SEROEPIDEMIOLOGY OF A NOVEL ETEC VACCINE: THE KINETICS OF THE IMMUNE RESPONSE TO A NOVEL VACCINE IN A RABBIT MODEL

By

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ABSTRACT

SEROEPIDEMIOLOGY OF A NOVEL ETEC VACCINE: THE KINETICS OF THE IMMUNE RESPONSE TO A NOVEL VACCINE IN A RABBIT MODEL

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Diarrhea is the third most common cause of diseases in the world. Enterotoxigenic E. coli (ETEC) is responsible for over a quarter of reported cases of diarrheal diseases. ETEC produces two toxins that are capable of inducing diarrhea in humans, heat-labile (LT) and heat-stable enterotoxins (ST). It is seen in developing countries that infections with ETEC that produce ST cause the most severe forms of diarrhea. ETEC causes 280-400 million cases of diarrhea annually and is responsible for 380,000 deaths. Having an effective vaccine against this diarrheal disease is currently a high priority.

This study describes the kinetics of the immune response to a recently developed vaccine that is comprised of ST attached to the carrier bovine serum albumin. The vaccine was administered to ten rabbits. This study showed that the vaccine was able to induce significant immune response to ST in eight of the rabbits tested, and a positive neutralization of ST in seven of the ten rabbits. Binding capability was shown to increase over time. After achieving the peak, neutralization capacity, however, was not shown to increase over time at a statistically significant level. A model was constructed to predict neutralization capacity from binding data. In this study seven of ten rabbits mounted an immune response that was able to neutralize 10,000 mouse units or more, as opposed to previous studies that have only showed maximal neutralization upwards of 5,000 mouse units.

DEDICATION

Dedicated to Addison Rae Kathleen Francis.
AMDG

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DD	Diarrheal Diseases
DC	Developing Countries
WHO	World Health Organization
NIH	National Institute of Health
TD	Traveler’s Diarrhea
ETEC	Enterotoxigenic Escherichia coli
EHEC	Enterohemorrhagic Escherichia coli
STEC	Shiga Toxin Producing Escherichia coli
EIEC	Enteroinvasive Escherichia coli
EPEC	Enteropathogenic Escherichia coli
EAEC	Enteroaggregative Escherichia coli
DAEC	Diffuse-Adherence Escherichia coli
LT	Heat-Labile
ST	Heat-Stable
STa	Heat-Stable Toxin
CF	Colonizing Factors
cGMP	Cyclic Guanosine Monophosphate
cAMP	Adenosine Monophosphate
VPO	Vaccine Preventable Outcomes
IgG	Immunoglobulin G
IgA	Immunoglobulin A

ELISA	Enzyme Linked Immunosorbent Assay
BSA	Bovine Serum Albumin
OD	Optical Density
GW:RBWR	Gut Weight to Remaining Body Weight Ratio
FCA	Freund Complete Adjuvant
FIA	Freund Incomplete Adjuvant
SMA	Suckling Mouse Assay
MU	Mouse Unit
NC	Neutralization Capacity
NET	Neutralization End Titer
OR	Odds Ratio
Da	Dalton

Background

Global Impact of Diarrheal Diseases:

Diarrheal diseases (DD) represent a significant proportion of childhood and infant morbidity and mortality in developing countries (DC), as well as a constant global health burden for adults as well as travelers in these areas. In 2006 it was estimated that DD caused 1.9 million deaths per year, ranking diarrheal diseases as the third cause of disease in the world (13). The WHO defines DD as those associated with 3 or more loose or liquid stools a day. The passing of multiple formed stools and the passing of regular paste-like infant stools do not constitute diarrhea. There is no set definition for newborn/infant diarrhea, but it is generally described as a sudden change in the stool of an infant that is typically more watery than normal. Diarrheal diseases are also a major source of morbidity and mortality in neonatal livestock populations effecting cows, sheep, and pigs (52-54). While DD is a major problem within this population it is hard to quantify because of variances in herds, climate, livestock management, and diagnosis of disease (53). Diarrheal diseases can be caused by a multitude of factors including viruses, bacteria, and parasites (3).

It has been reported that DD make up approximately 25% of infant and childhood deaths in developing countries (3). It was estimated that between 1950-1980 there were 3.7-4.6 million deaths per year in children that could be attributed to DD (3,29). Studies have shown that in developing countries there is an estimated childhood incidence rate of 2-10 episodes of diarrhea a year (3). Most cases occur in the first two years of life and then decline as the child ages (3). In 2003 it was estimated that for children under five years of age the mortality rate of DD was 8.2

per 1000 (29). Diarrheal diseases are a major concern for children 5 and under because they can also lead to serious health problems including death.

A study published by Guerrant et al. found that there are serious health impacts for children 2 years and younger who experienced cases of DD early in life. Guerrant et al. found that there was significant evidence for long-term disabilities in these children. Children were shorter than their peers, scored lower on physical fitness tests (Harvard step tests), showed signs of cognitive impairment, and also had started school later than their peers (14). Guerrant et al. attributed this to the cycle of malnutrition that is common with diarrheal illnesses, as well dehydration during the diseases course. The WHO states that malnutrition and dehydration lead to an increase in the likelihood of being infected by a diarrheal disease which in turn leads to dehydration and malnutrition. Dehydration is the most common cause of death in children who have a diarrheal disease (50). The NIH states that children can die within a day from severe dehydration caused by DD (51). While children 5 years and younger make up the majority of cases of DD, adults and elderly are also at risk.

Girard et al. estimated that in the U.S. alone in 2006 there were 250-350 million cases of DD, which hospitalized upwards of 450,000 adults. A major cause for DD in adults is traveler's diarrhea (TD) (2,3,4,30,41). Traveler's diarrhea is commonly defined as the passage of 3 or more loose or watery stools within 24 hours with symptoms presenting during or shortly after travel (2). Other common symptoms that are present with TD are nausea, vomiting, abdominal pain, blood or mucus in stool, sudden urgent bowel movements, and fever (2). Traveler's diarrhea is a disease that has a very significant impact not only on tourism, but a significant economic impact

on business and the military (2). In 2005 it was estimated that TD affected 11 million people annually (2). A 2004 study done using U.S. soldiers reported that more than 70% of soldiers reported having TD (35). The leading cause TD as well as an important pathogen in diarrheal diseases is Escherichia coli, specifically the Enterotoxigenic Escherichia coli strain (2,3,4,13,30,41,45).

Enterotoxigenic *Escherichia coli* (ETEC):

Escherichia coli (E. coli) is a Gram-negative rod-shaped bacterium that is commonly found in the intestines of most warm blooded organisms. There are six major strains of E. coli that cause diarrhea: Enterohemorrhagic E. coli (EHEC) which includes shiga toxin-producing E. coli (STEC) like E. coli 0157, Enterotoxigenic E. coli (ETEC), Enteroinvasive E. coli (EIEC), Enteropathogenic E. coli (EPEC), Enteroaggregative (EAEC), and Diffuse-adherence E. coli (DAEC) (49). ETEC is estimated to cause 280-400 million cases of diarrhea annually, as well as being responsible for 380,000 deaths annually (45). ETEC is the most common pathogen that leads to TD and is estimated at causing 30-60% of TD cases (41), as well as being one the most important enteropathogens affecting impoverished children (2,3,4,13,30,41,45). Globally, ETEC is responsible for 25% of persistent diarrheal episodes and 26% of diarrheal episodes that require hospitalizations (13). It is estimated that 30% of travelers who are stricken with TD caused by ETEC are confined to their room, and 40% are forced to change their travel plans (41). Travelers that return to places they have visited before have a 15% chance of reacquiring TD caused by ETEC (41).

ETEC produces two types of enterotoxins that cause sickness in humans, heat-labile (LT) and

heat stable (ST) toxin (4,7, 11-13,16,17,19-22,33,45). ETEC strains can produce LT only, LT/ST, or ST only (4,7, 11-13,16,17,19-22,33,45). The distribution is roughly equal between the three different types of enterotoxin carrying strains of ETEC (4,7, 11-13,16,17,19-22,33,45). The larger of the two enterotoxins is heat-labile toxin (LT). LT is a large toxin [84,000 Dalton (Da)] and is similar to the toxin produced by cholera in immunogenicity, size, structure, sequence, and mechanism (45). Many studies have shown that LT can be neutralized by the cholera vaccine and other LT specific vaccines; however they are not able to neutralize heat stable toxins (7,8,19-22). Heat stable toxin is a comparable smaller toxin (2,000 Da) and has two different forms, however, only one STa, has been found to produce diarrhea in humans (45). STa is nonimmunogentic because of its small size which makes producing a vaccine for it very difficult (4,7, 11-13,16,17,19-22,33,45). In developing countries it is seen that ETEC strains that contain ST, with or without LT, cause the most severe forms of diarrhea (45). Epidemiological data estimates that 75% of clinical cases are attributed to infection with ST-producing ETEC suggesting that a vaccine to neutralize ST as well as LT a very important goal for public health (45).

ETEC also produces over 25 different colonizing factors (CF) that are immunogentic, but show tight serotype-specificity (13, 45). Colonizing factors allow for ETEC to adhere to the epithelial cells in the small intestine (33). ETEC causes diarrhea by attaching to the epithelial cells in the small intestine using surface adhesions produced by CFs (35). After attaching to the epithelial cells one or more enterotoxins are released depending on the strain of ETEC (35). ST is able to bind to guanylate cyclase receptors on the brush borders of the intestinal villous cells where it causes activation of guanosine monophosphate to form cyclic guanosine monophosphate (cGMP) (9). This leads to an inability of the cells to regulate the absorption of water, sodium, and

chloride resulting in the onset of a diarrhea episode (17). LT works similar to cholera toxin by attaching to the GM1 ganglioside and activating adenylate cyclase (9,52). This then causes an increase in cyclic adenosine monophosphate (cAMP) which leads to a diarrheal episode (52).

