccinating dams with bacterial outer membrane proteins (OMPs) and *Actinobacillus equuli* Aqx toxin elicited measurable antibody response to both OMPs and Aqx, but this previous experimental vaccination had undesirable long-lasting side effects including injection site swelling and fibrosis. These reactions were thought to be a result of the adjuvant selected. The current project will replace the previously-used adjuvant with squalene or double mutant *Escherichia coli* heat labile toxin (dmLT). Squalene has been used successfully in BHV-1 vaccines, which suggests it is a candidate for avoiding negative injection site reactions. For this study, we will use previously established procedures to isolate and purify OMPs and Aqx and combine the purified proteins with the selected adjuvant. The adjuvant-protein combination will be administered to mares and the immune response will be assessed. Illness behavior and injection site reactions will also be evaluated. Follow-on studies will evaluate successful candidate vaccines for colostrum transfer to foals.

Keywords: *Actinobacillus equuli*, horses, vaccine, Aqx toxin

Introduction

Actinobacillus is a genus containing four different bacterial species (*A. pleuropneumoniae*, *A. suis*, *A. lignieresii*, and *A. equuli*) commonly found in animals. It is related to other pathogenic bacteria found in the *Pasteurellaceae* family, such as *Haemophilus*, *Mannheimia*, and *Pasteurella* (Holyoak 2007). *A. equuli* comes in two subtypes— hemolytic (*A. equuli* subsp. *haemolyticus*) and non-hemolytic (*A. equuli* subsp. *equuli*) (Skaggs 2011). *Actinobacillus equuli* causes sleepy foal disease, a septicemia found in neonatal foals. The first sign of the disease may be diarrhea, with

more serious illness following, such as meningitis, pneumonia, purulent nephritis, or septic polyarthritis. Mortality rates for foals infected with *A. equuli* are higher than those infected with other bacteria, and deaths are common in infected foals. *Actinobacillus equuli* originates in the mare's mouth, respiratory tract, and alimentary tract. It is normal flora in the mare's alimentary system, but is also associated with peritonitis, reproductive failure, endocarditis and pneumonia in adult horses. *A. equuli* is contracted by the foal during or immediately following birth, but

research has revealed the ability of antibodies in the mare's colostrum to ward off the disease (Rycroft 2000).

Researchers established that mares have detectable amounts of antibodies to *A. equuli* outer membrane proteins and these antibodies were transferred to their foals (Holyoak 2007). While these antibodies are naturally occurring, Skaggs hypothesized that immunizing mares with *A. equuli* outer membrane proteins and Aqx would increase colostrum antibody content. The toxin Aqx is the hemolysin of *A. equuli*. Aqx hemolytic activity can be inhibited by specialized antibodies raised in an immune response against Aqx. A vaccine was created that did elicit an immune response, but implications arose from vaccine injection site reactions such as long-lasting swelling and fibrosis regardless of the site preparation (Skaggs 2011). The injection site reactions were hypothesized to be due to the Freund's incomplete adjuvant used in the study. In this project, the adjuvant used by Skaggs will be replaced with squalene in water or double mutant *Escherichia coli* heat labile toxin (dmLT). Used in conjunction with sulfolipo-cyclodextrin for BHV-1 vaccines, squalene did not create injection site reactions in cattle (Romera 2001). With respect to this evidence, we hypothesize we will elicit a similar immune response to the previous studies but that vaccine site reactions will be within acceptable limits.

Materials and methods

The *A. equuli* subsp. *haemolyticus* strain 0211192 for this vaccine was originally isolated from a septicemic neonatal foal at the Oklahoma Animal Disease Diagnostic Laboratory. This strain was grown for 8 hours in Brain Heart Infusion broth at 37°C on a rotary shaking incubator. The whole cells from this are then prepared as described below.

The first phase in our project was to prepare the cell envelopes via sonication with the method developed by Dabo, with minor modifications (1997). First, plates were streaked with *Actinobacillus equuli* for isolation, and incubated overnight at 37°C. To insure culture purity, one colony of the *A. equuli* was inoculated into 5 mL of BHI and grown to an OD of ~0.6 at 37°C in a shaker incubator. This culture aliquot was inoculated into 1L of fresh BHI for overnight growth at 37°C in a shaker incubator. Cells were pelleted at 6,000 rpm in a GSA rotor for 20 minutes at 4°C. Products were washed in half-volume sterile phosphate-buffered saline, pellets of the same culture were combined, and pelleted again at 6,000 rpm in a GSA rotor for 20 minutes at 4°C, then supernatant was poured off.

