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Production and testing of *Clostridium difficile* toxin A monoclonal antibody (αCdA Bella-1) and toxin A specific polyclonal antibody (IRP 583)

Teri Lynn Thiele

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Production and testing of Clostridium difficile toxin A monoclonal antibody (αCdA Bella-1) and toxin A specific polyclonal antibody (IRP 583)

by

Teri Lynn Thiele

A thesis submitted to the graduate faculty in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE

Major: Veterinary Microbiology

Program of Study Committee:
Ricardo Rosenbusch, Major Professor
Louisa Tabatabai
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Paul Hauer

Iowa State University
Ames, Iowa
2011

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ABSTRACT

*Clostridium difficile* Toxin A (TcdA) monoclonal (Mab) and polyclonal (Pab) antibodies were produced for use by the USDA-Center for Veterinary Biologics (CVB) in anticipation of vaccine product licensing requests. The Virus-Serum-Toxin Act which requires potency evaluation of bacterins and toxoids prior to sale is implemented by the CVB which produces the reagents necessary for product testing. The monoclonal antibody was isotyped and determined to be IgG1, k. The antibodies were also tested using indirect enzyme-linked immunoabsorbent assay (ELISA), sandwich ELISA, western blot and toxin neutralization tests to determine their neutralizing effects on TcdA. Both the Mab and Pab had neutralizing activity against TcdA in mice and they reacted with TcdA in western blots and ELISA. The Pab reacted with TcdA in the sandwich ELISA and neutralized the toxin in mice. The Pab was also shown to be specific for TcdA in mouse tests because it would not neutralize crude *C. difficile* toxin. Both antibody preparations reacted with native (non-denatured) *Clostridium sordellii* Toxin HT (TcsH) in ELISA.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

*Clostridium difficile* is the most common nosocomial pathogen of the gastrointestinal tract and has increased in frequency over time. Symptoms of *C. difficile* infection include diarrhea, which is usually non-bloody, or colitis associated with severe abdominal pain, fever and/or gross or occult blood in stools. *C. difficile* produces several virulence factors including toxin A (TcdA), toxin B (TcdB), tissue-degrading enzymes (proteases, collagenase, hyaluronidase, heparinase and chondroitin-4-sulfatase) and fimbriae. The two major virulence factors are TcdA, a potent enterotoxin and cytotoxin, and TcdB, a potent cytotoxin and mild enterotoxin. The toxins are co-produced, are important in pathogenesis and are usually found in patients with *C. difficile*– associated pseudomembranous colitis (PMC) [13].

*Clostridium sordellii* lethal toxin (TcsL) and hemorrhagic toxin (TcsH) have often been compared to TcdA and TcdB of *C. difficile*. The biological and antigenic properties of TcdA and TcdB are very similar to those of TcsH and TcsL, respectively [3].

*Clostridium sordellii* is a species of anaerobic, spore forming bacteria that is the etiologic agent of clostridial myonecrosis (gas gangrene) in humans as well as domestic animals. It can also cause enterotoxemia in cattle and sheep [1-3]. The pathogenesis of the disease is due to two separate toxins, TcsL and TcsH [3]. Although there are common preventative vaccines used for *C. sordellii*, it is unknown if these vaccines protect against both TcsL and TcsH.

Results from an experiment in which guinea pigs vaccinated with toxoids containing toxoided TcsL, TcsH, or both TcsL/TcsH were challenged with virulent strains of *C. sordellii* that expressed both TcsL and TcsH toxins indicated that animals may need immunity to both the lethal and hemorrhagic toxins to be completely protected from *C. sordellii* because both are essential protective antigens [3]. Currently, it is unknown whether currently licensed animal vaccines confer protection against both toxins because the vaccine potency test requirement only
requires that the vaccines protect against TcsL. By developing a test to measure the potency of current vaccines against TcsH, I will be able to ascertain whether or not the vaccinated animals are protected against both toxins of *C. sordellii*.

Recent studies have shown that TcsH toxoid may play a considerable role in protection against challenge spores of TcsH – expressing *C. sordellii* strains [3]. The goals of the research contained in this dissertation are 1) produce reagents to test currently licensed vaccines to determine if they elicit anti-HT antibodies in vaccinated animals, and 2) Clone the TcsH gene to facilitate the development of molecular diagnostics and the production of highly purified reagents. Hopefully this research will lead to development of more appropriate regulatory tests to determine if better vaccines are needed that are capable of protecting against both TcsL and TcsH.

**Literature Review**

*Clostridium difficile* is the most common nosocomial pathogen of the gastrointestinal tract and has increased in frequency over time. Symptoms of *C. difficile* infection include diarrhea, which is usually non-bloody, or colitis associated with severe abdominal pain, fever and/or gross or occult blood in stools. Pseudomembranous colitis (PMC) in humans and antibiotic-associated colitis (AAC) in experimental animals is the most severe form that results from a severe inflammatory response to *C. difficile* toxins [13, 14]. The disease has been diagnosed in domestic and wild animals including: Kodiak bear, penguin, captive ostriches, rabbits, hares and adult horses. *C. difficile* has been found in the gut of dogs and pups with chronic diarrhea. It has also been isolated from camels, donkeys, swine and cats [6]. *C. difficile* causes AAC in experimental laboratory animals, including hamsters, mice, guinea pigs, rabbits and rats. The disease progresses rapidly to cecitis and hemorrhage, ulceration and inflammation are evident in the intestinal mucosa. The animals become lethargic and develop severe diarrhea, and a high percentage of the animals die from the disease [15].
**Clostridium difficile** is an anaerobic bacterium that was first isolated from normal microbial flora in the stool of infants. It has also been isolated from other sources, including wounds; war wounds; superficial infections; various human pathological specimens such as pus and other exudates; tissues exhibiting gas gangrene; abscesses; blood culture; pleural fluid; peritoneal fluid and the intestinal tract of Wendell seals [1]. *C. difficile* is unique in that the affected hosts have nearly always been exposed to antimicrobials. Clindamycin – associated PMC has been repeatedly documented. Hospitalization is considered a major risk of acquiring PMC due to the exposure of patients to a concentrated source of the pathogen. The length of stay increases the risk of infection [6].

**Clostridium difficile** is a sporulating gram positive anaerobic bacterium that is 6-8 microns in length and 0.5 microns in width. The spores are oval, terminal and don’t cause swelling in the rods. The gram positive characteristic can be lost after one day of incubation [1]. *C. difficile* produces several virulence factors including toxin A (TcdA), toxin B (TcdB), tissue-degrading enzymes (proteases, collagenase, hyaluronidase, heparinase and chondroitin-4-sulfatase) and fimbriae. The two major virulence factors are TcdA, a potent enterotoxin and cytotoxin, and TcdB, a potent cytotoxin and mild enterotoxin. The toxins are co-produced, are important in pathogenesis and are usually found in patients with *C. difficile* – associated PMC [13].

Severity of the disease, caused by *C. difficile*, ranges from benign diarrhea to PMC [13]. PMC is a serious colonic disease which can occur when antibiotics or other agents disrupt the normal flora. The disease rarely occurs without prior antibiotic therapy [17]. PMC is usually associated with watery diarrhea, fever, abdominal cramping and leukocytosis. These symptoms can occur after 4 days of antibiotic treatment, but may occur up to 6 weeks after antibiotics have been discontinued. PMC was first recognized as a manifestation of *C. difficile* infection and thought to be a side effect of clindamycin therapy. PMC results from the overgrowth of the large bowel by *C. difficile* usually after perturbation in bowel flora, caused by antibiotic therapy. An identical disease occurs in hamsters and guinea
pigs after antibiotic treatment [6]. Although the disease can occur in association with any antibiotic, it occurs most commonly with the use of penicillin, ampicillin, clindamycin and cephalosporin. Although PMC is most common in adults, especially elderly patients; there have been infrequent reports of pediatric PMC [13].