As stated earlier ETEC is estimated to cause 280-400 million cases of diarrhea annually, as well as being responsible for 380,000 deaths annually, with most cases of ETEC in children 0-5 years old in developing countries (45). Peak incidence for ETEC is seen within the first 2 years of life, with a significant drop off after 10 years of age (13). Peak incidence for TD caused by ETEC is the spring through summer months (2,32,33). Humans are the natural reservoir for ETEC strains that affect humans (49). Transmission is mainly thought to occur via ingestion of contaminated food and water, with direct human to human transmission with fecal contaminated hands thought to be rare (49). An important area of interest is the possibility of contaminated foods during the weaning period of infants that may lead to the increased incidence rate in that group (49). Incubation time is dependent on type of enterotoxin. Single enterotoxin producing strains, LT or ST only, have been shown to have a typical incubation time of as short as 10-12 hours, whereas the strains that contain both ST and LT enterotoxins have an incubation time upwards of 24-72 hours (49). Humans shed the bacteria for the full length of the sickness in their feces (49).

Literature Review of Vaccine Development:

There is no current FDA approved vaccine for the prevention of ETEC-caused diarrheal disease in humans. The majority of vaccine research had in the past been focused on preventing LT diarrheal episodes. This is mainly due to the fact that LT is a much larger molecule than ST and provides certain levels of immunogenicity, whereas because of the small size of ST it must be

coupled with a carrier in order to induce immunogenicity. Current efforts are focused on providing protection not only against LT strains, but also for ST and LT-ST producing strains, as well as research into the effects of colonizing factors. Current vaccine approaches for ETEC include using cholera toxin vaccine, using a modified semi-purified LT molecule, and using a synthetic ST molecule with different carriers including LT.

Cholera vaccines have shown in multiple studies to be at least partially effective against LT producing ETEC strains, but have shown no significant advantage against ST producing strains of ETEC. B subunit-whole cell cholera vaccine has been shown to produce short term protection against LT only ETEC strains that last during the 3 week booster cycle but drops off sometime with the three month follow up (7). The rate of protection against LT was around 67% which may have largely been due to the fact that while cholera toxin and LT are similar they are not identical (7). Clemens et al. stated that while LT protection fades quickly after boosters are given any protection may afford the patient with time to get medical attention to prevent rapid dehydration due to diarrhea (7).

Another cholera vaccine that has shown to produce short term protection from LT is the Dukoral inactivated vaccine, which is made up of four different heat killed cholera toxins (13). In a study done by Sack et al., researchers used a killed oral vaccine made up of the cholera B subunit and five common CF in the area (36). The study looked at two different outcomes and separated vaccine preventable outcomes (VPO) from other ETEC and (TD) causes (36). Vaccine preventable outcomes were defined as infections that contained the 5 CF that were included in the vaccine or having contained LT (36). The combined CF and cholera B subunit vaccine was

able to show that it significantly protected against VPO, p-value=.037, however, it did not provide protection against ETEC with CFs not included in the vaccine along with other probable causes of TD (36). A study done by Weidermann et al. on Australian travelers comparing a ETEC LT/CF vaccine, cholera vaccine and a placebo showed that there was clinically promising results, only 1 of 6 cases of diarrhea cased by ETEC in the ETEC vaccine group, against LT producing ETEC strains when using the LT/CF vaccine as compared to the cholera vaccine or placebo (70). None of these techniques provided protection against ST producing ETEC strains.

Another strategy of vaccine development has been to use a semi-purified LT to try and induce an immune response. Among these vaccines a fusion LT-ST vaccine has also been developed to try and prevent not only LT, but also ST producing strains of ETEC. Additionally, a purified LT vaccine has been shown to provide protection against LT producing ETEC but it was unable to provide protection against ST (19). The technique of fusion between LT and ST was also researched to see if LT was a suitable carrier for ST. Studies have found that fusion between the two toxins provides protection against LT but only provided protection against ST at low serum dilutions in suckling mice (5,8). Dilution for ST binding by enzyme-linked immunosorbent assay (ELISA) did not reach over a 1/2,048 dilution, with crude ST not showing ELISA binding over a 1/128 dilution (5,8). Vaccines using synthetic ST and different carriers have also been studied. Klipstein and Houghten have shown that synthetic ST acts like native ST and can be used with different carriers to provide immune protection against ST producing ETEC strains (45). In 1983 Klipstein, Engert, and Houghten showed that a synthetic ST peptide attached to porcine immunoglobulin G (IgG) can neutralize ST at about a 1:10 dilution (23).

Seroepidemiology:

Seroepidemiology is defined in the Merriam-Webster Dictionary as “of, relating to, or being epidemiological investigations involving the identification of antibodies to specific antigens in populations of individuals.” Seroepidemiology is an important facet of epidemiology and vaccine research. As stated in the definition, seroepidemiology focuses on antibodies to specific antigens which include not only identification, but also the kinetics, quantifying, and determining immune status of the population. Most seroepidemiological studies start with a baseline serum level of an antibody, usually IgG, and move forward over time looking at the change in the population with regard to antibody levels. This approach can also be used in vaccine testing trials.

Seroepidemiological approaches are useful in vaccine trials because they show the progression of the immune response that can be easily measured and do not always rely on challenging the subject with an antigen. Vaccine seroepidemiology studies are similar to other seroepidemiological studies by establishing a baseline level of an antibody. This can be done through a variety of ways including an ELISA, where antigens are presented in the wells of the plate and serum is allowed to bind. In most cases baseline levels are low if not nonexistent. After the vaccine is administered serum samples are obtained at set intervals. Depending on the vaccine, boosters may also be set up. Blood is then tested for antibodies to the specific antigen that the vaccine was prepared for. Many factors are looked at in a seroepidemiology vaccine study including binding kinetics, neutralization, cross-reactivity, time to protection, and time to antibody degradation.

Seroepidemiology does not focus on the immune response of one single subject. It is not a case

study as to the efficacy of the vaccine. Instead, seroepidemiological vaccine studies look at how the population as a whole responds to the treatment. Therefore, it is not unusual to pool serum samples to test. This is can be done after an assessment of good vs. normal vs. poor responders; it can also be done from the start. Pooling can be done for many reasons, including as a way to get results when there are low amounts of serum collected, or as a way to do multiple tests on the serum without fully bleeding out a rabbit.

Kinetics of Immune Response in a Rabbit Model:

Immunoglobulin A (IgA) along with IgG are primary antibodies in the intestines responsible for protecting the gut from infectious agents. A study by Keren et al. looking at *Shigella dysenteriae* showed that while both IgA and IgG are present in the mucus secreted by the gut, IgG shows up stronger in a serum sample than one taken from intestinal secretions, where as IgA levels in the serum are much lower than those found in a sample from an intestinal secretion when using rabbits (39). Keren et al. showed that when collecting serum samples for antibodies found in the gut, a better response is found by testing IgG instead of IgA since IgG is more common in the blood than IgA (39). When testing directly from isolated ileal loops IgA gives a better response (39). In a seroepidemiology study of *Helicobacter pylori* in children, Granström et al., researchers found that no samples were positive for IgA alone when collecting samples for IgG and IgA concentrations (61).

Various studies have looked at IgG and IgA kinetics with regards to various different antigens. Keren et al. demonstrated that IgG responses could be seen at around 10 days after immunization and followed for 30 days when they began to start to fall when looking at *Shigella dysenteriae*

(39). A study by Vitetta et al. using Trypanosomatidae showed that rabbit antibodies first peak 1-20 days after injection with the rise continuing for 10-40 days after initial peak (56). Vitetta et al. also found that antibody levels can plateau for at least 140 days (56). Another study done by Bauer et al. using diphtheria toxoid and a priming method using *Salmonella* showed that typical rabbit antibodies peaked at 8-15 days after toxoid injection (68).

In rabbits as well as with other models, binding of an antibody in an ELISA assay does not always correspond to neutralization of the toxin. Held et al. found that when vaccinating rabbits with an alkylated botulism toxoid that the ELISA titer was not predictive of potency of the ability of the antibody to neutralize the toxoid (48). The predictive ability of ELISA on neutralization is an important part of immune kinetics. If ELISA binding is predictive of neutralization capacity, then the vaccine can be studied at a reduced cost and without an increased use of lab animals such as suckling mice to determine efficacy. ELISA predictions would allow for a quick inexpensive way to test not only antibody levels in serum, but also the neutralization capacity of the vaccine. The inability to predict neutralization from binding does not correspond to an inferior or weak vaccine. Held et al. reported an increased ability to neutralize the toxoid even though no predictive model for neutralization could be reported.

Rabbits are used as a model for vaccine trials in many studies. In many studies the responses of rabbits are not uniform and some rabbits have been noted as being poor responders. In a study by Staats et al. the variability of the antibody responses from the rabbits that were immunized with two different botulinum neurotoxin A vaccine adjuvants lead to a finding of no significant difference between the groups although one adjuvant had a 10-fold greater antibody response

(65). A study done by Daniel et al. found that rabbit immune responses are not equal across the board (69). In 1965 Daniel et al. reported that in 3 of 4 groups of rabbits that were stimulated with different antigens only 50% of rabbits responded in an immunogenic way (69).

Study Objectives and Methods

STa producing ETEC strains account for roughly two thirds of all ETEC related diarrheal cases and there is a need for an effective vaccine that produces high binding and neutralizing titers against STa, specifically. More research into better ways of presenting the STa peptide to the immune cells is still needed. Previous studies have not shown an adequate dilution factor for protection or binding. Weak binding and neutralization end titers show that there is still much to be done before a usable vaccine against STa producing ETEC strains can be found. This study focuses on using an entirely different carrier than has been proposed in previous studies, bovine serum albumin (BSA).

Objective 1: To study the binding trends of antibodies induced by the vaccine.

