PASSIVE IMMUNIZATION FOR OEDEMA DISEASE
2C-108

Report prepared for the Co-operative Research Centre for High Integrity Australian Pork

By

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Executive Summary

The lack of effective measures to curb mortality rates due to Oedema Disease in affected herds in Queensland in 2010 through 2012 created a crisis for the affected farms.

Antimicrobial drugs were either ineffective from the beginning or became so after periods of treatment. The practice of exposing piglets to live E. coli (recovered from sick pigs on farm) before weaning proved, contrary to earlier experiences in Victoria, to be ineffective in the Queensland cases.

The toxic principle of oedema disease is a toxin expressed by specific E. coli. The toxin, known as STX2e, Shiga-like toxin or Vero-toxin is elucidated after the pathogenic E. coli attach to receptors on the surface of the small intestine. The toxin acts principally on small blood vessels resulting in fluid leakage and accumulation (oedema) in serosal surfaces of the gut, the larynx, centres of satiety in the brain and other tissues.

McLeod and Gyles (1990) demonstrated that inactivated STX2e (administered as a vaccine) was protective. While Paton et al. (2001) and Matsuis et al. (2011) used genetically modified organisms (GMOs) to produce large amounts of toxin; we elected to avoid genetic modification because we perceived a risk to market, consumer and regulatory acceptability of the final product.This led us to look for ways to leverage small amounts of toxin to protect large numbers of pigs in a cost effective manner. McLeod and Gyles (1990) also showed that the efficacy of horse-derived antibodies in protecting pigs from Oedema disease. They harvested plasma from horses vaccinated against STX2e and 1-2mL was shown to be protective in inoculated pigs. Exogenous antibodies have a half-life measured in weeks when administered to a recipient animal. This would allow a program of inoculation at weaning to protect pigs through the “danger period” 7-14 days post weaning.

Our plan was to produce toxin in modest amounts, then use the toxin as a vaccine for administration to horses, and then harvest the immune plasma for injection into piglets at weaning.

In our experiments we produced, from selected bacterial strains, satisfactory protein levels with good potency (toxicity) against Vero-cells. The toxicity of the Protein Extract, as demonstrated by vero-cell culture, was, as expected approximately 1000 fold less than researchers using GMOs technology.

Purification of the toxin from the Protein Extract was problematic. Large amounts of toxin were lost during the harvesting process. This was demonstrated by the equivocal results in the vero-cell assays that were done using the purified Protein Extract. This loss was significant because we started with low concentrations.Animal trials demonstrated that the purified Protein Extract was not toxic to mice. Therefore we have no evidence that the purified Protein Extract contained STX2e.

Despite the failure of this project, the concept of utilising passive immunity as a control method for oedema disease is still sound, subject to a better method of producing and harvesting vSTX2e. The concept provides a research opportunity forward for Australian pig producers in the face of oedema disease outbreaks.

Further efforts in this area should concentrate on increasing the efficiency of recovering toxin from the purification phase and/or increasing the absolute production of toxin such that losses during purification can be tolerated.

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1. Introduction

Oedema Disease has reappeared in pigs on Australian farms in 2010 and 2011. The disease was present in the Australian herd during the 1980-1990 period but was effectively controlled through the use of live autogenous E. coli vaccines given to piglets before weaning. In recent outbreaks in Queensland the disease has been unresponsive to the technologies from the 1980-90 era.

Oedema Disease is linked to the presence of specific toxigenic fimbriated *E. coli* together with changes in the protein characteristics of the diet. The *E. coli* attach via fimbriae to intestinal cell receptors, multiply and produce a toxin that is absorbed through the intestinal wall and disrupts small blood vessels. Tissue damage and fluid leakage follows. The fluid leakage is seen as oedema in different tissues. Damage to specific organs is manifested as a change in satiety, voice, gait and gut function all together or in part. The responsible toxin, STX2e, is also called vero-toxin, SLT2e or Shiga-like Toxin. The term vero-toxin was coined because of the effect of the E. coli toxin on a tissue culture cell line of African green monkey kidney epithelial cells otherwise known as Vero cells.

The disease is frightening in its onset with the triggering factors not well understood. The typical clinical signs are oedema of the eyelids, neurological disorders and sudden death. Growth retardation in survivors is noted in high mortality scenarios.

The impact of the disease depends on the quantity of toxin absorbed, the distribution of the toxin in the body and therefore the organs affected and the number of cells affected in the target organs. Survivors, because of the effect of the toxin on the satiety centres in the brain, fail to thrive.

The impact on affected herds is profound. Losses of between 10% and 20% in each batch of weaners are consistently reported; enough to threaten farm financial viability. The psychological impact on affected farmers is very significant as they have to deal with the loss of their animals without any effective means of control. The welfare of the animals is also obviously compromised. In addition, the disease creates an indication for a higher level of antimicrobial treatment response. The target bacteria have a well-developed capacity to develop resistance to antibiotics and multi-drug resistant strains of E. coli are not unknown.

Currently accepted control measures have failed to control the disease in many affected herds. Anti-microbial resistance of the STX2e producing E. coli to all available veterinary antibiotics is seen on many farms. Conventional immunological approaches are difficult due to the nature of the F18 attachment antigen (it is only expressed at 18-19 days of age) and the timing of the infection soon after weaning, before an immune response can be generated.

Due to the fact that the STX2e is absorbed into the systemic circulation, both active and passive immune approaches utilizing IgG are likely to be successful.

During the 1990s researchers in North America and Europe were able to successfully demonstrate the protective characteristics of a toxoid vaccine prepared from the STX2e (Imberechts et al., 1992; Bosworth et al., 1996; Johansen et al., 1997). As well, passive immunization using an antitoxic plasma derivative raised in horses with an inactivated STX2e (toxoid) has been shown to be effective (Johansen et al, 2000) .

Current practices of immunizing against Oedema Disease (and all *E. coli* diseases in general) rely on creating an immune response to proteins on the bacterial cell surfaces. These proteins are highly variable and so custom vaccines are required to be tailored to individual situations.

Targeting the STX2e has the potential to negate E. coli strain differences and enable a universal prophylactic method to be developed.

The first step in producing a passive immunization product is to establish a protocol to harvest the STX2e, deactivate it and demonstrate the safety of the deactivated toxin in animal models.