The pathophysiology of PMC may involve up to four distinct pathways. The first is the disruption of the normal intestinal micro flora by the inducing antibiotic or agent. The normal intestinal flora possesses 'colonization resistance' or the ability to resist the overgrowth of opportunistic pathogens through a variety of mechanisms, which include competition for nutrients, production of bacteriocins or toxin degrading proteases and receptor site competition. Broad spectrum antibiotics have a greater impact on normal flora and are associated with higher rates of intestinal disease [13].

The second mechanism is cellular distortion and subsequent diarrhea as a result of toxins produced by the pathogens. The actions of TcdA and TcdB are bimodal; one disrupting the cell cytoskeleton and the other involving the activation of the signal transduction pathways of the immune system. TcdA and TcdB are produced by *C. difficile* in the lumen during infection and bind to receptor sites on the surface of enterocytes and are then internalized by endocytosis. The toxins inactivate guanine-nucleotide-binding protein called *rho* A, leading to the disaggregation of F actin. *Rho* is involved in maintaining the cytoskeleton structure within the cell. Cytoskeleton disruption results in cell rounding which widens the tight junctions between enterocytes leading to fluid loss and diarrhea. The tissue damage by TcdA results in viscous hemorrhagic fluid response [13].

The third mechanism of PMC is the activation of components in the immune system. TcdA and TcdB cause mast cell degranulation, up-regulation of leukocyte adhesion and release of cytokines from granulocytes. TcdA attracts neutrophils and both toxins stimulate the release of cytokines, such as interleukin (IL) 1, IL-6, IL-8 and tumor necrosis factor from human monocytes. Both toxins act on mast cells to release histamine and may affect leukocyte, endothelial cell and platelet interactions through up-regulation of certain adhesion molecules [13]. Significant increases in
leukocyte adherence and emigration (LAE) and albumin leakage was noted with TcdA exposure. This response was accompanied by mast cell degranulation and the formation of platelet-leukocyte aggregates. TcdA induced increases in LAE and albumin leakage were significantly attenuated by pretreatment with either monoclonal antibodies directed against the leukocyte adhesion glycoproteins, CD11/CD18, intercellular adhesion molecule-1 (ICAM-1) and P-selectin or with sialyl Lewis X, a counter receptor for P-selectin. These observations indicate that TcdA induces a leukocyte-dependent leakage of albumin from postcapillary venules. Mast cell-derived histamine appears to mediate at least part of the leukocyte-endothelial cell adhesion and platelet-leukocyte aggregation by engaging H1-receptors on endothelial cells and platelets to increase the expression of P-selectin. The adhesion glycoproteins CD11/CD18 and the ICAM-1 also contribute to the inflammatory responses elicited by TcdA [24]. The profuse release of leukocytes, mucin, fibrin and cellular debris results in the formation of a pseudomembrane [13].

The fourth mechanism may involve the enteric nervous system. TcdA has been found to increase myoelectric activity before causing mucosal damage in rabbit colonic loop models. The involvement with the enteric nervous system and intestinal disease requires further study [13].

PMC is diagnosed by assessing the patient on three levels: clinical evaluation, stool assays for enteric pathogens and visualization of the colonic mucosa. A history of antibiotic use, recent hospitalization, intestinal surgery or residence in a chronic care facility may all predispose to PMC. Symptoms of watery diarrhea, abdominal pain or cramping and fever are typical. The ‘gold standard’ for C. difficile detection is the tissue culture assay for TcdB. The diagnosis of PMC includes the detection of C. difficile in the stool. Tests for detection of C. difficile include enzyme immunoassays, tissue culture assays and latex agglutination assays. C. difficile can also be detected by microbial cultures in the stool. The third level of diagnosis is colonoscopic or sigmoidoscopic visualization of pseudomembranes [13].
The initial evaluation should assess the level of hydration and severity of illness. The two most commonly used antibiotics to treat *C. difficile* infections are metronidazole and vancomycin. Recent recommendations from the Center for Disease Control strongly suggest the use of metronidazole as the first line drug of choice. The bile-salt-binding resin cholestyramine has also been used to treat *C. difficile* disease. The usual response to therapy is improvement in diarrhea within 1-4 days with resolution by 2 weeks [13].

Both TcdA and TcdB are believed to be involved in the pathogenesis of PMC. This is based on findings that (i) both toxins are tissue damaging and lethal when injected into animals (ii) experimental animals must be vaccinated against both toxins to be protected against the disease and (iii) both toxins are present in fecal specimens from patients with PMC [16].

In addition to humans, *C. difficile* can be found in the intestinal tracts of a variety of animal species, including food animals, such as pigs and cattle. *C. difficile* has also been found in retail meat. Concern about the role of food in epidemiology of community-associated *C. difficile* infection (CA-CDI) has been expressed [23]. So far it is not clear if these animals are a possible source of human *C. difficile* infections [21].

*Clostridium difficile* is well accepted as the etiological agent of *C. difficile*-associated disease (CDAD) in neonatal pigs and may contribute to enteritis in calves [19, 20]. Infected herds may experience morbidity as high as 97% and while most piglets recover, the mortality rate in some outbreaks may be as high as 16% [19].

Pseudomembranous colitis and large numbers of *C. difficile* were subsequently described in conventional pigs with naturally occurring CDAD. *C. difficile* and its toxins were detected in an outbreak of CDAD in pigs approximately 5 days of age; affected piglets were dyspneic, and had mildly distended abdomens, scrotal edema, diarrhea, ascites (>50ml), edema of the ascending mesocolon, and hydrothorax. Microscopically the distinctive lesions were severe ascending colonic submucosal and mesocolonic edema, with multifocal exudation of mucus, fibrin, and polymorphonuclear leukocytes (PMN) aggregates. Based upon examination of
approximately 2000 individual diseased piglets, the case definition for porcine CDAD includes piglets 1 to 7 days of age with a history of scouring since shortly after birth. Litters from both gilts and sows are affected. Gross lesions usually include moderate-to-severe edema of the mesocolon, and colonic serosal edema is common; pseudomembranous formation is uncommon, and the yellow plaques seen in human disease are not observed in affected piglets. Colonic contents are frequently pasty-to-watery and yellow, although some piglets are constipated or obstipated [22].

Clinically relevant strains of *C. difficile* usually produce toxins TcdA and TcdB, although *C. difficile* and its toxins can be found in non-diarrheic piglets. In one such group, 74% were TcdA/B positive, but had normal feces or were constipated. It is common to find typical lesions in these non-diarrheic piglets. Some piglets present with systemic signs, including mild depression, lack of appetite, cachexia and anorexia, without diarrhea. Most recover quickly. The only diagnostic findings are the toxins in cecum and colon and typhlocolitis consistent with CDAD [22].

As with human CDAD, diagnosis of porcine CDAD is based primarily upon detection of TcdA/B in stools or intestinal contents. Antibodies against TcdA prevent binding, eliminate secretion and inflammation, and prevent clinical disease in mice and hamsters; anti-TcdB antibodies also have a role in protection against CDAD. Active or passive immunization against TcdA prevents clinical disease. Thus, it may be that if a toxin-neutralizing response can be generated in sows, piglets can be passively protected by antibodies obtained from colostrum [22].

*Clostridium sordellii* toxins have often been compared to TcdA and TcdB of *C. difficile*. The biological and antigenic properties of TcdA and TcdB are very similar to those of TcsH and TcsL, respectively [3].