In order for the novel vaccine to provide immune protection against STa producing ETEC strains, the vaccine must elicit a strong measurable binding capacity. In addition, for the vaccine to provide lasting immunogenic properties, antibodies must mature over time. The vaccine should also increase antibody concentrations over time instead of staying the same at each vaccination point. This shows that the body is responding to the vaccine and the immune system is able to target and produce more antibodies specific to what the vaccine is presenting. Binding will be measured using ELISA techniques. Binding measures the ability of the antibody to detect the antigen and attach to it. However, it does not mean that the antibody is able to neutralize the toxin.

Binding was done using a serial dilution model. Serum was diluted out 1/1,000, 1/10,000

1/100,000 and 1/1,000,000. Each time serum in sufficient quantities was collected from a rabbit; an ELISA test with that serum was run to determine its binding potential. A serial dilution model was used to make sure that at each time point binding could be measured. Serial dilution allowed for the ELISA to show the highest binding concentration based off dilution and optical density (OD) reading. Positive binding was defined as having an OD reading of two or more standard deviations above the negative control. The negative control was void of any rabbit serum.

Objective 2: To assess the neutralization capacity of the antibodies induced by the vaccine.

The ability to bind to the toxin as stated before does not mean that the antibodies are able to neutralize the toxin and prevent an incidence of a diarrheal episode. Neutralization of the toxin is the ability of the antibody to prevent the toxin from causing pathogenesis in the host by preventing binding of the toxin to the guanylate cyclase receptors. Neutralization does not mean destruction of the ETEC bacteria. ETEC would still bind to the intestinal tract of the host and produce enterotoxins, however, the toxins would be prevented from binding and activating guanylate cyclase, which would prevent diarrhea from developing in the host.

Neutralization was measured using a suckling mouse assay. This is a common method used to detect the ability of a toxin to induce a diarrheal state in a mammal. Suckling mice were injected orally, down their esophagus, to their stomach via a process called gavage. This method of delivery provides for a much more accurate delivery of toxin and serum to the stomach of the mouse. Injection through the skin to the stomach provides a way for the toxin and serum to leave the body via the puncture wound caused by the needle. Gavage relies on the inability of mice to vomit, or forcefully expel the contents of their stomachs. This means that little to no toxin or

serum is lost during oral injection, making it the preferred method. Mice were weighed and injected with a mixture of toxin and serum, about .5mL orally and then left for two hours at 22C. The mice were then euthanized and their intestines were removed and weighed. The cut off point for neutralization was set at a gut weight to remaining body weight ratio (GW:RBWR) of ≤ .083. Neutralization was considered positive if the GW:RBWR was ≤ .083 and was considered negative, meaning the toxin would produce a diarrheal episode if the GW:RBWR was >.083. A negative control was used for each set of assays.

Objective 3: To study the correlation of the binding kinetics of antibodies induced by the vaccine measured by ELISA and the neutralization capacity of the antibodies against STa in sucking mice.

Binding does not correlate to neutralization in all cases. However, when it does predict the ability of the vaccine to prevent the disease, it is of great epidemiological significance. If binding can predict neutralization then populations can be easily screened for vaccine effectiveness cheaply and quickly. This would be very important for travelers and also for military personnel. For this vaccine, as binding ability increases over time there should also be an increase in the ability of the antibodies to neutralize the toxin. Since the vaccine works by binding to the STa peptide, as the antibodies mature and become more avid to STa there should also be an increase in the ability to neutralize since neutralization is simply the binding of the toxin by the antibodies to prevent attachment of STa to its receptor. Over time as binding reaches positive values at higher dilutions there should be a correlation with neutralization potential at higher dilutions, end titers, as well.

Animals:

Ten female New-Zealand albino rabbits were used in this study. The rabbits were housed in approved single cages at the Containment Facility of Michigan State University. Rabbits were checked daily by qualified professionals for health status. Rabbits were kept at approximately 20°C with 55% humidity.

Swiss Webster Mice were used as the source for the suckling mouse assay. Suckling Mice used in neutralization assay were approximately 2 days old.

Vaccine:

The vaccine that was developed for this study is a novel approach to presenting the STa peptide. This vaccine uses STa and attaches it to the carrier BSA. Since STa is such a small molecule and does not produce an immune response it was attached to a larger molecule. Freund Complete Adjuvant (FCA) and Freund Incomplete Adjuvant (FIA) were added to the vaccine as well. FCA and FIA are used to help promoted the immune response of the vaccine. FCA contains dried dead mycobacteria in a water and mineral oil emulsion, where as FIA lacks the mycobacteria and is just the water mineral oil emulsion. FCA was only used at the initial vaccination and the remaining 7 boosters used FIA. Table 1 shows the vaccination schedule of the rabbits.

Each rabbit was injected in 5 locations with 0.2mL of immunogen intradermally. Rabbits were restrained, shaved, and injected in the middle of the back, groin region, and both shoulders. Groin injections where made near to the prefemoral lymph node on both sides of the groin.

Shoulder injections where made near to the prescapular lymph node on each shoulder.

Blood Draws:

Blood draws were preformed on the rabbits at various times throughout the study. Table 2 shows dates of blood collection and which rabbits were collected. Blood/serum samples were not collected for all intervals for a few rabbits. Blood was drawn via the central ear artery. After collection blood was placed at 37°C for one hour to allow for clotting. Clot was allowed to contract overnight at 4°C. Serum was obtained by centrifugation at 2000 rpm for 30 minutes and stored at -20°C. This study focused on IgG and so therefore serum samples were collected. The design of the study was to look at the kinetics of the immune response in rabbits and the seroepidemiological changes over time. In this study rabbits were used as models and not challenged with toxin. Serum was collected from the rabbits and used on suckling mice to determine neutralization and binding of IgG was determined by ELISA. Ileal loops to collect IgA were not used on the rabbits as this was a longitudinal study where each of the 10 rabbits were bleed at intervals to better observe their immune kinetics.

ELISA Procedure:

An indirect antibody-capture enzyme-linked immunosorbent assay (ELISA) was used to measure the binding capacity of the antibodies produced by the vaccine. STa antigen was attached to the solid phase of the well plates and serum was then presented to the wells for reaction purposes. Serum was serially diluted from 1,000 to 1,000,000 by factors of 10.

ELISA procedures were taken from *Design and Evaluation of Immunogenic Escherichia Coli*

Heat-Stable Enterotoxin (STa) and Characterization of the Immune Response in Laboratory Animals by Nasr-Eldin Mohamed M. Aref. Plates were coated with 2.5 µg STa and 100 µg of 0.05M carbonate buffer at a pH of 6.9. Plates were allowed to incubate at 4°C overnight. Plates were then washed four times with a solution of 0.01M PBS in 0.05% Tween-20, then blotted dry. Then each well was injected with 100 µL of a solution of 0.5% cold fish gelatin, 0.01M PBS, and 0.05% Tween-20 to block nonspecific binding sites on the plastic well. Plates were then incubated at 37°C for 30 minutes. Plates were then washed with 0.01M PBS in 0.1% Tween-20 and blotted dry.

Serum was serially diluted from 1,000 to 1,000,000 by factors of 10 and 100µL of diluted serum was added to each well in triplicate. Plates were then incubated at 37°C for 45 minutes. Plates were then rewashed with 0.01M PBS in 0.1%Tween-20 and blotted dry. 100µL of a substrate solution made up of one tablet set of *p*-nitrophenyl phosphate and 5mL of 0.1M diethanolamine buffer at a pH of 9.8 was added to each well. Plates sat for 30 minutes at 37°C to allow for the reaction to develop. Plates were then read using an ELISA plate reader (Molecular Devices "ThemoMax" Microplate Reader with SOFT max Pro 2.6.1) at λ 405 nm. Positive binding threshold was set at an optical density (OD) reading greater than 2 standard deviations above the mean of the negative control. A negative control was set up for each plate. Antibody response to binding was reported as the reciprocal of the end titer, highest dilution factor that produced a positive binding, and by the mean of the triplicate.

Suckling Mouse Assay:

Suckling Mouse Assays (SMA) were used to measure the ability of the antibody to neutralize the

toxin, stopping the onset of the symptoms of ETEC infection, specifically symptoms caused by STa toxin. The majority of SMAs were done in triplicate, however, some serum dilutions were only tested on one mouse, while others where tested against 4-5 mice. Means were taken where able and positive neutralization threshold was set at GW:RBWR of less than or equal to 0.083. Positive neutralization was defined as the ability of the antibodies to neutralize the STa toxin to completion, resulting in a GW:RBWR of less than or equal to 0.083.

Literature on the subject of SMAs looking at intestinal inflammation shows that there are 2 closely related thresholds for neutralization of a toxin and RBWR. The higher of the 2 is set at GW:RBWR less than or equal to 0.09 for neutralization (1,8). The other group uses a threshold of GW:RBWR less than or equal to 0.083 (12,16,21-24, 38). In a study by Giannella et al. looking at optimal conditions for SMA, GW:RBWRs greater than or equal to 0.083 were stated as being positive for toxin activity, meaning that the toxin was able to induce a diarrheal incident.

Suckling Mice Assays were performed by diluting the serum and STa stock solution in various concentrations, brought to a final volume of ~.5 mL, and then orally injected into the suckling mouse. Oral injection to the stomach was determined by Dr. Saeed as superior to the traditional method of injecting through the skin into the stomach. Since rodents are unable to vomit, forcefully expelling the contents of their stomachs, very little of the injection is lost when administered orally, as compared to injecting through the skin into the stomach where abdominal contractions may expel some of the injected solution.

Serum was diluted to various concentrations to test neutralization capacity of the antibodies

produced by the vaccine. Similarly, STa stock solutions concentrations were also used and diluted to get various levels of mouse units (MU). Mouse units are defined as the amount of toxin needed to raise the GW:RBWR of a mouse to greater than 0.083. The combination of serum dilution and STa dilution allowed for testing of neutralization capacity (NC), defined as the amount of MU of STa that would be neutralized by 1 mL of serum. Neutralization end titer (NET) is defined as the highest dilution of serum that can neutralize 1 MU of STa.