As well as the clinical and field experience of the principal investigator, the research team includes Dr Sharon de Wet (Biosecurity Queensland) who has extensive experience with the production of a Botulinum toxoid vaccine. The other team members - Drs Conny Turni and Justine Gibson (UQ) - bring expertise in on-farm pig vaccine trials (Turni), experience with AVPMA (Turni) and research experience with pathogenic forms of *E. coli* (Gibson).

2. Methodology

Laboratory

Identification and characterization of STX2e producing Escherichia coli (STEC)

To obtain a porcine disease-associated STEC isolate expressing STX2e *E.coli,* isolates from the collection at Biosecurity Queensland were screened for STX2e and other virulence genes using multiplex PCR. A total of 126 porcine isolates were analysed.

Detection of virulence genes by PCR

PCR was used for the identification of enterotoxin (STX1, STX2, STa, STb and LT) and fimbrial genes (F4, F5, F6, and F18) as well as the virulence genes for enterohaemolysin (ehxA) and intimin (eaeA).

The assays were carried out as previously described (Ojeniyi *et al.*, 1994; Franklin *et al.*, 1996; Blanco *et al.*, 1997; Paten *et al.*, 1998; Osek *et al.*, 1999) with some modifications. In summary, we used the QIAGEN Multiplex PCR *Plus* kit with 2× QIAGEN multiplex PCR master mix (final concentration, 1×), a 0.16 µM of each primer, and 2 uL of crude template DNA. The volume of this mix was adjusted to 20 uL with sterile water. Crude bacterial DNA template was prepared by picking a single colony from tryptic soy agar (TSA) into 250 uL of 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA and heating at 95-100^{o}C for 10 minutes. Four sets of primer mixes were prepared: set 1 consisted of STX1, STX2, eaeA and ehxA; set 2 of STX2 (a, b, c, d), STX2e, STX2f, and STX2e2g; set 3 of LT, STb, F6 and F18; and set 4 of STa, F4 and F5. The DNA sequences, melting temperatures and predicted size of amplified products for specific primers used in this study are shown in Tables 1 and 2.

Amplification procedures were carried out in a thermal cycler (Perkin-Elmer-Cetus).Reactions for primer sets 2, 3 and 4 were incubated at 94^{o}C for 5 min for 1 cycle, then 94^{o}C for 1 min, then melting temperature for 1 min, and 72^{o}C for 1 min for 30-35 cycles, followed by a final incubation at 72^{o}C for 7 min. Reactions for primer set 1 were incubated at 94^{o}C for 5 min for 1 cycle, then 94^{o}C for 1 min, then melting temperature for 1 min, and 72^{o}C for 1 min for 30-35 cycles, followed by a final incubation at 72^{o}C for 7 min.Reactions for primer set 1 wereto 35 PCR cycles, each consisting of 1 min of denaturation at 95°C; 2 min of annealing at 65°C for the first 10 cycles, decrementing to 60°C by cycle 15; and 1.5 min of elongation at 72°C, incrementing to 2.5 min from cycles 25 to 35. Following the completion of the PCR, 8 µL of amplified product was subjected to gel electrophoresis (2% agarose in 1 x Tris–acetate- EDTA (TAE) with 1 mg ethidium bromide/mL) in running buffer (1 x TAE) at 7.5 V/cm. DNA fragments were

visualized using ultraviolet light (300 nm) and photographed using the Gel Doc 2000 documentation system (Bio-Rad, Australia).

A negative control (all reagents, except template DNA) and positive control (bacterial DNA from strains carrying virulence genes) were included for each PCR. A 1 kb DNA Ladder (Promega) was used as a molecular size marker in each agarose gel.

In Tables 1 and 2, below the terminology stx_{2e} **is** used, which is the name of the gene that transcribes the STX2e toxin

Table 1 - Primer sequences and predicted size of amplification products

Virulence gene	Primers	Oligonucleotide sequences (5'-3')	Tm (°C)	Amplified Products (bp)	Reference
stx_1	STX2e1-F	ATAAATCGCCTATCGTTGACTAC	65/60	180	Paton and Paton (1998)
	STX2e1-R	AGAACGCCCACTGAGATCATC			
stx_2	STX2e2-F	GGCACTGTCTGAAACTGCTCC	65/60	255	Paton and Paton (1998)
	STX2e2-R	TCGCCAGTTATCTGACATTCTG			
eaeA	eaeA-F	GACCCGGCACAAGCATAAGC	65/60	384	Paton and Paton (1998)
	eaeA-R	CCACCTGCAGCAACAAGAGG			
ehxA	ehxA-F	GCATCATCAAGCGTACGTTCC	65/60	534	Paton and Paton (1998)
	ehxA-R	AATGAGCCAAGCTGGTTAAGCT			
LT	LT-1	GGCGACAGATTATACCGTGC	58	696	Blanco *et al.*. (1997)
	LT-2	CCGAATTCTGTTATATATGTC			
F4	F4-F	GGTGATTTCAATGGTTCG	53	764	Franklin *et al.* (1996)
	F4-R	ATTGCTACGTTCAGCGGAGCG			
F5	F5-F	TGGGACTACCAATGCTTCTG	53	450	Ojeniyi *et al.* (1994)
	F5-R	TATCCACCATTAGACGGAGC			
F6	F6-F	TCTGCTCTTAAAGCTACTGG	58	333	Ojeniyi *et al.* (1994)
	F6-R	AACTCCACCGTTTGTATCAG			
STa	STa-1	TCTTTCCCCTCTTTTAGTCAG	53	166	Osek *et al.* (1999)
	STa-2	ACAGGCAGGATTACAACAAAG			
STb	STb-1	ATCGCATTTCTTCTTGCATC	58	172	Blanco *et al.* (1997)
	STb-2	GGGCGCCAAAGCATGCTCC			
F18	FedA-1	GTGAAAAGACTAGTTTATTTC	58	510	Osek *et al.*

Virulence gene	Primers	Oligonucleotide sequences (5'-3')	Tm (°C)	Amplified Products (bp)	Reference
	FedA-2	CTTGTAAGTAACCGCGTAAGC			(1999)
F41	F41-F	GAGGGACTTTCATCTTTTAG	58	431	Ojeniyi *et al.* (1994)
	F41-R	AGTCCATTCCATTTATAGGC			

