Identifying and purifying protective

immunogens from cultures of

Clostridium chauvoei

by

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DEDICATION

I would like to dedicate this thesis to my children, Matt and Jill, as a "thank you" for allowing me to relive the simplest joys of science again through their eyes.

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ACKNOWLEDGMENTS

GENERAL INTRODUCTION

Clostridium chauvoei is a species of anaerobic, spore forming bacteria that is the etiologic agent of a disease commonly referred to as "blackleg". Blackleg affects primarily cattle and sheep, and occurs in livestock producing areas throughout the world. The disease is characterized by a rapidly fatal course of muscle necrosis and toxemia. Animals affected are usually found dead without any clinical symptoms having been observed. No data documenting the economic losses caused by blackleg have been published, however, the disease is so prevalent in the United States that vaccines containing *Clostridium chauvoei* are the most frequently used in the cattle industry.

In the United States, vaccine manufacturers are regulated by the United States Department of Agriculture's, Animal and Plant Health Inspection Service. To maintain a license, manufacturers are required to test a fraction of each serial of vaccine for potency using acceptable methods outlined in the Code of Federal Regulations. The current potency test for products containing *C. chauvoei* involves vaccinating guinea pigs with the test vaccines then injecting them with virulent *C. chauvoei* spores. Nonvaccinated control guinea pigs also receive the virulent challenge.

Two paramount disadvantages are inherent to this test:

the high cost and, more importantly, the pain and suffering that is inflicted on live animals. Development of a suitable alternative potency test that measured the serological response of rabbits to protective immunogens of *C. chauvoei* would alleviate both drawbacks of the current test. Since clostridial vaccines are always marketed as combination products, containing fractions that must be assayed for potency in rabbits, no additional animals would be required to perform this type of *C. chauvoei* potency test. Before such an alternative test can be developed however, specific protective immunogens of *C. chauvoei* must be identified and characterized.

The objective of this study was to identify, purify, and partially characterize protective proteinaceous immunogens from *C. chauvoei* cultures for potential use in a potency assay.

Thesis Organization

This thesis consists of an introduction, a literature review, a separate manuscript, a general summary, a list of references cited in the literature review, and an appendix. The masters candidate, Paul Joseph Hauer, is the senior author and principal investigator for the manuscript.

LITERATURE REVIEW

Clostridium chauvoei

Clostridium chauvoei was first described by Arloing, Cornevin, and Thomas[1] in 1887 as the etiological agent of a rapidly fatal myonecrotic disease of cattle and sheep commonly referred to as blackleg. The organism was named in honor of Jean-Baptiste Auguste Chauvoei, a prominent nineteenth century French medical researcher who worked extensively with it in studies on infectious diseases. *Clostridium chauvoei* is an anaerobic, Gram positive, pleomorphic bacillus with peritrichous flagella. Cell dimensions are variable, particularly in older cultures, and typically range from 0.5 to 1.7 μm in width by 1.6 to 9.7 μm in length. Cells are frequently swollen by central to subterminal oval spores.[2] The organism is more fastidious than most other clostridia. It is a strict anaerobe that grows best in media such as chopped meat or brain-heart infusion which contain complex peptides and is noted for producing large amounts of gas. It has a requirement for high levels of cysteine.[3] *Clostridium chauvoei* resembles *C. septicum* closely enough that some researchers have proposed that they be classified in a single species[4,5,6]. However several unique characteristics make them readily distinguishable.[7]

Culture filtrates of *C. chauvoei* contain an array of extracellular enzymes including: oxygen-stable hemolysin,

oxygen labile hemolysin, hyaluronidase, neuraminidase, and deoxyribonucleases.[8,9,10] The oxygen-stable hemolysin, designated as α toxin is hemolytic and causes necrosis when injected intradermally into guinea pigs.

Clostridium chauvoei can be found in the soil, water, and the intestinal tracts of many species of living and dead animals. The organism has been shown to persist in loam for up to 11 years,[11] and Barnes et al.[12] correlated occurrence of the disease to newly excavated soil. Likewise, the presence of *C. chauvoei* in aquatic habitats is well established.[13,14]

Blackleg

The disease caused by *C. chauvoei* occurs commonly in livestock production areas throughout the world. Although cattle and sheep are most often affected, goats, swine, deer, mink, guinea pigs, and mice are also susceptible.[7,15] Outbreaks in freshwater fish causing high mortality have been documented,[13] and recently a paralytic-like disease in ostriches has been associated with *C. chauvoei* infection.[16] Humans appear quite resistant since no cases of human infection or disease have been reported. The disease is most often characterized by a rapidly fatal necrotizing myositis. Typically, the lesions are located in the large muscle masses of the fore- and hindquarters, hence the name "blackleg" or "blackquarter". The disease progresses swiftly and affected

animals are often found dead with no prior clinical signs. Early disease manifestations include elevation in temperature and marked swelling at the site of the lesion. In sheep the infection usually results from wound contamination, but in cattle there is often no evidence of trauma. *Clostridium chauvoei* has been isolated from spleens and livers of normal cattle[17] suggesting entry via the hepatoportal pathway. Muscle and liver tissue may be seeded with viable organisms, possibly spores, which remain dormant until the environment is altered in some way to permit growth. The disease has been reproduced in animals by oral administration of organisms followed by an intramuscular injection of either saponin[18] or calcium chloride.[19] Entry of the organism via the alveoli when deciduous teeth are lost has also been proposed.[20] This hypothesis is supported by the fact that young cattle, 6 months to 2 years old, are most often affected. However, cases of blackleg have also been reported in neonates[21] and *in utero*.[22] Attack rates are usually quite low, but case fatality rates approach 100%.[23]

Gross lesions observed with blackleg are usually quite characteristic. Typically the affected muscles are blackened, dry, and crepitant with a rancid odor. A pale yellow fluid which eventually becomes bloodstained surrounds the lesion. Myocarditis and pericarditis or "blackleg of the heart" has also been described in both cattle and sheep.[24,25] Malone et

al.[26] reported finding pericarditis in 48% of 29 cases, and the pericardium was the primary focus of infection in 21% of these cases. A single case of purulent meningitis in a calf was described from which a pure culture of *C. chauvoei* was obtained.

Diagnosis of blackleg usually involves analysis of the history, clinical signs, and postmortem lesions. Culture results alone are not reliable for diagnosing clostridial infections due to the fastidious nature of some species, including *C. chauvoei*. The rapidity with which clostridia invade the tissues from the gastro-intestinal tract after death may also complicate diagnosis.[27] Batty et al.[28] suggested the criteria necessary for a diagnosis of clostridial infection should include analysis of the clinical picture, post mortem lesions, and state of decomposition of the animal. A fluorescent antibody staining technique is the most widely used diagnostic procedure to demonstrate clostridial species in lesions.[29] The utility of this method was demonstrated by Williams[30] who was able to demonstrate the presence of *C. chauvoei* in 97 cases of suspected blackleg using fluorescent antibody staining while culturing the organism from only 89 of those cases.