*Clostridium sordellii* is an anaerobic bacterium that causes clostridial myonecrosis (gas gangrene) in humans, as well as, domestic animals. It has also been recognized as a causal agent of diarrhea, enterotoxemia and enteritis in cattle and sheep [1-3]. It is an important veterinary pathogen that causes bloat, hemorrhage, ulcers, liver disease, fatal myositis and sudden death in sheep, cattle,
and horses. *C. sordellii* has also been reported in human maternal deaths with a disease resembling toxic shock-like syndrome. The etiology of *C. sordellii* is poorly understood. Myonecrosis is rather infrequent, and death may be due to septicemia, as well as the expression of several exotoxins [4, 7]. *C. sordellii* is pathogenic for man, cattle, sheep and laboratory animals. Death can be attributed to the lethal and hemorrhagic toxins [1, 3]. The symptoms generally resemble those produced by *C. novyi* because of the marked edema that occurs. This edema is blood-tinged or rose-colored instead of being colorless, as with *Clostridium novyi*. In guinea pigs; postmortem, the most pronounced symptom is a subcutaneous, gelatinous edema, usually rose-colored, lying between the abdominal muscles and the skin. The muscles are red and either uniformly hemorrhagic or discolored by hemorrhagic streaks. Gas is also normally evident at site of inoculation. *C. sordellii* is sometimes responsible for liver infections in cattle and sheep. Infection seems to be initiated by the liver fluke because the primary lesion is adjacent to the necrotic track left behind by the fluke burrowing through the liver [1].

The bacterium, initially described as *Bacillus oedematis sporogenes* by Sordelli in 1922, was isolated from cases of acute edematous wound infections in South America. In 1927 Hall and Scott suggested the name *Bacillus sordellii* after further study. Meleny *et al* isolated the same organism, originating from infected surgical material, from cases of postoperative infection in a man in 1928. Unaware that it was the identical strain as that named by Hall and Scott they named it *Clostridium oedematiodes*. As more strains were studied it was found that *C. sordellii* closely resembled *Clostridium bifermentans*, but that the *C. sordellii* isolates were simply the pathogenic strains. Tataki and Huet concluded that they were two distinct species in 1953. *C. sordellii* can now be distinguished from *C. bifermentans* by its ability to produce urease and by its characteristic spore antigen [1].

*Clostridium sordellii* habitats in nature have not been clearly established. The principal habitat seems to be soil and not the animal body because of the differences in the incidence of infection caused by it in many different parts of the world. Cases are most often found in the western United States, Latin America, and in Asia Minor;
all areas of relatively low rainfall. They are uncommon in Great Britain and the rest of Europe [1].

*Clostridium sordellii* is a gram positive sporulating rod and an obligate anaerobe [1, 5]. The bacterium is 2-4 microns long and 0.6-1 micron in diameter. Spores are sub-terminal to central in position and oval to cylindrical in shape. Cultures 3 days old or more will be found to be chalky white and composed almost entirely free of spores [1].

It appears that all strains of *C. sordellii* share some antigens, but it is not certain that all strains are identical in antigenic structure. Agglutination tests, however, cannot be used to definitively distinguish between *C. sordellii* and *C. bifermentans*. Studies have shown that there are three soluble antigens that are common to strains of *C. sordellii* and are not produced by strains of *C. bifermentans* [1].

*Clostridium sordellii* is one of the causative agents of gas gangrene. It produces more edema and induration of the local lesion and less gas than other Clostridial species. The incubation period for clostridial myositis may vary from 18 hours to 6 days [9].

All ages and species of animals are affected. In most cases a wound is the portal of entry and a dirty environment which permits contamination of wounds with soil is the common predisposing cause. The infection is usually soil-borne and the resistance of spores of the causative clostridia to environmental influence leads to persistence of the infection for long periods in a local area. Potent toxins are produced in the local lesion and cause death when absorbed into the bloodstream. Locally the exotoxins cause extensive edema and necrosis followed by gangrene [11].

The local lesion differs from that in the usual type of pyogenic infection. In gas gangrene, erythema is not the most conspicuous finding; rather signs of circulatory inadequacy are present, due to the rapid formation of edema and gas. The wound does not discharge pus, but brownish watery exudates [9].
During the next phase the skin becomes discolored, the lesion taking on a reddish-brown tint. This discoloration progresses rapidly in a cranial direction. Complete cutaneous necrosis is observed at the wound edges, usually indicating a deeper-seated myonecrosis [9].

During this phase the “gas boundary” may advance ahead of the causative lesion. In gas gangrene caused by *C. sordellii* formation of gas may be a less prominent feature. This variation is frequently recognized too late or not at all. This indicates the inadequacy of crepitation due to gas in establishing an early diagnosis [9].

*Clostridium sordellii* infection presents with unique clinical features including edema, absence of fever, leukemoid reaction, hemoconcentration, and later shock and multi-organ failure in humans. Often infections develop after childbirth or after gynecological procedures, and most represent endometrial infection. Rarely, cases have occurred at sites of minor trauma such as lacerations of the soft tissues of an extremity. The absence of fever and the lack of signs or symptoms delay diagnosis. The elaboration of a potent toxin is related to the mechanisms of diffuse capillary leak, massive edema and hemoconcentration [8].

The clinical diagnosis is based on typical signs and lesions. Rapid spreading crepitus painful swelling around a wound in a febrile animal is significantly important. The presence of gaseous serosanguineous fluid in and around necrotic tissue as is revealed by an incision of swollen tissue and at necropsy is additional evidence of malignant edema [10]. There are also other symptoms and signs. Pain is a prominent symptom. It is usually extremely acute, probably due to the rapid occurrence of swelling due to edema and gas formation. Hypotension can be a sign at serious degrees of toxicity. A psychiatric-neurologic complex of symptoms is also one of the toxic manifestations of gas gangrene [9]. When *C. sordellii* is the causative agent for gas gangrene a foul, putrid odor is often present in infections at necropsy [11].

Since malignant edema is associated with wound contamination it can be partially prevented by minimizing opportunities for injuries in a feedlot. Protruding
nails, wire or other sharp objects should be removed from fences and walking areas. Vaccination of young cattle gives some protection [10].

In early stages of disease it can be treated daily with penicillin at a rate of 2,000-4,000 units per pound of body weight. After suppression or elimination of infection the necrotic tissue should be incised to provide drainage and to accelerate healing [10].

*Clostridium sordellii* produces two toxins. The pathogenic *C. sordellii* strains produce lethal toxin (TcsL) alone or both TcsL and hemorrhagic toxin (TcsH). TcsL has edematizing and cytotoxic activities in addition to its lethal toxicity. TcsH has much weaker lethal and cytotoxic activities than TcsL. Immunizing guinea pigs with toxoid containing TcsL, TcsH and both TcsL/TcsH has shown that only the TcsL/TcsH toxoid gave complete protection against bacterial challenge with strains of *C. sordellii*. The result shows that TcsL toxoid, as well as, TcsH toxoid are essential protective antigens of *C. sordellii* [3].

*Clostridium sordellii* antitoxin cross-neutralizes TcdA and TcdB of *C. difficile*. This suggests the two toxins of *C. sordellii* are immunologically related to the *C. difficile* toxins. Intradermal injections of cell-free extracts into guinea pigs caused edema and hemorrhage shown to be the result of two separate toxins, the toxin causing edema being the more lethal TcsL than the hemorrhagic TcsH. The toxins have been separated by ion-exchange chromatography and are usually large. The MW for TcsH is 300,000 and TcsL is 260,000 [12]. Purification of TcsH by ultrafiltration and immuno-affinity chromatography with a monoclonal antibody (MAb) to TcdA can be used as well [7].