Table 2 - Primer sequences and predicted size of amplification products for STX2e subtypes

Virulence gene	Primers	Oligonucleotide sequences (5'-3')	Tm (°C)	Amplified Products (bp)	Reference
stx$_{2a,b,c,d}$	STX2-F	GGCACTGTCTGAAAACTGCTCCTGT	55	180	Schmidt *et al.* (2000)?
	STX2-R	ATTAAACTGCACTTCAGCAAATCC			
stx$_{2e}$	STX2e-F	GGCACTGTCTGAAACTGCTCC	55	255	Schmidt *et al.* (2000)
	STX2e-R	TCGCCAGTTATCTGACATTCTG			
stx$_{2f}$	STX2f-F	AGATTGGGCGTCATTCACTGGTTG	55	428	Schmidt *et al.* (2000)
	STX2f-R	TACTTTAATGGCCGCCCTGTCTCC			
stx$_{,2g}$	STX2g-F	GTTATATTTCTGTGGATATC	55	573	? Schmidt *et al.* (2000)
	STX2g-R	GAATAACCGCTACAGTA			

Strain Selection for optimal toxin production in-vitro.

The strains were analysed by PCR as above (Table 2) to determine the presence of the gene sequence capable of producing STX2e. Five strains expressing only the STX2e virulence marker were grown in different media (Tryptone Soy Broth and (MacLeod and Gyles, 1989) at 37° and 42° C, a Polymyxin B extraction performed, and the toxic supernatants titrated and tested on Vero cells. The strains killing the Vero cells at the highest dilution were chosen as candidates for toxin production. Furthermore, when the list of strains was assembled, in consultation with the herd veterinarian, the strains with the highest in vitro toxin level and corresponding to the most severe clinical outcomes were selected.

Toxin Production

A single colony from a TSA culture grown at 37°C was inoculated into eight 100 mL Schott bottles containing 80 mL of glucose syncase broth (MacLeod and Gyles, 1990). Cultures were incubated at 37°C for 18 h shaking at 200 rpm. Eight one-litre Schott bottles containing 800 mL of glucose syncase broth were inoculated with 80 mL of the overnight cultures. Cultures were incubated at 37°C for 18 h shaking at 200 rpm.

Purification of STX2e

STX2e is composed of an A subunit of 27-33kDa in size and a B subunit of 7.5kDa. Purification of the STX2e involved extraction of bacterial cells with Polymyxin B, followed by differential $(NH_4)_2SO_4$ precipitation, anion-exchange fast-protein liquid chromatography, and filtration through a concentrator with a 50 kDa molecular weight cut-off (MWCO).

Polymyxin B extraction

The cultures were then centrifuged at 3,500 x g for 20 min at 4°C, and the bacterial pellet washed twice in phosphate buffered saline, pH 7.4 (PBS). The bacterial pellet from the 6.4-litre culture was suspended in 500 mL of PBS containing 0.2 µg of Polymyxin B sulphate (P4932; Sigma-Aldrich, St Louis, MO, USA) per mL and gently shaken (75 rpm) at 37°C for 2 h before centrifugation at 25,000 x g for 20 min at 4°C. The resulting supernatant (Polymyxin B extracts) was passed through a 0.45 µm-pore-size cellulose membrane filter (Millipore, Bedford, MA, USA). The filtered supernatant was then assayed on Vero cells for STX2e activity.

Ammonium sulphate precipitation

Solid $(NH_4)_2SO_4$ was added to the Polymyxin B extract to 40% saturation, and the mixture was stirred at 4°C for 2 h. The precipitate was removed following by centrifugation at 25, 000 x g for 30 min at 4°C, and the supernatant was adjusted to 60% saturation and stirred at 4°C for 2 h. The second precipitate was collected by centrifugation as described above and dissolved in 20 mL of PBS. The solution was passed through a 0.45 mm filter and desalted by passage through 2 x HiPrep 26/10 Desalting columns (GE Healthcare) equilibrated with 10 mM Tris-HCl, pH 7.6. The sample was then dialyzed at 4°C for 18 h against one change of 250 volumes of 10 mM Tris-HCl, pH 7.6 (dialysis membrane MWCO = 3.5 kDa). Immediately following dialysis, the sample was filtered through a 0.22 µm membrane to remove any precipitated protein.

Anion-exchange chromatography

The dialyzed pooled material from the ammonium sulphate precipitation procedure was applied at 0.5 mL/min to a fast-protein liquid chromatograph anion-exchange column (Mono Q 5/50 GL from GE Healthcare) equilibrated in 10 mM Tris-HCl, pH 7.6. The column was washed with 4.5 mL of the same buffer and developed at 0.5 mL/min with a 45 mL 0.0 to 0.5 M NaCl gradient in 10 mM Tris-HCl, pH 7.6. Elution of the protein was monitored at 280 nm and 1 mL fractions were collected and tested for STX2e activity by Vero cell assay. All steps were performed on an AKTAxpress FPLC system (GE Healthcare) at 4°C. Fractions with peak levels of STX2e activity were pooled and buffer exchanged into PBS using a HiPrep 26/10 Desalting column on an AKTAxpress FPLC system (GE Healthcare) at 4°C. To remove higher molecular weight contaminants, the sample was then filtered using an Amicon Ultra concentrator with a 50 kDa MWCO (Millipore). The final sample was concentrated using an Amicon Ultra concentrator with a 3 kDa MWCO (Millipore) and stored at -80°C in 50% glycerol.

Cytotoxicity assay for STX2e

African green monkey kidney cells (Vero cells; ATCC CCL81; American Type Culture Collection, Rockville, MD) were grown in Eagle modified essential medium (EMEM) with Earle salts supplemented with 2 mM l-glutamine, 5% foetal bovine serum, 100 U of penicillin (Sigma), 100 mg streptomycin/mL (Sigma), and 1mg/mL of fungizone/mL (Sigma). To remove confluent monolayers the cells were washed with PBS, incubated with trypsin-EDTA for 5 minutes at 37°C, and suspended to 4

x 10^5 cells/mL in EMEM. Toxin preparations were serially diluted twofold with EMEM in 96-well tissue culture plates. A 100 µl volume of Vero cells was then aliquoted into each well, and the plates were incubated in 5% CO_2 at 37°C for 72 h. The cells were then washed twice with PBS and incubated with supplemented EMEM containing 10% Alamar blue (Invitrogen) for 2 h in 5% CO_2 at 37°C. The fluorescence of the Alamar blue using a FLUOstar OPTIMA fluorimeter with excitation and emission filters of 544 nm and 590 nm, respectively. Fluorescence readings were used to calculate the cell viability and 50% cytotoxic dose (CD_{50}). Each sample was tested in triplicate and the mean fluorescence of three assays was determined. Each experiment was conducted in duplicate.