Treatment of the disease is usually futile, but has been successful with parenteral administration of high levels of antibiotics in conjunction with drainage and H_2O_2 lavage of

affected tissues.[31]

Immunity to Blackleg

Humoral immunity is considered most important in protecting an animal from infection with *C. chauvoei*. Oxer et al.,[32] demonstrated that lambs from vaccinated ewes were passively protected against challenge by colostral antibodies. An *in vitro* plate agglutination assay[33] was used to demonstrate maternally derived anti-*C. chauvoei* antibody in newborn calves,[34] ewes, and buffaloes.[35] Macheak et al.[36] used the plate agglutination assay to correlate antibody level to protection in cattle vaccinated with vaccines of varying potency. Awad et al.[37] demonstrated a correlation between agglutination titers and protection in guinea pigs and sheep. Passive protection has also been demonstrated using immune serum. Data from mice which were administered varying doses of immune equine serum revealed that maximum protection against challenge was afforded the mice receiving the largest doses.[38] Other researchers demonstrated passive protection in mice which were administered rabbit antibody directed specifically at the flagella of *C. chauvoei*.[39] In related experiments, Tamura and Tanaka[40,41] showed that cyclophosphamide treatment of mice that had been administered anti-flagellar rabbit serum dramatically increased the fatality rate caused by a

C. chauvoei challenge, while treatment with carrageenan did not affect the fatality rate at all. This led to the conclusions that anti-flagellar serum exerts its protective effect by opsonic activity, and polymorphonuclear neutrophils are more important effector cells than macrophages in protection against the organism. Recently, a monoclonal antibody has been described which recognizes an epitope located on the surface of *C. chauvoei* flagella and also passively protects mice.[42]

Research into the relationship between cell-mediated immune responses and immunity to blackleg is limited. One study showed that neither delayed hypersensitivity nor leukocyte migration inhibition could be detected in vaccinated guinea pigs prior to challenge, even though all of the animals were protected against challenge.[43] Agglutination titers in these protected guinea pigs were at high levels. In another study, cobra venom was used to deplete the C3 component of the complement system in subsequently challenged mice.[44] It was concluded that C3 did not play a significant role in the resistance of mice against *C. chauvoei* infection.

The Protective Antigens of *C. chauvoei*

Vaccination for the prevention of blackleg has been practiced for over 100 years. Arloing, Cornevin, and Thomas prepared the first blackleg vaccine in 1881, before the

causative agent had been isolated and identified. Early vaccines were prepared by heating or drying muscle tissue from infected animals. A later version of the vaccine commonly referred to as "aggressin" was prepared by collecting and filtering edema fluid from lesions. As one would expect from such crude preparations, local and systemic reactions were common side effects of vaccination.[7] In 1925, Lechlainche and Vallee[45] showed that formalinized whole culture was an effective immunizing agent, and that form of vaccine was soon widely used by cattle producers. Blackleg vaccines currently licensed in the United States consist of formalinized whole cultures, differing little from the 1925 version.

Immunity to blackleg is generally considered to involve antigens other than toxins.[46,47] This is in contrast to the other common clostridial pathogens of animals in which protective immunity is equated to the level of specific toxin neutralizing antibody.

The first work which identified individual antigens in *C. chauvoei* was done by Roberts[48] who devised a typing scheme based on heat stable somatic (O) and heat labile flagellar (H) antigens. He concluded that the O antigen was common to all strains, but the H antigens of ovine and bovine isolates were unique. Henderson[49] later proposed that only English ovine isolates possessed a unique H antigen. This was confirmed by Moussa[4] who also demonstrated a spore antigen which was common

to all *C. chauvoei* and *C. septicum* isolates. Kerry[38] confirmed
an immunological difference between strains and concluded that
animals showed a greater resistance to challenge by the
homologous strain than by heterologous strains.

Studies which tried to identify the specific protective
immunogens of *C. chauvoei* have resulted in controversy and
contradictions. Henderson[50] was the first to demonstrate
protection using boiled whole cells and reported that
protection was due to the heat stable somatic O antigen.
Subsequently the presence of protective antigen was
demonstrated in culture supernatant.[51] That work has since
been confirmed independently by several researchers.[52,53,54]
However, the relative contribution to immunity made by soluble
immunogens remains unclear. The protective soluble immunogen
is most likely proteinaceous since it can be precipitated with
ammonium sulphate and is destroyed by treatment with trypsin,
pepsin, hydrochloric acid, or heat.[53,54] A purified soluble
antigen has been described as being non-lethal to mice and
distinct from an oxygen-stable hemolysin (α toxin). This
soluble antigen was approximately twice the molecular weight
of the α toxin, however it did elicit anti-hemolysin titers
when injected into rabbits. This raised the possibility that
a larger soluble immunizing agent dissociates into the
hemolytic alpha toxin which is not capable of stimulating
protective immunity.[53] In contrast, Bergman[55] reported that the

hemolysin of *C. chauvoei* was capable of inducing protective immunity, and the level of hemotoxoid in a vaccine correlates to the immunizing power of the vaccine. Hemolysin of *C. chauvoei* has recently been partially characterized, but its ability to induce protective immunity has not been determined.[56,57]

Many years after the early investigations on cellular antigens, attention was focused on flagella as a possible protective immunogen when it was reported that a highly protective strain of *C. chauvoei* differed from less protective strains only in flagellar antigen type. The observation was also made that the degree of cross-reactivity between O antigens was constant between strains regardless of their protective abilities and sera from highly protected guinea pigs failed to show O agglutination with either homologous or heterologous strains. These findings cast some doubt on the importance of the common heat stable O antigen as an immunizing agent in whole culture vaccines.[58] In a subsequent investigation, flagella from *C. chauvoei* were purified using a procedure which included treatment with trypsin and sodium dodecyl sulfate.[59] A flagellar preparation produced in this manner was not protective, but it did stimulate high agglutination titers when injected into rabbits. Conversely, deflagellated cells induced no measurable agglutination titers but were protective.[60] It was also demonstrated that

protoplasts of *C. chauvoei* were not protective but spheroplasts maintained protective antigenicity.[61] The conclusion from these studies was that the protective antigenicity of *C. chauvoei* was not of flagellar origin, but due to a heat labile, pH sensitive, non-agglutinogenic somatic antigen, possibly the peptidoglycan component of the cell wall.[61]

Despite the preceding findings, recent investigations into the protective immunogens of *C. chauvoei* have focused on flagella. *Clostridium chauvoei* anti-flagellar antibody, both monoclonal and polyclonal, have been shown to protect mice against challenge. A partially purified *C. chauvoei* flagellar preparation was shown to be 100-fold more protective in mice than a non-flagellated mutant strain.[62] The protective effect of antisera against whole cells and partially purified flagella was lost after absorption with the parent strain, but no loss was shown after absorption with the non-flagellated mutant strain.[39] An acid extract antigen which possessed many characteristics of flagella was also shown to be highly protective in guinea pigs.[63] Data from another study, however, showed no protection from homologous challenge when guinea pigs were immunized with acid extracted flagellar antigen.[64] Sanousi et al.[65] tried to resolve the apparent discrepancy between protection induced by flagellar vs somatic antigens by

growing *C. chauvoei* cultures in media of varying pH. More flagella were observed in cultures grown at pH 7.3, and vaccines containing equal numbers of cells provided greater protection when more flagella were present. The authors noted, however that somatic antigen may also have been influenced by pH changes.

Investigators have cited various factors which may contribute to contradictions in these studies designed to identify protective immunogens. Some purification procedures may have been better than others in maintaining the native conformation of the antigens under investigation. Strain differences may also have played a role in the conflicting results. It appears likely that multiple antigens contribute to the protective immunogenicity of *C. chauvoei* and the relative contribution of each antigen may depend on the strain of both the immunizing agent and challenge.

Assaying the Potency of *C. chauvoei* Vaccines

The current standard for assaying the potency of *C. chauvoei* vaccines is a guinea pig protection test.[66,67] Host animal challenges have been conducted successfully in both cattle[36] and sheep,[68] and correlation to the guinea pig model has been established. Results of the guinea pig assay can be influenced by factors including the strain and diet of the guinea pigs.[69] Comparison of responses to vaccination between

guinea pigs and hamsters led to the observation that hamsters are immunized more readily by the cellular antigens and guinea pigs by the soluble antigens. Therefore, the two animal species cannot be used interchangeably in protection tests.[70] Mice are less suited to serve as a laboratory models because they appear to have some degree of natural immunity to *C. chauvoei*.[39,71]

Initial attempts to monitor the serological response of animals to *C. chauvoei* vaccines were directed at quantitating antibody with an agglutination assay.[72] While this test appeared to correlate to animal protection tests, difficulties were encountered with autoagglutination. A later version of the agglutination test which used washed, formalinized whole cells as the antigen was proposed by Claus and Macheak.[33] This test also demonstrated strong correlation to animal protection tests, but controversy over the protective ability of flagellar antigen precluded widespread acceptance since the test primarily measured flagellar antibody.[73] A flocculation assay, was developed to measure the level of protective antibody in sheep sera.[74] The purpose of this test, however, was for selection of negative sheep for challenge studies and for use as a diagnostic aid, not for potency testing vaccines.