The washed, whole pellets of *A. equuli* were transferred to pre-weighed centrifuge tubes for a SS34 rotor, then centrifuged at 12,000 rpm in a SS34 rotor for 20 minutes at 4°C. The supernatant was discarded and the pellets were weighed and resuspended in 20 mL 20% sucrose in 0.01 M HEPES. This was split between two 50 mL sterile plastic centrifuge tubes, then brought to a volume of 20 mL each. To each tube, 0.24 mg RNase and 0.24 mg DNase per gram of cells and 1.5 mM of phenylmethylsulfonyl fluoride (PMSF) was added. Each aliquot was sonicated five times for 30 second intervals with 30 second rests. Each tube was in a plastic beaker filled with ice and the opening of the tube covered with parafilm through which the microtip sonicator probe was inserted with the limit setting at 7 and 50% duty. The aliquots were incubated for 40 minutes at 37°C, centrifuged at 7,000 rpm in a SS34 rotor for 20 minutes at 4°C, and the supernatant was removed and saved. The supernatant was ultracentrifuged using 55.2 Ti rotor at 50,000 rpm for 70 minutes at 4°C. Pellets

were washed three times in cold, sterile, distilled water, then spun at 50,000 rpm for 70 minutes at 4°C. The final pellet was resuspended in 1 mL of sterile, distilled water. At this stage in the procedure, pellets from the same cultures were combined.

Next was the extraction of the outer membranes of the cells. An equal volume or greater of 0.5% Sarkosyl was added to the washed total membrane preparation, mixed for 30 minutes at room temperature and ultracentrifuged using a 55.2 Ti rotor at 50,000 rpm for 70 minutes. The supernatant, which contained inner membrane proteins, was discarded. The pellet was washed three times in cold, distilled water and centrifuged for 70 minutes at 50,000 rpm. The pellet was resuspended in 1.0 mL cold distilled water and the protein concentration was determined, then the pellet was frozen at -20°C.

For the Aqx toxin production, an *Escherichia coli* transformed with a plasmid containing the Aqx gene was grown in LB broth with 40 micrograms/mL kanamycin in a 37°C shaker incubator to an A₆₀₀ of 0.5-1.0 at which time the expression of poly-His-tagged Aqx was induced by adding *iso*-propyl thiogalactopyranoside (IPTG) to a final concentration of 1mM. Expression was continued for 3 hours and cells were harvested. The cell pellet was then lysed by 5 freeze-thaw cycles and the cell suspension was centrifuged. Pellet and suspension were assayed for the presence of Aqx. The crude extracts were run on an SDS-PAGE gel and probed with anti-poly-His antibody. A Western Blot was then used to confirm the presence of insoluble Aqx protein in the pellet.

We will repeat the OMP isolation and Aqx toxin

production until sufficient quantities of each protein are acquired. We will then inject 18 adult breeding mares at the Center for Veterinary Health Sciences Ranch with a vaccine comprised of 200 ug OMPs and 200 ug Aqx combined with either 50ug squalene or 50 ug dmLT, qs. to 1ml with phosphate-buffered saline. Mares will be vaccinated intramuscularly on Day 0 and boosted on Day 21. Serum samples will be collected on days 0, 7, 14, 21, 35, 42, 56, 70, 154 and 238 following the first immunization for determination of specific immune responses to both OMPs and Aqx. Injection sites will be monitored daily for the first 7 days and weekly thereafter through Day 238.

Progress to date

With the time allotted for this research project, outer membrane proteins and aqx expression were produced. OMP yield was 0.275 mg per liter of bacterial culture. OMP purity was verified via SDS-PAGE gel (Figure 1). Synthesis of OMPs is continuing. Aqx expression was verified via Western Blot (Figure 2 and 3), quantification is pending.