During the development of the method each fraction obtained during each step in the process was tested for toxicity, however, the readings were done by eye. Only once the method was established was every fraction read with the Alamar blue staining and all final products were analysed by Alamar blue.

Note on alteration of purification

The method of purification was taken from McLeod and Gyles (1990), which had a cation chromatography step. Using the method with the described method did not achieve binding to the column and a lot of toxin was lost in this step without obtaining the purification of the product. This step was replaced with the 50kDa cut-off filter.

Testing toxicity and safety after inactivation of Protein Extract in mice

Inactivation

The Protein Extract was inactivated by incubating with 0.1% of glutaraldehyde (25% solution, Sigma Aldrich) for 30 minutes at 37°C and then 24 hours at 4°C.

Method

Eighty eight mice (5 weeks old) were housed for five days before starting this experiment. The mice were held in groups of three and five and were allocated randomly to 10 groups.

Toxin group (60 mice)

10 mice injected IP with 0.2 mL of toxin at

1. 0.45 pg/ mouse
2. 0.9 pg/ mouse
3. 1.25 pg/ mouse
4. 1.8 pg/ mouse
5. 3.6 pg/ mouse
6. 7.2 pg/ mouse

Control group (10 mice)

10 mice injected IP with 0.2 mL of phosphate buffered saline with 50% glycerol (storage buffer of the toxin)

Inactivated toxin group (18 mice)

6 adult mice are injected IP with 0.2 mL of inactivated toxin at

1. 1.80 pg./ mouse.
2. 3.6 pg./ mouse.
3. 7.2 pg./ mouse.

Each mouse was scored at 4 hourly intervals and at a combined score of 10 the mouse was euthanased (Table 3).

Table 3 - Scoring criteria: General Health

Criteria	Score and description
Eating	0. Eating and drinking well
	1. Change in eating or drinking habit
	2. Inappetence
	3. Not eating/drinking and severely dehydrated
Locomotion	0. Walking normally
	1. Limping, stiffness
	2. Swollen limbs
	3. Severely restricted mobility
Behaviour	0. Normal
	1. Away from littermates
	2. Aggressive or huddled in the corner
	3. Severe distress
Appearance	0. Normal
	1. Ruffled fur
	2. Animal appears hunched, depressed and reluctant to move.
	3. Animal appears severely depressed
Weight Loss	0. Normal
	1. 5-10%
	2. 10-15%
	3. >15%
Breathing	10. Respiratory distress indicated by open mouth breathing and/or pronounced chest movement
Neurological signs	10. As soon as paralysis observed.
	10. Tremor, convulsion, circling, head tilt, coma

Each animal to be individually scored every 4 hours and score recorded

In the event that an individual animal achieved a score of 10 in total or a score of 3 within a category, then that animal was euthanased.

3. Outcomes

Laboratory

Isolation of isolates picked for purification process

33 isolates out of the 126 isolates tested were positive for *stx2e* virulence gene. Strains with only *stx2e* and no other toxins were selected in order to create assurance that isolated toxin was not contaminated with other toxic factors. *E. coli* strains that came from herds with acute outbreaks were selected from these 33 isolates, and from several strains small amounts of supernatant were obtained and tested with the Vero assay. The isolate with the highest toxicity in the Vero-cell assay was chosen for production and purification of toxin. Unsurprisingly, this isolate came from a herd with an extremely serious clinical picture and was isolated at a time when clinical disease was at its worst.

Production of toxin

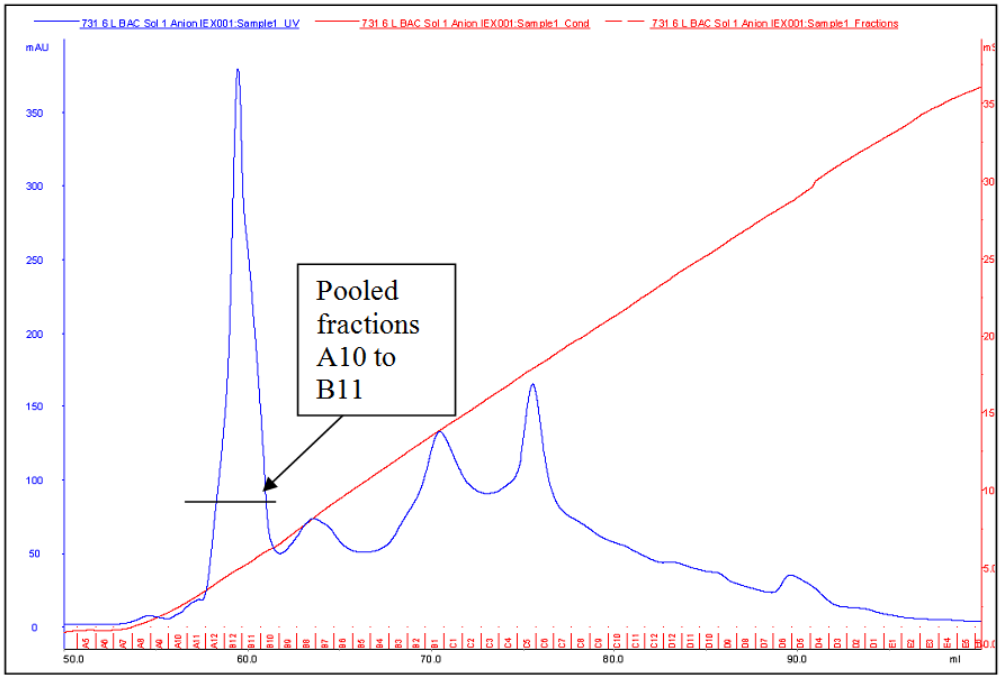
Toxin was produced as per the method ofMcLeod and Gyles (1990) that used GMO *E. coli*. The Vero cell results at this stage indicated that the levels of toxin recovered were 1000 fold less than the reference paper's GMO yields.