Several other assay procedures for *C. chauvoei* vaccines have been proposed. An indirect hemagglutination assay in which sonicated antigen, mainly of flagellar origin, was

adsorbed to treated red blood cells demonstrated good specificity.[75] A strong correlation between results of this test, the agglutination assay, and a mouse protection test was established.[76] Results from an enzyme-linked immunosorbent assay (ELISA) which used culture filtrate as the solid phase antigen to quantitate the antibody response of vaccinated mice were shown to parallel the results obtained in a mouse protection assay.[77] Another ELISA which used whole culture as the solid phase antigen also correlated with the guinea pig protection test.[78] However, the specificity of the test was not addressed. Specificity is important since cross-reactivity between clostridial antigens from different species has been frequently observed.[5,79,80]

Flagella as Immunogens and Test Antigens

Flagella are major antigenic determinants in many bacteria and have been identified as virulence factors in some species such as *Vibrio cholerae*, *Campylobacter jejuni*, and *Salmonella typhimurium*.[81] The motility imparted to bacteria by flagella is important in establishing infections. For example, motility in *Treponema pallidum*, the etiological agent of venereal syphilis, has been shown to contribute to its ability to invade host tissue.[82] Antibodies to the flagella of *Pseudomonas aeruginosa* have been shown to be passively

protective, and non-motile variants exhibited reduced virulence in animal models.[83] Also, aflagellate variants of *Helicobacter pylori* were, in contrast to flagellate organisms, unable to colonize the gastric mucosa of gnotobiotic piglets.[84] Evidence suggests flagella may also act as adhesins, thus influencing pathogenesis in ways other than through motility.[85] Even the direction of flagellar rotation relative to the bacterial cell has been shown to influence pathogenicity in *Salmonella typhimurium*.[86]

Flagellar antigens frequently exhibit species or even strain specificities making them attractive antigens for use in diagnostic tests. Enzyme-linked immunosorbent assays using purified flagellar antigen have been described for diagnosis of congenital syphilis,[87] Tyzzer's disease,[88] and Lyme borreliosis.[89,90] In each of these three cases, the specificity and sensitivity of the diagnostic test was enhanced when flagellar antigen was used in place of whole cell antigens.

PAPER:

IDENTIFYING AND PURIFYING PROTECTIVE IMMUNOGENS

FROM CULTURES OF *CLOSTRIDIUM CHAUVOEI*

Identifying and purifying protective immunogens from cultures of *Clostridium chauvoei*

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INTRODUCTION

Clostridium chauvoei is the etiological agent of a disease commonly referred to as "blackleg." Blackleg most commonly affects cattle and sheep and is characterized by a necrotizing myositis, which is rapidly fatal. Vaccination for prevention of this and other clostridial diseases is routinely practiced with the use of multivalent bacterin-toxoids. Humoral immunity has been shown to be the major defense against infection with *C. chauvoei*[1,2], and passive immunity transfer through colostrum and immune serum has been demonstrated.[3,4]

Specific protective antigens of *C. chauvoei* have not been well characterized. Both cellular and soluble antigens have been shown to elicit a protective immune response in guinea pigs[5,6], but controversy exists as to which specific antigens are responsible. Chandler and Gulasekharam[7] reported that the protective cellular antigenicity of *C. chauvoei* was not of flagellar origin but due to a heat-labile, pH sensitive, nonagglutinogenic, somatic antigen. In contrast, Tamura et al.[8] used a partially purified flagellar preparation and a non-flagellated mutant to show that flagella were important for protective immunity in mice. Flagella-specific monoclonal antibodies which are passively protective in mice have also been produced[9].

The protective antigens present in the supernatant of
C. chauvoei cultures also remain ambiguous. Bergman[10] reported
that the hemolysin of *C. chauvoei*, designated as α toxin, was
capable of inducing protective immunity. Bergman stated that
level of hemotoxoid in a vaccine correlates to the immunizing
power of the vaccine. An investigation by Verpoorte et al.[11],
however, revealed that purified hemolysin was poorly
protective but a larger molecule, which itself was not
hemolytic, could induce protective immunity and high
antihemolysin titers. This led to speculation that an
important soluble protective antigen for *C. chauvoei* was a
molecule which dissociated into hemolysin. Claus et al.[12] also
studied the immunizing properties of culture filtrate but made
no attempt to separate individual protein antigens.

Many of the antigens contained in a multivalent
clostridial bacterin-toxoid are cross-reactive between
species,[13,14] making the development of a serological potency
test to evaluate the *C. chauvoei* fraction difficult. The
objective of this investigation was to identify and purify
specific protein antigens of *C. chauvoei* which are capable of
eliciting a protective immune response in guinea pigs.
Identification of specific protective antigens may facilitate
development of a serological test that could be used in place
of the current guinea pig challenge for assaying the potency
of *C. chauvoei* bacterins.

MATERIALS AND METHODS

Bacterial Strains

Clostridium chauvoei, IRP 206 was used for preparation of all antigens as well as spore challenge in this experiment.

Culture Methods

The culture medium consists of 55.5 g/l of dehydrated Brain Heart Infusion (BHI) and 3.5 ml/l of a 25% glucose solution in 0.01 M phosphate buffered saline (PBS), pH 7.2. Dialysis tubing (12-14 Kd) containing 200 ml of 0.01 M PBS was suspended in the BHI, and the solutions were allowed to equilibrate overnight in an anaerobic incubator. Four ml of a *C. chauvoei* culture, which was actively growing in liver-egg-brain medium, was inoculated into the dialysis tube. The culture was incubated anaerobically at 37°C. After 22 hours, the cells were harvested from the dialysis tubing and pelleted by centrifugation at 20,000 x g for 30 minutes. The pellet was resuspended in sterile saline containing 0.5% formalin and stored overnight at 4°C. Cells were then removed from the formalin solution by centrifugation at 9000 x g for 30 minutes. The pellet was resuspended in 0.1 M Tris buffer, pH 8.0.

Soluble Antigen

Separation of the Soluble Protein Antigens

The supernatant was added to an equal volume of saturated ammonium sulfate solution to precipitate the soluble protein. One mM EDTA and 1 μM leupeptin were added, and the solution was stored overnight at 4°C. The precipitated proteins were removed from the culture supernatant by centrifugation at 15,000 x g for 30 minutes. The pellets containing the precipitated proteins were resuspended in sterile water, placed in dialysis tubing, and dialyzed against deionized water. Concentrated protein from the soluble phase of 3 liters of culture grown in this manner was combined after dialysis, and the volume was increased to 50 ml with the addition of deionized water. Two ml of carrier ampholyte, pH gradient 4-6, was added to the protein solution. The solution was placed in a preparative isoelectric focusing chamber for 2.5 hours at 12 watts. Twenty fractions were harvested, and the pH of each was recorded. The solutions were adjusted to neutral using either NaOH or HCl. Eighty μl of each fraction was boiled with sodium dodecyl sulfate (SDS) for 3 minutes, then electrophoresed on 10-15% polyacrylamide gels (PAGE). The contents of the gels were electro-transferred to nitrocellulose membranes and stained with a gold colloid stain to ascertain the protein content and purity.

Initial Guinea Pig Protection Tests

Each of the 20 fractions was combined with an equal volume of aluminum hydroxide gel adjuvant and used to immunize groups of two guinea pigs. The guinea pigs received 2 doses (1 ml/dose) at 3-week intervals. Fourteen days after the second dose, one of the two guinea pigs was challenged by injecting virulent *C. chauvoei* spores. Guinea pig deaths were recorded. Serum was collected from the second guinea pig in each group and used to identify protective immunogens through immunoblotting techniques.