Serum/STa dilutions were taken to a final volume of approximately 0.5 mL, and then orally injected into the stomach of the suckling mouse. Suckling mice were then left at room temperature (~22°C) for 2 hours. Mice were then euthanized and weighed. Mice intestines were then removed and weighed and a remaining body weight was recorded. The remaining body weight was divided by the weight of the intestines, and a ratio was obtained. Neutralization as stated before was considered to be positive if the GW:RBWR was less than or equal to 0.083.

Statistical Analysis:

Data was collected from a previous study by Nasr et al. and taken directly from lab-notebooks associated with the study provided by Dr. Saeed. The previous study analyzed the data using Microsoft Excel identifying the trends of responses of the immune kinetics of rabbits. No statistical modeling was used.

SAS proc lifetest was used to test binding and neutralization data. Proc lifetest was used because the data was collected over time like a longitudinal study; however, there were not enough subjects to analyze the data properly as a longitudinal data set. Data was instead viewed as

survival data.

For data collected for objective 1 looking at the ability of the antibody to bind to the toxin, censoring was defined as either having the ability to bind or not. Data was stratified into 4 levels of dilution; 1,000, 10,000, 100,000, 1,000,000. Proc lifetest was used to analyze the time to event (binding) for each of these four levels.

Data for objective 2 looking at the neutralization capacity of the antibodies was also analyzed using proc lifetest. Censoring was defined as having neutralized or failed to neutralize. Data was stratified by neutralization capacity into 8 levels; 800, 2,000, 5,000, 8,000, 10,000, 20,000, 30,000, 40,000. Proc lifetest was used to analyze the time to event (neutralization) for each neutralization capacity.

SAS proc genmod was used for analysis of the ability for binding to predict neutralization capacity.

Results

Rabbits 207 and 208 were classified as poor responders by their inability to neutralize the toxin at any time point. No binding data was ever collected for these 2 rabbits, due in some cases to the poor immune response shown by lack of ability at any level to neutralize, and the failure to collect significant amounts of blood during draws for testing. The previous study by Nasr et al. also classified rabbits into 3 groups on decreasing levels of immune response potential. Three rabbits were placed into group 1, which had the best immune responses of the 10 rabbits (71). In the paper published by Nasr et al. the three rabbits that were assigned to this group were not listed, however, when looking at the data collected for this study as compared to the data in the Nasr et al. paper it is clear that the rabbits in group 1, the best responders, are rabbit ID 204, 209, and 210.

Binding Capability:

Figure 1 shows the survival curve of the ability of the toxin to remain unbound by the antibodies induced by the vaccine. A survival model was used to show the change in time of the ability of the antibodies to bind to the toxin as they mature in the rabbits. Figure 1 represents the survival of the unbound toxin. As time progresses more and more of the toxin is being bound by antibodies, and overtime the ability of diluted serum concentrations to bind to the toxin also increases. No data was collected for rabbit IDs 207 and 208.

At a dilution of 1:1,000 we see it takes very little time for binding to occur. Binding was measured as the time point when first positive binding occurred. For a serum dilution of 1:1,000

binding occurred one week after injection for all rabbits whose blood was collected and run against an ELISA.

At a serum dilution of 1:10,000 we are able to see the progression of the group of rabbits becoming immunocompetent with regards to ability to bind. Figure 2 shows the survival curve for serum dilution 1:10,000, with binding at this level increasing over time. The mean time to event (ST binding) for serum dilution 1:10,000 is 6.750 weeks. At serum dilution of 1:100,000 we see that the first event of binding does not occur till week 14. The mean time to event for this dilution is 18.375 weeks. Figure 3 shows the survival curve for serum dilution 1:100,000, with binding at this level also increasing over time. At serum dilution 1:1,000,000 first event to binding occurs at week 14 similar to 1:100,000 and mean time to event is 18.1667 weeks which is also similar. Figure 4 shows the survival curve for serum dilution 1:1,000,000, again with binding increasing over time. Table 3 shows time to first binding and mean time for each of the serum dilutions.

Neutralization Capacity:

Neutralization capacity (NC) was obtained by serum dilution and dilution of STa stock solution. Eight different NCs were tested on various rabbits. Not every rabbit's serum was subjected to the same potential NC end point. Weak responders such as 207 and 208 were tested at much lower NC, lower serum dilutions/higher STa stock dilutions, whereas rabbits such as 204, 209, and 210 were tested at much higher levels, higher serum dilutions/lower STa stock dilutions. Table 4 shows the maximum NC for each rabbit ID that was reached and the time at which it was reached. For those rabbits that were unable to produce any antibodies that were capable of

neutralizing the toxin the time point is 0. Rabbits considered to be the best responders, 204, 209, and 210, were pooled together toward the end of the study and tested against higher than normal NC end points. The pooled samples started at week 26 and were tested against NC levels of 20,000, 30,000, and 40,000 MU. The pooled sample was able to neutralize 30,000 MU but was unable to reach the 40,000 MU end point.

A survival curve was used to represent the change over time in the ability of the vaccine to produce antibodies that can neutralize the STa toxin. While the survival curve shows that the mean time to neutralization are not significantly different from each other, a clear trend can be seen in figure 5. Table 5 shows the time to first event and mean time for each of the potential NC.

Since good and poor responders are not separated in this analysis due to the small number of observations in the data set, poor responders cause the mean time to event for the NC to trend together. Since poor responders were tested with low NC potentials at later weeks the mean tends to be around week 20 for each NC category. Good responders like rabbit IDs 204, 209, and 210 were never tested at the lower NC because at week 14 they were able neutralize 8,000 MU. Poor responders like 206 and 212 did not start to neutralize the toxin until week 22, where their NC end points were 10,000 MU, as opposed to the good responders which neutralized 20,000 MU by week 15 and rabbit ID 209 who neutralized 30,000 MU by week 21.

Predictive Ability of Binding Kinetics:

A successful model was constructed to predict the neutralization capacity of a serum sample based on its binding characteristics. The model contains a measurement for the OD reading based

on the dilution. OD reading was multiplied by the specific dilution factor for which the reading was taken. This however led to significant values of the estimators being in the range of 10^{-12}, so the products of the OD reading* dilutions were divided by 100. Time was removed from the model because of it being a confounding variable. Time was selected as a confounding variable since as time increased so did the OD reading and dilution factor. The variable time could be used to predict the value of dilution, which would allow for an estimate of OD reading*dilution, so it was removed to prevent confounding. OD reading*Dilution was set at four categories, one for each dilution factor and labeled ODR1-4. ODRs were then looked at to see if there was any interaction between them and a fourth order hierarchical model was formed that included the variable ODR1*ODR2*ODR3*ODR4 and all lower order corresponding variables as well. The full model contains 16 parameters. The fourth order variable of ODR1*ODR2*ODR3* ODR4 was statistically significant at a P-value of <.0001.

The complete prediction model for neutralization capacity is:

NC = (-1528044) + (60143.42*ODR1) + (5643.132*ODR2) + (7304.449*ODR3) + (596.005*ODR4) + (-214.758*ODR1*ODR2) + (-327.662*ODR1*ODR3) + (-23.3212*ODR1*ODR4) + (-34.1582*ODR2*ODR3) + (-.2893*ODR2*ODR4) + (-1.6367*ODR3*ODR4) + (1.527*ODR1*ODR2*ODR3) + (.0016*ODR1*ODR2*ODR4) + (.0707*ODR1*ODR3*ODR4) + (.0064*ODR2*ODR3*ODR4) + (-.0003*ODR1*ODR2*ODR3*ODR4)

A second model was constructed to predict the probability of neutralization being greater than or equal to 10,000 MU. A successful model was constructed that used OD reading, dilution, and binding. The variable of weeks was removed from the model since it was a confounding variable

when looking at OD reading, dilution, and binding. Table 6 shows the odd ratios (OR) and the corresponding statistical values for the model. The threshold of 10,000 MU was selected for this model since 7 of the 10 rabbits were able to achieve neutralization at this capacity and the majority of previous studies were unable to achieve this range of neutralization. The model uses serum dilution 1:1,000 as the reference serum dilution and binding as a binary outcome as seen in objective 1.

The complete prediction model for neutralization greater than or equal to 10,000 MU is:

$$P(\text{Neutralization} \geq 10{,}000\ \text{MU}=1) = (-2.3956) + (-.0001\text{OD}*\text{Reading}) + (.3483*\text{Dilution}10{,}000) + (1.2281*\text{Dilution}100{,}000) + (1.6956*\text{Dilution}1{,}000{,}000) + (2.3350*\text{Binding})$$

Discussion

Rabbits were never challenged with STa, antibody production was only facilitated by vaccination and booster inoculations. As stated earlier not all rabbits were adequate responders in this study. Rabbits 207 and 208 did not produce any data for binding capability and their serum was unable to neutralize the STa toxin at the lowest MU capacity that was tested (800 MU). Serum from rabbit 205 was also unable to neutralize the lowest amount of MU that the serum was tested against (8000 MU). It is unknown why some rabbits where better responders than other, and why some rabbits seemed to fail at mounting an immune response at all.

Binding Capability:

Survival curves for binding data shows that over time there was an increase in the ability of the antibodies produced by the vaccine to bind to the STa toxin. Even though there were differences in the individual rabbits the mean time to event for each of the 4 dilutions was statistically different from one another.

The mean time for binding for serum dilution of 1:1,000 was one week after injection. This was followed by a lag time to the next mean binding time for 1:10,000 as expected and seen in other studies. While some rabbits reached binding at week one for serum dilution 1:10,000 the mean time to binding was not until week 6. Mean time to binding at serum dilution 1:100,000 occurred at week 18. All 8 rabbits that were tested against an ELISA for serum dilution 1:100,000 reached that dilution factor. Only 6 rabbits were able to bind at a serum dilution of 1:1,000,000 resulting in the mean time for this group at week 18 as well.

