Development and testing vaccine(s) for epizootic bovine abortion (EBA; foothill abortion): current status

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Introduction

Epizootic bovine abortion (EBA) is commonly referred to as "foothill abortion" by cattlemen. The disease was first recognized in the early 1950's and characterized by near-term abortions or birth of weak calves that were borne by dams grazed in foothill regions in California during their 1st or 2nd trimesters of pregnancy (Howarth et al, 1956) . Diagnosis was based upon unique fetal pathology and supported by elevated levels of serum immunoglobulin and geographically placing the dam in foothill terrain during the 1st half of pregnancy (Howarth et al, 1956; Kennedy et al, 1960, 1983; Sawyer et al, 1973; Blanchard et al, 2014). The geographic distribution of EBA is now known to extend into Nevada and Southern Oregon. EBA continues to be the leading cause of abortion in beef cattle in California.

Ornithodoros coriaceus, a soft-shell tick commonly known to Native Americans and early immigrants as the pajaroello tick, was established as being the vector of EBA in the 1970's (Schmidtmann et al, 1976). The observation that, the geographic distribution of this tick was coincident with areas considered endemic for EBA, lead researchers to undertake studies of this tick which ultimately established them as being vectors of the disease. The tick inhabits foothill regions and typically lives in the duff under trees and shrubs, an area commonly frequented by wildlife and livestock. The tick is attracted to animals by the CO_2 they expire, penetrates the skin using their hypostome, engorge with blood in less than 30 minutes and then leaves the host. This feeding behavior is unlike hard-shelled ticks; the latter embed in the host for extended periods of time (days to weeks). *O. coriaceus* remains relatively stationary and must await the arrival and bedding of a warm-blooded mammal to obtain a blood meal. This behavior undoubtedly contributes to maintenance of the unique geographical distribution.

The etiologic agent of EBA was not identified until 2005. Development of a reproducible laboratory method to transmit the disease using cryopreserved thymus homogenate from select necropsied EBA-positive fetuses in 2000 allowed researchers to establish that the etiologic agent was antibiotic susceptible, and thus a prokaryote (bacteria) (Stott et al, 2002). Armed with this knowledge, and access to advanced molecular biological technologies, investigators identified a unique bacterium within the Myxococcales order as being the etiologic agent of EBA (King et al, 2005). This bacterium has tentatively been coined *Pajaroellobacter abortibovis*. Identification of the etiologic agent has led to a variety of assays that have enhanced diagnosis, including immunohistochemistry (IHC; Anderson et al, 2006) and PCR (King et al, 2005; Brooks et al, 2012) for bacterial detection and an indirect fluorescence assay test (IFAT) to detect bacteria-specific antibody (Blanchard et al, 2014). To date, this bacteria has not been successfully propagated using synthetic media or cell culture systems. Mice with severe combined immunodeficiency (SCID) have been the only non-bovine fetal host in which the bacteria can be propagated (Blanchard et al, 2010).

Controlling EBA Losses using Herd Management

Control measures to minimize the impact of EBA on beef producers have been historically limited to those associated with management of breeding schedules and animal movement. Replacement heifers represent the most susceptible animals in closed herds as they typically have not yet acquired natural immunity via infection by tick bite. Relative to the manipulation of breeding programs, fall calving has the potential to reduce EBA-associated losses as compared to spring calving; this is especially true producers that are geographically located in areas with hard winters (as compared to Central and Coastal California). The tick vector is minimally active during periods of cold and/or wetness, and thus is not feeding on livestock. This seasonality, combined with the fetal bovine's window of susceptibility being limited to the first five months of pregnancy, can be effectively used to reduce EBA losses. Fall calvers typically breed November through early January, thereby placing the pregnant heifers at risk in February through April; this is typically a period of minimal *O. coriaceus* activity. Additional approaches to reduce EBA-associated losses include: i) intentional exposure of replacement heifers to tick bites prior to breeding, ii) keeping heifers in areas not considered endemic for EBA during the first 5 months of pregnancy and iii) grazing stockers or non-susceptible cattle on endemic regions for a limited period of time immediately prior to the introduction of susceptible pregnant heifers. This latter approach is based upon the "occasional" feeding habits of the tick; *O. coriaceus* fed under experimental conditions did not refeed for at least one month (Hokama et al, 1987). While all three approaches can reduce EBA losses, their relative efficacy can be compromised or rendered mute by yearly variations in spring temperatures and rainfall, both of which will dictate yearly variation in tick feeding patterns. Lastly, the greatest EBA-associated losses are experienced when naïve pregnant cattle are moved into EBA endemic areas from regions in which the disease and the tick vector do not exist; losses can be devastating and approach 100%.