Purified Immunogen Protection Test

One protein was chosen as a likely protective immunogen from the initial guinea pig test. The protein was purified by isoelectric focusing, followed by ultrafiltration through a 300-Kd molecular weight cut-off (MWCO) filter. The protein was then dialyzed in a 50-Kd MWCO dialysis tubing. To validate the results of the initial guinea pig protection test, a group of 13 guinea pigs wase vaccinated twice with 100 μg of purified soluble protein at 3-week intervals. Five nonvaccinated controls were also included in the test. Two weeks after the second injection, eight vaccinates and five controls were challenged with an injection of virulent *C. chauvoei* spore suspension. Serum was collected from the remaining five vaccinated guinea pigs and used to prepare

immunoblots.

Immunoblots

Precipitated protein from the culture supernatant and the purified immunogen were electrophoresed on 10% SDS-PAGE gels and electro-transferred to nitrocellulose membranes. Immunoblots were performed using serum collected from the guinea pigs in the guinea pig protection tests. Other immunoblots were made using serum from cattle and rabbits which had been vaccinated with *C. chauvoei* bacterin-toxoids.

Amino Acid Sequencing

The purified protein immunogen was separated by an initial preparative isoelectric focusing of the supernatant as described above, followed by electrophoresis on a 10 % native PAGE gel. The separated proteins were electro-transferred to a PVDF membrane. A portion of the membrane was used for amino acid analysis and N terminal amino acid sequencing. The sequence was compared to the GENE BANK data base using GCG software (Genetics Computer Group, Inc., Madison, WI).

Hemolysin Assay

Samples of culture supernatant, precipitated culture supernatant as well as each isoelectric focused sample were assessed for hemolytic activity. Two-fold serial dilutions of

each of the samples were made in 0.01M PBS, starting at 1:2 and ending at 1:1024. Fifty μl of each dilution was combined with 100 μl of a 1% suspension of bovine red blood cells in 0.01M PBS on a 96 well, round-bottomed microtitration plate. The plates were incubated at 37°C for two hours then transferred to a 4°C refrigerator for 12 hours. The titers were reported as the reciprocal of the highest dilution in which the red blood cells were completely hemolyzed.

Hemolysin Inhibition Assay

Pooled serum samples from groups of guinea pigs vaccinated with the purified soluble antigen, the purified flagellar antigen, and the whole culture bacterin-toxoid were assayed for their ability to neutralize the hemolytic activity of *C. chauvoei* culture supernatant. Two-fold serial dilutions of each serum sample were made in 0.01M PBS, starting at undiluted and ending at 1:1024. Fifty μl of each dilution was combined with 50 μl of *C. chauvoei* culture supernatant and allowed to neutralize for 1 hour at room temperature. One hundred μl of a 1% suspension of bovine red blood cells were then added to each toxin-serum mixture. The 96 well, round-bottomed microtitration plates which contained the mixtures were incubated at 37°C for two hours, then transferred to 4°C for an additional 12 hours. The antihemolytic titers were reported as the highest dilution of serum which completely

neutralized the hemolytic activity of the culture supernatant.

Cellular Antigens

Flagella

Flagella were sheared from the cells by homogenizing the cell suspension for 5 min at 20,000 RPM in a high speed homogenizer. The flagella was purified by differential centrifugation and density gradient ultracentrifugation, as previously described.[6] The flagella was evaluated for purity using electron microscopy and SDS-PAGE. Cross-reactivity with other clostridial antigens was evaluated by enzyme-linked immunoassay (ELISA) using the partially purified flagella as the solid phase antigen. Mono-specific rabbit antiserum samples against *C. novyi*, *C. perfringens* types C and D, *C. septicum, C. tetani, C. hemolyticum,* and *C. sordellii* were tested for reactivity with flagellar protein.

Further purification of the flagella was accomplished by isoelectric focusing of the crude flagellar extract prior to density gradient ultracentrifugation. The technique for isoelectric focusing of the flagellar preparation was the same as that previously described for the soluble antigen. The flagella migrated to an isoelectric point at a pH of approximately 4.25.

Purified flagella was used to immunize a group of 13 guinea pigs. The animals were given two 100-μg doses at 3-

week intervals. Two weeks following the second vaccination, nine of the guinea pigs along with five nonvaccinated control animals were challenged with virulent spores of *C. chauvoei*. Serum was collected from the remaining four vaccinated guinea pigs for use in preparing immunoblots.

Cell Wall Antigen

Deflagellated whole cells were checked for cross-reactivity among clostridial species using an indirect ELISA. Dilute whole cells were used as the solid-phase antigen and coated onto a microtitration plate. Mono-specific rabbit antiserum samples against the clostridial species used to test flagellar antigen were assayed.

RESULTS

In the initial guinea pig protection test, animals vaccinated with soluble fractions having isoelectric points of 4.25 and 4.74-5.15 all survived challenge. The immunoblots done with serum from the guinea pigs in this test indicated that, for the pI 4.74-5.15 samples, a protein with molecular mass of approximately 88 Kd was most likely responsible for conferring protective immunity (Figure 1). The fraction with a pI of 4.25 contained a protein band with a molecular mass of approximately 46 Kd. This pI and molecular mass are identical to that of the flagellar protein. None of the other fractions conferred protective immunity.

In the second guinea pig protection test, the purified soluble protein protected six of eight vaccinated guinea pigs. All control guinea pigs died as a result of the challenge.

Immunoblots of the soluble antigens made using serum from the guinea pigs that were vaccinated but not challenged, demonstrated that protected guinea pigs produced antibody to a single protein which had a molecular mass of approximately 88 Kd and a pI of 4.96 (Figure 2). This protein was present in immunoblots made using serum from both the initial guinea pig protection test and the test using purified protein. No other single protein present in culture supernatant could be determined to be a protective immunogen from these tests; however, the fraction with a pI of 4.25 containing a 46 Kd and

FIGURE 1:   Immunoblots made using serum from guinea pigs
             vaccinated with separated soluble antigens. Each
             lane contains proteins with differing isoelectric
             points from *C. chauvoei* culture supernatant.
             Immunoblots were prepared using the serum from a
             guinea pig vaccinated with the corresponding
             fraction. Guinea pigs vaccinated with fractions 1
             and 3 were not protected. The guinea pig
             vaccinated with fraction 2 (pI 4.25) was protected;
             guinea pigs vaccinated with fractions 4 (pI 4.74)
             and 5 (pI 4.80) survived challenge but were very
             sick. Guinea pigs vaccinated with fractions 6 (pI
             4.91), 7 (pI 5.03), and 8 (pI 5.15) withstood
             challenge with no clinical signs. No other
             fractions demonstrated the 88 Kd protein in an
             immunoblot, and no other fractions were protective
             in guinea pigs.