Binding increased over time as the vaccine was able to stimulate antibody development in the rabbits. Since the rabbits were never challenged with STa, the immune kinetic response is very linear for most of the rabbits. As time increased the antibodies that were produced were able to more readily bind to the STa toxin because of both an increase in quantity and maturity of existing IgG antibodies. The lag between mean times for serum binding can be accounted for by the time it takes for the immune system to mount a response to a vaccine when the body is not being stimulated by the antigen. There was no difference in mean time for serum dilution 1:100,000 and 1:1,000,000 because of the difference in the amount of rabbits in these groups. Rabbit IDs 211 and 213 were unable to positively bind to the toxin at a serum dilution of 1:1,000,000 during the course of the study.

Binding characteristics are an important part of seroepidemiology and clinical vaccine trials. Binding characteristics show how the antibodies will bind to the toxin in the body. Presumably if IgG does not bind to the toxin it will not be able to neutralize the toxin either, and therefore another antibody or mechanism would have to be looked at to assess the body's ability to recognize the antigen. The time it takes for the body to form antibodies specific to an antigen is a very important area of study. If a vaccine is unable to quickly produce antibodies then the effectiveness of it becomes a major issue. The issue of when the vaccine should be administered before it becomes useful to the body is a major factor in the successful use of the vaccine in the real world. The antibodies produced by the vaccine also must remain active in the body for some time. If antibody levels drop to undetectable counts within a week or two after vaccination then the protection afforded by the vaccine has a very limited range of effectiveness.

This study was able to show that not only did the novel vaccine being tested here produce antibodies that were able to bind to the specific antigen they were designed for, that the antibodies that were produced also remained and increased over time without the bacteria having to infect the body and produce STa toxin to stimulate proliferation of IgG. The study also showed that the vaccine, along with a series of boosters can promote a very substantial immune response as evident by the antibodies in the serum being high enough to maintain positive binding at dilution factors of 1:1,000,000.

Neutralization Capacity:

Suckling mice were all naïve to the STa toxin and had no innate immunogenic abilities to neutralize the toxin as seen by the use of controls from the same liter for each SMA preformed. For the 8 MU end points, the mean time to neutralization did not significantly differ between the groups. This resulted in a failure to reject the null hypothesis, and the inability to accept the alternative hypothesis that mean times to neutralization for the 8 MU end points increases over time. The survival curve, fig. 5, does show a clear trend of NC end points increasing over time, however, as stated the means of the individual NC end points are not statistically different. There is also a clear trend that the neutralization capacity increased over time when looking at GW:RBWR for different NC end points. Figures 6-8 show the decreasing trend of the GW:RBWR for NC end points of 8,000 10,000 and 20,000 MU respectively. The decrease in GW:RBWR over time for each end point shows that the antibodies in the serum injected into the suckling mice increased over time in its ability to neutralize the STa toxin and prevent inflammation of the intestine, which is a marker for a diarrheal episode. In figure 6 the trend line passes through the threshold of GW:RBWR 0.083 around week 17 for NC end point 8,000 MU

where as in figure 7 for NC end point 10,000 MU the trend line passes through the threshold of 0.083 at around week 19. The trend line passes through the threshold of GW:RBWR 0.083 at around 21 for NC end point 20,000 MU. Neutralization capacity end points 8,000 10,000 and 20,000 MU were selected because they were the most common end points tested against. Only rabbits in the good responder category were tested against NC end points of 30,000 and 40,000 MU. For NC end point 30,000 MU the trend line passed through the threshold of GW:RBWR 0.083 around week 24. There was no positive neutralization result for serum samples tested against NC end point of 40,000 MU.

The results obtained from neutralization data were not statistically significant for a few reasons. The main reason is the process by which serum samples were analyzed for neutralization capacity. Samples were not pooled like in other studies, instead they were tested individually. This lead to a great degree of variation between the rabbits, which may have been solved if the samples where pooled. There were 3 good responders out of the 10 rabbits who produced serum capable of neutralizing over 10,000 MU, 4 rabbits that were able to neutralize up to 10,000 MU, and 3 rabbits that failed to neutralize the toxin at any neutralization capacity that their serum was tested against. When the good responders were pooled at the end of the study, weeks 26-28, the pooled serum is able to effectively neutralize 30,000 MU and was tested against 40,000 MU but failed to neutralize. The progression of NC end point challenges may have also lead to the lack of difference in mean time points. Neutralization capacity end points were not done in a logical progression resulting in serum from some rabbits being tested one week at a high MU end points and giving a positive result, and then being tested for the following 2-3 weeks at lower MU end points resulting in a grouping of the means around the same time points.

Another factor that may have led to the mean times being statistically non-significant is that the rabbits were not all tested against the same MU end points. When looking at only the good responders, who were tested against all the same end points, using a survival curve for neutralization the mean time points are statistically different from each other. Figure 9 shows the neutralization survival curve for rabbit IDs 204, 209, and 210. These 3 rabbits were only tested against NC end points 8,000, 10,000, 20,000, 30,000, and 40,000 MU. No neutralization attempt was positive at NC end point 40,000 MU. The mean time to neutralization of NC end point 8,000 MU for the 3 rabbits was 14 weeks. At NC end point 10,000 MU the mean time to neutralization was 16 weeks, and for NC end point of 20,000 MU the mean time was 17.75 weeks. The mean time to neutralization for NC end point 30,000 MU for the 3 rabbits was 24.5 weeks. The 4 rabbits that were not in the good responders group, but still produced a positive neutralization response were tested against 800, 8,000, and 10,000 MU NC end points, but not all four were tested against each of the end points. When looking at these 4 rabbits the survival curve for mean time is not significant.

Despite the overall survival curve being non-significant, this study was able to produce 3 good responders that were able to produce higher than normal values for neutralization capacity. Other studies have reported maximum NC end points of under 5,000 MU for the majority of studies with a few obtaining NC end points of 10,000 MU (71). Our study was able to induce 3 rabbits that were able to neutralize 20,000 MU. One of these rabbits was able to neutralize 30,000 MU, and when the 3 were collectively pooled together that the end of the study, the serum from these 3 were able to constantly neutralize 30,000 MU. This is 6 times greater than most studies and 3

times larger than the largest study previously reported.

Predictive Potential of Binding Kinetics:

The ability of binding kinetics to predict neutralization potential is not seen in all antibody responses. In this study we were able to fit a statistically significant model that allows for the prediction of neutralization capacity by using the data gained by an ELISA. ELISA data and the interactions between the data allow for a quick and easy way to predict neutralization without having to run suckling mice assays. This reduces the uses of animals in the testing of the vaccine. The first model was constructed using the product of the 4 dilutions and the corresponding OD readings. These were then tested to see if interactions between these values were significant, resulting in the 4^{th} order interaction between all of the variables being significant (p value <.0001).

The second model was used to better quantify the effect of the ELISA data. The ORs that were produced by the model, in figure 6, show that as binding increases so does the odds of neutralization capacity being ≥ 10,000 MU. The OR for serum dilution 10,000 versus serum dilution 1,000 is 1.4166 meaning that at serum dilution level 1:10,000 the odds of neutralization capacity being ≥ 10,000MU is 1.4166 times greater than it is at serum dilution level 1:1,000. Similarly there is an increase in OR at serum dilution 1:100,000. The OR for serum dilution 100,000 versus serum dilution 1,000 is 3.4148 and 5.4499 for serum dilution 1,000,000 versus serum dilution 1,000. These OR show that as binding capability increases so does the odds that the serum will be able to neutralize at least 10,000 MU.

Time was not used as a variable in either model for two distinct reasons. First and foremost time is a confounding variable when using ODR, OD reading*dilution. The function of time on the variable ODR is linear. As weeks increase so does the dilution factor, which would allow for an estimation of the variable ODR based on a certain time point. The second reason time was not considered for this model is because the ability of the antibodies to predict NC does not rely on the function of the time from vaccination, which is what the variable of time actually represents. The model is designed to predict NC end titer from the results of the binding profile of an ELISA. This formula holds true outside of the scope of the vaccination trial. It is the predictive ability of the antibody, not the vaccine that is modeled. The vaccine is only a stimulant for the immune system to produce antibodies to this antigen by presenting it in an immunogentic way.

The ability of binding kinetics to predict neutralization capacity is of epidemiological and seroepidemiological significance for many reasons. The ability of an ELISA test to predict neutralization capacity means that large populations can easily be tested against STa to obtain neutralization capacity. This allows for larger studies without the increased use of mice in neutralization assays for each vaccinated subject. The use of ELISA does not only mean that there will be a reduction in animal usage, it also greatly reduces the time component of the study. Serum can be drawn, run on an ELISA plate and the results will not only show binding information, but also the current neutralization potential of the sample.

The ability of binding kinetics to predict neutralization end titers (NET) for antibodies associated with this vaccine is a very positive step in its development. Prediction of NET can be very useful in real world applications of the vaccine. In practice, travelers who received the vaccination

schedule against STa would be able to go to a health care facility and get a simple blood test to see if they are currently protected, and also their current antibody level. The benefit of this being that physicians would be able to compare titer results and see if they are dropping and if the patient needed a booster before going to an endemic area. Similar methods could also be used by military facilities to help to alleviate the burden of TD on soldiers going to endemic areas. Depending on the vaccine availability and the current health structure of a country, this tool for diagnosis of immune protection against STa could be used to screen children to set up a working prevention model for early childhood protection from STa infection and the cycle of malnutrition and re-infection that is associated with it. This may lead to a reduction not only in the burden of DD in the child population, but also a reduction in the negative health outcomes associated with early childhood infection with DD, especially in STa producing ETEC strains.

This application of the vaccine and the binding kinetics associated with the antibodies produced is not only limited to human populations. Animal populations are also infected with STa producing ETEC strains and would benefit from the ability to predict NET using ELISA readings. Herd immune status could be looked at quickly and without the use of sucking mice to determine the NET and protection status.

