Monoclonal antibody production: a comparison of *in vitro* and *in vivo* methods and their use in Clostridial vaccine manufacture.

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Abstract

The genus *Clostridium* contains rod-shaped, endospore forming, gram-positive bacteria that are obligate anaerobes (Delano, Mischler, & Underwood, 2002; Hatheway, 1990; Rood, 1997). Clostridial diseases are important diseases of livestock in New Zealand, and are considered by some to be the most economically important diseases of sheep and other livestock (Walker, 1992). They are characterized by systemic vascular failure and/or necrotizing enteritis within hours after exposure—a speed that out-paces a naïve individual's ability to control and effectively counteract the toxin's effects. Vaccination is an important management practice that can decrease the morbidity and mortality associated with Clostridial infections, and vaccination has been used safely and successfully in New Zealand livestock for many years.

Vaccine manufacture and quality assurance (QA) often involves production of monoclonal antibodies (MAbs) derived from culture of hybridoma cells. Traditionally, large numbers of animals have been used to support research, development, and manufacture of Clostridial vaccines (for use in toxin neutralization, vaccine challenge studies, and potency determination) and for MAb manufacture (ascites model). There is currently a great emphasis on finding ways to reduce, refine, and replace animal use in research.

Studies were undertaken that involved MAb production techniques and assay development related to *C. perfringens* Type C beta toxin and Type D epsilon toxin. Two different methods for MAb production were evaluated: a traditional *in vivo* murine ascites method and an *in vitro* method based on use of a commercially available two chamber plastic bioreactor system (CELLine; Becton Dickinson). Two hybridoma lines with historic MAb activity against the lethal and dermo-necrotizing effects of epsilon toxin (EP82) and beta toxin (CP68) were cultured in each production system. In addition to comparing the quantity of MAbs produced by each method, a sandwich ELISA based on use of the anti-epsilon MAbs was developed. This ELISA was implemented for use as a diagnostic tool for internal investigation of batches of epsilon toxoid-containing vaccines that were identified as having low potency in the standard QA test (rabbit antibody induction) for potency.

These studies showed that an *in vitro* method of hybridoma culture was more time and cost effective than conventional live animal ascites production, based on the total quantity of

MAb produced for both of the hybridoma lines that were studied. Additionally, the sandwich ELISA that was developed was effective in detecting very small amounts of toxoid.

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Abbreviations

BA/BSA	Bovine albumin, bovine serum albumin
CCM	Cell compartment media
CDC	United States Centers for Disease Control and Prevention
CMI	Cell-mediated immunity
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FPLC	Fast protein liquid chromatography
IgG	Immunoglobulin G
MAb	Monoclonal antibody
mAU	Absorbance units
MHC	Major histocompatibility complex
NCM	Nutrient compartment media
PET	Polyethylene terephthalate
PBS	Phosphate buffered saline
OD	Optical density
QA	Quality assurance

Chapter 1: Introduction

Historical importance of Clostridial diseases

The bacterium

The genus *Clostridium* contains rod-shaped, endospore forming, gram-positive bacteria that are obligate anaerobes. Vegetative organisms, endospores, and some toxins are typically found in soil, sewage, marine sediments, decaying animal and plant products, and the intestinal tracts of humans and other animals (Delano, et al., 2002; Hatheway, 1990; Rood, 1997). Of the approximately 100 species in the genus, 14 are known to be pathogenic. When vegetative clostridia encounter unfavourable environments, (e.g. contact with oxygen) they form endospores that are resistant to desiccation and heat which enables them to persist in the environment (e.g. pastures or housing areas) for extremely long time periods and to resist food safety measures (retorting, etc.).

Clostridial diseases

The genus' ubiquitous nature makes it a common food and wound contaminant, and contaminated foodstuffs are often responsible for toxin-mediated illness (Hatheway, 1990). Clostridial diseases generally fall into one of two categories. In the first category, the vegetative cells themselves are responsible for creating a disease process by either active invasion or local dormancy in tissues. Toxins act locally to facilitate bacterial multiplication and localized disease spread, or acts extensively to cause toxaemia and systemic disease, often including shock and death. Examples of Clostridial diseases include black leg (*C. chauvoei*), infectious necrotic hepatitis (i.e. black disease, caused by *C. novyi* infection), and malignant oedema (*C. septicum* and other *spp.*). The second category of Clostridial diseases includes those whereby the bacterial toxins alone are responsible for causing the disease. As examples, absorption of toxins present in the digestive tract (*C. perfringens* enterotoxins), environment (*C. botulinum* toxins), or contaminated wounds (*C. tetani* toxins) can result in systemic intoxication and clinical disease (Kahn & Line, 2010)

C. perfringens is a widely occurring pathogenic bacterium (Hatheway, 1990; Rood, 1997). *C. perfringens* strains are categorized into one of five 'toxin-producing types' (types A, B, C, D, and E) according to their ability to produce one or more of four major lethal Clostridial toxins (Table 1).

The current studies described in this dissertation were concerned specifically with Type C beta toxin and type D epsilon toxin. These toxin types are responsible for various diseases in livestock—typically involving young animals, but also affecting adults. These diseases are characterized by systemic vascular failure and/or necrotizing enteritis (Table 2.). Clostridial diseases are important diseases of livestock in New Zealand, and are considered by some to be the most economically important diseases of sheep and other livestock (Walker, 1992); importantly, humans are also susceptible to Clostridial infection and toxaemia. In people, clinical signs of Clostridial disease can range from abdominal discomfort and watery diarrhoea (gastroenteritis form) to necrotizing enteritis ('pig-bel' disease). The Centers for Disease Control and Prevention (CDC) in the United States estimates that there are approximately 966,000 *C. perfringens* foodborne illness cases annually in the U.S , second only to *Salmonella* when considering bacterial causes of foodborne illness (Al-Khaldi, 2012). The vast majority of Clostridial infection cases are thought to go unreported due to mild gastrointestinal signs and infrequency with which patients are tested for either the bacteria or its toxins.

The course of the clinical disease for both Type C and Type D infections in animals is usually dramatic and acute. Type C beta toxin causes intestinal epithelial necrosis that typically results in severe gastrointestinal signs (enteritis and dysentery) toxaemia, neurological signs (convulsions and opisthotonos), and high mortality in young animals. In adult animals, the infection causes an enterotoxaemia without dysentery. Similarly, the disease course for *C. perfringens* Type D enterotoxaemia is often extremely short; animals are frequently found dead without any signs of prior illness. When affected animals are observed prior to death, neurological signs are not uncommon and include ataxia progressing to opisthotonos, convulsions, and recumbence (Delano, et al., 2002; Kahn & Line, 2010).

Disease and immunity

Host, pathogen, and environment interaction

Disease is not a random event but is the result of a complex interaction between a host, pathogenic agent, and an environment that brings the two together. Certain requirements must be met so that a pathogen can cause disease in the host, namely that the pathogen must be present in numbers above the minimal infectious dose and that those organisms must be presented to an ecological niche that allows successful infection to occur (e.g. abraded skin, penetration wound, contact with a mucous membrane, etc.). An additional important criterion

for infection to successfully occur is that the host must be susceptible to infection. Given the ubiquitous nature of many Clostridial organisms, this often means the animal must be immunologically naïve or have compromised immunity. Host factors that can affect disease susceptibility include age, nutritional status, reproductive status, immune status, and level of stress and recent life events (i.e. weaning, transport, castration, etc.). Immunologically naïve animals may not have enough time to produce an adequate immune response to neutralize or eliminate pathogens before significant disease or death occurs. Immune compromise can come in many forms—innate immune deficiency in some component (failure of passive transfer of maternal-derived immunity in a neonate, T-cell or B-cell deficiencies), malnutrition that impairs the immune system's ability to manufacture acute phase proteins, chronic stress with high circulating levels of cortisol that suppress immune function, and others. Environmental factors that affect the likelihood of infection occurring include environmental hygiene (increased pathogen load), high animal population density (increased likelihood of contact between pathogen and host), direct contamination of feedstuffs by the organism or its toxins, and the occurrence of environmental temperature and humidity conditions favourable for pathogen survival or toxin elaboration (Deitemeyer & Rollin, 2005).

Mechanism of immunity

A fundamental principle of the immune response is the ability of animal's immune system components to differentiate between 'self' and 'non-self' antigens. In general, an animal's immune system can respond to infection through two broad functional pathways, an innate or 'passive' response and an adaptive or 'active' response.

The innate pathway is comprised of anatomic, physiologic, phagocytic, and inflammatory barriers that provide a non-specific first line of defence against pathogens. Innate immunity has no ability to target specific pathogens and prior exposure to a pathogen does not produce any immunologic memory. As examples of the innate system, keratinized skin and acidic sebum provide a waterproof barrier and inhospitable environment for bacterial multiplication; mucous membrane secretions trap and prevent adhesion of pathogens; and commensal organisms competitively inhibit colonization by pathogenic organisms on skin and mucosal surfaces. Phagocytic cells and the inflammatory cascade (i.e. non-self-antigen triggering protein-mediated events that change vascular permeability and result in recruitment of granulocytic cells) provide a physiologic defence that work in concert with complement to

efficiently neutralize foreign antigens. Cell mediated immunity (CMI) is an important part of the innate immune system. CMI involves immune cells that phagocytize pathogens (i.e. neutrophils, macrophages, and dendritic cells) and sometimes present them to other immune cells, others that release cytokines and chemotactic substances (mast cells, basophils, and eosinophils) to attract immune cells, and others that recognize when 'self' cells are diseased or abnormal (e.g. natural killer cells). The innate system can be directly affected by the animal's environment. As examples, poor hygiene and living conditions, poor nutrition, and injuries can disrupt the skin barrier and decrease the effectiveness of the innate arm of the immune system and which will in turn lead to a greater reliance on the adaptive immune system.

The adaptive immune system generates a highly specific response to invading pathogens, and prior exposure generates highly specific immunologic memory. Generally adaptive immunity involves several classes of cells: naïve cells that are mature but have not yet encountered cognate antigen, effector cells that are actively involved in eliminating pathogens and memory cells that persist after past infections. Naïve cells become effector cells when they encounter cognate antigen in the context of a major histocompatibility complex (MHC) Class I or Class II molecule on the surface of an antigen presenting cell. Lymphocytes are the major cell of the adaptive immune system and B cells are a subset of lymphocytes that produce antibody. When presented with cognate exogenous (i.e. bacteria or toxins) antigen in the context of an MHC Class II molecule, a B cell will be activated to produce immunoglobulins (Ig). There are several types of Ig in the body - IgA, IgM, IgG, and IgE, all of which have different areas of activity. In an initial exposure, IgM and some IgG are produced by effector cells. IgG is the antibody of primary concern when we talk about immunity secondary to vaccination. In addition to making Ig, activated B cells will also create clones that either produce antibody or become memory cells. These memory cells remain after the immune response is over, and await secondary exposure to the same antigen. Upon secondary exposure the humoral response to neutralize antigen is faster, larger (more memory cells produce more clones and greater quantities of antibody - predominantly IgG), and stronger (Deitemeyer & Rollin, 2005).

In addition to production of immunoglobulins, the adaptive immune system also induces T-cells (another subset of lymphocytes) to either secrete cytokines to regulate or direct the immune response (helper T-cells), or to become killer T-cells that are involved in killing

'self' cells that are infected with intracellular pathogens. Endogenous or intracellular pathogens (generally viruses, intracellular bacteria, or parasites) are presented to T-cells in the context of an MHC Class I molecule. Like-B cells, T-cells also produce a memory population that circulates and waits to encounter cognate antigen again.

Vaccines and vaccine manufacture

Vaccine principles and types

Development of vaccines for diseases depends on several factors—cost, risk of adverse reactions vs. benefit of immunity, ability to eradicate disease through vaccination vs. management practices (improved husbandry, biosecurity measures, etc.), ability of vaccine to induce protective immunity, and many others (Deitemeyer & Rollin, 2005). Different vaccines can stimulate different types of immune responses. However, the goal of vaccination is the induction of both an antibody and CMI response (Plotkin & Plotkin, 2011).

There are two main types of vaccine: live attenuated and inactivated. Live attenuated vaccines generally produce immunity similar to that of a natural infection (because the vaccine agent replicates in the host and will be processed by the immune system in a manner similar to that of the non-attenuated agent; this is likely to include both a CMI and humoral response. Because of this, a single dose of an attenuated vaccine is able to generate a protective immune response. Inactivated vaccines may be comprised of whole or subunits of bacteria or viruses, and may also include inactivated toxins or 'toxoids'. Inactivated vaccines primarily stimulate a humoral immune response as the antigen is predominantly recognized as an extracellular pathogen and processed through the MHC Class II pathway in a manner that is not likely to mimic a natural infection. Inactivated vaccines generally require multiple doses in order to provide protective immunity and may also require periodic 'booster' doses to ensure that humoral immunity does not wane to a non-protective level (Beckenhaupt, et al., 2012). Inactivated vaccines are often combined with adjuvants to stimulate inflammation and create a stronger CMI response.

Vaccination: key component of disease prevention

Vaccination is widely used to protect animals against disease and to exploit a powerful anamnestic response to antigen exposure, and is widely used in human and animal medicine. Vaccines provide a means of exposing naïve animals to immunogenic material related to a pathogen, in the form of inactivated or weakened organisms, toxins, or specific immunogenic epitopes, in a controlled manner so as to induce immunologic protection without inducing

disease. By training the immune system to recognize pathogens, vaccines produce protective immunity in a naïve animal with the assumption that the animal will be immunologically competent and able to mount an effective immune response against the same agent when encountered in the future. This is accomplished by the vaccine creating a memory bank of B and T-cells that can recognize and respond to pathogens quickly and efficiently (Deitemeyer & Rollin, 2005; Plotkin, 2003). Vaccination provides the animal with a reasonable ability to combat an infection but it is not guaranteed to protect the animal completely from clinical signs of disease. Vaccinated animals may still become sick, though the course and intensity of the disease are often diminished as compared to that experienced by an unvaccinated animal. Vaccines can fail if the animal has not mounted an appropriate immune response to the vaccine (i.e. was not boostered appropriately, vaccine was given too early in life, or the vaccine was blocked by maternal antibodies, etc.), if the vaccine was handled or administered improperly, or if other life events (transport, weaning, environmental stress, malnutrition, etc.) impaired the animal's ability to mount an immune response. Vaccination should represent only one part of a preventive health program that also describes the necessary management practices for minimizing disease exposure and maximizing animal health.

Clostridial vaccines

Most effective Clostridial vaccines include both cellular 'inactivated vaccine' and toxin 'toxoid' components. The first safe and effective inactivated vaccine with a toxoid component was developed in 1924 by Gaston Ramon for the prevention of diphtheria (Ramon, 1923). Ramon was the first to use formaldehyde in combination with heat to inactivate the bacterial toxin and kill the bacteria, making it safe for use as a human vaccine; formaldehyde inactivation remains a procedure that is still commonly used today.

Modern Clostridial vaccine manufacture begins with culture of the desired organism using a fermentation process designed to maximize toxin production by the organism. Clostridial species are fastidious anaerobes and will only produce toxin under appropriate conditions. For example, *C. perfringens* Type D actively produces toxin when it enters the acidic environment of the stomach and proximal small intestine. In vaccine manufacture, this process is simulated when strains are moved from culture to fermentation vats where the pH is then lowered allowing the bacteria to remain in a logarithmic growth and producing high levels of toxin. In natural infections, *C. perfringens* Type D toxin is activated when it encounters trypsin. This activation can be induced when *in vitro* toxicity effects are studied

(Knight, Queminet, Blanchard, & Tilleray, 1990). Inactivated toxin is used in vaccine manufacture. When cultures produce the desired concentration of toxin, formaldehyde is added to inactivate the toxin (rendering it into a toxoid) and kill the bacterial cells.

Toxoids are inactivated toxins that remain immunogenic but do not cause disease in the target species. Inactivated vaccine-toxoids are expected to be non-toxic (the harmful effects of the toxin have been mitigated), potent (able to generate a protective immune response in vaccinated individuals), and non-viable (no cellular components of the vaccine are capable of further growth or production of toxin). The efficacy of the formaldehyde inactivation is usually demonstrated using a validated indicator system. For example, some toxins cause lysis of red blood cells. If the toxin is mixed with red blood cells and the red blood cells remain intact, then the toxin can be assumed to have been inactivated and is safe for use in vaccines.