*Clostridium difficile* toxin A, a large protein of 308,000 MW, binds to human carbohydrate antigens I, X and Y on human epithelial surfaces, but its exact intestinal receptor sites in humans are unknown. *Clostridium difficile* toxin B, a large protein of 270,000 MW, is heat labile, and its receptor site has not been identified in either humans or animals [13]. A number of methods have been developed for purification of TcdA and TcdB. The toxins can be separated by anion exchange chromatography and affinity chromatography [12]. TcsH and TcsL are related to
TcdA and TcdB, respectively, and are responsible for refractory shock observed in some animal and human infections [1, 3, 5].

*Clostridium sordelli* Toxin HT has biological activities and immunological properties similar to TcdA. TcdA has been cloned and sequenced [18]. TcsH is also cytotoxic and lethal with minimum doses similar to those reported for TcdA [12]. The N-terminal amino acid sequence of TcdA and TcsH also share close identity (Figure A [7]. The lethal toxin of *C. sordelli* is immunologically related to TcdB. However, TcdB is 10,000 times more cytotoxic than the TcsL. In contrast, TcsL is tenfold more lethal than TcdB. The genes for TcsL and TcdB have been cloned and sequenced [12]. See table A for comparison of the four different toxins.
References


A Ser Leu Ile Ser Lys Glu Ser Leu Ile Ser Lys
HT Ser Leu Ile Ser Lys Glu Ser Leu Ile Ser Lys Leu Ala Tyr Ser Ile Arg Lys Pro Pro

**Figure A.** Amino-terminal sequences of *Clostridium difficile* TcdA and *Clostridium sordellii* TcsH.

<table>
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<tr>
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<th><em>Clostridium sordellii</em></th>
<th><em>Clostridium difficile</em></th>
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<tr>
<td>Lethal Toxin (TcsL)</td>
<td>Hemorrhagic Toxin (TcsH)</td>
<td>Toxin B (TcdB)</td>
</tr>
<tr>
<td>MW 260,000</td>
<td>MW 300,000</td>
<td>MW 270,000</td>
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<tr>
<td>Similar to TcdB</td>
<td>Similar to TcdA</td>
<td>Similar to TcsL</td>
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<tr>
<td>Has been sequenced</td>
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<td>10 fold more lethal</td>
<td>Similar cytotoxicity</td>
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<td>and lethality to TcdA</td>
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**Table A.** Comparison of *C. sordellii* and *C. difficile* toxins.
CHAPTER 2. PRODUCTION AND TESTING OF CLOSTRIDIUM DIFFICILE TOXIN
A MONOCLONAL ANTIBODY (αCdA Bella-1) AND TOXIN A SPECIFIC
POLYCLONAL ANTIBODY (IRP 583)

An unsubmitted paper

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Abstract

Clostridium difficile Toxin A (TcdA) monoclonal and polyclonal antibodies were produced for use by the USDA-Center for Veterinary Biologics (CVB) in anticipation of vaccine product licensing requests. The Virus-Serum-Toxin Act which requires potency evaluation of bacterins prior to sale is implemented by the CVB which makes the production of reagents necessary for product testing. The monoclonal antibody was isotyped and determined to be IgG1, κ. The antibodies were also tested using indirect enzyme-linked immunosorbent assay (ELISA), sandwich ELISA, western blot and toxin neutralization tests to check for the neutralizing effects they had on TcdA. Both the Mab and Pab had neutralizing activity against TcdA in mice and they reacted with TcdA in western blots and ELISA. The Pab reacted with TcdA in the sandwich ELISA and neutralized the toxin in mice. The Pab was also shown to be specific for TcdA in mouse tests because it would not neutralize crude C. difficile toxin which contained TcdB as well. Both antisera reacted with native (non-denatured) Clostridium sordellii toxin HT (TcsH) in ELISA.

Keywords

Clostridium difficile; Toxin A (TcdA); Monoclonal antibody
1. Introduction

*Clostridium difficile* is a sporulating gram positive anaerobe and is the most common nosocomial pathogen of the gastrointestinal tract and has a high rate of occurrence. Symptoms of *C. difficile* infection include diarrhea, which is usually non-bloody, or colitis associated with severe abdominal pain, fever and/or gross or occult blood in stools. Pseudomembranous colitis (PMC) in humans is the most severe manifestation that results from a severe inflammatory response to *C. difficile* toxins [1, 13, 14].

*Clostridium difficile* has recently emerged as a pathogen or commensal in food animals such as neonatal pigs and dairy and beef calves; most of these animal isolates are toxigenic [2]. *C. difficile* causes enteritis in neonatal pigs and calves and is the etiologic agent of *C. difficile*-associated disease (CDAD) in neonatal pigs [1, 3, 4]. Infected herds may experience morbidity as high as 97% and while most piglets recover, the mortality rate in some outbreaks may be as high as 16% [1]. The symptoms of porcine CDAD include piglets 1 to 7 days of age with a history of scouring since shortly after birth. Gross lesions usually include moderate-to-severe edema of the mesocolon, and colonic serosal edema is common; pseudomembranous formation is uncommon, and the yellow plaques seen in human disease are not observed in affected piglets. Colonic contents are frequently pasty-to-watery and yellow, although some piglets are constipated or obstipated [4].

*Clostridium difficile* and its toxins can also be found in non-diarrheic piglets. In one such group, 74% were TcdA/B positive, but had normal feces or were constipated. It is common to find typical lesions in these non-diarrheic piglets. Some piglets present with systemic signs, including mild depression, lack of appetite, cachexia, and anorexia, without diarrhea. Most recover quickly. The only diagnostic findings are the toxins in cecum and colon and typhlocolitis consistent with CDAD.

Clinically relevant strains of *C. difficile* usually produce toxins A and B and comprehensive diagnosis of porcine CDAD includes clinical history, gross and microscopic pathology, bacteriologic culture, detection of TcdA and/or TcdB in feces or colonic contents. Toxin detection is the primary confirmatory CDAD diagnostic
test for specimens obtained ante mortem or postmortem [4, 1]. Antibodies to TcdA have been shown to eliminate binding, secretion and inflammation, and prevent clinical disease in mice and hamsters; anti-TcdB antibodies also have a role in protection against CDAD. Active or passive immunization against TcdA prevents clinical disease. Thus, it may be that if a toxin-neutralizing response can be generated in sows, piglets can be passively protected by antibodies obtained from colostrum [4].

_Clostridium difficile_ has also been found in retail meat resulting in concerns about the role of food in epidemiology of community-associated _C. difficile_ infection (CA-CDI) [5]. So far it is not clear if food animals are a possible source of human _C. difficile_ infections, although there is a high level of overlap between _C. difficile_ types present in humans and animals. Two ribotypes associated with outbreaks of severe disease in humans have been found in animals also. Modes of transmission between human and animal reservoirs could be retail meat, dog food and contact with the hospital environment [3]. The goals of the research contained in this study are to produce reagents to test the effectiveness of current and/or future licensed vaccines.

2. Materials and Methods

2.1 Bacterial Strain and Culture Filtrates.

_Clostridium difficile_ strain VPI 10463 (received from Dr. Glenn Songer, University of Arizona) _Clostridium sordellii_ VPI 9048 (received from Dr. Robert Carman at TechLab, Inc.), and _Clostridium sordellii_ #7502 (CVB strain obtained from Dr. Bairey, Jensen-Salsbury Laboratories, Montana) was grown in brain heart infusion media in 1-liter dialysis flasks for 24 hours at 37°C. After 24 hours the growth in the dialysis bag was collected and centrifuged at 7,300 x g for 60 minutes. Prior to filtration Protease Inhibitor (Sigma Co., St. Louis, MO) was added to the culture filtrate. Culture supernatant was filtered through a 0.22-μm Millipore Sterivex filter. _C. difficile_ strain VPI 10463 expresses TcdA and TcdB, _C. sordellii_ Strain VPI 9048 expresses HT and LT, and _C. sordellii_ strain #7502 expresses LT but not HT.
Toxin expression was determined using a monoclonal antibody kit that detects the presence of TcdA (BioStar OIA CdTOX A from Inverness Medical-BioStar Inc., Louisville, CO).