Conclusion

Diarrheal disease due to infection with ETEC is a global burden that requires not only educational preventative measures, but also prophylactic steps such as a vaccine that is designed to protect against LT and ST containing strains. This study is the first step in the development of a vaccine that is capable of protecting individuals against STa toxin producing ETEC strains. This novel vaccine out preformed all existing trials on the two most important aspects of vaccine development, binding potential and neutralization capacity. These two factors together show that the novel vaccine approach used here is a viable contender for becoming the first useful vaccine against STa producing ETEC strains. The antibodies produced were also able to elicit a predictive model that would allow for the rapid testing of serum samples with only an ELISA to determine both binding potential and neutralization capacity, reducing the amount of animals needed in clinical trials.

The high binding potential that was seen in this study shows that the body is able to make antibodies that are very specific to the STa toxin. This study was able to show that using boosters and an initial inoculation, very high levels of antibodies can be reached in a relatively short period of time. The antibodies that were produced increased over time and remained after the boosters were stopped for a period of time.

Binding was reached at dilutions levels of 1:1,000,000 in 7 of 10 rabbits. This is well over any other studies reporting ELISA binding results. The ability to bind at high levels of dilution shows that the vaccine and the corresponding boosters that were given are eliciting an excellent immune response in the rabbits. The new carrier, BSA, was able to present the STa antigen to the immune

cells of the body and cause an immunogenic response.

Neutralization was also higher than any other study had previously reported. A final neutralization end titer (NET) was consistently neutralized at 30,000 MU, 1 MU being the amount of toxin needed to raise the gut weight to remaining body weight ratio to greater than 0.083 in a naïve suckling mouse. Neutralization capacity also increased over time, but due to the immune kinetics in the individual rabbits, mean times to neutralization for each of the 8 NET were statically equal. Taking only the three top responders a clearer trend toward an increase over time in NET can clearly be seen and the mean times to neutralization for the NETs tested within this group were statistically different.

This study was also able to show that binding kinetics of the serum are able to accurately predict the NET of the same serum sample. This is a very useful finding for this study and any future studies looking at the novel vaccine for STa producing ETEC strains using a BSA carrier. Use of the prediction model would allow for the reduced use of animals in future testing which is a goal in all studies. The prediction model may also have benefits outside of the reduction use of animals in studies. It may also allow for a useful tool once the vaccine is distributed for use by the population. Use of ELISA binding kinetics would show not only how well the individuals antibodies are binding to the toxin, but because of the statistical significance of the model, neutralization capacity can also be measured to see how well the person would be protected from STa producing ETEC infection.

This vaccine is of great epidemiological significance. Diarrheal diseases are a major global

burden that targets all ages, especially children, and has an impact not only on the indigenous population living in the endemic areas, but also impacts travelers to those places. Diarrheal diseases also have an impact on economics through TD and on soldiers stationed in endemic areas. Since a major contributor to DD is ETEC a vaccine that may be able to prevent STa related sickness is of great interest. Studies show that around 75% of all clinical cases of ETEC caused DD contain STa (45). Since roughly two-thirds of ETEC strains contain STa producing capability, with roughly one-third of all strains containing only STa production, the development of a novel vaccine against STa specifically is greatly needed. This study shows the first steps to the development of a working STa vaccine. The conjugation of STa and BSA proved to be not only capable of producing antibodies that were able to bind to the toxin, but also was able to produce antibodies that were capable of producing the highest neutralization end titers (NET) seen in any STa vaccine development study, 30,000 MU. This coupled with the ability of ELISA binding kinetics to predict NET makes the BSA conjugated vaccine the top candidate for further research.

Table 1: Schedule of vaccine injections for the ten by date and adjuvant added.

Dose	Date	Adjuvant
1	08/30/07	FCA
2	09/20/07	FIA
3	10/13/07	FIA
4	11/12/07	FIA
5	12/04/07	FIA
6	12/26/07	FIA
7	01/19/08	FIA
8	02/12/08	FIA

Table 2: Blood collection intervals by date and ID for the ten rabbits. Blood/serum samples were not collected for a few rabbits on certain dates.

Date	Rabbits
08/30/07	204, 205, 207-213
09/20/07	204-213
10/12/07	204-213
11/30/07	204-213
12/04/07	204-213
12/15/07	204-206, 209-212
12/31/07	204-213
01/19/08	204, 209, 210
01/25/08	204, 206, 207, 209, 210, 212, 213
02/17/08	204-213
02/28/08	204, 209, 210

Table 3: Time to First Binding Event (IgG Binding to ST) and Mean Time of Serum Dilutions

Serum Dilution	First Binding (Week)	Mean (Week)	Median (Week)
1:1,000	1	1	1
1:10,000	1	6.75	7
1:100,000	14	18.38	17.5
1:1,000,000	14	18.17	17.5

Table 4: Variation in the level of maximal neutralizing immune response mounted by the ten rabbits by week of detection. Mouse Units (MU) are defined as the amount of toxin needed to raise the RBWR of a mouse to greater than 0.083.

Rabbit ID	Max NC (MU)	Week
204	20000	15
205	8000	0
206	10000	22
207	800	0
208	800	0
209	30000	21
210	20000	15
211	10000	26
212	10000	22
213	10000	22
Pooled 204,209,210	30000	28

Table 5: Neutralization Capacity in MU and Time to First Event (ST Neutralization) and Mean Time to Event

Neutralization Capacity (MU)	Time to First Event (Week)	Mean Time (Week)	Median Time (Week)
800	22	22	22
2000	0	0	0
5000	0	0	0
8000	14	17	15
10000	16	20	22
20000	15	17.75	15
30000	21	24.5	24
40000	0	0	0

Table 6: Odds Ratios for Predictive Potential of Binding Kinetics

	Odds Ratio	p-Value
10,000 MU vs 1,000 MU	1.4166	<.0001
100,000 MU vs 1,000 MU	3.4148	.0072
1,000,000MU vs 1,000 MU	5.4499	.0103

Figure 1: IgG-STa Binding ELISA @ 405nm

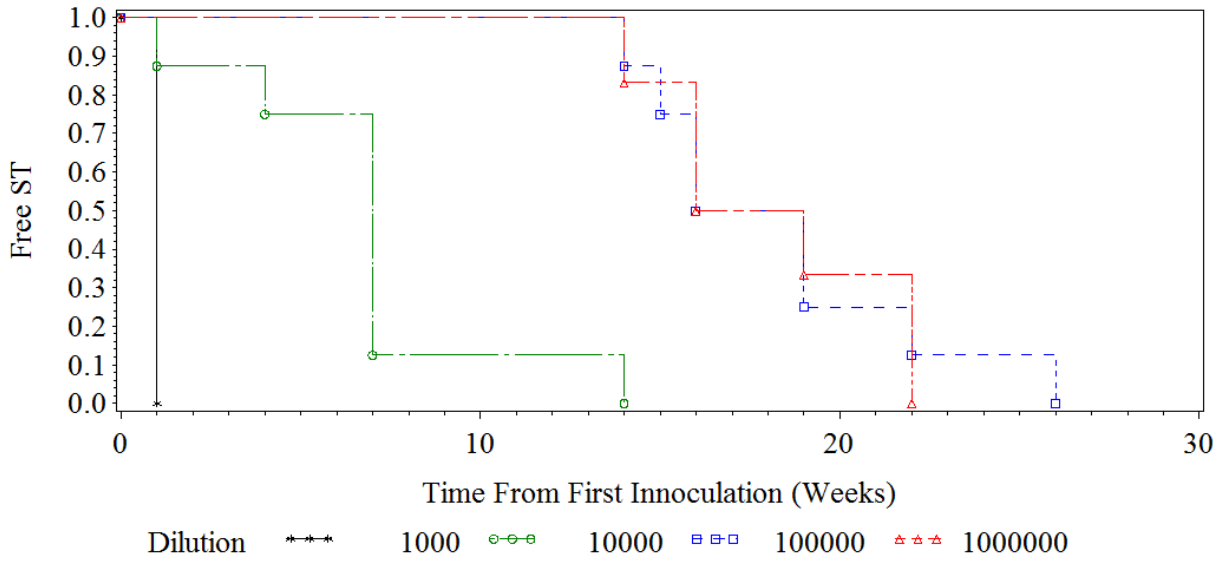

Figure 1: Survival curve for IgG-STa binding for all rabbits at λ 405nm. Curve represents the time until complete IgG binding of STa in the serum. As time increases the ability of the toxin to remain unbound reaches zero for each serum dilution. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

Figure 2: IgG-STa Binding 1:10,000 ELISA @ 405nm

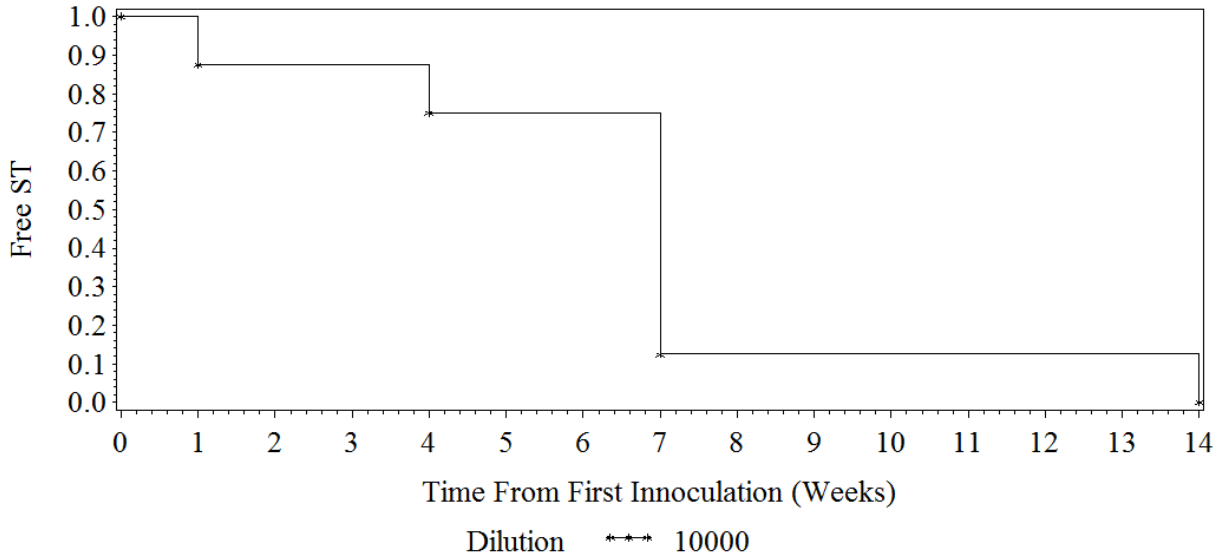

Figure2: Survival curve for IgG-STa binding at serum dilution 1:10,000 for all rabbits at λ 405nm. Curve represents the time until complete IgG binding of STa in the serum at a 1:10,000 dilution.