Purification of toxin

During the first ammonium sulphate precipitation step (40% saturation), the precipitated protein was not fully pelleted, with a large amount of precipitate remaining in the supernatant. However, when the solution was brought to 60% saturation with ammonium sulphate, the majority of the precipitated protein was pelleted by centrifugation. This behaved similarly to the previous large scale purification.

The method of purification yielded an ion exchange elution profile that was the same as in the previous trial runs. In these trial runs we established the fractions that had to be collected and pooled from the anion exchange chromatography. The pooled fractions are marked in the ion exchange profile Picture 1

Picture 1 – Anion Exchange elution Profile
Fraction collected and pooled are marked



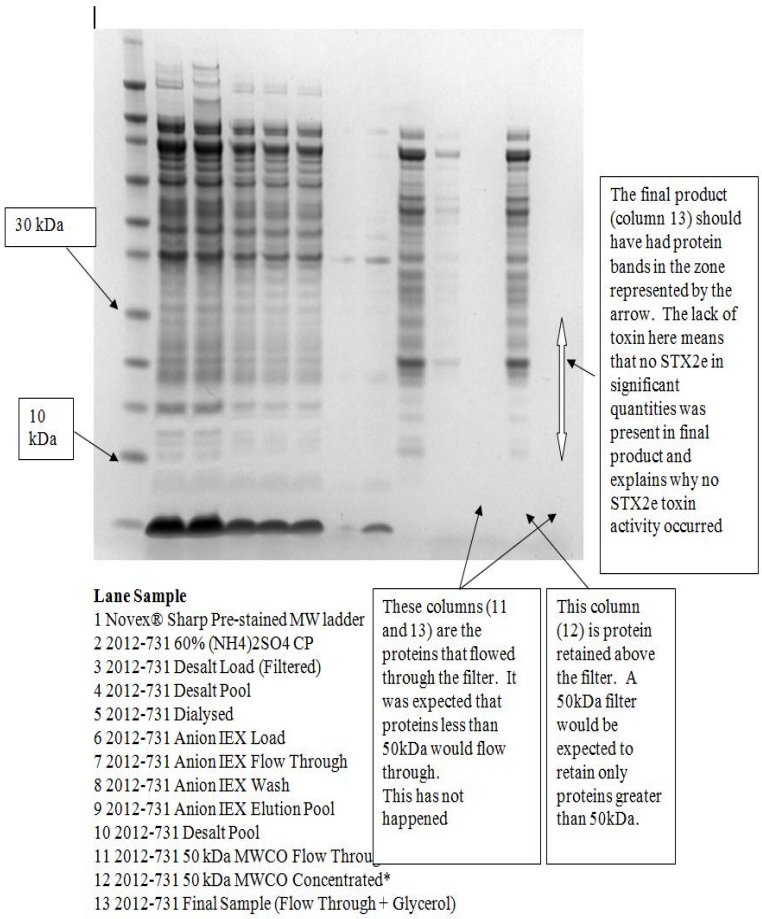
E. coli from 6.4 litres of culture was harvested and incubated with 500mL of PBS containing 0.2 µg of Polymyxin B sulphate. Bacterial residue was removed by ultracentrifugation and filtering. The 500 mL of PBS containing the toxin was then purified. The purification yielded a pure toxin that was much diluted (Picture 2), as can be seen by the protein assay result (<0.1 mg/mL) (Table 4).

Table 4 - The volume, concentration and total protein is given for each sample taken from all steps throughout the purification

Sample	Volume	Concentration of protein	Total Protein
60% (NH4)2SO4 CP	20 mL	1.40 mg/mL	28.0 mg
Desalt Load (Filtered)	20 mL 1.04 mg/mL	1.04 mg/mL	20.8 mg
Desalt Pool	36 mL	0.48 mg/mL	17.3 mg
Dialyzed	37 mL	0.47 mg/mL	17.3 mg
Anion IEX Load (Filtered)	37.5 mL	0.46 mg/mL	17.3 mg
Anion IEX Flow Through	40 mL	<0.1 mg/mL	
Anion IEX Wash	4 mL	0.12 mg/mL	0.48 mg
Anion IEX Elution Pool	5 mL	0.48 mg/mL	2.4 mg

Sample	Volume	Concentration of protein	Total Protein
Desalt Load (Filtered)	5 mL	0.44 mg/mL	2.2 mg
Desalt Pool	20 mL	<0.1 mg/mL	
50 kDa MWCO Flow Through	19 mL	<0.1 mg/mL	
50 kDa MWCO Concentrated (proteins above 50 kDa)	500 uL	2.89 mg/mL	1.45 mg
Final Sample stored in 50 % glycerol	38 mL	<0.1 mg/mL	

Picture 2 - SDS PAGE results from the purification of 500 mL toxin in culture supernatant produced from 6 litres of culture.
Samples were taken at each step of the purification and analysed by SDS Page. Description of Lanes underneath the picture



It appears the majority of proteins did not pass through the 50 kDa molecular weight cut-off filter according to the SDS-PAGE results and proteins were concentrated in the upper chamber of the filtration device (Picture 2, lane 12). On the SDS-PAGE no proteins are visible in the flow through sample (Picture 2, lane 11), although this was not concentrated afterwards. Even if it had been concentrated, the key proteins appear to be in a different section