2.2 Preparation of immobilized thyroglobulin.

Bovine thyroglobulin (Sigma Co., St. Louis, MO) was dissolved in coupling buffer at a concentration of 10 mg/mL using AminoLink Coupling gel (Thermo Fisher Scientific Inc., Rockford, IL) and allowed to incubate overnight at 4°C. The remaining active sites on the gel were blocked with quenching buffer (1 M Tris/HCl) and cyanoborohydride solution (1 M NaCNBH3 in 0.01 N NaOH) for 30 minutes. The column was then washed with 5 bed volumes of 1 M NaCl, to ensure that free thyroglobulin did not remain ionically bound to the gel. The immobilized thyroglobulin was then used to purify TcdA from *C. difficile* VPI 10463.

2.3 Affinity Chromatography.

TcdA was purified from culture filtrates of *C. difficile* VPI 10463 as previously described [6]. The resulting concentration of TcdA was 57µg/mL.

2.4 Production of monoclonal antibody (Mab).

(i) **Preparation of TcdA toxoid.** The purified toxin was inactivated with 0.1% formalin and 20% aluminum hydroxide was added prior to immunization. A commercially available toxoid was also used (List Biological Laboratories Inc., Campbell, CA).

(ii) **Immunization.** BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN) were injected subcutaneously with 0.4 mL of toxoid with adjuvant. Two more injections of 0.2 mL toxoid with adjuvant were given; each 14 days apart. Each animal received a 0.2 mL injection intravenously of purified TcdA toxoid without adjuvant 3 to 4 days before splenectomy.

(iii) **Fusion.** Spleen cells from immunized mice were fused with Sp2/0 cells using the polyethylene glycol method. Hybrid cell lines were selected and their
culture supernatant was analyzed for TcdA antibody by screening ELISA described below. Hybrid cells that were positive for TcdA were expanded and subcloned as described [7].

(iv) **Production of ascites fluid.** About $2.0 \times 10^6$ cells/mouse of the selected clones were injected intraperitoneally into BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN) primed with pristane (Sigma Co., St. Louis, MO). The ascites fluid was clarified by centrifugation, filtered through a 0.45µm membrane, labeled αCdA Bella-1 and stored at 4°C.

2.5 **Production of TcdA specific polyclonal antibody (Pab).**

A male goat was injected with TcdA toxoid mixed with 2.0 mg/ml of Rehydrogel L.V. (Reheis Inc., Berkeley Heights, NJ). The goat received 1.0 ml injections subcutaneously every two weeks for 14 weeks; it was then injected with 0.5 ml of a commercial TcdA (List Biological Laboratories Inc., Campbell, CA). Seven days later blood was collected totaling 4.0 ml/lb of body weight. An additional injection with 0.5 ml of a commercial TcdA (List Biological Laboratories Inc., Campbell, CA) was given and blood was collected again totaling 4.0 ml/lb of body weight. The blood was centrifuged and filtered through bottle top 0.45 µm membrane filters, labeled as IRP 583 and stored at 4°C.

2.6 **ELISAs**

(i) **Screening ELISA.** A screening ELISA was performed on supernatant from all wells containing hybridoma cells. Microtiter plates were coated with the appropriate amount of antigen in carbonate buffer (pH 9.6). The plates were incubated for 1 hour ± 15 minutes at 37°C then washed four times with 0.01 M phosphate buffered saline (PBS) with 0.05% polyethylene glycol sorbitan monolaurate (Tween 20). The plates were then blocked for 1 hour ± 15 minutes at 37°C with blocking solution 0.01 M PBS with 2% Non-fat dry milk (NFDM). Tissue culture supernatant from each hybridoma (producing clusters) was added to each well of the test plate diluted 1:1 in blocking solution and incubated for 1 hour ± 15
minutes at 37°C. The plates were washed 4 times with 0.01 M PBS with 0.05% Tween 20 and 0.1 mL of affinity purified antibody peroxidase labeled goat anti-mouse IgA, IgG, IgM (heavy and light chains; Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted 1:1000 in blocking solution 0.01 M PBS with 2% NFDM was added to each well. After incubation for 1 hour ± 15 minutes at 37°C and washing four times with 0.01 M PBS with 0.05% Tween 20, 0.1 mL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to each well and the plate was incubated at room temperature for 10 minutes. The reaction was terminated after 10 minutes by adding 100 µL sulfuric acid (2.5 M H₂SO₄) to each well prior to being examined for positive reactions. The plates were read with a SpectraMax spectrophotometer (Molecular Devices Inc., Sunnyvale, CA). Absorbance was measured at wavelengths 450 and 650. Controls consisted of mouse serum that was collected from the mouse prior to splenectomy (positive) and ascites containing an unrelated Mab (negative).

(ii) **Sandwich ELISA.** The BCA Protein Assay (Thermo Fisher Scientific Inc., Rockford, IL) was used to estimate the total protein of purified TcdA and the αCdA Bella-1. A titration of αCdA Bella-1 was analyzed by sandwich ELISA. Wells of an Immulon II high binding plate (Thermo Scientific, Waltham, MA) were coated with 50 mg/ml and 81 mg/ml of TcdA specific polyclonal antibody diluted in carbonate buffer (pH 9.6). The plates were incubated for 1 hour ± 15 minutes at 37°C then washed four times with 0.01 M PBS with 0.05% Tween 20. The plates were then blocked for 1 hour ± 15 minutes at 37°C with blocking solution 0.01 M PBS with 2% NFDM. The antigen, TcdA, was then added to the plate diluted in 0.01 M PBS with 2% NFDM. Ascites fluid was diluted starting at 1:100 to 1:30,000 in blocking solution then added to the plate and incubated for 1 hour ± 15 minutes at 37°C. The plates were washed 4 times with 0.01 M PBS with 0.05% Tween 20 and 0.1 mL of affinity purified antibody peroxidase labeled goat anti-mouse IgA, IgG, IgM (heavy and light chains; Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted 1:1000 in blocking solution 0.01 M PBS with 2% NFDM was added to each well. After incubation for 1 hour ± 15 minutes at 37°C, the plates were washed four times with
0.01 M PBS with 0.05% Tween 20, then 0.1 mL of TMB substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to each well and the plate incubated at room temperature for 10 minutes. The reaction was terminated after 10 minutes by adding 100 µL sulfuric acid (2.5 M H₂SO₄) to each well prior to being examined for positive reactions. The plates were read with a SpectraMax spectrophotometer (Molecular Devices Inc., Sunnyvale, CA). Absorbance was measured at wavelengths 450 and 650. Controls consisted of mouse serum that was collected from the mouse prior to splenectomy (positive) and ascites containing an unrelated Mab (negative). An additional sandwich ELISA was performed as described above using TcsH as the antigen.

2.7 Neutralization assay.

(i) TcdA LD₅₀ dose. The toxicity of TcdA was determined by titrating TcdA in mice and calculating the 10LD₅₀. Three mice were injected IV with 0.2mL of various dilutions of TcdA diluted in Bacto-Peptone diluent (10gm Bacto Peptone, 2.5gm Sodium chloride per 1L).

(ii) αCdA Bella-1. A neutralization assay was performed to check the neutralizing effect of the monoclonal antibody in-vivo. The TcdA was diluted to the 10LD₅₀ dose and αCdA Bella-1 was diluted 1:10, 1:20, 1:40, 1:80, 1:160 (protein concentration of 41.6 mg/mL). Each mixture was injected intravenously (0.2 mL dose) into 3 Swiss Webster mice (Harlan Sprague Dawley, Indianapolis, IN). The mice were observed for 72 hours and deaths were recorded.