— 88 Kd
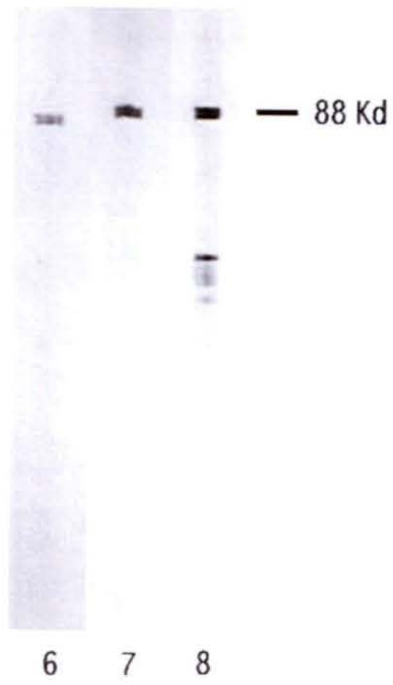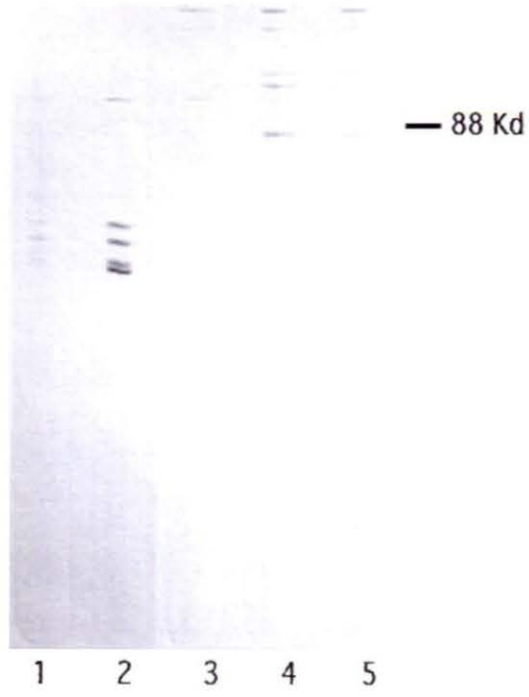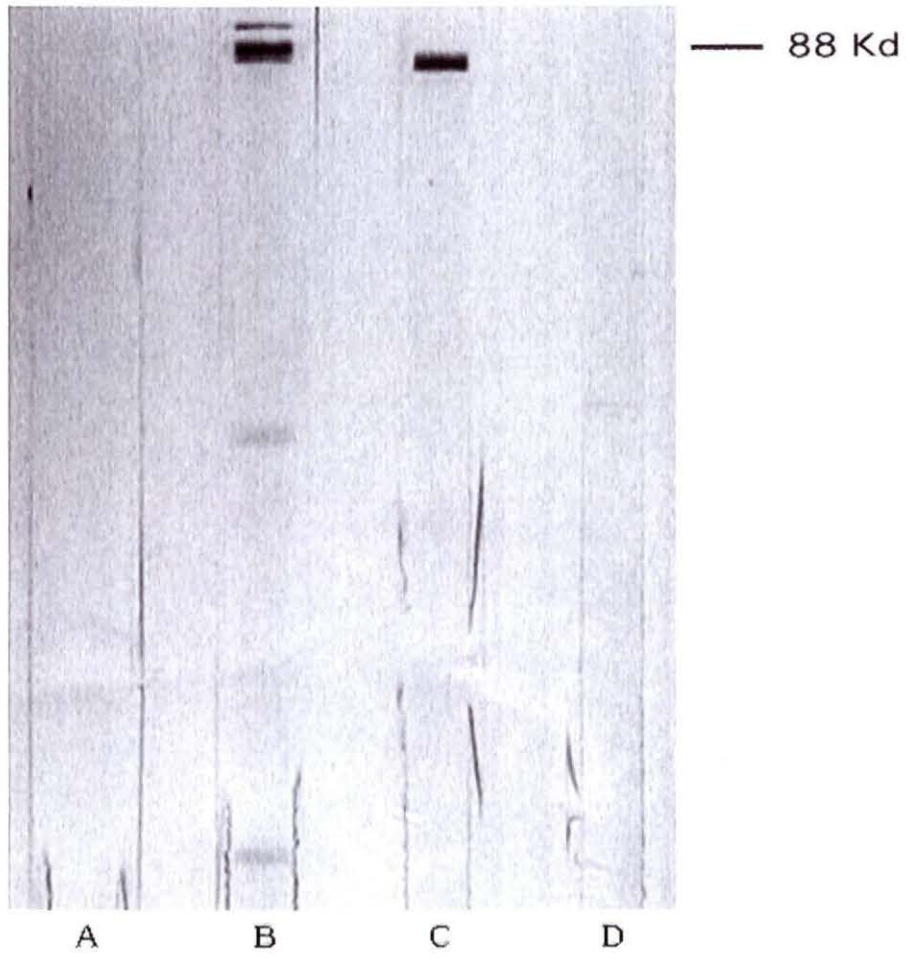
1   2   3   4   5

— 88 Kd

6   7   8

FIGURE 2: Immunoblot made using serum from guinea pigs vaccinated with 88 Kd immunogen of *Clostridium chauvoei*. Lane A: Blank, Lane B: Precipitated toxin, Lane C: Purified 88 Kd immunogen, Lane D: Purified flagellar antigen. Serum from protected guinea pigs reacts almost exclusively with the 88 Kd protein.

other proteins was protective.

Immunoblots made using immune serum from rabbits and cattle demonstrated a major band at the 88-Kd molecular weight location (Figures 3 and 4).

The results of the amino acid analysis of the 88 Kd protein are summarized in table 1. The following N terminal sequence was determined for the 88 Kd protective immunogen:

Gly-Gly-Pro-Val-Gly-Leu-Leu-Gly-XXX-Thr-Arg

This sequence was compared to the GENE BANK data base using the GCG computer program. No homologous or closely homologous sequences were found.

The hemolytic activity of the supernatant, precipitated proteins from the supernatant, and the isoelectric focused fractions of the supernatant proteins are summarized in table 2. The hemolytic activity of the supernatant remained with the precipitated proteins. The isoelectric focusing procedure significantly decreased the hemolyzing ability of the precipitate, however some hemolytic activity remained in fractions grouped around the isoelectric point of 5.90.

The pooled serum samples from guinea pigs vaccinated with whole bacterin-toxoid had an anti-hemolysin titer of 32, while serum from guinea pigs vaccinated with both the purified 88 Kd immunogen as well as the flagellar antigen had no anti-hemolysin titer.

Electron micrographs of the partially purified flagella

FIGURE 3:   Immunoblot demonstrating antigens of *C. chauvoei*
            made using immune bovine serum from cattle
            vaccinated with a commercial bacterin-toxiod.   Lane
            A: Purified flagella, Lane B: Precipitated
            supernatant, Lane C: Whole cells.   All antigens of
            C. chauvoei are represented in the immunoblot.

88 Kd ——

46 Kd ——

A   B   C

FIGURE 4: Immunoblot demonstrating antigens of *C. chauvoei* made using immune rabbit serum from rabbits vaccinated with a commercial bacterin-toxoid. Lane A: Purified flagella, Lane B: Precipitated supernatant, Lane C: Whole cells. All antigens of *C. chauvoei* are represented in the immunoblot.
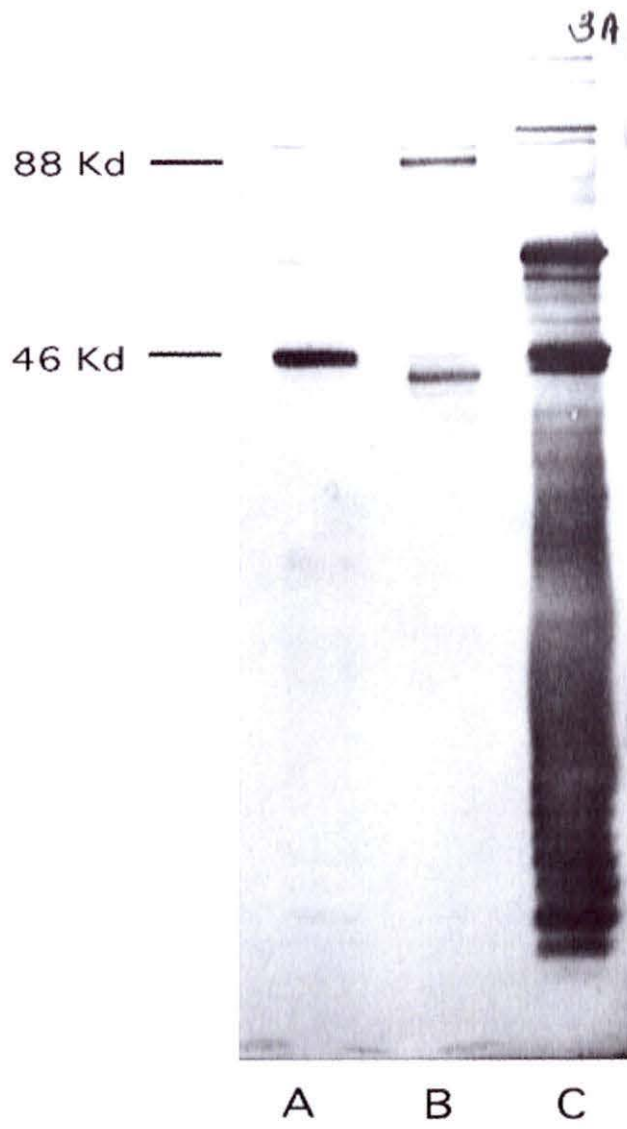
TABLE 1: **Amino acid analysis of 88 Kd protective immunogen from the culture supernatant of *C. chauvoei* culture.**[a,b]

Amino Acid	Mole %	Number of Amino Acids per Molecule
Asparagine and Aspartic Acid	12.8	114
Glutamine and Glutamic Acid	9.0	77
Serine	9.7	81
Glycine	12.5	107
Histidine	0.1	1
Arginine	1.6	11
Threonine	8.6	63
Alanine	6.8	60
Proline	2.5	29
Tyrosine	2.1	17
Valine	7.6	66
Methionine	0.3	5
Isoleucine	7.9	67
Leucine	8.1	69
Phenylalanine	3.2	28
Lysine	7.3	55

[a] The amino acid analysis was done by the Iowa State University Protein Facility.
[b] Tryptophan and Cysteine not included in analysis.