Vaccine potency is usually tested using live animal models. For clostridial vaccines this includes antitoxin (vaccine-induced antibody level) response in rabbits, paired with toxin neutralization tests. The toxin neutralization test is conducted by collecting serum from rabbits that have been immunised with vaccine/toxoid then measuring the titer of antitoxin antibodies by comparing it to the number of units of standard antitoxin that is required to elicit the same level of neutralisation activity (Mowat & Rweyemamu, 1991). Safe vaccine-toxoids are manufactured using processes designed to completely inactivate all the toxins that were produced during culture of the vaccine seed. Toxoids are inactivated toxins that remain immunogenic but do not cause disease. Manufacturers must formulate vaccines containing enough toxoid to generate a protective response in the target animal. To test this, the candidate toxoid (of unknown concentration) is first mixed with various known amounts of antitoxin (immunised rabbit serum). The toxoid in the mixture will then be bound and neutralized by the antitoxin that was added to the mixture. Second, lethal (non-inactivated) toxin is then added to the mixture and administered to test animals; the test animals are then monitored for the occurrence of clinical signs consistent with harmful effects of the toxin. If the initial toxoid preparation had a very low toxoid concentration at the time the antitoxin was added, there should have been excess antitoxin available to neutralize the lethal toxin that was added to the mixture in the second step, thereby minimizing (or eliminating) the occurrence of clinical disease in the test animals. If the initial toxoid preparation had a high toxoid concentrations, little or no of the antitoxin that was added in step one would have been

available to neutralize the effects of the lethal toxin that was added in step two, thereby allowing severe toxic disease to occur in the test animals. More clinical disease observed in the test animals indicates higher potency in the toxoid preparation. Purposive induction of clinical disease in the test animals is one of the undesirable side effects of live animal-based potency testing.

After verifying potency, non-toxicity, and non-viability, the toxoids are combined with an adjuvant to formulate the final clostridial vaccine preparation. The vaccine can then be packaged for distribution and use *in vivo* after passing various additional quality assurance tests, including potency tests (Mowat & Rweyemamu, 1991; O'Hara & Bauer, 2008).

Monoclonal antibodies

History and definition

Monoclonal antibodies (MAbs) are antibodies directed against a single antigen. By definition, a plasma cell produces monoclonal antibody but because the blood is populated by many different B-cells, the blood collected from animals includes many different antibodies and is considered 'polyclonal'. In a laboratory setting, large amounts of monoclonal antibodies can be generated from hybridoma cells; hybridoma cells are a fusion of a myeloma cell (an immortal B-cell line) with B-cells harvested from the spleen of mice that have been immunized with the antigen for which the desired MAbs are targeted. After a population of hybridomas is created during the fusion process, steps are undertaken to remove non-fused cells (spleen cells and myeloma cells) through culture in selective media. Cells remaining after this step represent a polyclonal population of hybridoma cells, most producing antibodies directed against non-target antigens or epitopes. To identify the hybridomas that are producing the antibody of interest, the population is diluted across wells on clonal selection plates at a concentration expected to result in no more than one hybridoma per well; each of these single clones are then cultured and the supernatant from each culture is screened for the antibody of interest. The clone(s) producing the antibody of interest can then be further cultured to produce large quantities of the MAb of interest (Sheehan, 2007). Hybridomas were first described in 1975 (Kohler & Milstein, 2005). Prior to the development of the hybridoma system for generating MAbs, it was difficult to obtain specific antisera from animals because of contamination with non-specific or non-target antibodies (polyclonal antiserum) and which led to unpredictable measurements in the response to vaccination (Brodeur, Tsang, & Larose, 1984).

Production process

Monoclonal antibodies are produced for a variety of purposes. Production of small quantities of less than 0.1 g of purified MAb is often adequate to meet the needs of many research projects. However, medium-scale production systems are required to create the 0.1-1.0 g quantities necessary for manufacturing diagnostic testing kits or developing assays. Even larger scale production systems are required to produce quantities greater than 1.0 g necessary for therapeutic and diagnostic procedures (Anonymous, 1999).

Traditionally, small to medium scale MAb production has been done almost exclusively through an *in vivo* technique that makes use of a murine ascites model. In this technique, hybridoma cells known to be capable of producing the desired MAb are injected into the peritoneal cavity of experimental mice (and sometimes rats) whereby their numbers are expanded and they begin producing MAb-containing ascites fluid in significant quantities (Goding, 1980; Mikami, Makishima, & Suzuki, 1991). Known downsides to this *in vivo* technique includes the potential for pain and distress in the mice, contamination of the ascites fluid with non-target mouse-derived IgG, and the possibility of contamination of the ascites fluid with pathogenic agents of the mouse (Marx, 1998).

Alternatives to *in vivo* production have been attempted over the years which include a variety of *in vitro* production techniques. However, many of these techniques have been found to be impractical due to their high cost, slow production speed, requirements for specialized laboratory equipment, or their tendency to become contaminated. Low-density culture techniques (static or agitated flasks) generally produce only low concentrations of MAb and are prone to contamination with non-specific animal serum proteins (from FBS in the culture media). High-density culture techniques such as hollow fibre reactors (Jackson, Trudel, Fox, & Lipman, 1996), while producing high concentrations of MAb comparable to that which could be achieved through the ascites fluid technique are cumbersome to operate, can only work with one hybridoma line at a time, and are not cost-effective for general research laboratories. Over time, higher-density (compared with static culture) and comparatively more economical matrix- and membrane-based solutions to hybridoma culture have been developed including autoclavable tumbling chamber systems (Jaspert, et al., 1995), dialysis tubing (Falkenberg, et al., 1993), modular mini-fermenters (Falkenberg, et al., 1995), and glass cylinder-containing packed-bed reactors (Moro, et al., 1994). These methods offer the advantages of being able to work with more than one hybridoma line at a time, but are still

prone to contamination and are not a viable option for all research laboratories as they require relatively significant setup and training procedures before use (Trebak, Chong, Herlyn, & Speicher, 1999). One particular product, a membrane-based culture system that utilizes a facilitated gas exchange (CO_2 and oxygen) system as well as nutrient and metabolite exchange has been developed for use in conventional CO_2 incubators. These membrane-based flasks called 'CELLine' flasks have the advantage of being easy to set up and require no special equipment outside that which would be used for traditional mammalian tissue culture.

In all cases, hybridoma culture required purification of the culture supernatant over sepharose gel columns to remove contaminants from culture medium (or the ascites fluid), prior to use of the MAb.

Enzyme-linked immunosorbent assays (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a diagnostic tool first conceptualized and published independently by Engvall and Perlman (Engvall E, 1971) and Van Weemen and Schuurs (Van Weemen BK, 1971) in 1971. These publications were the first that combined several techniques used in working with immunoglobulins and were geared primarily towards working with human serum in a diagnostic setting. There are several types of ELISAs, all of which rely on the lock and key nature of antigen-antibody interaction. All involve either antibody or antigen adsorbed to a microtitre plate, primary antibody (directed against the antigen), and a secondary antibody (directed against the primary antibody and linked to an enzyme), and an enzyme substrate. The substrate creates a detectable reaction (usually by colour change) which can be read and interpreted. ELISAs can be constructed in several manners depending on the availability of reagents and whether the user wishes to detect an antigen or an antibody:

1. Indirect ELISA. Tests for the presence of antibody in a sample. Antigen (toxin, virus, etc.) is adsorbed to a microtitre plate. Primary antibody is added and binds to the adsorbed antigen. Secondary enzyme linked antibody is added and followed by substrate. Colour change is greater when antibody is in high concentration.
2. Sandwich ELISA. Tests for the presence of antigen in a sample. Capture antibody is adsorbed in a known concentration. Antigen-containing sample is added. A primary antibody (directed against the antigen and thus creates a 'sandwich') and then a secondary and enzyme-linked antibody (directed against the primary antibody) are

added, and followed by an enzyme substrate. Colour change is greater when antigen is in high concentration.

3. Competitive ELISA. Tests for the presence of antigen in a sample. Antigen is adsorbed to a microtitre plate. Antibody is incubated with a sample containing unknown antigen concentration and then put in the presence of adsorbed antigen. 'Free' antibody (not bound in the incubation step) binds to adsorbed antigen and any incubate-bound antigen is removed with a wash step. Secondary enzyme-linked antibody is added and followed by an enzyme substrate. The colour change is greater when antigen in the sample is in low concentration.

Clostridial organisms are ubiquitous in many livestock environments and are responsible for causing several economically important livestock diseases in New Zealand's extensive and pasture-based agrarian system. Clostridial organisms and their toxins can cause clinical signs within hours after the animal is exposed, a speed that out-paces a naïve individual's ability to control and effectively counteract the toxin's effects. Vaccination with a Clostridial vaccine or vaccine-toxoid prepares the immune system to respond rapidly after an animal is exposed to a pathogen or toxin and thereby decreases the morbidity and mortality normally associated with Clostridial disease. Vaccines against various Clostridial diseases have been used in New Zealand for many years and they routinely included as part of a preventive herd health programme making their efficacy imperative.

Study objectives

During the manufacturing process for Clostridial vaccines, occasionally some batches of vaccines do not meet quality assurance (QA) criteria for potency (i.e. unable to induce levels of vaccine-induced titers in rabbits, failure of toxin neutralisation tests in mice, etc.). Reasons for a vaccine batch failing a potency-related QA test are not always understood but may include having an inadequate concentration of toxoid in the vaccine preparation or having inadequate adsorbence between adjuvant and toxoid components of the vaccine which can result in a diminished immune response to the toxoid. To assist in investigation of potency-related QA tests of Clostridial vaccines, creation of an assay capable of detecting differences in the concentration of unbound toxoid found in known high potency vaccines and known low potency vaccines was desired.

MAbs from cell lines CP68 and EP82 (Knight, et al., 1990) known to be directed against two important Clostridial toxins were available for use in developing this type of an assay but

were not available in great enough quantities to permit large-scale diagnostic test development (and on-going use). Therefore, a series of studies was conducted with three Objectives. First, three archived hybridoma lines held by the project sponsor were revived from long term storage, confirmed to be producing the MAbs of interest, and then scaled-up in a quantity sufficient to produce large amounts of MAbs for use in Objectives two and three. Second, a sandwich ELISA using MAbs produced in Objective one was developed to determine the reason for low potency in selected vaccine batches as described above. Third, in an effort to move away from animal-based MAb production, the efficiency and feasibility of an *in vitro* MAb production technique (CELLine flasks) was compared to an *in vivo* MAb production technique (murine ascites) using the hybridoma lines of interest.

Chapter 2: Materials and methods

Production and assessment of ascites-derived MAbs

Hybridoma preparation and MAb harvest from culture

Two immortal cell lines were selected for use in the current project and were created in 1990: Hybridoma line CP68 produces a murine MAb that neutralizes the dermonecrotic and lethal activities of the *C. perfringens* beta toxin, and hybridoma line EP82 produces a murine MAb that neutralizes the dermonecrotic and lethal activities of *C. perfringens* epsilon toxin (Knight, et al., 1990). Small quantities of purified CP68 and EP82 MAbs produced from these lines were available for use in the current project and had been in use for purifying beta and epsilon toxins and as reagents for use in an in-house ELISA. These MAbs had been produced by an Australian company that is no longer in business (Bioquest Ltd.) in October, 2000 using a mouse ascites model.

The class 2 laminar flow hood was sprayed with 70% ethanol and turned on for 20 minutes prior to its use. Cell growth media (MAb Cell Media Serum Free; Becton Dickinson, Auckland) was stored at 4°C until use. Fetal bovine serum (FBS; Sigma Aldrich, Auckland), 200 mM concentration L-alanyl-L-glutamine dipeptide (Glutamax solution; Invitrogen, Victoria, Australia), and penicillin-streptomycin solution with 10,000 units penicillin and 10 mg/mL streptomycin (Sigma Aldrich, Auckland) were stored at -20°C until use. In the class 2 laminar flow hood, 160 mL MAb Cell Media, 40 mL thawed fetal bovine serum, 4 mL Glutamax solution, and 2 mL antibiotic solution were combined and gently agitated in a sterile polyethylene terephthalate (PET) media bottle using sterile technique. Once all components were combined, the completed media solution was labelled with the date of manufacture and the expiry date (one week from the date of manufacture) and stored at 4°C.

For convenience, aliquots in working volumes of all components were made, labelled, and stored for future use. MAb media was stored at 4°C in 160 mL aliquots. FBS, Glutamax, and penicilin-streptomycin colutions were all stored at -20°C in aliquots of 40 mL, 2 mL and 2 mL, respectively. Storage in aliquots reduced the opportunity for contamination of components from repeated manipulation, minimized potential ill effects of repeated temperature fluctuation (i.e. repeated freezing and thawing cycles), and improved the efficiency and ease of media production.

Forty minutes prior to manipulating cells, pre-prepared complete media was placed in a 37°C water bath and allowed to warm. In a Class 2 Laminar Flow Hood, 10 mL of warmed media was pipetted into a sterile 15 mL centrifuge tube. A single vial of hybridoma cells was retrieved from vapour phase liquid nitrogen storage and thawed by partial immersion in the water bath. Cells were not agitated during thawing and were removed from the bath just prior to being completely thawed, sprayed with 70% ethanol and transferred to the class 2 laminar flow hood. When the alcohol evaporated from the vial, contents were added, drop-wise, into a sterile 15 mL conical vial (BD Biosciences, Auckland, New Zealand) containing 12 mL warmed media.

The cell-containing media was centrifuged at 170g for five minutes and transferred back to the class 2 laminar flow hood. Taking care not to disturb the cell pellet in the bottom of the vial, media was pipetted off and discarded. The cells were then gently re-suspended in another 5 mL of warmed media and transferred into a 25 cm^2 vent-capped, canted neck flask (BD Biosciences, Auckland, New Zealand). The flask was sealed, labelled with the date, cell line, and passage number, and placed in a 5% CO_2 incubator set at 32°C.

Every one to two days for fourteen days, the percentage of viable cells in the culture media was determined using a trypan blue exclusion method. Using a pipette, 100-200 μL of cell suspension was removed from the culture container and placed into a sterile bijou; the culture media was gently agitated to distribute cells evenly throughout the media prior to pipetting. A volume of 0.4% trypan blue solution (Sigma Aldrich, Victoria, Australia) equal to the volume of culture material in the bijou was added and the contents gently mixed. The mixture was loaded into both chambers of a Neubauer haemocytometer and placed on a light microscope stage. Dead (blue stained) and live (unstained) cells were enumerated in four, one mm^2 rectangles in the counting chamber and recorded. Cells spanning across or touching the lower and right hand lines of a counting square were not counted; the concentration of viable cells per milliliter of culture solution was determined by the formula shown below:

$$\text{Dilution factor} \times \frac{\text{Average number of live cells}}{\text{Number of squares counted}} \times 10^4$$

Forty minutes prior to cell manipulation, pre-prepared complete media was placed in a 32°C water bath to warm. Twenty minutes later the class 2 laminar flow hood was sprayed with 70% ethanol, turned on and allowed to run. After removing flasks from the incubator for examination under an inverted microscope, cultures were examined grossly for evidence of

contamination including turbidity and the presence of bacteria or fungi. The overall health of the culture was assessed subjectively and objectively. Subjective measurements included cell density (based on cell crowding, cell appearance (shape, size, and refractivity), and media colour (normal media began at a deep orange-red colour and become progressively more yellow as by-products of metabolism accumulated in the media solution). Objective measurements of the health of a culture included calculation of cell concentration and viability. Both objective measurements were calculated by performing trypan blue exclusion (method detailed above). The concentration of cells in a flask was maintained (by dilution with fresh media) between $6x10^5$ and $3x10^6$ viable cells per mL, and viability was considered good if maintained above 85%. Special care was taken when more than one cell line was being manipulated or cultured simultaneously in the laboratory. Only one cell line was manipulated at a time, the hood was sprayed with ethanol and allowed to stand at least ten minutes between lines, flasks were clearly labelled, and media bottles were designated and clearly labelled for use in one cell line only to avoid cross contamination.

Cells were in the logarithmic phase of growth and determined to have at least 85% viability (determined the day before, and confirmed again at the time of freezing) at the time of freezing to ensure maximum recovery of cells when revived for culture at a later date. Twenty minutes prior to cell manipulation the class 2 laminar flow hood was sprayed with 70% ethanol, turned on and allowed to run. Cells to be frozen were removed from the incubator and observed grossly. Cell count and viability check were performed to confirm the culture was suitable for freezing. Sterile, 2 mL cryovials (Nalgene, Thermo Fischer Scientific, Rochester, New York, USA) were labelled with the cell line (EP82 or CP68), the number of cells in the vial, passage number, and the date, then placed into a foam-lined controlled cooling rate device called (Mister Frosty; Thermo Fischer Scientific, Rochester, New York, USA) that had been filled with isopropanol and chilled overnight at -4°C. This ensured that the vials would be pre-chilled and ready for cell receipt.

Freezing media comprised of 8-10% dimethyl sulfoxide (DMSO) was made in sufficient quantity using a ratio of 1.7 mL DMSO (Hybri-Max; Sigma Aldrich, Auckland), 4 mL FBS, and 16 mL MAb cell media serum free.