Figure 3: IgG-STa Binding 1:100,000 ELISA @ 405nm

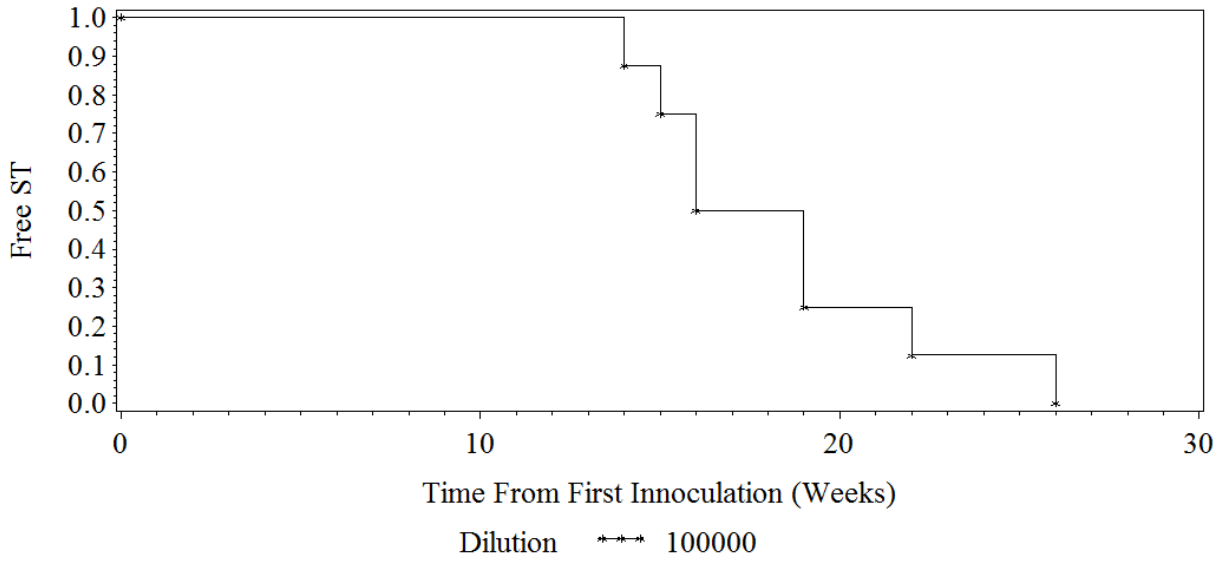

Figure 3: Survival curve for IgG-STa binding at serum dilution 1:100,000 for all rabbits at λ 405nm. Curve represents the time until complete IgG binding of STa in the serum at a 1:100,000 dilution.

Figure 4: IgG-STa Binding 1:1,000,000 ELISA @ 405nm

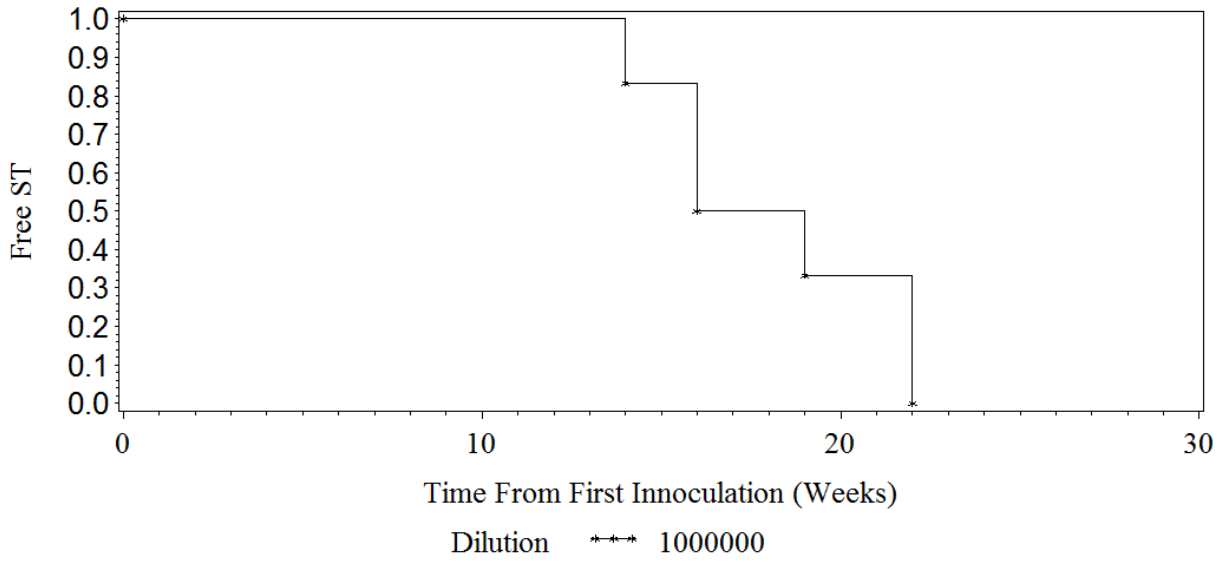

Figure 4: Survival curve for IgG-STa binding at serum dilution 1:1,000,000 for all rabbits at λ 405nm. Curve represents the time until complete IgG binding of STa in the serum at a 1:1,000,000 dilution.

Figure 5:IgG-STa Neutralization Capacity

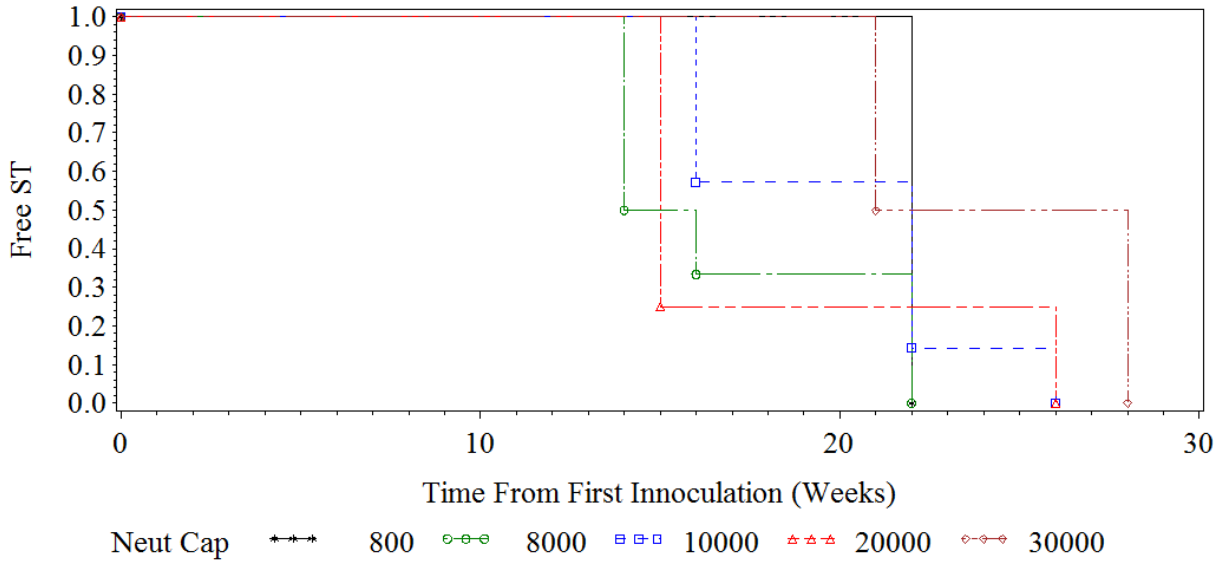

Figure 5: Survival curve for IgG-STa neutralization capacity. Mean times to neutralization for each of the 8 strata were not statistically different. Curve shows the ability of the STa toxin to produce a gut weight to remaining body weight ratio greater than 0.083. As time increases the antibodies produced were able to neutralize higher MU concentrations preventing sickness as defined by gut weight to remaining body weight ratios of less than or equal to 0.083.