(iii) IRP 583. A toxin-antitoxin neutralization test was performed to check for the antitoxin units per mL (AU/mL) contained in IRP 583. TcdA was diluted to the 10LD₅₀ dose in Bacto-Peptone diluent (10gm Bacto Peptone, 2.5gm Sodium chloride per 1L). IRP 583 was diluted at various levels (1:10, 1:20, 1:40, 1:80, 1:160 and 1:320) and 1.0 mL of diluted antibody was added to 0.8 mL of diluted TcdA and 0.2 mL Bacto-Peptone diluent. The mixtures were allowed to neutralize for 1 hour at room temperature then placed on ice. Each mixture was injected intravenously (0.2 mL dose) into 3 Swiss Webster mice (Harlan Sprague Dawley, Indianapolis, IN). The
mice were observed for 72 hours and deaths were recorded. Once the AU/mL in IRP 583 was determined a toxin-antitoxin neutralization test was performed to check the neutralizing effect of the toxin A specific polyclonal antibody and determine the 10 Lo and L+ doses of TcdA. The 10Lo dose is defined as the greatest amount of toxin that, when mixed with 10 antitoxin units (AU), results in 100% survival of all mice inoculated intravenously with 0.2mL of the mixture. The 10L+ dose is defined as the least amount of toxin that, when mixed with 10 AU antitoxin, results in the death of 80-100% of all mice.

(iv) An additional mouse test was performed using crude C. difficile toxin to check for specificity to TcdA. The mouse test was run the same as the previous test with TcdA.

2.8 SDS-PAGE and Western Blot.

A western blot was done to check the specificity of the monoclonal antibody. TcdA, crude C. difficile toxin (VPI 10463) and crude C. sordellii toxin Strain #7502 (TcsL +, TcsH -) and VPI 9048 (TcsL +, TcsH+) were all evaluated. Aliquots of each sample were mixed with 1X NuPAGE® LDS buffer (Invitrogen, Carlsbad, CA) and heated for 10 minutes at 70°C. Tris acetate SDS running buffer (Invitrogen, Carlsbad, CA) was added to deionized water. The proteins were separated in duplicate NuPAGE® Tris-acetate 7% gels (Invitrogen, Carlsbad, CA) in Tris acetate SDS running buffer under reducing conditions. A molecular weight calibration lane was loaded with Hi-Mark pre-stained molecular weight standards (Invitrogen, Carlsbad, CA). Gels were assembled into a sandwich with nitrocellulose membranes and transferred in an iBlot® Western Detection Kit (Invitrogen, Carlsbad, CA). Membranes were equilibrated in 20 mM Tris—500 mM NaCl (TBS, pH 7.5) for 10 minutes prior to being blocked in 20 mM Tris—500 mM NaCl—0.5% Tween 20 (TTBS, pH 7.5) and incubated with agitation at room temperature for 30 to 60 minutes. Membranes were then immersed in αCdA Bella-1 subclone supernatants C2 and C3 diluted to 1:100 in 20 mM Tris—500 mM NaCl—0.5% Tween 20 (TTBS, pH 7.5) and incubated with agitation at room temperature for 1 to 2 hours. One
membrane was immersed in CPD IRP 578 (Clostridium perfringens Type D Mab) diluted 1:100 as a negative control and one membrane not immersed in a Mab, only in conjugate (neither pictured). Following four 10 minute washes in TTBS the membranes were immersed in affinity purified antibody peroxidase labeled goat anti-mouse IgA, IgG, IgM (heavy and light chains; Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted 1:1000 and incubated with agitation at room temperature for 1 to 2 hours. After four 10 minute washes in TTBS, bound antibodies were detected with 3,3',5,5'-tetramethylbenzidine (TMB) and TMB Membrane Enhancer (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Separated total protein of each sample was visualized by staining a duplicate nitrocellulose membrane with AuroDye™ forte (GE Healthcare, Buckinghamshire, UK).

2.9 Determination of isotype.

Monoclonal antibody αCdA Bella-1 was tested using a Pierce® Rapid Isotyping kit (Thermo Fisher Scientific Inc., Rockford, IL).

3. Results
3.1 ELISA.

Several hybridomas that reacted with purified TcdA were isolated. One of these, designated αCdA Bella-1, revealed a high sensitivity of the monoclonal antibody for the antigen TcdA, and reactivity extended to the 1/5000 dilution of ascites fluid. Similar results were obtained when using 81 or 53 mg/ml of IRP 583 as capture antibody (Figure 1). αCdA Bella-1 also showed sensitivity for the antigen TcsH with reactivity exceeding the 1/200 dilution of ascites fluid (Figure 2).

3.2 Neutralization assay.

The 10LD₅₀ dose of the TcdA was 1:11 (protein concentration of approximately 5µg/mL). The monoclonal antibody was shown to neutralize TcdA and protect mice from 10 LD₅₀ lethal effects at all dilutions tested (Table 1). The
polyclonal antiserum IRP 583 contained 160 AU/mL (Table 2). The Lo and L+ dose of purified TcdA was determined to be 0.5 ml and 0.7 ml, respectively (Table 3). The Lo dose of 0.5 mL was chosen to ensure 100% survival rate in the mice. The L+ dose of 0.7mL was the least amount of toxin that resulted in the death of 100% of all mice. The Pab was shown to be specific for TcdA when the mice injected with crude toxin, which contained lethal levels of TcdB, and various dilutions of Pab all died.

3.3 SDS-PAGE and Western Blot.

The monoclonal antibody αCdA Bella-1 subclone C2 and IRP 583 (Figure 3 and 4) reacted with TcdA. There was also a slight reaction of αCdA Bella-1 subclone C2 and IRP 583 to the Bovine thyroglobulin in Lane 6, but this was also present with the conjugate only control (not shown). There was insufficient data to determine if αCdA Bella-1 subclone C2 and IRP 583 also detected TcsH because the TcsH band was not visible on the Western blot or the membrane blotted with Aurodye indicating that an insufficient amount of toxin may have been present in crude preparation to allow detection.

3.4 Determination of isotype.

The monoclonal antibody αCdA Bella-1 was shown to be of mouse IgG1, κ isotype.

4. Discussion

This study has shown the produced Mab, αCdA Bella-1 is specific for and neutralizes TcdA. It specifically recognized TcdA in Western Blotting and was shown to be protective in mice injected with the toxin. αCdA Bella-1 also reacted strongly to the TcdA and TcsH antigen in an indirect and sandwich ELISA.

αCdA Bella-1 did not cross react with TcsH on Western Blot. Previous work has shown that Mab’s for TcdA can cross react with TcsH [8]. This difference may be due to the different procedures used to purify the TcdA or a difference in the preparation of the toxoid that was used to immunize mice [8, 9]. DEAE-Sepharose
CL-6B columns with toxins A and B eluted by NaCl gradient as described by Sullivan et al [9] was used to produce a cross reacting Mab, unlike this study which used TcdA purified using bovine thyroglobulin. Although αCdA Bella-1 did cross react to TcsH with the ELISA, it did not cross react with TcsH on the Western blot. The reason for this may be that the Mab is specific to a conformational epitope or was specific to an epitope that was blocked on Western Blot. This would explain why it reacts with the native toxin and not the de-natured toxin.

The TcdA specific Pab (IRP 583) was also shown to neutralize TcdA in mice, it cross reacted with TcsH in ELISA and reacted with TcdA in western blot. It was shown to be specific for TcdA since it would not protect mice against crude *C. difficile* supernatants containing both TcdA and TcdB.