After cell count and viability were determined, cell suspensions were removed from culture flasks, placed in 50 mL conical vials (BD Scientific, Auckland, New Zealand), placed in a refrigerated centrifuge, and spun at 200g for ten minutes. After centrifugation, supernatant

was removed and unless needed to verify antibody production or affinity, was discarded. Cells were then gently re-suspended in the freezing medium and transferred into sterile 2 mL cryovials in one mL aliquots, each containing between $5x10^5$ and $5x10^7$ viable cells. The cryovials were closed tightly, replaced in the Mister Frosty, and then transferred to a -80°C freezer and left overnight. Cells were removed from the Mister Frosty before 48 hours and then placed into vapour-phase liquid nitrogen for storage.

Assessment of MAb affinity by ELISA

Supernatant was collected from cell culture from three cell lines of interest: to ensure hybridomas were producing antibody and to determine if the antibody being produced was still specific to the antigen of interest. Using a previously established antibody-capture ELISA (indirect ELISA) protocol as a template, the affinity of antibody produced by line CP68 during the current project was compared to antibody produced from line CP68 in previous production lots (Bioquest, Ltd. 2000) and to the antibody produced by a non-Clostridium specific hybridoma (from Bioquest, Ltd 2000). The purpose of this test was two-fold: First, to determine if the hybridoma line was still actively producing MAb and second, to determine an appropriate dilution of enzyme conjugate that would permit detection of the MAb. If the concentration of conjugate was too high then non-specific binding could produce false-positive results; too low of concentration would be likely to produce false-negative results. After the appropriate conjugate dilution was determined and the assay shown to work using the CP68 cell line antibody, identical procedures were used to assess the MAb produced by the EP82 and CN04. Standard antibody concentration dilution curves were established for each of the cell lines that were still producing MAbs of interest.

A 10 µg/mL antigen coating buffer was prepared by combining coating antigen with 0.05M carbonate buffer, and adjusted to pH 9.6. *C. perfringens* type C (antigen for CP68) was diluted 15 µL in 12 mL, *C. perfringens* type D (antigen for EP82) was diluted 100 mL in 12 mL, and *C. novyi* (antigen for CN04) was diluted 120 µL in 12 mL antigen buffer to create an antigen coating solution containing 10 µg per mL of the antigen. Each well of a 96 well NUNC-Immuno Microwell plate (Sigma Aldrich, Auckland) was coated with 100 µL of the antigen/buffer mixture, covered with plate sealers, placed in a humid storage box, and refrigerated overnight at 4°C (minimum of eight hours). Meanwhile, a solution of ELISA antibody buffer was created by adding 1.0 g of bovine albumin (BA; Sigma Aldrich, Auckland, New Zealand) to 100mL ready-made washing buffer (phosphate buffered saline

(PBS)/0.05% v/v Tween-20 (Sigma Aldrich, Auckland, New Zealand). The beaker was labelled, covered, and stored at 4°C until used.

Initially, the ELISA plate was designed to determine two facts: First, to confirm that the cultured hybridoma lines that had been recovered from long-term storage were still producing MAbs; and second, to estimate an appropriate dilution of conjugate for use in other MAb based assays (i.e. whether the hybridoma lines were producing the desired MAb) and standard dilution curve ELISAs. The plate was laid out to allow evaluation of three different conjugate dilution factors (1:1000, 1:2000, 1:3000) commonly used in ELISA protocols. Within each block of conjugate, dilution factors were supernatant samples collected from different dates during the CP68 culture protocol, positive and negative controls, and blanks. Positive and negative controls were components used in previous cell expansion/hybridoma production by Bioquest, Ltd in October of 2000. These components were cell culture supernatant and ascites fluid from mice used to produce CP68 antibody (positive controls), and stored non-specific hybridoma antibody and unused cell culture media (negative controls). All samples were diluted with antibody buffer prior to removing coated plates from refrigeration (Table 3). A schematic of the ELISA plate setup is shown in Figure 1.

To complete the ELISA, antigen-coated plates that had incubated overnight were removed from the humidity box and washed four times with wash buffer using a manual strip washer. During washing, plates were orientated with well A1 in the upper left position. Each well was filled to the top with wash buffer, allowed to stand for two minutes, then the plate was inverted onto a clean paper towel and tapped gently to remove excess wash buffer. Plates were then oriented with well H12 in the upper left position, and the described washing process was repeated; these two steps were repeated one time for a total of four washing steps. After the final wash, 100 µL of antibody buffer was pipetted into blank wells and the wells where serial dilutions were to be made (Figure 1); supernatant samples were then pipetted into the plate wells as per the ELISA plate diagram. Where samples were ‘Neat’ (undiluted), 100 µL of sample was applied. Where samples were part of a serial dilution, 200 µL of sample was applied. Serial dilutions were completed in rows across the plate by aspirating 100 µL of sample from the appropriate well, transferring it to the adjacent well, mixing gently with the 100 mL antibody buffer already present in the well, and then moving the pipette plunger up and down at least three times (taking care to avoid creating bubbles). Repeating this process sequentially, serial two-fold dilutions were created. After the final

dilution was made, 100 µL was removed from the final wells and discarded so that all wells on the plate contained 100 µL of diluted sample, positive or negative controls, or antibody buffer. Plates were then covered and incubated at 25°C for 1.5 hours.

During the first incubation step, 12 mL of substrate buffer (1M diethanolamine, pH 9.8) was allowed to come to room temperature. The conjugate (alkaline phosphatase-conjugated goat affinity purified antibody to mouse IgG whole molecule; Product number 59296, Cappel, MP Biomedical, Auckland, New Zealand) was prepared for storage and then used to make 1:1000, 1:2000, and 1:3000 dilutions for use in the assay. Conjugate storage buffer was created by dissolving 365 mg Tris (BDH, Auckland, New Zealand), 1.0 g BA, and 822 mg NaCl (BDH, Auckland, New Zealand) in 80 mL distilled water. Twenty mL of distilled water was added to the solution and pH was adjusted to 8.0 with the addition of eight drops of 36% HCl. Conjugate was prepared by adding one mL of conjugated goat-anti-mouse AP antibody to 12 mL of the prepared conjugate buffer. The buffered conjugate was then added to 12 mL glycerol for storage and divided into one mL aliquots in sterile two mL cryovials for storage at -10°C. This 1:25 dilution was used to make further working dilutions of 1:2000 and 1:3000. The 1:1000 dilution was made by combining 300 µL of the 1:25 solution with 11.7 mL of antibody buffer, the 1:2000 dilution was made by combining equal parts 1:1000 solution and antibody buffer, and the 1:3000 dilution was made by combining 1 part 1:1000 with two parts antibody buffer.

After dilutions of 1:1000, 1:2000, and 1:3000 were prepared, 100 µL of the appropriate conjugate was pipetted into each well on the plate per the plate plan schematic: 1:1000 applied to columns 1-4, 1:2000 to columns 5-8, and 1:3000 to columns 9-12. The plate was then covered and incubated at 25°C for 1.5 hours. At the end of the incubation period, the conjugate was removed and plates washed four times using the procedure described above.

One p-nitrophenylphoshate (p-NPP) (Sigma Aldrich, Auckland, New Zealand) substrate tablet was dissolved into 12 mL of pre-prepared substrate buffer (1M diethanolamine, pH 9.8). As the substrate was light sensitive and must be used immediately, 100 µL was pipetted into every well and the plate was then incubated in the dark at 25°C for 30 minutes.

After the 30-minute incubation, 50 µL of 3M NaOH stopping solution was added to each well and plates were read in a plate reader at 410 nm (reference 630nm).

Using an identical procedure, the ELISA was repeated with the remaining two cell lines to verify MAb reactivity and create standard dilution curves. Conjugate dilution for remaining

assays was 1:2000. **Error! Reference source not found.** illustrates plate plans for determining standard dilutions.

Ascites production

Ascites production in this project was completed in two stages. The first stage was designed as a small pilot study to determine an estimate of the MAb yield from each mouse. The second stage of the project was the full-scale MAb production experiment. Adult, aged male mice from an unrelated project were available at low-cost and so were used in the first stage experiment (BALB/C, Sourced from the Small Animal Production Unit at Massey University) For the second stage experiment, 7-11 week old BALB/C mice of mixed sex obtained from AgResearch, Hamilton, New Zealand were used. The procedures related to ascites production were identical for both stages (Table 4.).

Mice were individually weighed, placed in individually numbered cages, and then randomly assigned to receive either CP68 or EP82 cells. CN04 cells were not used after finding archived cells had lost specificity and were not producing the MAb of interest. Fourteen days prior to inoculation with hybridoma cells, each mouse was primed with Pristane (Sigma Aldrich, Auckland, New Zealand), an inflammatory substance that is injected intraperitoneally to increase the quantity of ascites fluid that is produced. To complete the intraperitoneal injection, each mouse was restrained by firmly grasping the scruff of its neck in one hand and its tail in the other hand, and then applying gentle traction lengthwise along the body to elongate its abdomen; 70% ethanol was then applied to clean the injection site on the abdomen. Next, the mouse was tilted so that the abdominal contents shifted cranially and a 21-gauge needle was inserted at a 35-45° angle, bevelled edge up, into the lower right hand abdominal quadrant (caudal to the umbilicus and right of midline Figure 3). To ensure that the needle was placed in the peritoneal space and that no vessel or hollow viscous was punctured, the syringe plunger was withdrawn slightly and the hub of the needle checked for blood, urine, or intestinal contents. When certain the needle was in the peritoneal space, 0.5 mL of Pristane was injected, the needle removed, and the mouse placed back in its cage. Mice were observed daily for the next 5-7 days to monitor for adverse reactions to the Pristane.

Culturing of the hybridoma cells was coordinated so that 14 days after mice were primed with Pristane the cells would be at 90-100% viability and still growing in a logarithmic growth phase. On Day -2 and -1 pre-inoculation, hybridoma cells were counted and viability

confirmed using trypan blue exclusion; cell cultures were split (with fresh culture medium) at that time to ensure cells would be in log phase growth at the time of inoculation. On Day 0, cells were counted again and when confirmed there were enough for inoculation, removed from flasks using sterile technique, put into conical vials, and spun at 200g for 10 minutes. Cells were removed from the centrifuge, supernatant was removed, and the cell pellet was re-suspended in a volume of media that would permit each mouse to receive 2-4 x 10^6 cells suspended in a 0.5 mL volume. The 0.5 mL inoculums were placed into sterile cryovials and transported on ice to the mouse facility.

Just prior to inoculation, each mouse was weighed and then the same process as was used for priming was used to inject the hybridoma cells intraperitoneally. Mice were then replaced into their cages and observed daily.

After inoculation, mice were observed and weighed daily to monitor for health and wellness, and to check for the level of ascites production. Approved guidelines were established to determine when mice would be euthanized (Table 5.). All experimental animal work was approved by the Massey University Animal Ethics Committee (Approval number 08/17, February 7, 2008).

When mice met criterion for euthanasia, they were euthanized using carbon dioxide asphyxiation. After euthanasia, the abdomen was cleaned using 70% ethanol, the skin was incised with scissors along the midline from pubis to xiphoid, reflected laterally, and removed to expose the abdominal musculature; care was taken not to puncture the abdominal cavity at this point. Next using thumb forceps, the abdominal musculature was tented and an incision made at the midline near the umbilicus to open the abdominal cavity. The mouse was then transferred to a small glass filter funnel held over a 15 mL conical tube labelled with the mouse's number and hybridoma line, so that ascites fluid could drain from the abdominal cavity. Once all fluid had drained, the tube was immediately capped and placed on ice until it could be processed.

Mice that developed solid tumours, or those that did not appear to develop ascites at the end of the 21day study period were euthanized (method above), and after euthanasia had 4-6 mL of sterile saline injected into the intraperitoneal space to maximize collection of any ascites fluid that might be present. Ascites was then collected from each mouse using the previously described method then the abdominal fluid that was collected was processed, and stored in a manner identical to that used for the true ascites.

Within six hours of collection, ascites fluid was spun in a refrigerated centrifuge at 1500g for ten minutes. The ascites fluid was aspirated from the centrifuged tubes with sterile pipettes and pooled (by hybridoma line, one day's collection per pool) into sterile 50 mL conical tubes. After pooling, fluid was filtered sequentially through 0.8 µm, 0.45 µm, and 0.20 µm Millipore syringe filters and then stored at -20°C until purification.

Production of CELLine-derived MAbs

Seeding and monitoring CELLine culture

Media was prepared on the same day as flask seeding. Forty minutes prior to cell manipulation, components for CELLine media (both nutrient and cell compartments) were gathered and placed in the water bath to warm (Table 6.). Twenty minutes later the class 2 laminar flow hood was sprayed with 70% ethanol, turned on, and allowed to run. In the class 2 laminar flow hood using sterile technique, components for cell compartment media (CCM) were combined in a 50 mL conical tube (BD, Auckland, New Zealand) and components for nutrient compartment media (NCM) were combined in the original one litre media bottle.

The CELLine Flask (BD, Auckland, New Zealand) is a specially designed dual chamber system that has a thin membrane that separates the cell compartment (accessed through a port under a small white cap) from the nutrient compartment (accessed through a large blue cap). The membrane is semi-permeable and allows nutrients to flow into the cell compartment and waste products to diffuse out while keeping antibodies and cells in the compartment. The membrane is very delicate and easily torn when dry, so the flask must be handled gently (i.e. must not be tapped or shaken). Precautionary notes from the manufacturers were followed when working with the flask: the blue nutrient compartment cap was always loosened or removed when adding or removing contents from the cell compartment; and the nutrient compartment was always pre-wetted with 25 mL of media prior to adding media to the nutrient compartment when using a new flask (allowed for expansion of the membrane and to support nutrient media and avoid rupturing the membrane when nutrient media was added). Using sterile technique in the class 2 laminar flow hood, the blue cap was removed and 25 mL of NCM was pipetted into the nutrient compartment. The blue cap was replaced and the flask left in the hood while cells were prepared.

Using a trypan blue exclusion test, static cell cultures were confirmed to be in logarithmic growth phase, present in sufficient numbers, and at 90-100% viability prior to seeding into the CELLine flask. For each flask, a total of 3-7.5 x 10^7viable cells were removed from static

culture, usually taken from two to three 175 cm^2 flasks, placed into a conical tube and centrifuged at 170g for 10 minutes. The supernatant was then removed and the cells were gently re-suspended in 15 mL of pre-warmed CCM. The CELLine's blue cap was loosened and the white cap was removed. Re-suspended cells were collected in a 25 mL pipette and the tip was pushed securely into the port for the cell compartment. The cells were slowly pipetted into the cell compartment. After the entire volume was expelled, it was immediately drawn up again, including any air bubbles that were trapped in the cell compartment. Air bubbles were allowed to rise to the top of the pipette and cells were gently pipetted back into the cell compartment without them. The flask was placed in a CO_2 incubator. Cell viability was checked 48 hours after seeding using trypan blue exclusion.

CELLine culture flasks were maintained continuously for 28 days. On Day 14 of culture, new NCM and CCM were prepared and the hybridoma cells were removed from the cell compartment, counted, and centrifuged at 170g for 10 minutes. The supernatant (which contained antibody of interest) was collected and the cell pellet re-suspended in 15 mL of new CCM at 2-5 x 10^6 viable cells per mL for re-seeding. Nutrient media was changed by carefully pouring out used media into a large beaker.

On Day 28 of culture, hybridoma cells were removed from the cell compartment, centrifuged at 170g for 10 minutes, and the supernatant was collected. Nutrient media was poured out and the flask was discarded.

Culture supernatant was filtered through 0.45 μm and 0.20 μm syringe filters and then stored in sterile conical tubes at -20°C prior to purification.

Development and optimization of an ELISA for vaccine toxoid potency testing

An ELISA was developed for testing the potency of a commercial Clostridial vaccine toxoid based on a 'sandwich' ELISA protocol that had been previously developed by the sponsor for other clostridial toxin and toxoid quantification projects. Reagents and assay conditions were varied from those established for the previous assay (antibody coating concentration, detecting antibody concentration, conjugate concentration, and spectrophotometric absorbance) to optimize the performance of the new assay based on use of the MAbs that had been produced as described above.

Optimization steps included assessments of the relatively binding affinity of the MAbs for toxin versus toxoid, determination of the appropriate concentration of antigen (toxin or

toxoid) that would result in absorbance values that fell within the limits of the assay, determination of the appropriate concentration of anti-sera for use in the assay, and determination of the appropriate concentration of capture antibody that was required.

MAb protein derived from both *in vitro* and *in vivo* production methods needed to be purified prior to use in assay development. Fast protein liquid chromatography (FPLC) was used for this purpose.

Fast protein liquid chromatography (FPLC) purification of MAbs

Prior to development of the ELISA, the MAb containing fluids that had been previously collected and stored were purified. Ascites fluid and supernatant samples collected from the CELLine flasks were removed from -20°C storage, thawed, and separated into 5 mL aliquots for ease of processing. Sample aliquots were diluted with an equal amount of phosphate buffered saline (PBS) buffer (i.e. 5 mL of buffer was added to each aliquot). When not being manipulated on the bench top, all samples were kept at 4°C.