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TABLE 2: Isoelectric points and hemolysin titers of
fractions of *Clostridium chauvoei* culture
supernatant separated by isoelectric focusing.

Fraction		pH	Hemolytic Titer
Supernatant		6.8	32
Precipitated protein		7.0	1024
IEF Fraction #	1.	3.98	4
	2.	4.25	2
	3.	4.44	2
	4.	4.74	2
	5.	4.80	2
	6.	4.91	2
	7.	5.03	2
	8.	5.15	2
	9.	5.25	4
	10.	5.38	8
	11.	5.46	8
	12.	5.54	8
	13.	5.67	16
	14.	5.73	32
	15.	5.80	32
	16.	5.90	64
	17.	5.99	64
	18.	6 15	32
	19.	6.45	16
	20.	6.78	8

FIGURE 5: Scanning electron micrograph of partially purified
flagella from *Clostridium chauvoei* cells (35,000X).
Some cellular debris is visible between the
palisading flagellar fibrils.
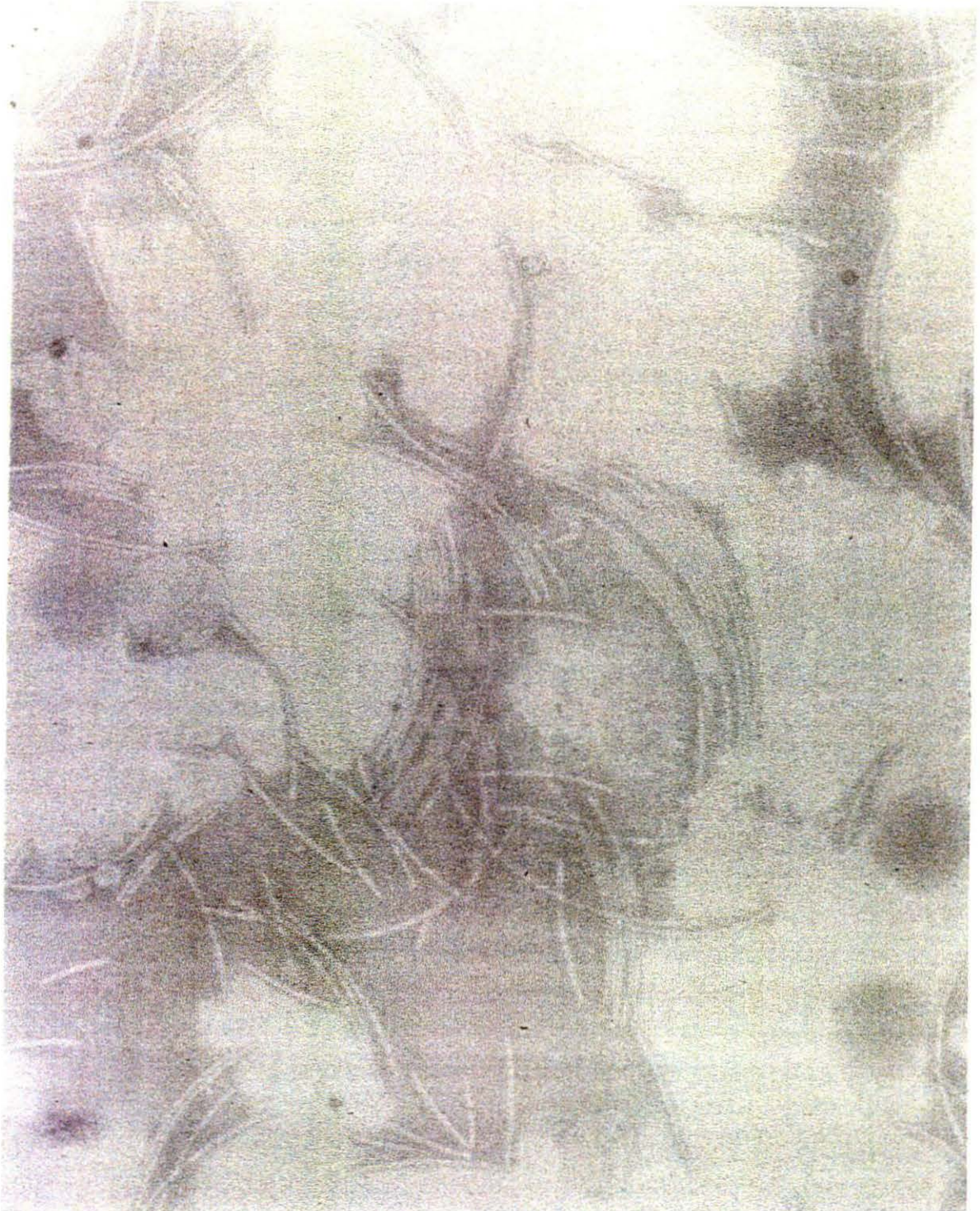
revealed some cellular debris in the preparation (Figure 5). The material was confirmed as cellular debris by the presence of serological cross-reactivity with *C. perfringens* type C antiserum. Since *C. perfringens* is not flagellated, the cross-reactivity was most likely due to cellular components other than flagella. Further purification of the flagella by isoelectric focusing eliminated the cross-reactions and resulted in a pure flagella preparation as evidenced by single bands on SDS-PAGE gels. Purified flagella protected eight out of nine guinea pigs, while none of the non-vaccinated control animals survived challenge. Immunoblots made using the serum from guinea pigs vaccinated with purified flagella demonstrated the purity of the flagellar protein (Figure 6). Protein bands at 46 and 92 Kd were recognized by antibodies of protected guinea pigs.

FIGURE 6: Immunoblot demonstrating the purity of the *Clostridium chauvoei* flagellar preparation. The blot was made using serum from guinea pigs vaccinated with the flagellar preparation. Lane A: Purified flagella, Lane B: Precipitated supernatant, Lane C: Whole cells. All antigens of *C. chauvoei* are represented in the three lanes. The 88 Kd band is visible in lane B even though the protein is not present in sufficient amount in lane A to be visible.

92 Kd ⎯⎯   88 Kd ⎯⎯

46 Kd ⎯⎯

A   B   C

DISCUSSION

Several investigations have been previously undertaken in an attempt to elucidate which immunogens of *C. chauvoei* are protective[6,7,8,11,12,15]. Partially purified flagella, which consisted of a mixture of proteins as demonstrated on SDS-PAGE gels, was shown to be protective in both guinea pigs and mice[8,15]. However, other studies showed that flagella purified by enzymatic treatment or acid extraction are unable to produce protective immunity in guinea pigs[6,7]. In an attempt to clarify these discrepant results, part of this study evaluated the ability of a highly purified flagellar preparation to induce protective immunity in guinea pigs. Interestingly, the serum from guinea pigs that had been vaccinated with purified flagella also contained antibody which reacted with the 88 Kd protein which was concentrated from culture supernatant. The 88 Kd "soluble" antigen either has epitopes in common with flagella, or it is co-purified with the flagella. This raises the possibility that the 88-Kd protein may actually be part of the flagella since enough of this protein may be present in the pure flagellar preparation to induce an immune response in guinea pigs; however, not enough is present to be detected on a stained SDS-PAGE gel or Western blot. It seems likely that a protein with a much higher isoelectric point and molecular weight would be separated by the flagellar purification procedures unless it was physically linked to the flagellum.