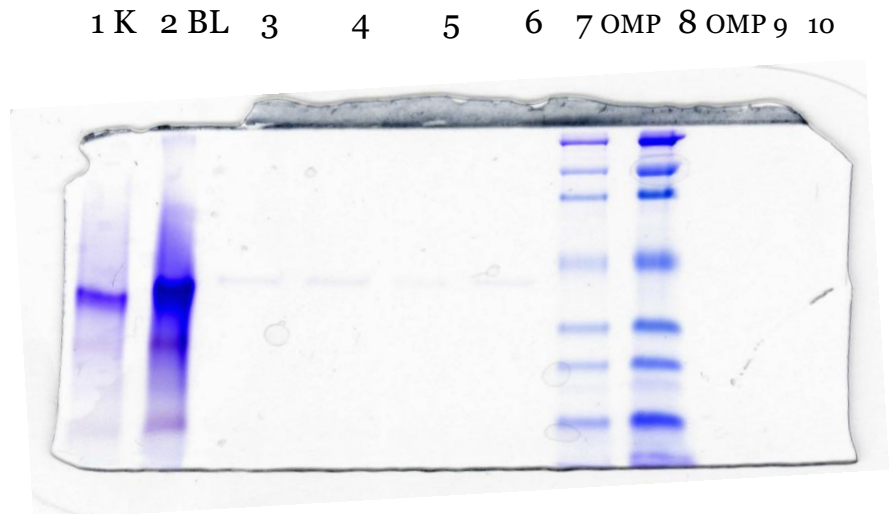


Figure 1 - SDS-PAGE gel for OMPs. Lane 1 is Kaleidoscope ladder. Lane 2 is broad-range ladder. Lanes 3-6 are empty. OMPs are in lanes 7 and 8 (OMP). Lanes 9 and 10 are empty. Ladders failed to denature prior to gel loading.

1 BL 2 IP 3 IP 4 IP 5 C 6 IP 7 IP 8 IP 9 IP 10 K

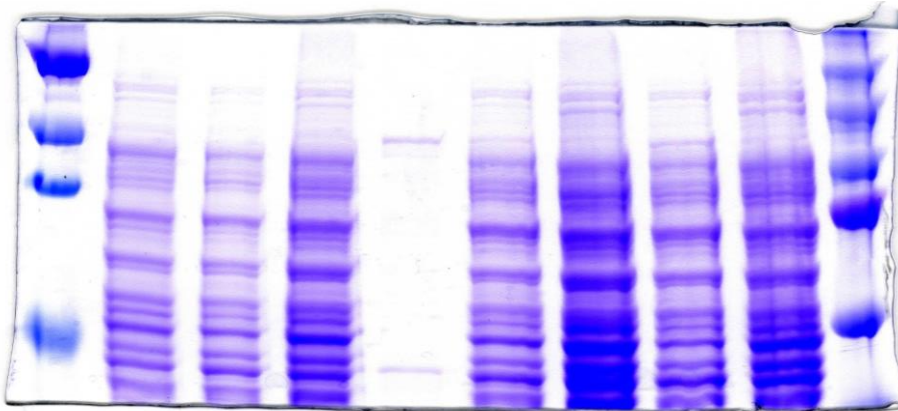


Figure 2 - SDS-PAGE Gel for Western blot. Lane 1: broad-range ladder (BL) with molecular weights of 200, 120, 100, and 51. Lanes 2-4 are pellets from induced construct (IP). Lane 5- purified Aqx protein (positive control) (C). Lanes 6-9 are pellet from induced c construct (IP). Lane 10- Kaleidoscope ladder (K) with molecular weights of 250, 150, 100, 75, and 50. Aqx protein has a size of approximately 110 kDa.

1 2 3 4 5 6 7 8 9 10

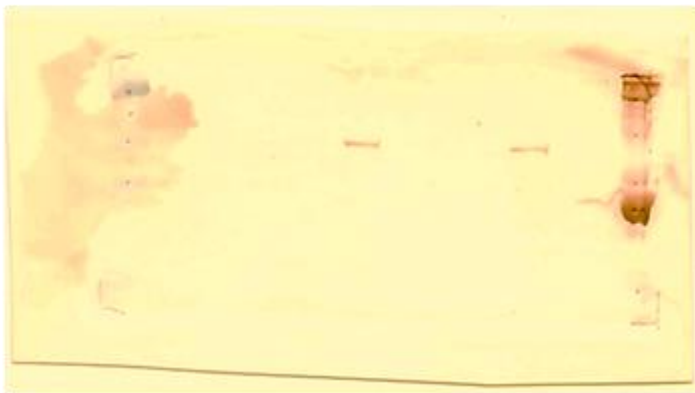


Figure 3 - Western blot of gel from Fig 2. The lanes remain the same as labeled above. Positive results are seen in lane 5 (positive control) and lane 8 (induced construct).

Discussion

Production of both OMPs and Aqx is ongoing for the current project. For OMPs, our procedure states that at 200 micrograms per dose and two doses per horse a total of 36 doses will be required for the 18 horses. Therefore, 10,800 micrograms (10.8 mg) of OMPs will be required. To obtain this quantity, an estimated 40L of bacterial culture will be required. As each isolation takes approximately a week, we are still in

the process of producing and purifying OMPs. Optimal Aqx expression conditions are still being determined through variation of expression time and IPTG concentration. Once optimal expression conditions have been selected, Aqx will be purified via affinity chromatography.

The progress of this project was hindered due to equipment malfunction and facility inefficiency, namely that OSU-CVHS does not


house the equipment needed to produce mass volumes of bacterial cultures. Our minimal progress is also a result of limited time, as only 14 weeks of research could be utilized.

Acknowledgements


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