The FPLC machine was turned on and a pre-set wash program was run with PBS buffer. A sepharose gel column (HiTrap™ Protein G Sepharose Gel 5 mL Column; GE Healthcare, Auckland) was attached to the FPLC machine and 50 mL of PBS buffer was run through the column to wash it. The FPLC was programmed with steps to load the sample, inject the sample into the HiTrap™ column, wash the column to remove unbound protein when absorbance at 280 nm reached 0 absorption units (mAU), elute bound protein with glycine/HCl pH 2.2 elution buffer, collect 5 mL aliquots (collection started and stopped when absorbance at 280 nm reached 10mAU), and finally to wash the column in preparation for the next sample. 250 µL 1M Tris-HCl pH 9.0 buffer was added manually to each aliquot after elution to bring the pH to approximately pH 7 and avoid the potential for antibody denaturation at low elution pH.

The concentration of IgG antibody in each sample was unknown, so sample run volumes (the amount of diluted ascites or supernatant injected into the column each run) were systematically increased to determine when all binding sites in the column became saturated with IgG antibody, and what the largest (and most efficient) run volume would be for that lot of antibody to be purified (i.e. a single lot of ascites or cell culture supernatant). To determine a saturation point for the antibody source being manipulated, computer-generated successive elution curves were compared. In theory, each batch of unpurified antibody will have a different amount of IgG uniformly distributed throughout solution. Systematically increasing

the amount of unpurified antibody loaded onto the column and comparing the height and area of the elution curves (purified IgG is removed from the column) should indicate when the column becomes 'saturated' with IgG during the loading phase (i.e. all IgG binding sites are occupied). Prior to the saturation point, height and area for successive elution curves should increase proportionally to the increased volumes loaded onto the columm—indicating that all IgG was bound and eluted. If the volume loaded onto the column is increased and the elution curve height and area are not increased in the same proportion, it can be assumed that IgG was lost because it could not bind due to column saturation. For example, Table 7 shows that the column reached saturation somewhere between the third and fourth run volumes because the area and height ratios were decreased compared to the sample volume ratio.

When the appropriate run volume for a sample was determined, the entire sample was purified, the pH checked so that it was between 7.2 and 7.6, divided into labelled 15, 5, and 0.5 mL aliquots, and frozen at -20°C. The purification column was washed with PBS buffer and then 20% ethanol before being stored at 4°C.

After MAbs were purified to remove non-specific proteins including albumin and non-IgG molecules, the protein concentration was determined. A commercially available colorimetric assay for estimating total protein concentration (BCA Protein Assay Kit™; Thermo Scientific, Rochester, NY) containing a bovine serum albumin (BSA) standard, and two reagents (A and B) that are combined to create 'Working Reagent' was used for assessing the protein concentration of purified antibody. Two hundred µL of the 2.0 mg per mL BSA standard and PBS were combined to make 400 µL of a 1.0 mg per mL standard. Serial doubling dilutions were then performed until four protein standards were created. Final concentrations for each standard were: 1.0 mg per mL, 0.5 mg per mL, 0.25 mg per mL, and 0.125 mg per mL.

Four dilutions of each purified antibody (neat, 1/5, 1/10, 1/20) were created. 400 µL of each was needed. For the 1/5 dilution, 80 µL of MAb was combined with 320 µL PBS; for the 1/10 dilution 200 µL of the 1/5 dilution was combined with 200 µL PBS; and for the 1/20 dilution 200 µL of the 1/10 dilution was combined with 200 µL PBS. Working Reagent was created by combining one part Reagent B with 50 parts Reagent A. An ELISA plate plan was made and 25 µL of each standard and each unknown was pipetted onto the plate according to that plan. Working reagent (200 µL) was added to each well and the plate agitated thoroughly for 30 seconds. The plate was covered and incubated at 37°C for 30 minutes. After incubation

the plate was allowed to cool to room temperature and the absorbance was read at 562nm on a plate reader.

ELISA setup

Plate maps were made for assay planning. Four 96-well plates were labelled 1 to 4. Plates were assigned different antigens (toxin or toxoid) and different detecting antibody concentration (1:50 or 1:100). Assignments were as follows: toxin antigen at 1:50 dilution for plate 1, toxin antigen at 1:100 dilution for plate 2, toxoid antigen at 1:50 dilution for plate 3, and toxoid antigen at 1:100 dilution for plate 4. All plates had identical capture antibody concentration (doubling dilutions from starting concentration of 10 µg per mL); and all plates had identically applied antigen (doubling dilution from starting dilution of 1:1) (Figure 17).

Capture antibody coating buffer was prepared by combining purified EP82 ascites with coating buffer (0.05M carbonate buffer, pH 9.6). Based on an average concentration of 210 µg per mL of purified EP82 ascites MAb (Table 10), a 1:19.8 (antibody:coating buffer) solution was made for plate coating at 10 µg per mL.

Coating buffer (100 µL) was pipetted into all wells in columns three to 12. Capture antibody (100 µL) was then pipetted into all wells in column one and 200 µL of capture antibody was pipetted into all wells in column two. Using an eight tip multi-pipette, 100 µL of capture antigen was removed from all wells in row two, pipetted into row three, and mixed gently three times. The process was repeated between rows three and four and doubling dilutions were performed down the plate. The remaining 100 µL in the wells from row 12 were discarded. Plates were covered with plate sealers, placed in a humidified box, and incubated overnight at 4°C (at least 8 hours).

ELISA wash buffer was prepared from PBS, Tween-20, and bovine albumin as described previously. Wash buffer was labelled and stored at 4°C when not in use.

ELISA technique

Coated plates that had incubated overnight were removed from humidity box and washed four times with wash buffer using a manual strip washer in the manner described previously. After washing, 100 µL of antibody buffer was dispensed into every well on all four plates.

Two antigens were used in this assay: *C. perfringens* D toxin PI, concentration 400 U/mL and toxoid PII, concentration 500 U/mL. All plates had one column of non-specific toxin to serve as a negative control (*C. perfringens* C toxin).

One hundred µL of non-specific toxin was pipetted into well A1 on all plates, and 100 µL of toxin or toxoid (plate-dependent) was pipetted into wells A2-A12. Using a 12-tip multi-pipette, antigen was mixed three times with antibody buffer. One hundred µL was removed and transferred to the row below (B). Serial doubling dilutions were performed down the plate and the last 100µL from row H was discarded. Plates were then covered and incubated at 25-27°C for 1.5 hours. After toxin/toxoid addition, anti-clostridial rabbit antiserum (Schering Plough, created for in-lab use) was removed from -20°C storage and thawed. Two dilutions were used in this assay: 1:50 and 1:100. At the end of incubation, plates were washed four times per protocol and 100 µL of antiserum was dispensed in all wells according to plate plan. Plates were covered and incubated at 25-27°C for 60 minutes. Thirty minutes prior to the end of incubation, 120 µL anti-rabbit alkaline phosphatase-conjugated antibody (Schering Plough, created for in-lab use) was removed from storage at -20°C and combined with antibody buffer to create a 1:400 (conjugate:buffer) solution. After incubation, plates were washed, 100 µL of the diluted conjugate was pipetted into each of the empty wells, and plates were incubated for 60 minutes at room temperature. Ten minutes prior to the end of incubation, four 5 mg pNPP Substrate (Sigma, Auckland, New Zealand) were combined in a clean beaker with 48 mL of antibody buffer and mixed with a stir bar. After incubation, plates were washed and 100 µL of mixed substrate was added to every well. A 30 minute incubation step was started as soon as the first wells had substrate added, and plates were incubated in the dark at 23°C. The substrate reaction was ended by pipetting 50 µL of 3M NaOH Stop Solution into each well. Plates were read on a VERSAmax microplate reader at 410/630 nm.

Capture antibody concentration for future assays was determined by absorbance between positive and negative controls/blanks, and capture antibody concentration with the largest difference and still within a maximum cut-off adsorbance (<1.4).

Optimisation

The ELISA procedure was repeated with blanks, and two non-specific toxins: *C. septicum* toxin, and *C. perfringens* C toxin. *C. perfringens* D toxin and toxoid starting concentrations for serial dilutions were decreased from 1:1 dilution to 1:32. Capture antibody coating concentration was increased for reference and ranged from 20 µg per mL to 0.3 µg per mL. Assay was run as previously with detection antibody at 1:50.

Coating buffer containing capture antibody was prepared by combining purified EP82 ascites (average concentration of 210µg per mL, Table 10) with coating buffer (0.05M carbonate

buffer, pH 9.6) at a concentration of 8µg per mL. Plate plans were made. Two plates were used, one each for toxin and toxoid. Plates were coated by pipetting 100 µL coating buffer into all wells in Rows C to H. Coating buffer containing capture antibody at 20µg/mL was pipetted into all wells in Row A (100µL) and B (200µL). 100µL of buffer was transferred from wells in Row B to C and mixed thoroughly. The process was repeated from Row C to D, and serial doubling dilutions were performed down the plate and the remaining 100 µL from all wells in Row H was discarded. Plates were incubated overnight as previously described. The next day, 100 µL of each of the four antigens (both non-specific toxins, and *C. perfringens* D toxin and toxoid) to be used in the assay was combined with 3.1 mL of antibody buffer to create a 1:32 (antigen:buffer) dilution. Plates were removed from incubation and washed as previously described. One hundred µL of diluted *C. septicum* was pipetted into each well in Column 1 and 100 µL of diluted *C. perfringens* type C was pipetted into each well in Column 2. Column 3 had 100 µL of plain antibody buffer pipetted into each well, and Column 4 had 200 µL of either *C. perfringens* type D toxin or toxoid (plate dependent) pipetted into each well. Columns 5-12 had 100 µL of plain antibody buffer pipetted into each well. Using 100 µL volumes, serial doubling dilutions were performed across the plate from Column 4 to 12. The remainder of the ELISA protocol was followed as previously described.

Implementation

After optimization, the ELISA was used in a blinded test to differentiate between vaccine supernatant (provided to the canditate by Schering Plough project supervisors with knowledge of sample identity) that had low potency for the *C. perfringens* type D antigen and supernatant that did not.

Coating buffer containing capture antibody was prepared by combining purified EP82 ascites (average concentration of 210µg per mL, Table 10) with coating buffer (0.05M carbonate buffer, pH 9.6) at a concentration of 8µg per mL. Plate plans were made. 100 µL of 8 µg per mL capture antibody coating was pipetted into each of the wells on both plates; the plates were sealed, and then incubated overnight as previously described. The next day *C. perfringens* type D toxin and toxoid (positive controls), *C. septicum* and *C. perfringens* type C toxins (negative controls) were each combined with antibody buffer and made to 1:250 (antigen:buffer) dilution. Plates were removed from overnight incubation and washed as previously described. One hundred µL of plain antibody buffer was pipetted into each well on

two plates. Antigen was then applied only to Plate 1. One hundred µL of each *C. septicum* toxin, *C. perfringens* type C toxin, plain antibody buffer, *C. perfringens* type D toxoid, and *C. perfringens* type D toxin were pipetted into wells A1, B1, D1, and E1, respectively. Row C was left blank on both plates. Three vaccine samples were used in this assay: A, B, and C. One hundred µL of 'neat' vaccine samples were pipetted into the remaining wells in Column 1 (Vaccine A in F1, Vaccine B in G1, and Vaccine C in H1). Serial doubling dilutions were made across Plate 1 and the final 100 µL volume from Column 12 was carried on to Column 1 of the Plate 2. A total of 23 serial doubling dilutions were made. ELISA protocol was followed as described earlier. Positive values were those similar to positive control (*C. perfringens* D toxoid), and negative values were conisered those similar to the negative controls (blanks, non-specific *C. septicum* toxoid).

Chapter 3: Results

Verification of hybridoma viability, reactivity, and specificity

All three hybridoma lines were still viable after several years of storage, and all grew easily in culture once protocols were established. Archived lines were expanded and enough hybridoma stock has been stored to ensure large quantities of MAb can be created when needed. This project was initiated by receipt of five vials of EP82 original seed stock from which 14 first passage cultures were created; nine of the first passage stocks remained at the end of the study (all original seed stock was used). For the CP68 line, three vials of original seed stock were received from which 21 first passage cultures were created; ten vials of cells remained at the end of the study (one original seedstock and nine first passage). For the CN04 line, four vials of original seed stock were received from which ten first passage cultures were created; 11 vials of cells remained at the end of the study (one original seed stock and 10 first passage). Upon thawing and use, percent viability of cell aliquots produced and frozen during this project was generally between 55 and 80%. The ELISA used to determine if the hybridoma lines were actively producing the correct, specific MAb was optimized and the conjugate dilution found to work best was 1:2000. At this conjugate dilution all samples and positive controls (Bioquest ascites and cell culture supernatant, project cell culture supernatant) at similar dilutions were closely associated and negative controls (Bioquest non-specific hybridoma and unused media, project unused media) and blanks showed no non-specific conjugate binding. At higher conjugate concentration (1:1000) there was more variation in optical density (OD) values for known positives (Bioquest ascites, cell culture supernatant), and the negative controls had a tenfold increase in OD in half of the wells, suggesting non-specific conjugate binding (Figure 4)

Cell culture supernatant from hybridoma lines EP82 and CP68 showed the cells were active and producing their respective MAbs of interest (Figure 5 and **Error! Reference source not found.**): Cell culture supernatant samples had similar OD values to positive controls (Bioquest ascites and culture supernatant). The CN04 hybridoma line was not producing the MAb of interest: Cell culture supernatant samples had OD readings identical to negative controls and blanks on the plate (Figure 7).

Ascites and CELLine culture production

Ascites production

Table 2 shows the number of mice used in each ascites production group, the average days to collection, and the average collection volume per mouse. Some mice (6/29 in Group 1, 8/95 in Group 2) never developed clinically apparent ascites or never met criterion for euthanasia (Table 5.) during the study period and so were euthanized at the end of the study period. Mice in the first group in this category had 5 mL of sterile saline injected intraperitoneally prior to collection in an effort to recover as much MAb as possible. Mice in the second group in the same category did not have saline injected intraperitoneally prior to collection. In Group 1, 30 percent of all mice that were inoculated developed solid subcutaneous/peri-serosal tumours instead of or in addition to the free-cell disseminated type intraperitoneal type that was expected. Consequently, ascites fluid recovery (and resulting monoclonal antibody yields) was low (Table 9). Figure 8, Figure 9, Figure 10, and Figure 11 show the distribution of mice harvested by hybridoma line and group, and the volume of processed ascites collected per study day by line and group. Table 9 shows the total volumes of ascites collected from each group after processing and filtering, prior to purification.

CELLine culture

The CELLine 1000 Flask was used for both hybridoma lines to evaluate its use as an alternative to mouse ascites in manufacturing the antibodies of interest. Cells thrived in the flasks, and even though viability sometimes dropped to very low levels, still produced antibody. Figure 12 and Figure 13 illustrates how the EP82 hybridoma line progressed through 28 days of continuous culture. Cell viability was low, but was still producing antibody.

Both ascites and cell culture supernatant were purified using a HiTrap™ Protein G column. Table 9 shows the volume of fluid obtained from processed and filtered antibody sources.

Four dilutions (neat, 1:5, 1:10, & 1:20) of purified MAb were compared to a standard protein curve. Using a SOFTmaxPro application, the mean OD values at 562 nm for the standards were used in a linear regression to establish a line of best fit. Undiluted (neat) purified MAb mean OD values were then plotted against this line to establish their concentration. Three assays were run to determine the concentrations of the various lots of purified MAb (Figure 14, Figure 15, and Figure 16). After determining concentration, total quantity (in milligrams) of purified MAb obtained was calculated (Table 9).

ELISA potency assay

The ELISA potency assay was used only with the EP82 anti-epsilon toxin MAb. By systematically manipulating the concentrations of the antibody coating, toxoid, and antiserum, we were able to determine the optimal concentrations of each to give the best readings for the assay. Optimal MAb coating was eight μg per mL (Figure 18, Figure 19), and optimal antiserum concentration was 1/50 (data not shown). Toxoid dilution was more difficult to determine, as there was what appeared to be non-specific binding that produced high background OD readings even at very low toxoid concentrations (i.e. more dilute than 1:2000) (Figure 20, Figure 21). There was some 'titration out' at a MAb coating of 2.5 μg per mL when toxin was used as the antigen, however this was not consistent with the values observed with the toxoid at that MAb concentration.

For the differentiation between vaccine supernatants that had low potency compared to those that did not, the non-specific binding made it difficult to differentiate between the two. Contrary to what was expected, vaccines with higher potency had higher OD readings than the single vaccine that did not (Figure 22).