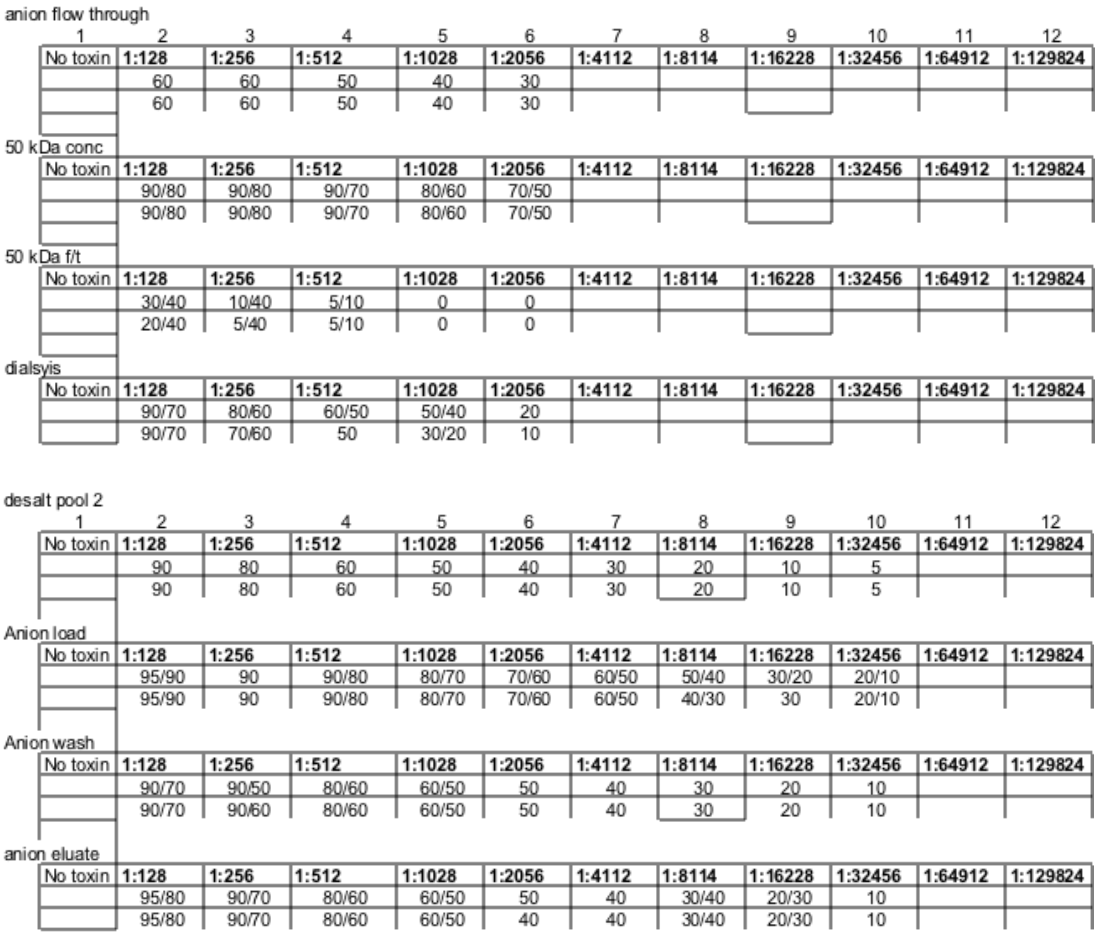
All the samples were tested for toxicity with the Alamar blue test (Table 5) after being analysed by eye (Picture 3)

Picture 3 Analysis of fraction with the Vero cell assay and read by eye.

A) All fractions read by eye. Desalt pool 2 is the extract after ammonium sulphate precipitation and the dialysis sample is the sample after the ammonium sulphate precipitation and dialysis overnight. The anion load is a sample tested that was loaded onto the anion column. The wash of the anion column was measured (Anion wash) and well as the elute coming off the column. A flow through sample of the anion column was also analysed. The sample above the 50 kDa filter (5-kDa conc.) and the sample flowing through the filter (50 kDa f/t), which should contain the toxin was also tested. The last was the testing of the final product diluted in 50% glycerol

B) Retesting of the final product (after purification, addition of glycerol and another concentration with a 3 kDa cut- off filter), and the original supernatant after Polymyxin B extraction that was used for the purification.

A



anion flow through

	1	2	3	4	5	6	7	8	9	10	11	12
No toxin		1:128	1:256	1:512	1:1028	1:2056	1:4112	1:8114	1:16228	1:32456	1:64912	1:129824
		60	60	50	40	30						
		60	60	50	40	30						

50 kDa conc

No toxin		1:128	1:256	1:512	1:1028	1:2056	1:4112	1:8114	1:16228	1:32456	1:64912	1:129824
		90/80	90/80	90/70	80/60	70/50						
		90/80	90/80	90/70	80/60	70/50						

50 kDa f/t

No toxin		1:128	1:256	1:512	1:1028	1:2056	1:4112	1:8114	1:16228	1:32456	1:64912	1:129824
		30/40	10/40	5/10	0	0						
		20/40	5/40	5/10	0	0						

dialsyis

No toxin		1:128	1:256	1:512	1:1028	1:2056	1:4112	1:8114	1:16228	1:32456	1:64912	1:129824
		90/70	80/60	60/50	50/40	20						
		90/70	70/60	50	30/20	10						

It appears this was unsuccessful Equal amounts of toxin were retained and lost

desalt pool 2

	1	2	3	4	5	6	7	8	9	10	11	12
No toxin		1:128	1:256	1:512	1:1028	1:2056	1:4112	1:8114	1:16228	1:32456	1:64912	1:129824
		90	80	60	50	40	30	20	10	5		
		90	80	60	50	40	30	20	10	5		

Anion load

No toxin		1:128	1:256	1:512	1:1028	1:2056	1:4112	1:8114	1:16228	1:32456	1:64912	1:129824
		95/90	90	90/80	80/70	70/60	60/50	50/40	30/20	20/10		
		95/90	90	90/80	80/70	70/60	60/50	40/30	30	20/10		

Toxin sits here. Lane 12.