*Clostridium difficile* has recently emerged as a pathogen or commensal in food animals such as neonatal pigs and dairy and beef calves; most of these animal isolates are toxigenic [2]. Active or passive immunization against TcdA prevents clinical disease. Thus, it may be that if a toxin-neutralizing response can be generated in sows, piglets can be passively protected by antibodies obtained from colostrum [4]. Since it has been shown that antibodies to TcdA eliminate binding and prevent clinical disease in animal models; immunization against TcdA can prevent clinical disease [4]. *C. difficile* has also been found in retail meat and concerns about the role of food in the epidemiology of community-associated *C. difficile* infection (CA-CDI) have been expressed and due to the fact that *C. difficile* is gaining importance as an animal pathogen, there is now a potential for product licensing requests [3, 5]. Since αCdA Bella-1 has been shown to be specific and protective against TcdA it may be used as a reagent for product testing that is required by the CVB prior to licensure of new animal vaccines.

5. **Acknowledgements**

This work was supported by the Center for Veterinary Biologics Graduate Training Program.
Thank you to all the knowledgeable professional and technical staff in the Biologics Bacteriology section at the Center for Veterinary Biologics for all the assistance that was provided with this project.
References


Figure 1. Sandwich ELISA Titration results for αCdA Bella-1 using TcdA as the antigen.

Figure 2. Sandwich ELISA titration results for αCdA Bella-1 using TcsH as the antigen.
**Figure 3** Western Blot using the monoclonal antibody αCdA Bella-1 subclone C2

**Figure 4** Western Blot using the Toxin A specific polyclonal antibody IRP 583
Figure 1 Western Blot using the monoclonal antibody αCdA Bella-1 subclone C2. Lane 1: Hi-Mark Protein ladder; Lane 2: Clostridium difficile purified Toxin A (VPI 10463); Lane 3: Clostridium difficile crude toxin (VPI 10463); Lane 4: Clostridium sordellii purified Toxin HT (VPI 9048); Lane 5: Clostridium sordellii crude Toxin (VPI 9048); Lane 6: Clostridium sordellii crude toxin (#7502).

Figure 2 Western Blot using the Toxin A specific polyclonal antibody IRP 583. Lane 1: Hi-Mark Protein ladder; Lane 2: Clostridium difficile purified Toxin A (VPI 10463); Lane 3: Clostridium difficile crude toxin (VPI 10463); Lane 4: Clostridium sordellii (VPI 9048); Lane 5: Clostridium sordellii crude Toxin (#7502); Lane 6: Bovine Thyroglobulin.
Table 1 Neutralization assay for αCdA Bella-1 against TcdA toxin

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<th>αCdA Bella-1 Dilution</th>
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<tr>
<td>1:20</td>
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Table 2 Neutralization assay to determine AU/ml in IRP 583 against TcdA toxin

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<th>IRP 583 Dilution</th>
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Table 3 Results from Neutralization assay for IRP 583 to determine $L_0$ and $L_\infty$ of TcdA.

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<td>0.5ml</td>
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CHAPTER 3 GENERAL CONCLUSION

Discussion

In this study, the TcdA monoclonal antibody, αCdA Bella-1, and toxin A specific polyclonal antibody, IRP 583, were produced for use by the USDA-Center for Veterinary Biologics (CVB) in anticipation of vaccine product licensing requests.

Specificity of both antibodies was determined using indirect enzyme-linked immunoabsorbent assay (ELISA), sandwich ELISA, western blot and toxin neutralization tests. Through these tests it was shown that both antibodies had specificity to TcdA.

The monoclonal antibody, αCdA Bella-1 was then tested for specificity to TcsH by Western Blot, as well as, by sandwich ELISA since previous studies show that C. sordellii antitoxin cross-neutralizes TcdA and TcdB of C. difficile [1]. The results showed that αCdA Bella-reacted with TcsH in ELISA tests.

αCdA Bella-1 did not cross react with TcsH on Western Blot. Previous work has shown that Mab’s for TcdA can cross react with TcsH [8]. This difference may be due to the different procedures used to purify the TcdA or a difference in the preparation of the toxoid that was used to immunize mice [8, 9]. DEAE-Sepharose CL-6B columns with toxins A and B eluted by NaCl gradient as described by Sullivan et al [9] was used to produce a cross reacting Mab, unlike this study which used TcdA purified using bovine thyroglobulin. Although αCdA Bella-1 did cross react to TcsH with the ELISA, it did not cross react with TcsH on the Western blot. The reason for this may be that the Mab is specific to a conformational epitope. This would explain why it reacts with the native toxin and not the de-natured toxin.

Recommendations for future research

It would be beneficial to obtain the gene sequence of Clostridium sordellii Toxin HT. Previous studies have shown that when immunizing guinea pigs with toxoid containing TcsL, TcsH and both TcsL/TcsH only the TcsL/TcsH toxoid gave complete protection against bacterial challenge with strains of C. sordellii. The result
shows that TcsL toxoid, as well as, TcsH toxoid are essential protective antigens of
C. sordellii [2]. Obtaining the gene sequence would allow the development of a PCR
assay method that could be used for the detection of TcsH in the bacterial strains
that are used to produce vaccine products. If the TcsH gene is absent in the
bacterial strains used it could then be deduced that the resulting vaccine product will
not completely protect the animal against a C. sordellii infection because there would
be a lack of TcsH antibody response. This research could result in a change to
government regulations relating to vaccine products containing C. sordellii toxoid.
References


APPENDIX A. CLOSTRIDIUM SORDELLII HEMORRHAGIC TOXIN (TcsH) SEQUENCING

1. Procedures performed in an attempt to sequence *C. sordellii* Toxin HT (TcsH)

1.1 Polymerase Chain reaction (PCR)

Since the biological and antigenic properties of TcdA and TcdB are very similar to those of TcsH and TcsL and *C. sordellii* antitoxin cross-neutralizes TcdA and TcdB it is suggested the two toxins of *C. sordellii* are immunologically related to the *C. difficile* toxins [1, 2]. A published PCR protocol for *C. difficile* was used to attempt to get the TcsH sequence by using primers that were specific for TcdA [3]. The annealing temperature was reduced in an attempt to get the primers to bind even if several of the base pairs were mismatched. The annealing temperature ranged from 38 to 55°C. PCR products were run using gel electrophoresis. When bands were visible that looked equivalent to the 600bp band for TcdA they were cut out of the gel and DNA was extracted from the bands using the MinElute PCR Purification Kit (Qiagen, Valencia, CA). Once the DNA was extracted a TOPO TA Cloning® Kit (Invitrogen, Carlsbad, CA) was used and the resulting transformants were sent to the Iowa State University (ISU) DNA facility for sequencing.

1.2 Isoelectric focusing

It has been previously shown that TcsH has an isoelectric point (pI) of 6.10 [4]. Crude toxin was made from *C. sordellii* Strain VPI 9048. The resulting toxin was concentrated 10X using iCON™ concentrators from Pierce Scientific. The concentrated toxin was then isoelectric focused using a Biorad Rotofor™ Preparative IF cell (Biorad Laboratories Inc., Hercules, CA). SDS-PAGE was then performed on the sample that resulted in a pI closest to 6.10. The gel was then blotted onto a nitrocellulose membrane and stained with Aurodye™ Forte (GE Healthcare) and the resulting bands were analyzed.
1.3 Southern Blot

Previous research has shown that when running a Southern Blot using a TcdA specific probe *C. sordellii* strains will also show positive hybridization provided that the *C. sordellii* strains were positive on the TcdA and TcdB assays [5]. A published PCR protocol for TcdA was performed. The resulting gel was used to extract and purify DNA using a S.N.A.P™ Gel Purification Kit (Invitrogen, Carlsbad, CA) [3]. Once TcdA specific DNA was obtained it was labeled using a DIG High Prime DNA Labeling and Detection Starter Kit (Roche Diagnostics Corp, Indianapolis, IN). The restriction endonuclease Hind III was used to digest *C. sordellii* genomic DNA. The resulting gel was used for the Southern Blot and the *C. difficile* Toxin A probe was used for immunological detection.