Chapter 4: Discussion

Comparison between *in vitro* and *in vivo* MAb production methods

Table 11. shows the cost differential between the two production methods used in this project in a dollar per mg of purified antibody basis. This clearly illustrated that for the hybridoma lines used in this project, *in vitro* methods were more cost-efficient than *in vivo* methods. Traditionally, *in vivo* methods have been considered less expensive because many facilities had animals readily available for use in ascites production (Falkenberg, 1998). However, this situation is changing as Institutional Animal Care and Use Committees are systematically encouraging reduced numbers of animals to be used in scientific studies. Fewer facilities have animals on-site or readily accessible and as technologies to replace animals become more available, the use of animals will be less justifiable.

The average number of hours spent performing routine cell culture (static flask culture) was approximately 4-7 hours per week. This included manipulation of cells in the hood and creating media on a regular basis, but also thawing or freezing cells which is a very time-consuming task though performed more infrequently. While mice were being used for ascites production, the time commitment to this project went up significantly. In addition to the 4-7 hours per week with the cell culture, weighing and assessing the health status of 100 mice requires up to two to three hours per day, totalling 14-21 hours per week for the 21 days of the study after inoculation of mice with hybridoma lines. This time commitment generally decreased over the period of the study as the mouse population decreased. Duties when working with mice included: Changing cages, weighing and recording weights of mice, assessing and recording presence of ascites and/or solid tumour formation, euthanizing mice and harvesting ascites, processing ascites in the lab after harvest, and travel to and from the housing facility. In contrast, hours spent while working with CELLine system did not change at all from time spent with traditional static culture. Two harvests of CELLine supernatant occurred 2 weeks apart, with one week of traditional culture required to seed the flask. To produce MAb via CELLine, a total of 20-23 hours over a five week period was required. To produce MAb via the ascites system, a total of 50-77 hours over a five week period was required. In terms of mg MAb produced per hour of labour, CELLine had a higher yield at 2.3 to 2.7 mg per hour invested, while ascites was less productive at 1.09 to 1.69 mg per hour invested. This time calculation for ascites production did not include additional training needed by staff to handle mice. This competency already existed as staff used for this study

had previous humane animal handling experience and were experienced working with mice. If this experience was not already in place for a particular project, the necessary training would increase the cost for ascites production even further.

The CELLine system was extremely easy to set up and required no additional equipment aside from what is needed for traditional cell culture (Falkenberg, 1998; Trebak, et al., 1999). The flask was designed to fit in standard CO_2 incubators typically found in reasonably equipped tissue culture laboratories. If technicians are familiar with sterile technique and mammalian cell culture techniques, incorporating use of the CELLine flask would represent only a simple extension of their existing skills.

Ascites production can be very difficult to set up. In our study we were fortunate to have animal housing at a nearby satellite facility. Arranging transport of animals, arranging housing and care, coordinating with animal care staff, and gaining IACUC approval for the project were all time-consuming tasks that delayed the start of ascites production.

Monoclonal antibodies from both cell lines were produced in quantities sufficient for immediate laboratory use and for archiving for future use in assay development. Part of the objective of this project was to determine the viability of *in vitro* technology for production of large quantities of MAbs for the two cell lines of interest. Mouse ascites production is an established method of producing large quantities of MAb in a relatively small-scale setting. However, the technique is falling out of favour due to pressure to reduce the number of animals used in research (Antibodies, Research, & Council, 1999). New technologies developed in the 1990s were discussed in Chapter 1 above with regard to their relative ease of setup, appropriateness for bench top laboratory work, and expected antibody yield. Table 10 shows the antibody yield from various systems, as well as the yield from our experiments on a mg per mL of purified MAb basis. Both of the MAb productions systems, while producing less than expected from the related literature, were approximately equal to each other with respect to the yield of final purified antibody that was produced; possible reasons for low yield are discussed in following sections).

For the purposes, of this project, CELLine production of purified MAb is superior to production of ascites. It is more cost-effective on a dollar per mg basis as well as an hour per mg basis. Setup and training for CELLine culture is easier, no specialized training is required (i.e. humane handling courses for working with mice), and no special permission (via IACUC) is required to begin CELLine culture.

ELISA assay performance relative to live-animal testing

An ELISA was developed to test the theory that there was insufficient adsorption of antigen (toxoid) to adjuvant in vaccine lots that induced lower antibody titres in a rabbit (potency) model. If decreased adsorption was a causative factor in vaccine potency failure, higher concentrations of unbound toxoid in the supernatant would be expected. Increased concentrations of unbound toxoid would predictably correspond to higher OD values in the antigen capture ELISA that was developed and have similar OD values as positive controls (epsilon toxoid). The OD for the vaccine supernatant samples (inferred as free antigen bound to capture antibody) was lower in low potency vaccine samples when compared with the positive control. This was contrary to what we were expecting. OD values were high for non-specific toxoid (*C. septicum*) and blanks with OD values between 0.6 and 0.8; we hypothesized this was a result of non-specific binding and plans were made to evaluate the effect of adding a blocking step as the next prior to operationalizing the ELISA as part of routine QA follow-up testing in the vaccine manufacturers laboratory. BSA has been previously implicated in causing significant non-specific binding in ELISA reactions that can interfere with OD readings (Xiao & Isaacs, 2012). Time available on this project was constrained preventing completion of this further step in test optimization.

Live animal testing is the gold standard for evaluating the potency of many commercial vaccines, especially inactivated vaccines. *In vivo* potency tests are animal-intensive (usually requiring the use of more than one species), time-intensive, often involve zoonotic organisms, and are expensive. Efforts to reduce the numbers of animals needed for vaccine potency testing around the world with both the scientific and animal welfare communities encouraging rapid movement away from *in vivo* techniques and towards *in vitro* techniques. Specifically for Clostridial vaccines, ELISA in combination with *in vitro* cell culture toxin neutralization tests have been recommended as a feasible alternative to *in vivo* toxin neutralization (Stokes, et al., 2011). Currently, the project sponsor already incorporates use of indirect ELISAs as an alternative to *in vivo* toxin neutralisation tests. To ensure that these alternative toxin neutralization tests are accurate in determining potency, results should be compared to a reference vaccine deemed efficacious in a natural challenge in the target species (i.e. sheep or cattle) (Draayer, 2011). ELISA tests have been used to assess the effect of toxoid adsorption to adjuvant and showed no correlation between different vaccines and the amount of tetanus toxoid measured (or the degree of adsorption) and the potency of the tetanus component when compared across manufacturers (Coombes, Tierney, Rigsby,

Sesardic, & Stickings, 2012). However, that study did suggest a place for a validated ELISA to monitor vaccine production consistency (not potency). The predomoninant use of the assay that was developed through this project will be to objectively monitor consistent production of high quality Clostridial vaccines in a manner that substantially reduces use of animals for potency testing has yet to be determined but there is certainly potential with further work.

Problems encountered during the study

Purification problems

During the purification step of the final batch of MAbs, white particulate matter appeared in purified samples suggesting the material may have become contaminated sometime during the purification process. Gram stains of particulate-containing samples were negative for bacteria suggesting bacterial contamination was not the problem. However, this test would not have necessarily rule-out the presence of fungal contamination. If the precipitate was immunoglobulin protein, this would explain the lower than expected protein yields determined in the quantification assays. Whether the precipitated material was protein or microbial contamination was not finally determined during the project but the project sponsor was planning to undertake further testing of the samples to resolve the issue.

Low MAb yields

Purified MAb yield from ascites was lower than expected. Most likely, this was a result of the ascites harvest procedures used in the study relative to what has been reported in much of the scientific literature. Historically, mice were not euthanized at the time of ascites harvest. Instead, they were 'tapped' and allowed to continue producing ascites over time and thereby increasing the total protein yield per mouse. Logically, a single harvest would decrease the protein yield per mouse. In this study, ascites harvest was limited to only one harvest (performed at the time of euthanasia) as mandated in the animal ethics approval. This fact alone likely contributed substantially to the lower than expected yield of purified MAb from the ascites method.

In addition to constraints on yield contributed by using only a single ascites harvest, low yields may also have been due simply to the genetic capacity of the particular hybridoma lines used in the study. Hybridoma lines are known to vary in the amount of MAb they produce and their robustness in culture. Both of these factors would potentially decrease the amount of MAb produced and neither is a problem that could be readily overcome aside from simply increasing the number of mice used in the study.

Numerous mice in the studies produced very little ascites with most never reaching the target weight for euthanasia and harvest (data not shown). Furthermore, approximately 30% of the mice produced body wall tumours after inoculation with the hybridomas instead of the anticipated non-solid neoplastic event that would result in ascites production. Study personnel experienced in performing intraperitoneal injections into mice and therefore it was considered unlikely that injection technique (i.e. subcuticular or intramuscular rather than intraperitoneal injection) was responsible for the tumour development. The majority of these solid tumours formed in mice inoculated with CP68 hybridomas, consistent with past experiences of the project sponsor. The scientific literature provided no consensus regarding the most appropriate age of mice for ascites production with reported studies using mice that ranged in age from adult aged mice (Falkenberg, 1998) to mice as young as eight weeks old (Brodeur, et al., 1984; De Deken, Brandt, Ceulemans, Geerts, & Beudeker, 1994). The current study used both aged and juvenile mice and a comparison between the groups showed that fewer young mice produced solid tumours (7% compared with 30% of older group) and produced a greater yield of MAb per mouse.

The outcome of the first attempt of *in vitro* MAb production using the EP82 hybridomas in CELLine flasks was suboptimal, likely due to incubator failure. Subsequent to MAb harvest, it was discovered that the CO_2 sensor in the incubator was faulty and that actual CO_2 levels were well above the 5% (+/- 2%) range known to be optimal for hybridoma growth and MAb production in the CELLine system. The compromised growth environment likely contributed to the lower than expected yield of cell culture-derived EP82 MAb. Unfortunately, even after repair the CO_2 sensor was found to be intermittently failing during the time period just prior to the final round of ascites and CELLine culture. This issue was discovered when it was noted the hybridoma cultures (which had been previously thriving) began suddenly to lose viability despite any planned changes to the culture maintenance protocols or methods. Upon confirmatory testing of the CO_2 sensors, the actual incubator CO_2 levels were found to be in excess of 12%; moving the cultures to a different incubator resulted in immediate improvement in culture viability.

CELLine culture protocols provided by the manufacturer were used in this study. However, modified CELLine protocols have been published in the literature, including some that recommend more frequent harvest and nutrient media changes (Howard GC, 2007; Trebak, et al., 1999). Logically, changing media more frequently would remove metabolites and provide

cells with fresh nutrient sources and promote a longer time period in the logarithmic phase of growth when hybridoma cells are known to be a peak level of MAb production. Future use of the CELLine system by the project sponsor will include an evaluation of some of these modified protocols to determine their effect on improving MAb yields from the CP68 and EP82 lines.

Summary and conclusions

The series of projects undertaken for this study resulted in production of MAb in quantities sufficient for the immediate and near-term future use of the project sponsor. Two of the three archival hybridoma lines that were evaluated were found to be viable and confirmed to be producing the MAbs that were expected. Two techniques for production of MAbs were evaluated. It was shown that the *in vitro* CELLine system can be a viable alternative to the traditional ascites model for MAb production, and that the *in vitro* system requires less time and cost to produce an equivalent quantity of MAbs, a fact consistent with other studies in the scientific literature. Using the MAbs that were produced, an ELISA was developed that will be useful in investigating some of the causes of low vaccine potency and provide a non-animal tool that can substantially reduce the number of animals required for vaccine manufacture.

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Tables

Table 1. Major toxins produced by *C. perfringens* and their toxicological properties.

		Toxin type				
Toxin	**Biological activity**	**A**	**B**	**C**	**D**	**E**
Alpha	Lethal, lecithinase	+	+	+	+	+
Beta	Lethal, necrotizing, trypsin labile	-	+	+	-	-
Epsilon	Lethal, permease, trypsin activatable	-	+	-	+	-
Iota	Lethal, dermonecrotic, trypsin activatable	-	-	-	-	+

Adapted from (Hatheway, 1990)

Table 2. Important clinical diseases of livestock caused by infection with *C. perfringens* types B, C, or D.

Disease	Toxin type	Class affected
Calf enterotoxaemia	B and C	Well-fed calves ≤ 1month
Pig enterotoxaemia	C	Piglets ≤ 5 days
Struck	C	Adult sheep
Goat enterotoxaemia	C	Adult goats
Pulpy kidney (overeating disease)	D	Well-fed lambs < 2 weeks or at weaning

Adapted from (Kahn & Line, 2010)

Table 3. Dilution methods used for optimizing performance of ELISA. Positive controls included the Bioquest ascites and culture supernatant. Negative controls included Bioquest non-specific hybridoma and unused media.

Sample type	Volume of sample	Volume of Ab buffer	End dilution
Bioquest ascites	50 µL	4.95 mL	1/100
1:100 ascites	500 µL	4.5 mL	1/1000
Massey supernatants	-	-	Neat
Bioquest non-specific hybridoma	-	-	Neat
Bioquest unused media	-	-	Neat
Bioquest culture supernatant	-	-	Neat

Table 4. Timeline of experimental activities for MAb production using an *in vivo* ascites technique. Mice were monitored daily for signs of adverse reaction to Pristane injection for the first seven days after priming and then again after inoculation with hybridoma cells. Daily monitoring was performed at the same time each day and changes in weight or overall health were noted. If criterion for euthanasia were met then mice were euthanized and ascites harvested.

	Animal-related		Hybridoma-related	
Study day	**Activity**	**Frequency**	**Activity**	**Frequency**
-14	Pristane injection	Once	Begin hybridoma culture	Once
-14 to 0	Pristane monitoring (adverse reactions)	Daily [a]	Monitor culture	Daily
-2 to -1	N/A [b]	N/A	Split culture, ensure viability	N/A
0	Hybridoma inoculation	Once	N/A	N/A
0 to 21	Monitoring (bodyweight, health, behaviour)	Daily	N/A	N/A
	Euthanize mice per protocol	As needed	N/A	N/A
21	Euthanize surviving mice	Once	N/A	N/A

[a] Pristane monitoring stopped at Day -7.
[b] Not applicable, no activity undertaken.

Table 5. Criteria for mouse euthanasia during production of ascites.

Criterion for euthanasia
Significant weight gain or loss (gain or loss of 10-12% of Day 0 weight)
Impairment of mobility from ascites accumulation or solid tumour formation such that eating, drinking, or other normal grooming behaviours cannot occur as a result of either ascites accumulation or solid tumour formation.
Injection site infection
Signs of severe sickness or distress (hypothermia, significant lethargy, cyanosis, significant increased respiratory effort, persistently hunched posture, ruffled fur, dehydration or emaciation).
Adapted from (Antibodies, et al., 1999)

Table 6. Components of media used in CELLine nutrient compartment and cell compartment.

Nutrient compartment media (NCM)	Cell compartment media (CCM)
900 mL BD MAb cell media serum free	16 mL BD MAb cell media serum free
40 mL Glutamax 200 mmol solution	1.5 mL Glutamax 200 mmol solution
10 mL penicillin streptomycin solution	0.5 mL penicillin streptomycin solution
50 mL FBS	4 mL FBS

Table 7. Performance of MAb purification by fast protein liquid chromatography using sepharose gel column.[a] Maximum column saturation was determined by contrasting the area and height ratio of a sample elution curve between sequential runs of the same sample lot being purified. The column reached saturation between the third and fourth runs because the area and height ratios are decreased with respect to the volume and retention ratio.

Run	Volume ratio	Retention ratio	Area ratio	Height ratio
1				
2	1.67	1.67	1.69	1.68
3	1.40	1.42	1.40	1.41
4	1.43	1.43	1.26	1.24

[a] HiTrap™ Protein G Sepharose Gel 5 mL Column; GE Healthcare, Auckland.

Table 2. Results of *in vivo* MAb production of hybridoma lines EP82 and CP68 using an ascites production technique.

Group	Cell line	No. mice injected	No. mice collected	Avg. days to collection	Avg. ascites per mouse (mL)	Minimum and maximum ascites volume (mL)
1	EP82	15	15	17.6	2.06	0 and 2.5
	CP68	14[a]	14		1.86	0 and 4.5
2	EP82	47[b]	45[a]	14.8	1.07	0 and 3.0
	CP68	48[c]	47[b]		1.83	0 and 4.5

[a] One euthanized for humane reasons one hour post-injection with cells, one died during study prior to harvest.
[b] One died during study prior to harvest.

Table 9. Volumes of MAb-containing fluids harvested from ascites and CELLline production techniques and resultant quantity of purified MAb obtained from each.

Hybridoma line	Method of collection	Filtered harvest volume (mL)	Purified MAb volume (mL)	Total purified MAb (mg)
EP82	Group 1 Ascites[a]	31	94	18.8
	Group 2 Ascites[b]	48	115	23.2
	CELLine Harvest 1[c]	25	46.5	
	CELLine Harvest 2[c]	15	49	28.2[d]
	CELLine Harvest 3[c]	18	54.5	
CP68	Group 1 Ascites[a]	26	82	14.7
	Group 2 Ascites[b]	86	135	27.8
	CELLine[c]	18.5	40	23.1

[a] Assay results summarized in Figure 15.
[b] Assay results summarized in Figure 16.
[c] Assay results summarized in Figure 14.
[d] Fluid from all three CELLine harvests were combined prior to quantifying amount of MAb present, this value represents the combined yield from the three harvests.