A recently developed serologic test has been validated for *P. abortibovis* infection in aborted bovine fetuses (Blanchard et al, 2014). The specificity and sensitivity both exceed 97%, making it an excellent diagnostic tool. This assay is currently being tested as a method to detect P. *abortibovis* exposure in mature cattle. The assay should prove to be an invaluable tool in better establishing the geographic distribution of EBA and providing a tool to facilitate management decisions including movement of naïve animals and identifying prior exposure and associated immunity to reduce EBA-associated losses.

Live Virulent EBA Vaccine Currently in Field Testing

Efforts directed at developing a vaccine for EBA were thwarted early on by an inability to grow the causative agent, *P. abortibovis*, in culture. The demonstration that the bacteria could be propagated in SCID mice in 2010 provided the first possible pathway to a vaccine (Blanchard et al, 2010). The bacteria was identified inside of mouse spleen cells and antibodies bound to *P. abortibovis* served to enumerate the percentage of cell infected using flow cytometry. The latter accomplishment was instrumental in establishing a way to QC (quality control) an experimental live virulent EBA vaccine, thus controlling for the potency of the vaccine (i.e. # of infected cells). Further studies determined the infected splenocytes could be rate-frozen and stored in liquid nitrogen for in excess of three years with negligible loss of infectivity. Based upon the unique pathogenesis of *P. abortibovis*, its' use as a candidate vaccine was considered to be viable. The bacteria has no apparent impact on the health of an immunocompetent cattle; the bovine fetus, which has yet to develop an adaptive immune system, is susceptible to a fatal

infection. Thus, administration of the live virulent bacteria to a naïve non-pregnant cow should have no impact on health.

The stage was now set to initiate controlled vaccination trials using murine splenocytes containing the live virulent *P. abortibovis* bacteria. Injection of these infected cells into non-pregnant naïve replacement heifers "should" induce an immune response capable of blocking subsequent infections with the pathogen during subsequent pregnancies. Naïve heifers were provided one or two SQ injections of cryopreserved *P. abortibovis*-infected murine splenocytes followed by initiation of breeding approximately one month later; control heifers receiving saline placebo were included. Heifers were challenged with live virulent bacteria (fetal thymus homogenates derived from infected cows) at the peak of susceptibility (90-110 days gestation). All vaccinates gave birth to healthy calves while most controls aborted near-term fetuses that were subsequently diagnosed as being EBA. Experimental challenge studies were repeated over three years using various schemes that differed in bacterial dose and timing between vaccination and breeding; all studies gave similar excellent results. The experimental vaccine has had no apparent impact upon conception. No immediate adverse anaphylactic-type reactions have been observed and swelling at the injection site has not been noted during the first 21 days following vaccination. Variable injection site reactions develop 3 to 8 weeks post-vaccination and are characterized as a soft swelling that was identifiable only by palpation. Deep tissue biopsies of injection site reactions obtained at the site of swelling were characterized by an infiltration of lymphocytes into the skin but no involvement of the underlying muscle. Injection site biopsies from heifers at 90 days post-vaccination revealed no histologic evidence of inflammation. Based upon these studies, the USDA's Center for Veterinary Biologics (CVB) approved a 90-day withdrawal period for the experimental vaccine.

Field trials employing the experimental live virulent vaccine were initiated in 2011 in both University and private producer herds under the supervision of USDA/CVB. In excess of 3000 heifers have received the vaccine to date. The vaccine has provided excellent protection, most notably in those herds experiencing large yearly losses due to EBA. Preliminary studies suggest the vaccine will probably provide long-term immunity (2-3 years) and possibly serve as the basis of life-long immunity in the presence of sporadic exposure to the tick vector.

Vaccine trials using the experimental live virulent bacteria are being expanded in 2015 to a relatively large number of private producer herds in CA, OR and NV to better establish the following: i) efficacy in different geographical locations and different management schemes, ii) better establish the market for the vaccine, and iii) provide some immediate relief to producers currently suffering large losses due to EBA. All vaccinations are being administered to heifers a minimum of 60 days prior to breeding to negate any potential complications with early fetal mortalities. However, some early fetal mortalities associated with vaccination have been suspected (not statistically significant) in select herds that received vaccine in the vicinity of 30 days prior to initiation of breeding in association with a tight breeding window of approximately one month. Studies are in progress to statistically establish whether the EBA vaccine can induce early fetal losses when administered close (30 days) to initiation of breeding. No evidence of vaccine-associated fetal mortalities have been observed in cattle that were vaccinated a minimum of two months prior to initiation of breeding.