1.4 Affinity Chromatography

The Mab αCdA Bella-1 was coupled to AminoLink® Coupling gel (Thermo Fisher Scientific Inc., Rockford, IL) and affinity chromatography was used to purify TcsH as previously described [4]. The resulting eluate was run on a SDS-PAGE gel and blotted onto a nitrocellulose membrane. To determine if TcsH was present the membrane was stained using Aurodye™ Forte (GE Healthcare) and Coomassie stain. The resulting bands will be sent to the ISU Protein Facility and the protein sequence of TcsH can be obtained.

1.5 Subtractive Hybridization

Subtractive hybridization methods can be used to identify cDNA sequences that are present in one bacterial genome, but absent in another. The genomic DNA that contains the genes of interest is considered the tester and the reference sample is considered the driver. Tester and driver DNAs are hybridized and the hybrid sequences are removed. The remaining unhybridized DNAs represent genes that are expressed in the tester yet are absent in the driver. Using the Clontech PCR-Select™ Bacterial Genomic Subtraction Kit (ClonTech Laboratories Inc., Mountain
View, CA) as recommended by suppliers, a TcsH – strain #7502 was used as the driver and a TcsH + strain VPI 9048 was used as the tester.

1.6 Primer Walking

The whole genome for *C. difficile* strain 630 has been sequenced (GenBank accession AM180355). The location of the TcdB gene is from base 787,393 to 794,493 and the TcdA gene is from base 795843 to 803975. This shows that the two genes are only located 1350 bases apart. Assuming that TcsL and TcsH would be located a similar distance apart *C. sordellii* DNA was submitted to the ISU DNA facility for primer walking. A primer was designed from the published TcsL sequence (GenBank accession X82638) and primer walking to the TcsH gene was attempted.

2. Results from procedures performed in an attempt to sequence *C. sordellii* Toxin HT (TcsH)

2.1 Polymerase Chain reaction (PCR)

When the resulting PCR products were run on the E-gels (Invitrogen, Carlsbad, CA) bands that were approximately the same size as the TcdA band were present (Figure A.1 – A.3). When the PCR products were purified, inserted into a plasmid and sent to ISU for sequencing the results did not match TcdA when nucleotide BLAST was used to analyze them.

2.2 Isoelectric focusing

Isoelectric focusing showed a pI of 6.18. SDS-PAGE was run using this sample and blotted to a membrane. When stained with Aurodye TcsH migrated as a band with the molecular weight of approximately 300,000 kDa (Figure A.4). Unfortunately, I was unable to submit the band for sequencing to the ISU Protein Facility due to the fact that the Coomassie stain didn’t produce a visible band and the faint Aurodye band couldn’t be used for sequencing either.
2.3 Southern Blot

Hind III was successful in cutting *C. sordellii* VPI 9048 DNA (Figure A.5), but no bands were present after running the Southern Blot. After several tries I was unable to get any bands; which I believe was due to insufficient concentration of the *C. difficile* probe.

2.4 Affinity Chromatography

SDS-PAGE of the resulting eluate migrated as a band with the molecular weight of approximately 300,000 kDa (Figure A.6, A.7). The band was visible using Aurodye stain, not visible using Coomassie stain so I was unable to submit the band to the ISU Protein Facility even after several additional attempts to concentrate it further.

2.5 Subtractive Hybridization

The restriction enzyme, Rsa I, was successful in cutting both *C. difficile* and *C. sordellii* DNA (Figure A.7). Adaptors were then ligated onto the cut DNA and the final hybridization step was performed (Figure A.8). The hybridization step was unsuccessful in showing any genes of interest that may have been present in the Tester, but not the Driver.
References


Figure A.1 E-gel results from PCR Thermocycler run with 55°C annealing temperature. Lane 1: 1 KB Plus TrackIt DNA ladder; Lane 2: *Clostridium difficile* VPI 10463 (2.5µl); Lane 3: *Clostridium difficile* VPI 10463 (5µl); Lane 4: *Clostridium sordelli* VPI 9048 (2.5 µl); Lane 5: *Clostridium sordelli* VPI 9048 (2.5µl); Lane 6: *Clostridium sordelli* VPI 9048 (1µl); Lane 7: *Clostridium sordelli* VPI 9048 (5µl); Lane 8: Negative Control; Lane 9: 1 KB Plus TrackIt DNA ladder.

Figure A.2 E-gel results from PCR Thermocycler run with 45°C annealing temperature. Lane 1: 1 KB Plus TrackIt DNA ladder; Lane 2: *Clostridium difficile* VPI 10463 (2.5µl); Lane 3: *Clostridium difficile* VPI 10463 (5µl); Lane 4: *Clostridium sordelli* VPI 9048 (2.5 µl); Lane 5: *Clostridium sordelli* VPI 9048 (2.5µl); Lane 6: *Clostridium sordelli* VPI 9048 (1µl); Lane 7: *Clostridium sordelli* VPI 9048 (5µl); Lane 8: Negative Control; Lane 9: 1 KB Plus TrackIt DNA ladder.
Figure A.3 E-gel results from PCR Thermocyler run with 38°C annealing temperature. Lane 1: 1 KB Plus TrackIt DNA ladder; Lane 2: *Clostridium difficile* VPI 10463 (2.5µl); Lane 3: *Clostridium difficile* VPI 10463 (5µl); Lane 4: *Clostridium sordellii* VPI 9048 (2.5 µl); Lane 5: *Clostridium sordellii* VPI 9048 (2.5µl); Lane 6: *Clostridium sordellii* VPI 9048 (1µl); Lane 7: *Clostridium sordellii* VPI 9048 (5µl); Lane 8: Negative Control; Lane 9: 1 KB Plus TrackIt DNA ladder.
**Figure A.4** Aurodye Blot results for isoelectric focused samples. Lane 1: *C. difficile* crude toxin VPI 10463. Lane 2: *C. sordellii* VPI 9048 isoelectric focused sample 1, Lane 3: *C. sordellii* VPI 9048 isoelectric focused sample 2, Lane 4 *C. sordellii* VPI 9048 isoelectric focused sample 3, Lane 5: Hi-Mark Protein ladder.
Figure A.5 REA gel used for Southern Blot procedure. Lane 1: λ Hind III Ladder, Lane 2: C. difficile VPI 10463 cut with Hind III, Lane 3 C. sordellii VPI 9048 cut with Hind III, Lane 4: λ Hind III Ladder.
Figure A.6 Affinity Chromatography results. Lane 1: Hi-Mark Protein ladder, Lane 2: *C. difficile* VPI 10463, Lane 2 Concentrated *C. difficile* VPI 10463 Lane 3: *C. sordellii* TcsH, Lane 4: *C. sordellii* TcsH, Lane 5: Hi-Mark Protein ladder.

Figure A.7 REA gel from Subtractive Hybridization kit. Lane 1: 1 KB Plus TrackIt DNA ladder, Lane 2: *C. sordellii* VPI 9048 cut with Rsa I, Lane 3: *C. sordellii* 7502 cut with Rsa I, Lane 4: Control DNA (Escherichia coli) cut with Rsa I, Lane 5: 1 KB Plus TrackIt DNA ladder.
Figure A.8 Hybridization gel from Subtractive Hybridization kit. Lane 1: 1 KB Plus TrackIt DNA ladder, Lane 2: Subtracted experimental DNA, Lane 3: Un-subtracted tester control, Lane 4: Subtracted control DNA form E. coli, Lane 5: Un-subtracted tester control for the control subtraction Lane 6: PCR control subtracted DNA, Lane 7: 1 KB Plus TrackIt DNA ladder.
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