Table 10. Expected MAb yield from published *in vivo* and *in vitro* production methods for MAbs as compared to current results.

Method of production	Yield purified MAb (mg per ml)	Published reference
Ascites	3-15	(Goding, 1980)
Ascites	4.1-8.4	(Jackson, et al., 1996)
Hollow fibre bioreactors	0.7-11	(Jackson, et al., 1996)
Tumbling chambers	0.3-1.9	(Jaspert, et al., 1995)
Dialysis tubing	0.1-2.8	(Falkenberg, et al., 1993)
Glass cylinders	0.6	(Moro, et al., 1994)
miniPERM	0.24-4.5	(Falkenberg, et al., 1995)
CELLine	0.7-0.9	(Trebak, et al., 1999)
Static flasks (standard batch culture)	0.02	(Antibodies, et al., 1999)
EP82 ascites	0.2-0.21	Current study
EP82 CELLine	0.18	Current study
CP68 Ascites	0.18-0.21	Current study
CP68 CELLine	0.58	Current study

Table 11. Estimated costs of purified MAb produced by ascites and CELLine production methods. CELLine culture was less than half the cost of ascites production on a milligram basis.

Ascites	Amount[a]	CELLine culture	Amount[a]
Tissue culture (2 weeks)	$329	Tissue culture (1 week)	$165
Mouse cost (5 weeks housing, priming, transport, etc.) for 100 mice	$5,460	4 weeks CELLine culture (flask, media, etc.)	$1,303
Total cost	$5,789		$1,468
Total MAb produced (mg)	84.5		51.3
Total cost (per mg)	$69		$29

[a] New Zealand dollars

Figures

		1	2	3	4	5	6	7	8	9	10	11	12
	Conjugate dilution	1/1000				1/2000				1/3000			
A	Bioquest ascites	1/100		1/1000		1/100		1/1000		1/100		1/1000	
B	Massey CP68 supernatant from June 16, 2008	1/2	1/4	1/8	1/16	1/2	1/4	1/8	1/16	1/2	1/4	1/8	1/16
C	Massey CP68 supernatant from June 16, 2008 (duplicate)	1/2	1/4	1/8	1/16	1/2	1/4	1/8	1/16	1/2	1/4	1/8	1/16
D	Massey CP68 supernatant from June 18, 2008	1/2	1/4	1/8	1/16	1/2	1/4	1/8	1/16	1/2	1/4	1/8	1/16
E	Massey CP68 supernatant from June 18, 2008 (duplicate)	1/2	1/4	1/8	1/16	1/2	1/4	1/8	1/16	1/2	1/4	1/8	1/16
F	Bioquest non-specific hybridoma	Neat[a]	Neat[a]	B[b]	B[b]	Neat[a]	Neat[a]	B[b]	B[b]	Neat[a]	Neat[a]	B[b]	B[b]
G	Bioquest unused media	Neat[a]	Neat[a]	B[b]	B[b]	Neat[a]	Neat[a]	B[b]	B[b]	Neat[a]	Neat[a]	B[b]	B[b]
H	Bioquest cell culture supernatant	Neat[a]	Neat[a]	B[b]	B[b]	Neat[a]	Neat[a]	B[b]	B[b]	Neat[a]	Neat[a]	B[b]	B[b]

[a] Neat = no dilution with buffer solution
[b] B = blank cell

Figure 1. Schematic of ELISA plate used for conjugate dilution and diagnostic performance of CP68 assay. ELISA plates were divided into three blocks of conjugated antibody concentration (1/1000, 1/2000, 1/3000). Within each conjutage dilution 'block' samples known to CP68 antibody (positive controls--previous cell culture supernatant, previous ascites supernatant) were compared to those of the current project's cell culture supernatant for determination of production activity. To rule out

		1	2	3	4	5	6	7	8	9	10	11	12
A	Massey CP68 supernatant from June 16, 2008	1:1	1:2	1:4	1:8	X	X	X	X	1:256	1:512	1:1024	1:2048
B	Massey CP68 supernatant from June 18, 2008	1:1	1:2	1:4	1:8	X	X	X	X	1:256	1:512	1:1024	1:2048
C	Bioquest ascites	1:50	1:100	1:200	1:400	X	X	X	X	1:12800	1:25600	1:51200	1:102400
D	Bioquest unused media	1:1	1:1	1:1	1:1	X	X	X	X	1:1	1:1	1:1	1:1
E	Bioquest non-specific hybridoma	1:1	1:1	1:1	1:1	X	X	X	X	1:1	1:1	1:1	1:1
F		B	B	B	B	X	X	X	X	B	B	B	B
G		B	B	B	B	X	X	X	X	B	B	B	B
H		B	B	B	B	X	X	X	X	B	B	B	B

X = Unused well
B = Blank well

Figure 2. Template for standard dilution curve ELISA plate. Cell culture supernatant was diluted down the plate with 11 serial dilutions (starting with a 1:1 supernatant:buffer dilution). This was compared with ascites from Bioquest Ltd (positive control) as well as unused media and a non-specific hybridoma (negative controls). Ascites was started at a higher dilution (1:50).

Figure 3. Technique for intraperitoneal injections into mice. Image courtesy of Newcastle University's Assessing the health and welfare of laboratory animals (Anonymous, 2013; University, 2013).

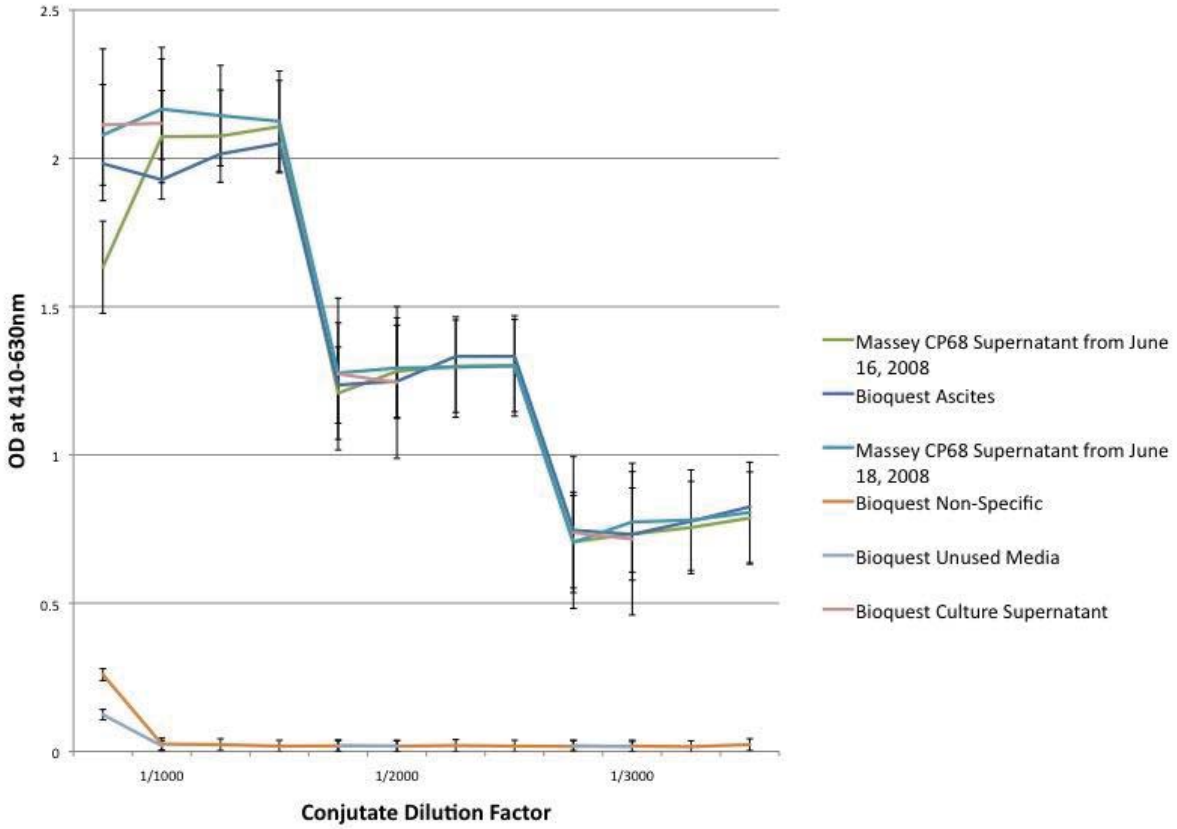
2.5
2
1.5
1
0.5
0
OD at 410-630nm
1/1000
1/2000
1/3000
Conjutate Dilution Factor
Massey CP68 Supernatant from June 16, 2008
Bioquest Ascites
Massey CP68 Supernatant from June 18, 2008
Bioquest Non-Specific
Bioquest Unused Media
Bioquest Culture Supernatant

Figure 4. Determination of optimum conjugate dilution for ELISA development. ELISA absorbance determined at 410-630 nm for conjugate dilutions of 1:1000, 1:2000, and 1:3000. Conjugate dilution factors were compared across Massey cell culture samples as well as Bioquest Cell Culture, ascites, non-specific hybridoma, and unused media. Optimum conjugate dilution for this assay was at 1/2000.

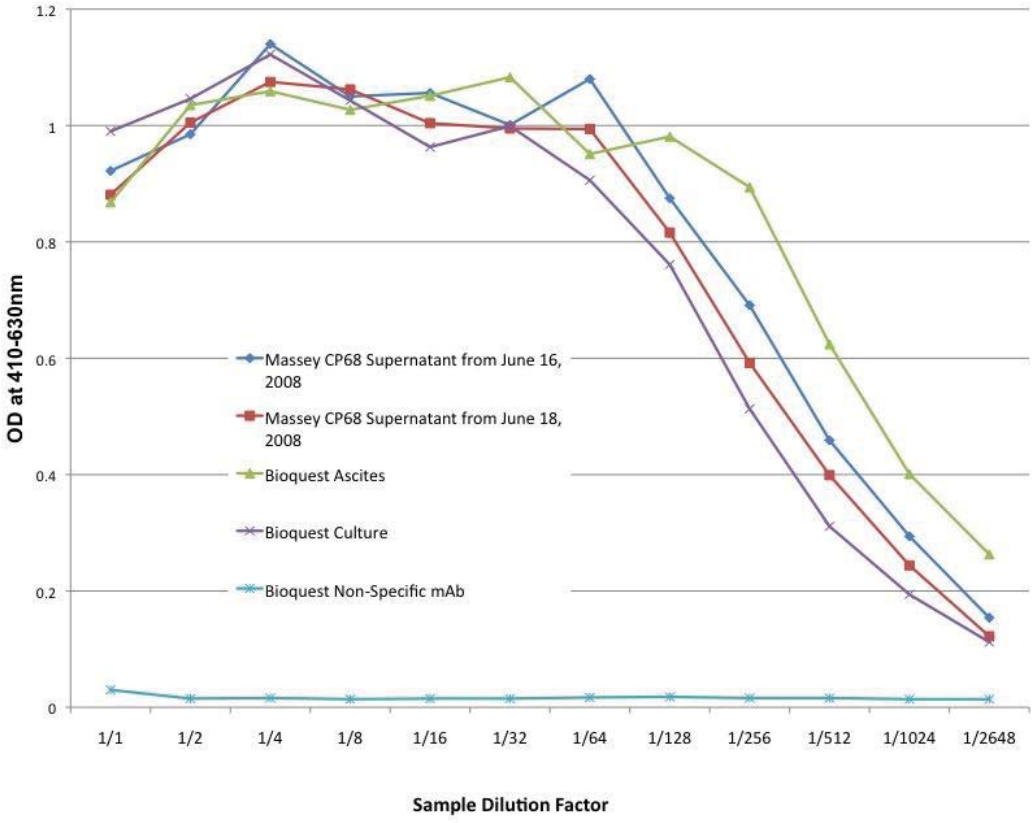
1.2
1
0.8
0.6
0.4
0.2
0
OD at 410-630nm
Massey CP68 Supernatant from June 16, 2008
Massey CP68 Supernatant from June 18, 2008
Bioquest Ascites
Bioquest Culture
Bioquest Non-Specific mAb
1/1
1/2
1/4
1/8
1/16
1/32
1/64
1/128
1/256
1/512
1/1024
1/2648
Sample Dilution Factor

Figure 5. Diagnostic performance of Massey CP68 culture compared to archived Bioquest control (OD at 410-630 nm). Massey CP68 hybridoma line retained acceptable specificity and cell culture supernatants were found to have similar OD values to positive controls (Bioquest Ascites and culture supernatant).

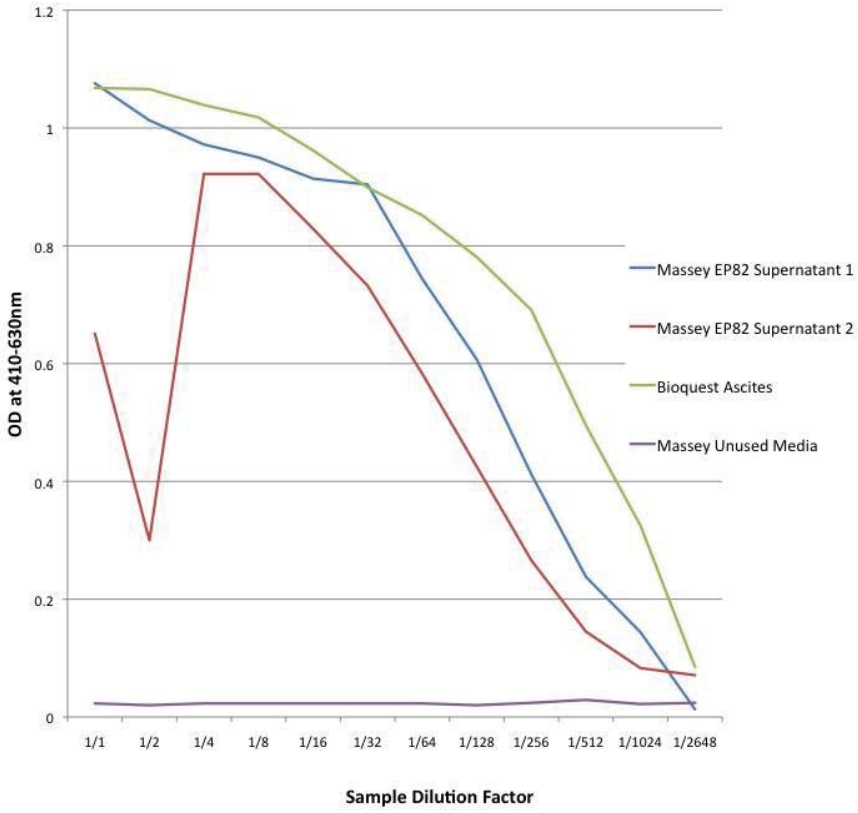
1.2
1
0.8
0.6
0.4
0.2
0
OD at 410-630nm
1/1
1/2
1/4
1/8
1/16
1/32
1/64
1/128
1/256
1/512
1/1024
1/2648
Sample Dilution Factor
Massey EP82 Supernatant 1
Massey EP82 Supernatant 2
Bioquest Ascites
Massey Unused Media

Figure 6. Diagnostic performance and specificity of Massey EP82 hybridomas compared to archived Bioquest controls (OD at 410-630 nm). Massey EP82 hybridoma line retained accpetable specificity and cell culture supernatants had similar OD to the archived positive control Bioquest ascites.