DNA-Based EBA Vaccine for the Future

Genomic analysis utilizing next generation sequencing technology and *in silico* bioinformatics approaches have been initiated to develop recombinant EBA vaccines. Recombinant vaccine

development depends on a thorough understanding of the genomic makeup of the pathogen, therefore, assembly and annotation of the *P. abortibovis* genome is the logical first step.

Genome assembly is the process of reconstructing the millions of sequencing reads into one contiguous sequence of base pairs (El-Metwally et al., 2013). DNA was extracted from the SCID mouse splenocytes containing *P. abortibovis*, therefore the *de novo* assembly of the bacterial genome was complicated by host-cell (mouse) DNA contamination. The DNA fragments of the different species were separated based on depth of sequencing coverage. Therefore, *de novo* genome assembly was accomplished using a de Bruijn graph assembler which resulted in 10 bacterial contigs (contiguous sequences of DNA) totaling approximately 1.815 Mb. The 10 contigs were manually scaffolded (ordering contigs and filling the gap with a series of unassigned (N) base pairs) through mate-pair mapping and were confirmed with a stand-alone scaffolding program. The resulting genome was a 1.822 Mb circularized genome with less than 0.01% unassigned base pairs, thus qualifying this assembly as a complete genome. Further gap filling was performed in conjunction with Sanger sequencing which reduced the number of unassigned base pairs and confirmed regions of genomic assembly. The completion of the circular bacterial genome indicates that there are no missing sections in the assembly and the relative genome size is accurate. When performing *de novo* assemblies, the relative size of the genomes of the most closely related species are often examined to confirm the size of the assembled genome. *Sorangium cellulosum*, a soil bacterium in the order Myxococcales is recognized as the closest phylogenetic relative and has a 13 Mb genome (Schneiker et al., 2007). Although the assembled *P. abortibovis* genome is much smaller, research indicates that microbes often experience a large-scale genome reduction in their evolutionary transition from free-living to obligate host-associated organisms (Moran, 2002).

The species annotation and phylogenetic analysis of *P. abortibovis* confirmed that this bacterium is most closely related to organisms of the order Myxococcales (myxobacteria) and the closest phylogenetic relative is *S. cellulosum.* The genome annotation predicted approximately 2200 genes with approximately 50 percent of the genes showing amino acid sequence similarity to genes found in *S. celluosum*. Further analysis indicated that this bacteria has maintained significant complexity within its genome while undergoing the large scale genome reduction. Myxococcales bacteria have been seen to exhibit social behaviors and have functions similar to multicellular organisms (Pathak et al., 2012). *P. abortibovis* maintained many of the genes associated with these social interactions and has maintained genes that enable pili-based motility in myxobacteria. The conservation of these genes and the phylogenetic analysis gives evidence that *P. abortibovis* is the first myxobacterial pathogen. *P. abortibovis'*genome reduction and loss of essential free-living genes suggests that this bacterium is obligatorily associated with a eukaryotic host. The phylogenetic analysis of this pathogen provides useful information for transmission and pathogenesis of EBA.

Upon the completion of the genome assembly and annotation, the next logical step is the prediction and testing of logical vaccine candidates (antigenic proteins) for a recombinant vaccine. The process of "reverse vaccinology" refers to the utilization of genomic sequence to make *in silico* predictions of vaccine candidates (Rappuoli, 2001). This process was developed to refine the selection criteria for a smaller group of candidate proteins to be expressed and tested using serology assays. The selection and ranking criteria utilized for vaccine candidate prediction included subcellular localization prediction, count of transmembrane helices, protein homology with host-proteins and commensal bacteria, adhesin (cell component that facilitates adhesion to

host cells) probability and RNAseq expression. Final candidate filtering led to the selection of 10 candidate proteins. These proteins have been experimentally expressed and are currently being immunologically tested utilizing serum from multiple *P. abortibovis* -infected and control hosts, including bovine fetuses, bovine cows, and mice. The serology assays should help provide information about the relative antigenicity of the candidate proteins.

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