This possibility may also explain previous discrepancies reported[7,8] as to whether the cell wall or flagella contained the protective immunogen for *C. chauvoei*. If a protein at the base of the flagellum, such as the hook protein, were a protective immunogen, some of it would be separated along with the flagella, and some would remain in the cell wall. The supernatant would contain flagellar proteins from dead and dying cells, which may dissociate. The 88-Kd protein is concentrated from the supernatant by precipitation and isoelectric focusing. This hypothesis would also be consistent with the observation that the soluble immunogen does not appear in the supernatant until very late in the growth cycle of the bacteria.[12]

Former investigations into the soluble immunogens of *C. chauvoei* also contain inconsistent results.[10,11,12] Data from this study demonstrated that anti-hemolytic activity of serum does not necessarily correlate to animal protection as reported by Bergman[10]. Guinea pigs vaccinated with the 88 Kd protein were protected from challenge, however sera from those animals contained no anti-hemolytic activity. The hemolytic activity of the culture supernatant remained with the precipitated fraction. Some hemolytic activity was lost in the isoelectric focusing procedure with the remaining activity focused around an isoelectric point of approximately 6.0. Guinea pigs vaccinated with protein fractions containing

hemolysin were not protected from challenge.

Deflagellated whole cells cross-reacted with antisera against *C. perfringens* and *C. septicum*. Since methods to separate the shared antigens from those unique to *C. chauvoei* were beyond the scope of this study, no further evaluation was conducted with the deflagellated whole cells.

The flagellum of *C. chauvoei* is a protective immunogen in guinea pigs. The flagella can be purified to an extent which eliminates the cross-reactivity caused by cellular antigens shared with other clostridial species. The immune systems of rabbits, guinea pigs, and cattle all recognize similar antigens of *C. chauvoei* with the flagellar protein being a dominant antigen. The primary protein of the flagella is a molecule with molecular weight of approximately 46 Kd and an isoelectric point of 4.25.

The culture filtrate of *C. chauvoei* contains a protein that is also a protective immunogen in guinea pigs. This protein has a molecular weight of approximately 88 Kd and an isoelectric point of 4.96. The tendency for this molecule to segregate with flagella during purification procedures as well as previous reports of protective immunity being induced by antigens located in the cell wall, leads to the speculation that it may be part of the flagellar base. Further studies are required to determine the cellular location and function of this molecule.

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GENERAL SUMMARY

Clostridium chauvoei is an important animal pathogen which causes a disease in cattle and sheep known as blackleg. Prevention of blackleg is routinely achieved by vaccination of susceptible animals. Vaccine manufacturers currently must test *C. chauvoei* bacterins by immunizing and challenging guinea pigs. An alternate potency test is desirable. Since many antigens in clostridial products are cross-reactive, an alternative test should measure the ability of a vaccine to induce an immune response directed only at protective immunogens of *C. chauvoei*.

Controversy exists in the literature as to which immunogens of *C. chauvoei* are protective. Data from this project identified flagellar protein as being a protective immunogen in guinea pigs. Data also indicated that a protein found in culture supernatant, which has a molecular weight of 88 Kd and a isoelectric point of 4.96, is also a protective immunogen in guinea pigs. This 88 Kd protein co-purifies with flagella and may be a flagella associated structural protein. Evidence was presented that demonstrated similar responses by the immune systems of rabbits and cattle to *C. chauvoei* antigens. Contrary to previous studies, data from this project showed that protective immunogens found in culture supernatant do not necessarily induce antihemolytic

antibodies.

In the appendix of this thesis the procedure for a proposed alternative test for measuring the potency of vaccines containing *C. chauvoei* is outlined. This test measures the immune response of rabbits directed specifically toward flagellar antigen.

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APPENDIX

PROPOSED SUPPLEMENTAL ASSAY METHOD FOR TESTING THE POTENCY

OF PRODUCTS CONTAINING *CLOSTRIDIUM CHAUVOEI* ANTIGEN

INTRODUCTION

This appendix contains the instructions for carrying out an indirect Enzyme-Linked Immunosorbent Assay (ELISA) which compares the *Clostridium chauvoei* flagella-specific antibody content of a sample of pooled rabbit sera to a standard rabbit serum sample. The solid phase antigen used in this test is purified *C. chauvoei* flagella. Flagellar antigen was chosen over the 88 Kd antigen for this test for several reasons. Pilot studies performed in our laboratory indicated that flagellar antigen was more stable than the 88 Kd antigen described in this thesis. Rabbits vaccinated with serials of fresh vaccine containing *C. chauvoei* produced antibodies to both the flagellar and 88 Kd antigen. However, when older vaccine was used to vaccinate rabbits, the antibody response to the 88 Kd antigen decreased in relation to the antibody response to the flagellar antigen. Also, flagella-specific diagnostic tests have been described in the literature making the flagella-based test more acceptable to industry.

More than 1 antigen is probably involved in eliciting a protective immune response to *C. chauvoei*. The ELISA procedure that follows measures the antibody response of a group of rabbits to a single, specific immunogen. Attempting to correlate the potency of *C. chauvoei* vaccines to the immune response of rabbits to a single protein may prove to be futile. It does, however, appear to be a logical place to start.

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SAM 200

9 CFR 113.106   December 31, 1992   Clostridium chauvoei
(Proposed)   Supersedes
                          July 14, 1980

SUPPLEMENTAL ASSAY METHOD FOR

TESTING THE POTENCY

OF PRODUCTS CONTAINING

CLOSTRIDIUM CHAUVOEI ANTIGEN

A. SUMMARY

This test is used to determine the potency of products containing
Clostridium chauvoei antigen. Rabbits are vaccinated twice 20 to 23
days apart and bled 14 to 17 days following the second vaccination.
The serum is assayed for *C. chauvoei* flagella-specific antibody by
indirect enzyme-linked immunosorbent assay (ELISA) and compared to a
reference rabbit serum sample using the USDA Relative Potency Program
(current version).

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B. TEST ANIMAL REQUIREMENTS

1. Nonpregnant New Zealand white rabbits weighing 4 to 8 pounds, free
    of external parasites, are used in this potency test.

2. At least seven rabbits are vaccinated per serial.

C. VACCINATION AND BLEEDING

1. Each sample bottle of product is thoroughly shaken before the
    syringe is filled.

2. Each rabbit is vaccinated subcutaneously in the shoulder region
    with one-half of the smallest host animal dose. 10-ml or 30-ml
    sterile disposable syringes with 20-gauge, 1-inch disposable
    needles are used.

3. A second vaccination is given 20 to 23 days after the first
    vaccination.

4. Fourteen to seventeen days after the second vaccination, rabbits
    are anesthetized with a mixture of xylazine and ketamine. The
    mixture is made by adding 3.6 ml xylazine (20 mg/ml) to 4.8 ml
    ketamine

    (100 mg/ml). The rabbits are given 0.15 ml/kg of this mixture,
    intramuscularly. The rabbits are bled from the heart, using a 13-
    ml evacuated serum separation tube with a 20-gauge, 1½-inch
    needle. Approximately 13 ml of blood is obtained from each
    rabbit. The rabbits are then euthanatized. The tubes of blood
    are gently inverted at least five times and held at room
    temperature until they are centrifuged (approximately 30 minutes).

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5. The tubes are centrifuged at 1000 x g for 10 to 20 minutes at 4°

C. Sera are poured into labeled 16 x 125 mm screw-capped tubes.

Equal volumes of serum from each rabbit vaccinated with the same

serial of product are pooled in a 17 x 100 mm snap-capped tube.

The pool must consist of sera from at least seven rabbits for a

valid test. A sample of the pooled serum may be held at 4-8° C up

to 7 days before the ELISA is run. The remaining serum is stored

at -20° C.