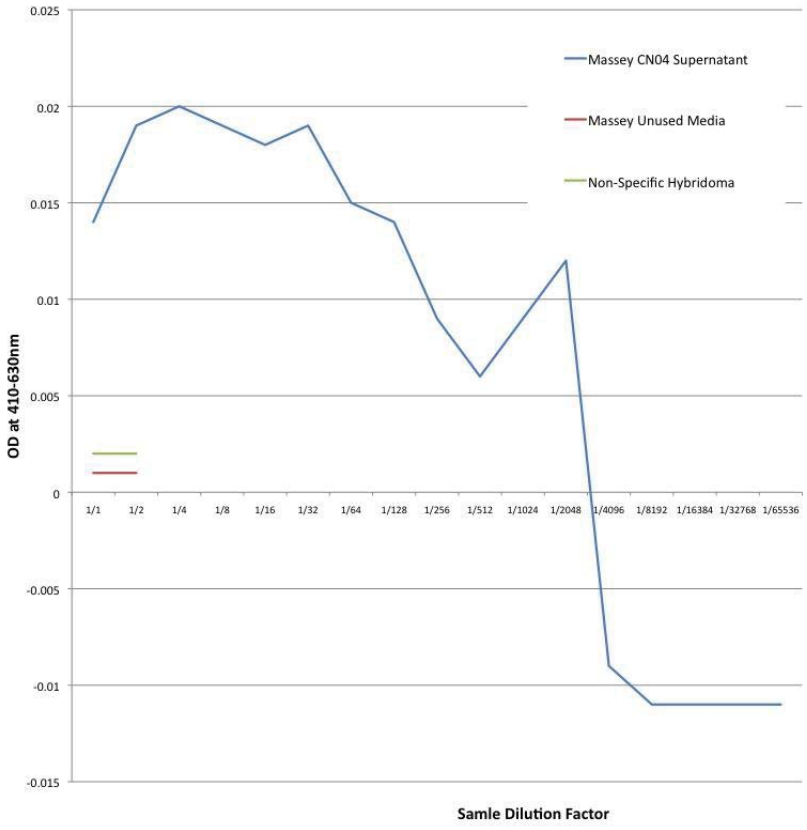
0.025
0.02
0.015
0.01
0.005
0
-0.005
-0.01
-0.015
OD at 410-630nm
Massey CN04 Supernatant
Massey Unused Media
Non-Specific Hybridoma
1/1
1/2
1/4
1/8
1/16
1/32
1/64
1/128
1/256
1/512
1/1024
1/2048
1/4096
1/8192
1/16384
1/32768
1/65536
Samle Dilution Factor

Figure 7. Diagnostic performance and specificity of Massey CN04 hybridoma (OD at 410-630 nm). Antibody capture ELISA showed that the *C. novyi* CN04 hybridoma had lost specificity for its target antigen and OD was similar at all dilutions to blanks and non-specific hybridoma.

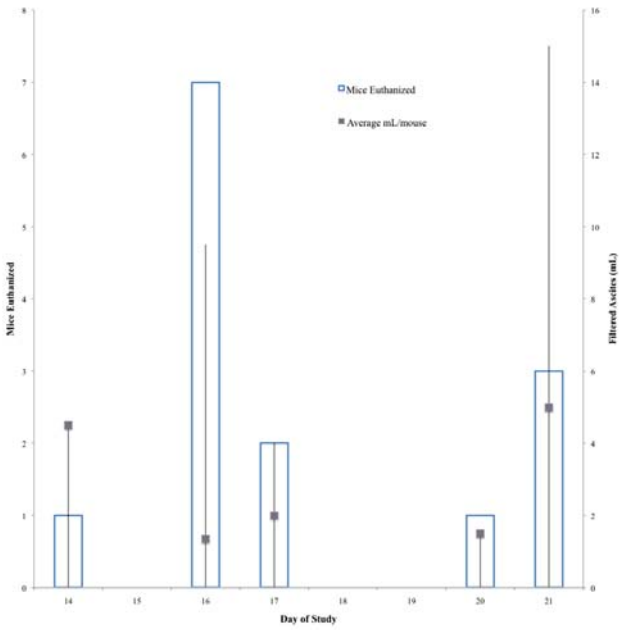

Figure 8. Volume of filtered ascites fluid collected from euthanized Group 1 CP68 mice. Most mice were euthanized early in the study (Day 16) due to formation of solid tumours that were yielding very low volumes of ascites fluid. Three mice did not meet euthanasia criteria during study and were euthanized on Day 21. Grey bars represent the range and average (grey square) of the volumed collected.

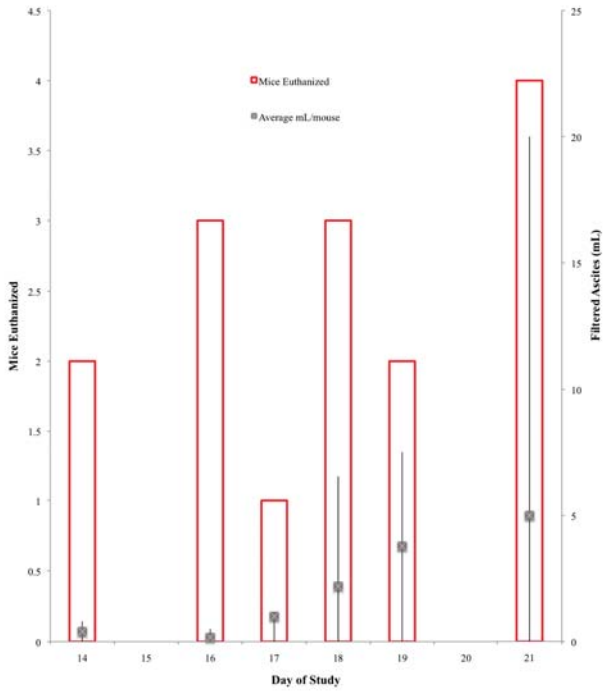
Mice Euthanized
Average mL/mouse
Mice Euthanized
Filtered Ascites (mL)
Day of Study
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
25
20
15
10
5
0
14
15
16
17
18
19
20
21

Figure 9. Volume of filtered ascites fluid collected from euthanized Group 1 EP82 mice. Most mice were euthanized early in the study (Day 16) due to formation of solid tumours that were yielding very low volumes of ascites fluid. Grey bars represent the range and average (grey square) of the volumed collected.

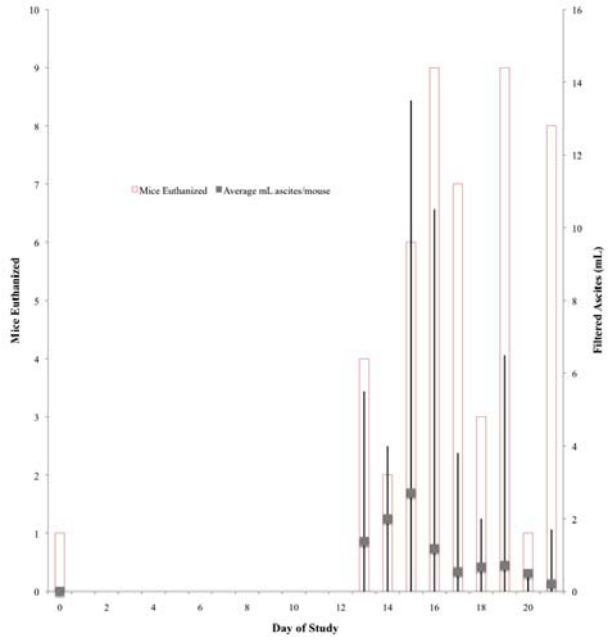

Figure 10. Volume of filtered ascites fluid collected from euthanized Group 2 EP82 mice. The single mouse euthanized on Day 0 was euthanized for humane reasons following innoculation. Average ascites volume collected per mouse peaked at Day 15, and then began to decline. Grey bars represent the range and average (grey square) of the volumed collected

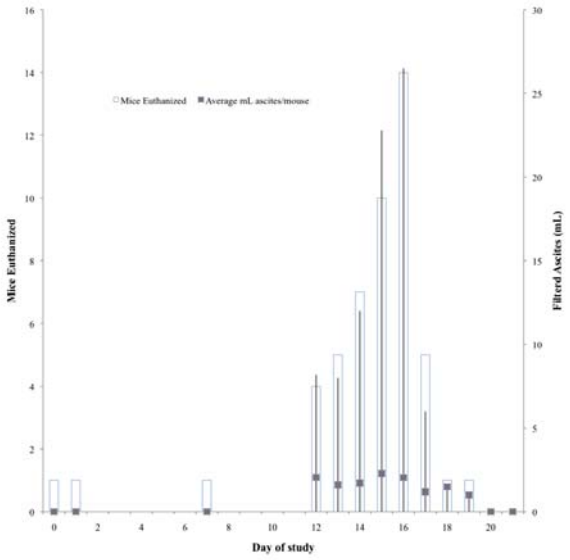

Figure 11. Volume of filtered ascites fluid collected from euthanized Group 2 CP68 mice. Overall average ascites production remained the same throughout the study period. Mice euthanized on Days 0, 1, and 7 were euthanized for humane reasons and no ascities was collected. Grey bars represent the range and average (grey square) of the volumed collected.

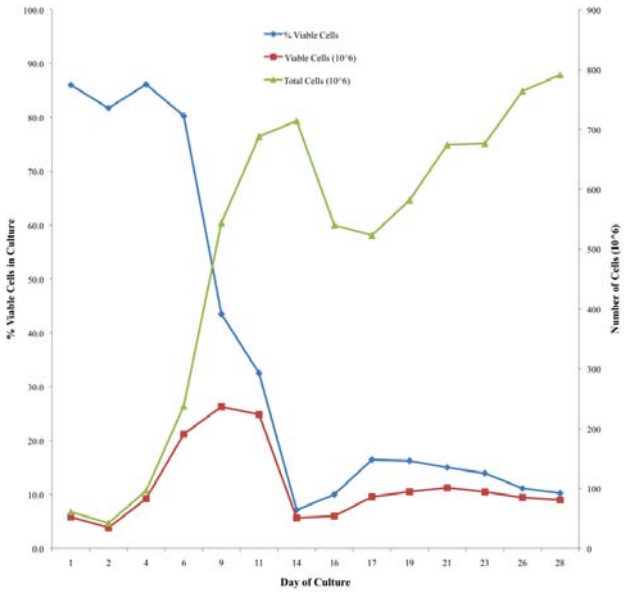

Figure 12. Cell viability and cell count versus culture time for EP82 hybridoma. As absolute cell number increased, cell viability decreased. Peak cell numbers occurred during the first two weeks of culture. After re-seeding at Day 14, overall cell numbers stayed high and continued to climb while viability remained steady and low.

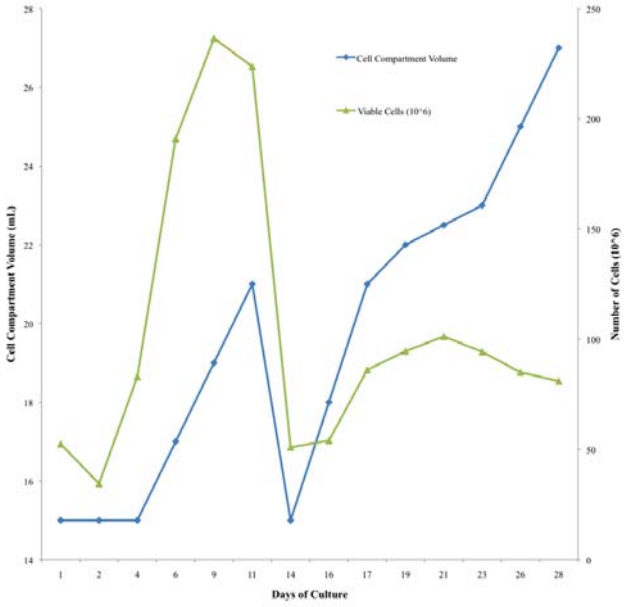

Figure 13. Cell compartment volume and viable cells in culture versus culture time for EP82 hybridoma. Even as viable cell numbers declined, antibody was still being produced. Cell compartment volume increased as antibody levels in that compartment increased and as the overall number of cells increased (both viable and non-viable).

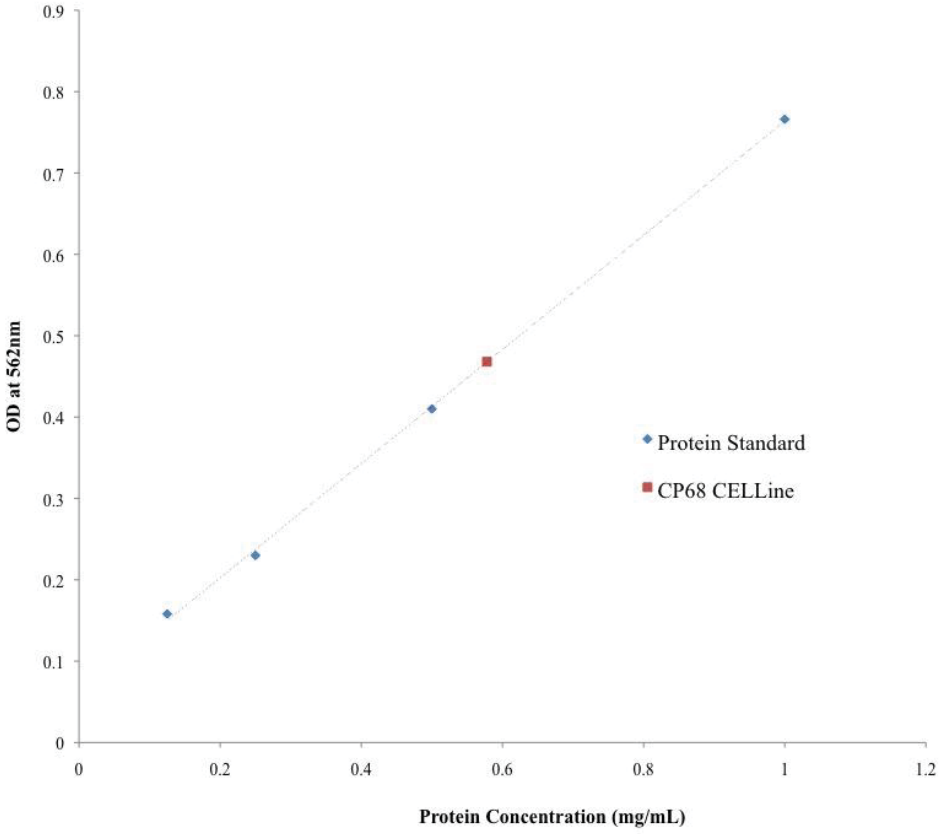
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD at 562nm
0
0.2
0.4
0.6
0.8
1
1.2
Protein Concentration (mg/mL)
Protein Standard
CP68 CELLine

Figure 14. CP68 CELLine culture protein assay. Protein concentration (mg per mL) vs. Mean OD at 562 nm. Linear regression curve shows that the CP68 hybridoma line produced an average of approximately 0.6 mg per mL MAb.

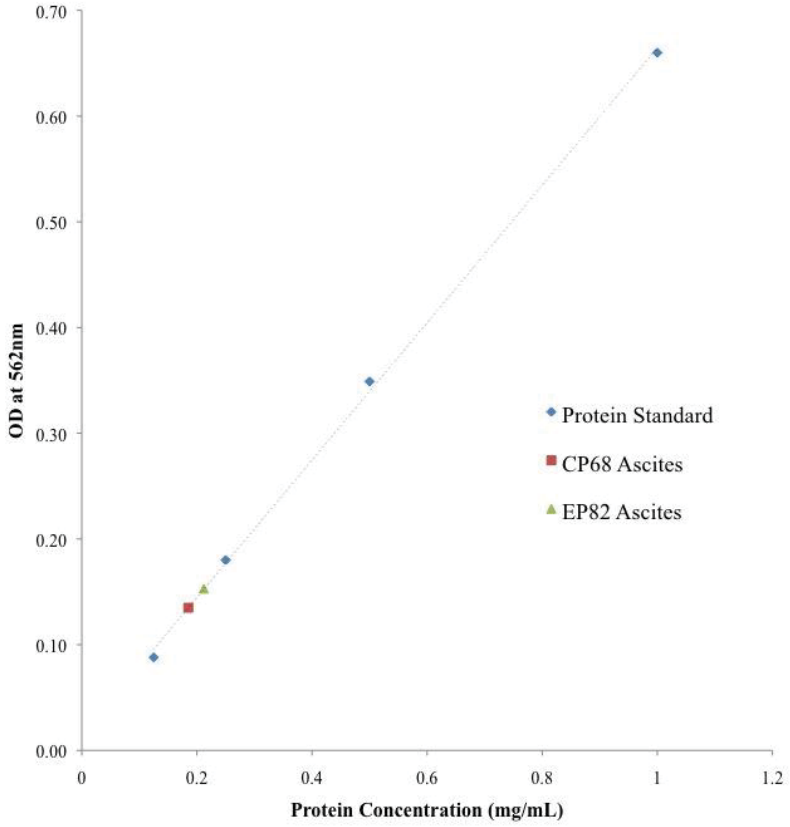
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
OD at 562nm
0
0.2
0.4
0.6
0.8
1
1.2
Protein Concentration (mg/mL)
Protein Standard
CP68 Ascites
EP82 Ascites

Figure 15. Group 1 ascites protein assay. Protein concentration (mg per mL) vs. Mean OD at 562 nm. Both cell lines produced low-concentration

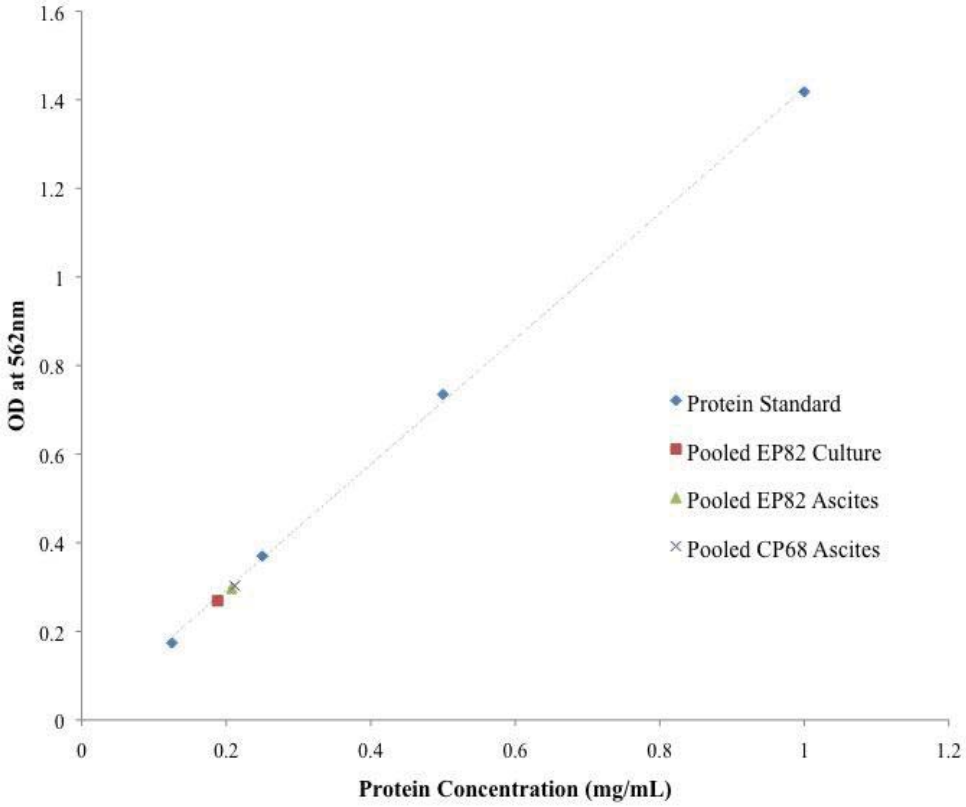
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD at 562nm
0
0.2
0.4
0.6
0.8
1
1.2
Protein Concentration (mg/mL)
Protein Standard
Pooled EP82 Culture
Pooled EP82 Ascites
Pooled CP68 Ascites

Figure 16. Group 2 ascites and EP82 protein assay. Protein concentration (mg per mL) vs. Mean OD at 562 nm. Both methods (cell culture and ascites) gave similar final concentration of MAb for the EP82 line, and both ascites yields were similar.