Anion wash

No toxin		1:128	1:256	1:512	1:1028	1:2056	1:4112	1:8114	1:16228	1:32456	1:64912	1:129824
		90/70	90/50	80/60	60/50	50	40	30	20	10		
		90/70	90/60	80/60	60/50	50	40	30	20	10		

Toxin not present here where it is expected to be

anion eluate

No toxin		1:128	1:256	1:512	1:1028	1:2056	1:4112	1:8114	1:16228	1:32456	1:64912	1:129824
		95/80	90/70	80/60	60/50	50	40	30/40	20/30	10		
		95/80	90/70	80/60	60/50	40	40	30/40	20/30	10		

final

	1	2	3	4
No toxin		1:128	1:256	1:512
		0	0	0
		0	0	0

This shows that we have no toxin in our final product. Lanes 11 and 13

B

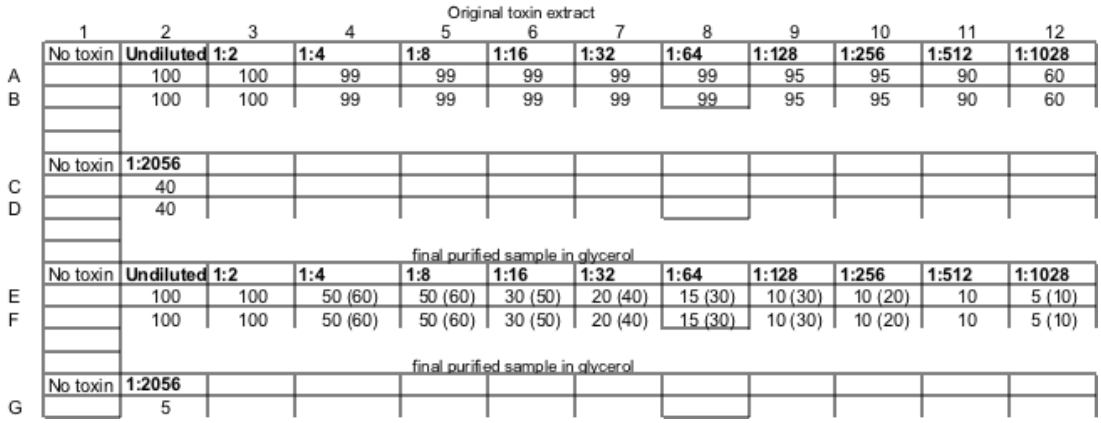


Table 5 - Results of the Vero cell cytotoxicity assay analysed with Alamar Blue for the different fractions expressed as the dilution exhibiting cytotoxic dose.

Sample	CD 50
60% (NH4)2SO4 CP	
Desalt Load (Filtered)	
Desalt Pool	
Dialyzed	
Anion IEX Load (Filtered)	1:768
Anion IEX Flow Through	
Anion IEX Wash	1:512
Anion IEX Elution Pool	1:512
Desalt Load (Filtered)	
Desalt Pool	1:128
50 kDa MWCO Flow Through	
50 kDa MWCO Concentrated	Could not determine the CD50, it is somewhere around 1:2056 (see figure above), but this is a very concentrated sample (only 500ul
Final Sample stored in 50 % glycerol	1:4

The final purified Protein Extract had a very low CD 50 in the Vero cell assay and had to be concentrated for the higher dose given to the mice (7.2 pg.) in group 6 of toxin treatment and group 3 for the toxoid (Table 6). The concentration was done with the 3kDa cut-off filter (Amazon concentration spin filter, Millipore). A total of 5 mL of toxin was concentrated into 3.5mL.

Table 6 - Alamar Blue reading of final product

	CD50 dilution	CD50 concentration
Final purified product in 50% glycerol	1:4	2 pg/100 uL
Concentrated again for high concentration to be given to mice (7.2 pg./mouse)	1:16	8 pg/100 uL

Animal Trials

Testing of toxicity and safety in miceafter inactivation of Protein Extract

Eight –eight mice were utilised for the animal trials. Mice were grouped in groups of ten (toxin and control) and six (inactivated toxin).

Doses of the toxin were increased from 0.45pg to 7.2pg in order to elucidate the LD50.

Doses of inactivated toxin were included to test the inactivation process. Hence only the 3 most concentrated groups were used so doses were 1.8, 3.6 and 7.2 pg.

A negative control group using glycerol storage medium was used.

No positive control group was possible due to the inability to source STX2e toxin.

The mice were injected with 200 µl of preparations that varied in their make-up according to the dose of toxin/toxoid for the group.

The toxin/toxoid batches used to make up the 7.2pg doses (both active and inactivated) had been treated to concentrate the protein fraction while reducing the glycerol content.

Within 1-2 hours, 23 mice showed signs of paralysis, agitation and ruffled fur. These mice were euthanased as per the scoring system and recorded. These are referred to as mortalities due to the inference that without euthanasia, they would have died regardless.

No other mouse showed any clinical signs and their health was maintained throughout. The remaining animals were euthanased 7 days after the administration of their doses.

The components of the doses for the different groups are shown in Table 7.

Table 7 – Dilutions and Results of Mouse Trial

Group	Group Name	No. of Mice	Toxin Preparation Concentration	Dose of Toxin/Toxoid (pg/mouse)	Glycerol Dose (mg)	Glutaraldehyde dose (pg)	PBS Volume (ul)	Mortalities total (no.)	Mortality Rate (%)
Toxin	TG-1	10	0.02	0.45	14.23	0.00	177.50	0	0.00
Toxin	TG-2	10	0.02	0.90	28.46	0.00	155.00	0	0.00
Toxin	TG-3	10	0.02	1.25	39.53	0.00	137.50	0	0.00
Toxin	TG-4	10	0.02	1.80	56.93	0.00	110.00	1	10.00
Toxin	TG-5	10	0.02	3.60	113.85	0.00	20.00	8	80.00
Toxin	*TG-6*	*10*	*0.08*	*7.20*	*56.93*	*0.00*	*110.00*	*0*	*0.00*
Toxoid	ITG-1	6	0.02	1.80	56.93	0.09	110.00	0	0.00
Toxoid	ITG-2	6	0.02	3.60	113.85	0.18	20.00	5	83.33
Toxoid	*ITG-3*	*6*	*0.08*	*7.20*	*56.93*	*0.09*	*110.00*	*0*	*0.00*
Control	C-1	10	0.00	0.00	126.50	0.00	100.00	9	90.00

The euthanasia of the mice was carried out as per Table 8. All euthanasia procedures performed on the 17th of April were performed on healthy mice and are not included in any of the subsequent data.

Table 8 –Euthanasia of Mice

Toxin/Toxoi	Group Name	Toxin/Toxoid lev	10-Apr	11-Apr	12-Apr	13-Apr	14-Apr	15-Apr	16-Apr	17-Apr
Toxin	TG-1	0.45								10
Toxin	TG-2	0.9								10
Toxin	TG-3	1.25								10
Toxin	TG-4	1.8	1							9
Toxin	TG-5	3.6	8							2
Toxin	TG-6	7.2								10
Control	C-1	0	9							1
Toxoid	ITG-1	1.8								6
Toxoid	ITG-2	3.6	5							1
Toxoid	ITG-3	7.2								6
		Total	23	0	0	0	0	0	0	65

4. Discussion

Strain Selection

The process for selection of strains for *in vitro* toxin production was twofold. The first step was the PCR method described above, seeking the presence of STX2e genes in strains isolated form clinical cases.