D. ELISA REAGENTS AND MATERIAL

1. **Antigen Coating Buffer**

Na_2CO_3 0.80 g

$NaHCO_3$ 1.45 g

Deionized water   500 ml

The pH is adjusted to 9.6 using 5 M NaOH. The buffer is stored at

4° C in a sealed container for no longer than 1 week.

2. **Antigen**

Clostridium chauvoei purified flagellar antigen (IRP 419),

provided by the National Veterinary Services Laboratories (NVSL),

is used to coat the well surface of the microtitration plate.

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3. **Blocking Solution**

    NaCl   4.25 g

    $NaH_2PO_4 \cdot H_2O$ 0.10 g

    Na_2HPO_4 0.75 g

    Deionized water   500 ml

    Nonfat dry milk   5 g

    Tween 20   .25 ml

    The pH is adjusted to 7.9 with 5 M NaOH. The solution is stored

    at 4° C for no longer than 1 week.

4. **Wash Solution**

    NaCl   8.50 g

    $NaH_2PO_4 \cdot H_2O$ 0.25 g

    Na_2HPO_4 1.19 g

    Tween 20   0.50 ml

    Deionized water   1000 ml

    The pH is adjusted to 7.2 with 5 M NaOH. The solution is stored

    at 4° C for no longer than 4 weeks.

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5. **Serum and Conjugate Diluting Solution** (SCDS)

    NaCl   8.50 g

    $NaH_2PO_4 \cdot H_2O$ 0.25 g

    Na_2HPO_4 1.19 g

    Nonfat dry milk   10 g

    Tween 20   1.0 ml

    Deionized water   1000 ml

    The pH is adjusted to 7.2 with 5 NaOH. The diluent is stored at

    4° C for no longer than 1 week.

6. **Conjugate**

    Peroxidase Conjugated Goat Anti-Rabbit IgG.

7. **Substrate**

    TMB (3,3'5,5'-tetramethylbenzidine) peroxidase substrate system,

    is purchased from Kirkegaard & Perry Laboratories, Inc., 2 Cessna

    Court, Gaithersburg, MD 20879.   (The use of this product by the

    NVSL is not an endorsement over other products not mentioned.)

8. **Stop Solution**

    Stop solution is prepared by adding 24.5 ml H_2SO_4 to 70 ml of H_2O.

    The volume is then raised to 100 ml with H_2O. The stop solution

    may be stored at room temperature for an indefinite period of

    time.

9. **Negative Serum**

    Negative rabbit serum (IRP 420) from the NVSL is used in the ELISA

    as a negative control.

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10. **Reference Rabbit Serum**

Clostridium chauvoei positive rabbit serum (IRP 421) is provided

by the NVSL. This reference was prepared from a serial of product

with the minimum acceptable potency.

D.   ELISA PROCEDURE

1.   **Antigen Immobilization (Coating)**

a.   *Clostridium chauvoei* purified flagella (IRP 419) is diluted in

Antigen Coating Buffer according to instructions that

accompany the antigen sent from the NVSL.

b.   100 µl of diluted antigen is dispensed into each well of a 96-

well microtitration plate. The plate is covered with a lid

and placed in a humid atmosphere.

c.   The plate is incubated overnight at 4-8° C. All incubations

are conducted with agitation on an orbital shaker revolving at

120 cycles per minute.

d.   After the incubation period, the plate is inverted and shaken

to remove excess antigen.

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Clostridium chauvoei

2. Blocking Well Surfaces

   a. Each well of the microtitration plate is filled with Blocking
      Solution (one plate requires approximately 40 ml of Blocking
      Solution).

   b. The plate is covered and incubated in a humid atmosphere at
      35-37° C for 2 hours.

   c. The plate is inverted and shaken to remove excess blocking
      solution. The plate is sealed, placed in a plastic bag, and
      stored at 4° C in an inverted position. Plates can be stored
      for approximately 2 weeks.

3. Addition of Serum Samples

   a. The plate is removed from the plastic bag, and each well is
      washed eight times using approximately 0.3 ml of wash solution
      per well. The inverted plate is tapped on absorbent material
      to remove all traces of residual wash solution.

   b. Negative rabbit serum (IRP 420), *Clostridium chauvoei* positive
      rabbit serum (IRP 421), and test serial rabbit serum are all
      run on the same plate. Only absorbance readings generated
      from the same microtitration plate can be compared for
      relative potency.

   c. The serum samples are diluted 1:100 by adding 100 μl of serum
      to 9.9 ml SCDS. A noncoated microtitration plate is used to
      make serial two-fold dilutions starting with 1:200. 125 μl of
      SCDS is added to each well in columns 2 through 11. 125 μl of

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the 1:100 dilutions of the serum samples is added to the corresponding well in the second column of the plate. Serial two-fold dilutions are made across the rows of the plate (starting with column 2) by transferring 125 μl from one well to the next through column eleven. 100 μl of each dilution is then transferred to the corresponding well on the antigen-coated microtitration plate. The negative serum is placed in row A, the positive reference serum is placed in row B, and test sera are placed in rows C through G. Also, 100 μl SCDS is transferred to each well in column 1. These wells are used to blank the microELISA autoreader. The remaining outside wells are not used. The desired dilution scheme on the seeded microtitration plate is illustrated in Figure 1.

d. The plate is covered and incubated in a humid atmosphere for 1 hour at 35-37° C.

4. Addition of Conjugate

a. The plate is removed from the incubator and washed as previously described in D.3.a.

b. Peroxidase conjugated anti-rabbit IgG is diluted in SCDS to approximately 1:4000.

c. 100 μl of diluted conjugate is added to each well of the plate.

d. The plate is covered and incubated in a humid atmosphere at 35-37° C for 1 hour.

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5. Addition of Substrate

    a. The plate is removed from the incubator and washed as previously described in D.3.a.

    b. 6.0 ml of TMB peroxidase substrate is added to 6.0 ml of peroxidase solution B (H_2O_2).

    c. 100 μl of substrate solution is dispensed into the wells of the plate, beginning with column 1 and continuing through column 12.

    d. The plate is covered and incubated at 20-24° C for 6-8 minutes.

6. Addition of Stop Solution

The reaction is stopped by adding 100 μl of stop solution to each well of the plate. (After stopping, the TMB substrate becomes yellow.)

7. Absorbance Readings

The plates are read using an ELISA reader with dual wavelengths (450 nm test, 650 nm reference). The mean optical density (O.D.) for the blank wells is calculated. The blank mean O.D. is subtracted from each of the test well O.D. values before any data are analyzed.

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E.   DATA PROCESSING AND INTERPRETATION OF TEST RESULTS

   1.   Relative Potency Calculation Method

        The USDA Relative Potency Calculation Program is used to calculate

        the relative potency of the test serial compared to that of the

        reference. The reference and test serial data are entered, and

        the program is executed as outlined in SAM 318 (current version).

        The relative potency value (RP) reported for the test serial will

        be the highest RP of the top scores from each test.

   2.   Requirements for a Valid Assay

        To be considered valid, an assay must meet all of the requirements

        of SAM 318 (current version), as well as requirements for a

        minimum slope and negative control.

        a.   Lines determined by first order linear regression must have a

             correlation coefficient (r) $\geq$ 0.95.

        b.   The reference line and the test serial line must show

             parallelism (slope ratio $\geq$ 0.80).

        c.   All of the negative serum dilutions must have O.D. values

             under 0.2.

        d.   Lines used in the relative potency calculation must have

             slopes with an absolute value greater than 0.2.

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Assays that do not contain valid lines may be repeated

up to a maximum of three times. If a valid assay cannot

be achieved with three independent assays, the serial

will be reported as unsatisfactory.

3.   Requirements for a Satisfactory Serial

To be considered satisfactory, a test serial must have an RP of $\geq$

1.0. Serials with an RP less than 1.0 on a valid assay may be

retested by conducting up to a maximum of three independent

replicate tests in a manner identical to the initial test. If 50

percent or more of the highest RP values of all tests are $\geq$ 1.0,

the serial is satisfactory.