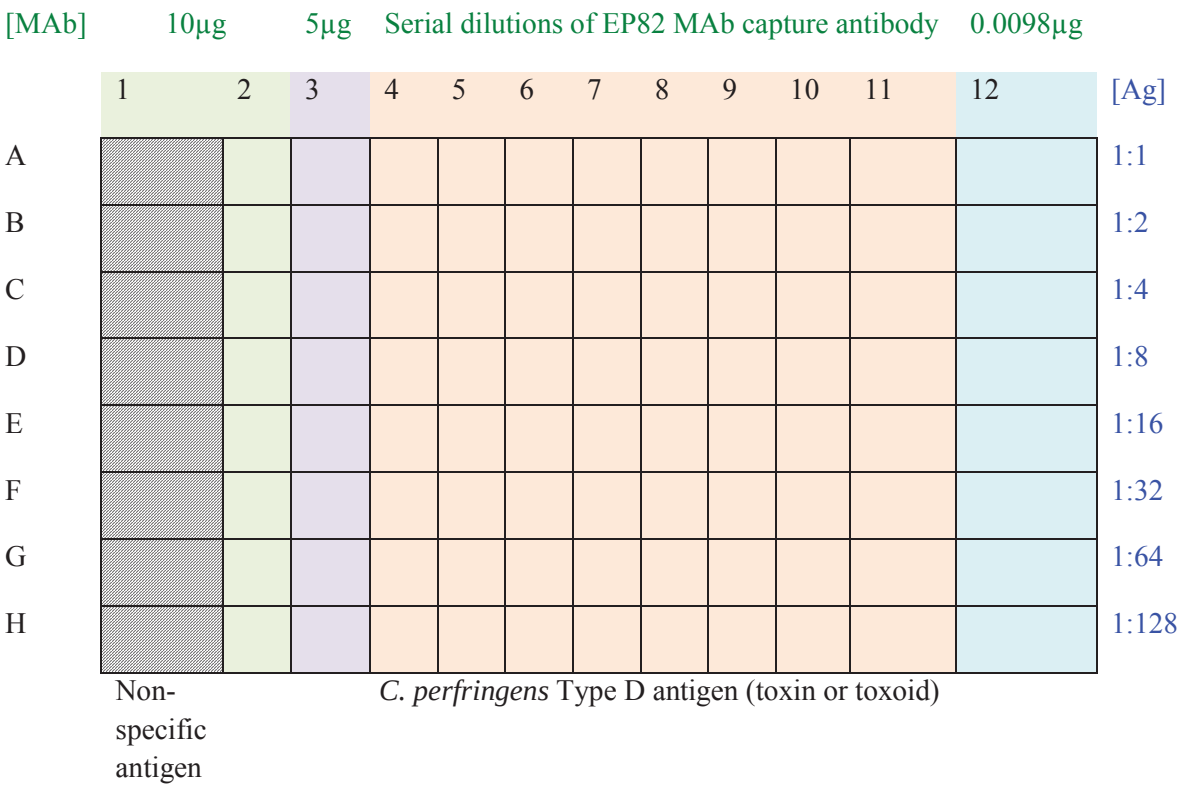

Figure 17. Schematic for plates used in ELISA development. Plates were assigned different antigen (toxin versus toxoid) and different detecting antibody concentration (1:50 or 1:100) to determine the optimal reagent conditions for the particular ELISA. All plates had identical capture antibody concentration (doubling dilutions across the plate from starting concentration of 10 µg per mL to an end concentration of $9.8x10^{-3}$ µg/mL); and all plates had identically applied antigen (doubling dilution from starting dilution of 1:1 and continuing down the plate to end in a 1:128 dilution). Column 1 was a non-specific antigen (*C. perfringens* type C toxin or toxoid), remaining columns were *C. perfringens* type D toxin or toxoid.

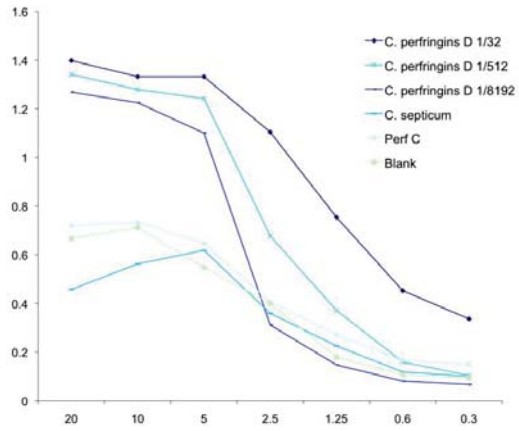

Figure 18. MAb coating concentration optimisation curve with toxin. MAb coating in µg per mL vs. OD at 410-630 nm. The MAb coat was determined to be 8 µg per mL based on the sigmoid shape of the curves produced. The shape of the curve is the same for the three dilutions of C. perfringins epsion toxin (1/32, 1/512, 1/8192).

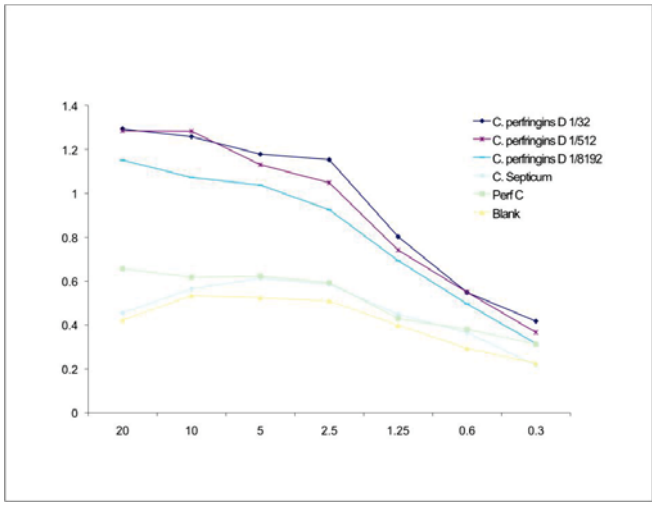

Figure 19. MAb coating concentration optimization curve with toxoid. MAb coating in µg per mL vs. OD at 410-630 nm.

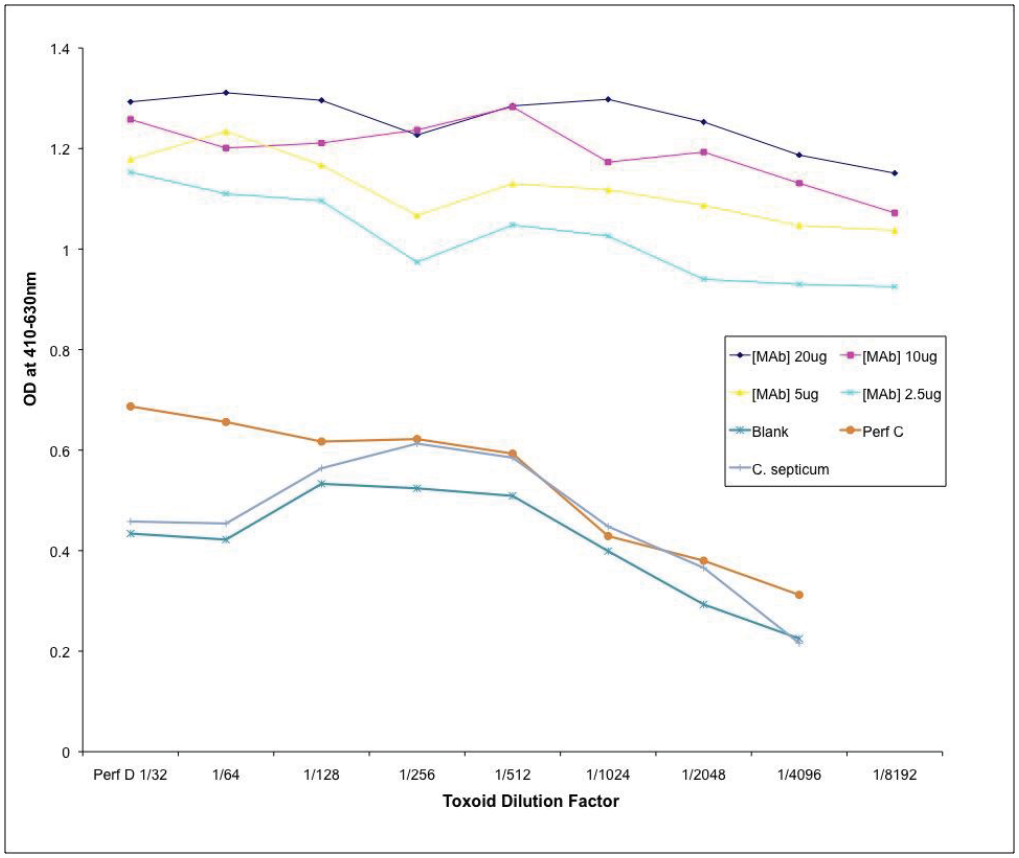
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD at 410-630nm
[MAb] 20ug
[MAb] 10ug
[MAb] 5ug
[MAb] 2.5ug
Blank
Perf C
C. septicum
Perf D 1/32
1/64
1/128
1/256
1/512
1/1024
1/2048
1/4096
1/8192
Toxoid Dilution Factor

Figure 20. Antigen dilution optimization curve. Toxoid dilution factor vs. OD at 410-630 nm. There is no obvious 'titration out' seen even with very dilute toxoid samples. There is no great effect of difference of MAb capture antibody concentration. It was determined that the toxoid was going to have to be more dilute for the assay to work.

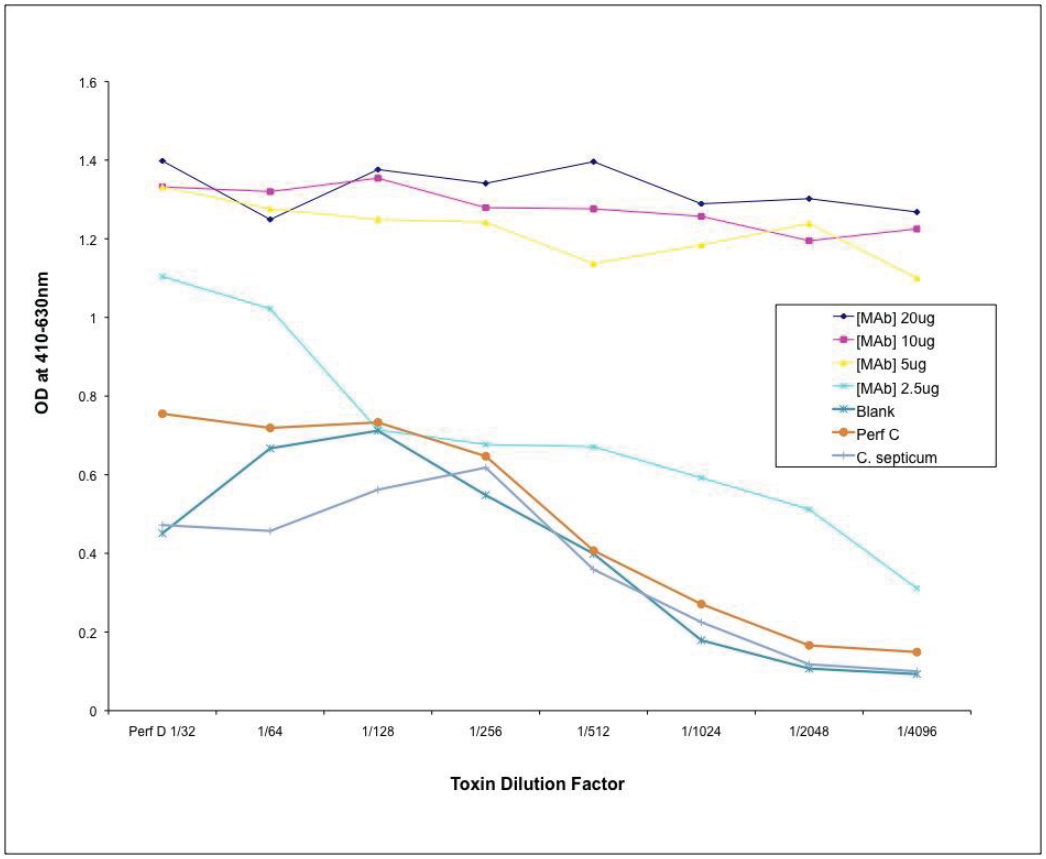
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD at 410-630nm
Perf D 1/32
1/64
1/128
1/256
1/512
1/1024
1/2048
1/4096
Toxin Dilution Factor
[MAb] 20ug
[MAb] 10ug
[MAb] 5ug
[MAb] 2.5ug
Blank
Perf C
C. septicum

Figure 21. Antigen dilution optimization curve. Toxin dilution factor vs. OD at 410-630 nm. There was some 'titration out' at MAb coating of 2.5 µg per mL, however this does not correspond with the pattern displayed in the toxoid curve. OD is still too high at these concentrations to be of use in this assay.

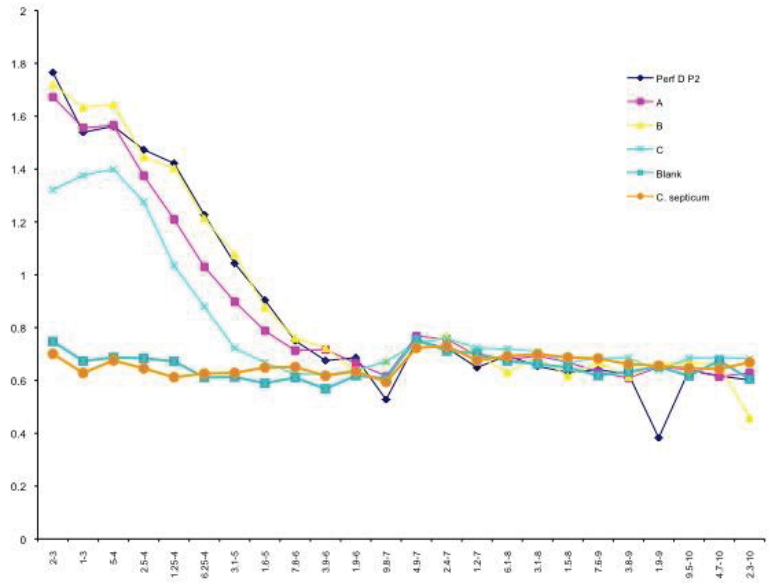

Figure 22. Vaccine potency determination curve. Vaccine sample concentraion vs. OD with plate MAb coat of 8 µg per mL. The single vaccine supernatant tested that did not pass QA process (C) had dissimilar binding to the positive control when less dilute, contrary to the presumed result.