Figure 6: Gut Weight to Remaining Body Weight(RBW) MU 8,000

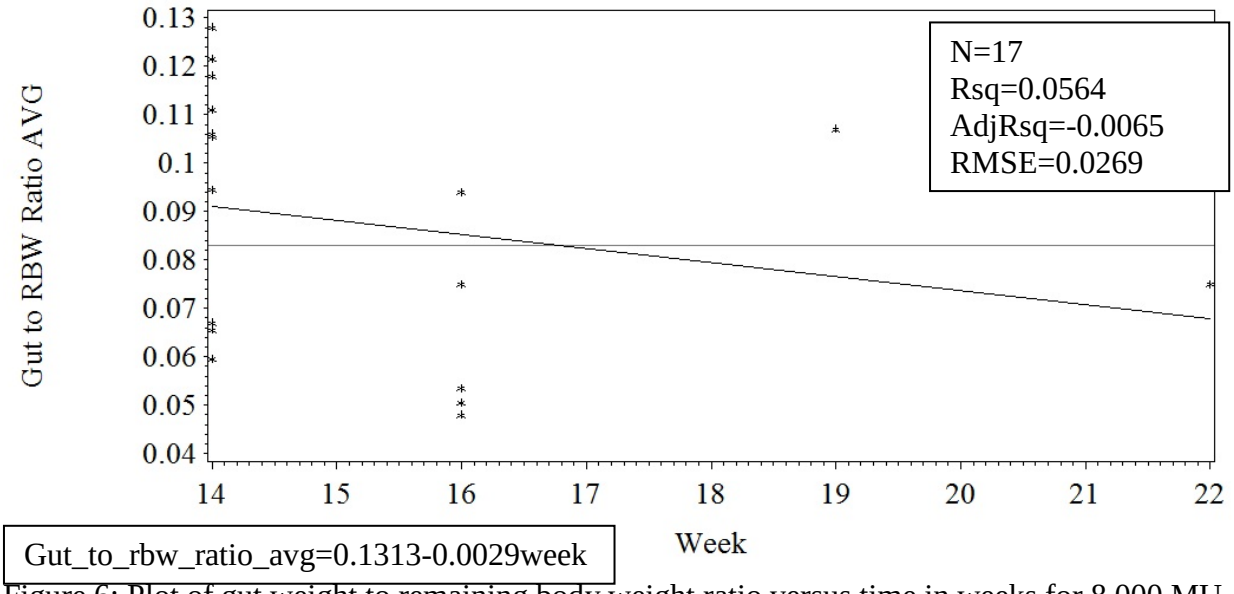

Figure 6: Plot of gut weight to remaining body weight ratio versus time in weeks for 8,000 MU. Ratios above the threshold line of 0.083 represent a failure of the antibodies to neutralize the STa toxin.

Figure 7: Gut Weight to Remaining Body Weight(RBW) MU 10,000

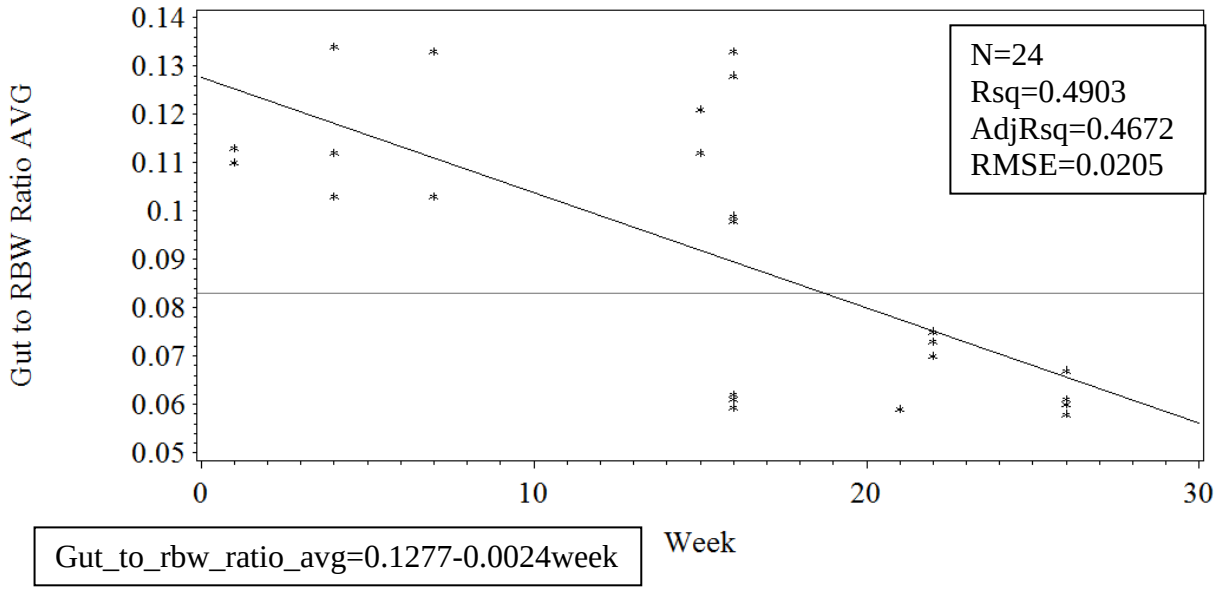

Figure 7: Plot of gut weight to remaining body weight ratio versus time in weeks for 10,000 MU. Ratios above the threshold line of 0.083 represent a failure of the antibodies to neutralize the STa toxin.

Figure 8: Gut Weight to Remaining Body Weight(RBW) MU 20,000

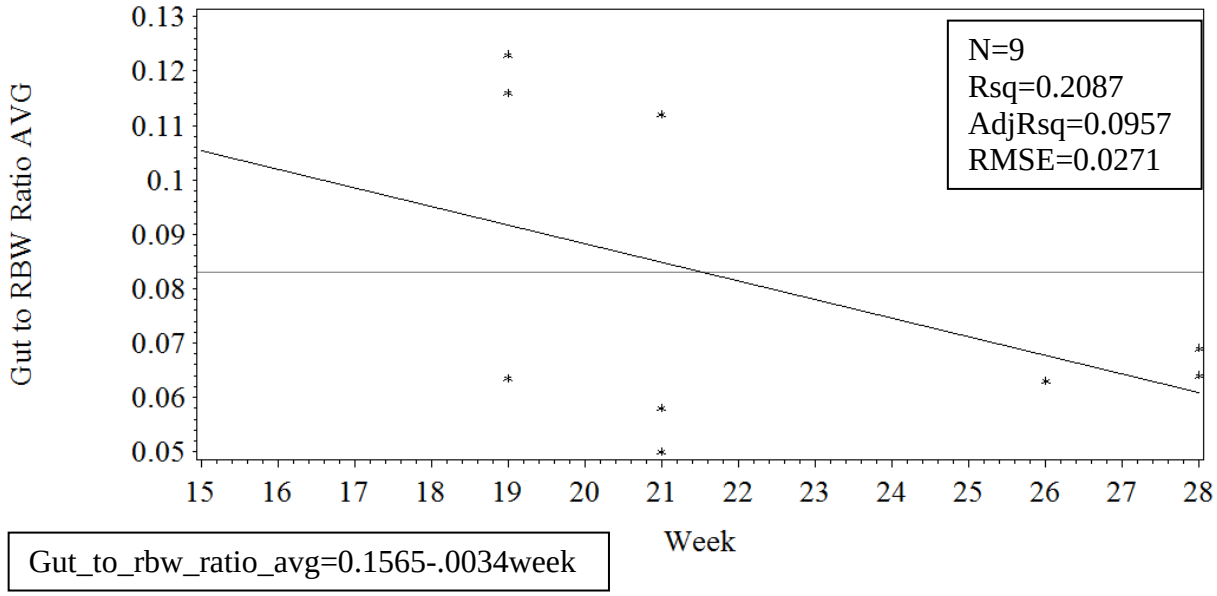

Figure 8: Plot of gut weight to remaining body weight ratio versus time in weeks for 20,000 MU. Ratios above the threshold line of 0.083 represent a failure of the antibodies to neutralize the STa toxin.

Figure 9: IgG-STa Neutralization Capacity Rabbit ID 204, 209, 210

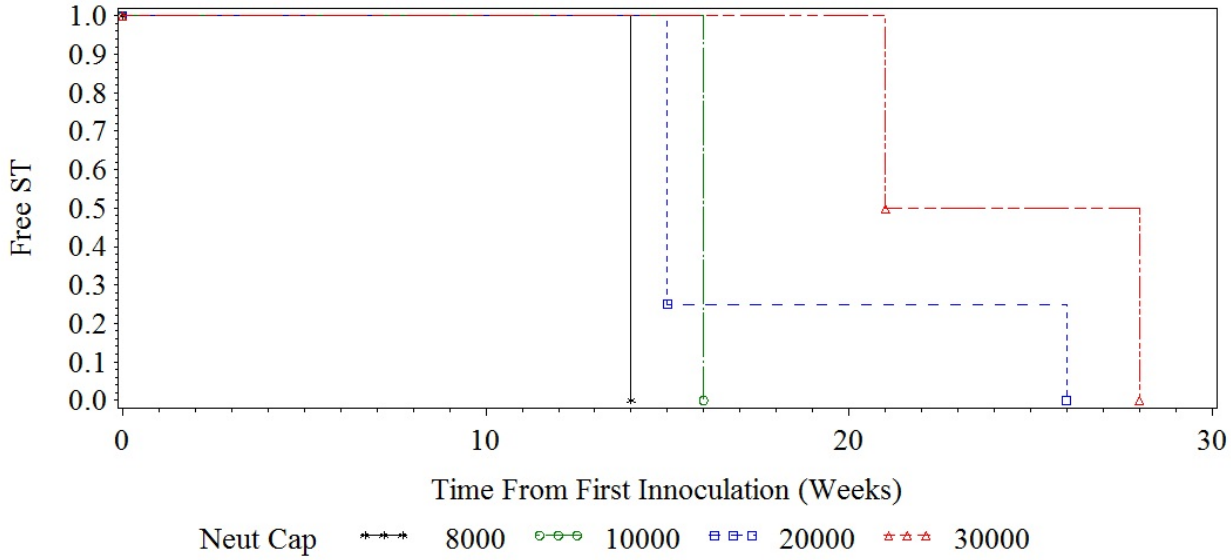

Figure 9: Survival curve of neutralization capacity of IgG-STa for the three good responding rabbits; 204, 209, 210. Mean times to neutralization for the 4 strata are statistically different. Curve shows the ability of the STa toxin to produce a gut weight to remaining body weight ratio greater than 0.083. As time increases the antibodies produced were able to neutralize higher MU concentrations preventing sickness as defined by gut weight to remaining body weight ratios of less than or equal to 0.083.

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