The second phase was to test supernatants from strains selected by PCR against Vero cells and the most potent were selected.

Extraction of Toxin

Purification results for the anion exchange chromatography were similar to the one in the paper of McLeod and Gyles (1990). Elution fractions were pooled based on toxicity measured by the STX2e assay performed previously. There was a high level of toxicity in the concentrated sample above the 50KDa cut-off. This toxicity was not due to other proteins. The gel shows that the 50 KDa filter did not work

and that toxins < 50 KDa were part of this fraction. The desired fractions were 27-33 KDa and 7.5 KDa, and bands corresponding with these sizes were visible.

The outcome of the purification was lower than achieved by McLeod and Gyles (1990), who had, after Polymyxin, $(NH4)_2SO_4$ precipitation and anion exchange chromatography, a protein concentration of 20.5 mg protein in 5 mL with an CD50 of 8.2 x 10^9/mL, while the final 38 mL purified protein in the current project had an CD50 of 4 x 10^1/mL with an un-measurable amount of protein. McLeod and Gyles (1990) started with a CD50 of 1.1 x 10^{10}, while the starting CD50 before the purification in this project was1.028 x 10^4. It is possible that the toxin purification itself did not work.

The anion exchange (lane 6) and elution pools (lane 9) are similar, so this step has not been optimized. There was only a very small amount of toxin actually in the 19 mL toxin + 19 mL glycerol sample supplied by the UQ group.

The low concentration of the starting material is where the problem llay. The level by McLeod and Gyles (1990) was produced from a genetically altered bacterium that had multiple copies of the *stx2e* gene on a plasmid, while ours was from a field strain.

Glutaraldehyde

The toxic level of glutaraldehyde for mice is 100mg/kg, which translates into 2mg per 20 g mouse. The glutaraldehyde we used had 1.06 kg/l, which if used at 0.1% translates to 1.06 µg/uL. Injecting 200 uL into a mouse would be 0.212 mg per mouse, which is well under the toxicity level so glutaraldehyde toxicity can be rejected as an issue.

Glycerol

The unexpected occurrence of severe neurological signs within two hours after injection can be associated with the level of glycerol in the final injection.

Glycerol has a density of 1.2656g/mL, which means that in 0.1 mL would be 126.6 mg of glycerol. The LD50 of glycerol injected intra-peritoneal into mice has been reported to be 10100mg/kg, which is 10.1 mg/g (http://webnet.oecd.org/Hpv/UI/SIDS_Details.aspx?id=BB8A47ED-67E4-42E5-AA5E-9C04222C4DE8). For a 20g that would be 202 mg/mouse.

Another contribution factor could be the high osmolality of the injection due to the glycerol. Glycerol is a non-electrolyte; therefore, its osmolality and molarity are the same. The molecular weight of glycerol is 92.09 and its weight is 126.6 mg. In a volume of 100 uL the molarity is 13.7474 molar. It is known that agents with widely non-physiologic osmolality administered via IM, SC, IP routes may result in localized tissue damage and associated pain upon injection. As the volume of injected compound increases the potential for tissue damage and pain due to non-physiologic osmolality also increases dramatically. Changes to blood osmolality will rapidly affect multiple organs. There has been a report in the literature that the symptoms of glycerol toxicity are tremors and convulsions (Hine, 1953).

Expected Response in mice

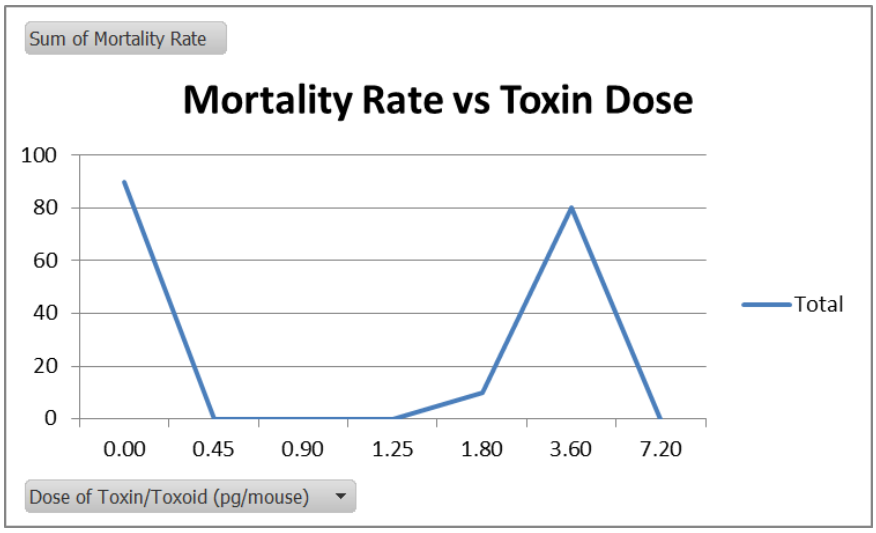
We did not see the response in mice from verotoxin injection recorded by others.

In mice given verotoxin intraperitoneally, the expected clinical signs are hind-limb paralysis followed by death. A study by Obata *et al.* (2008) showed that in mice the neurons are the primary target of verotoxin affecting the function and leading to paralysis. In a study by McLeod and Gyles (1990) the first sign of toxic activity appeared after 24h. These first signs included ruffled fur, huddling, and

disinclination to move. The first signs of severe illness were hind limb paralysis and rapid breathing and mice usually died within 12 hours of these signs. As the dose of toxin decreased, signs of intoxication developed more slowly and survival times increase. None of the mice showed any of these signs past the 24 hours leading to the conclusion that the level of toxin was not high enough to kill the mice. McLeod and Gyles (1990) stated that the 50% cytotoxic doses for Vero cells were 0.5pg. This was used as the base for calculating the dose injected into the mice. However, the low concentration of toxin and certainly the high level of glycerol might have had an effect on the cytotoxicity and distorted the results for the Vero cells cytotoxicity.

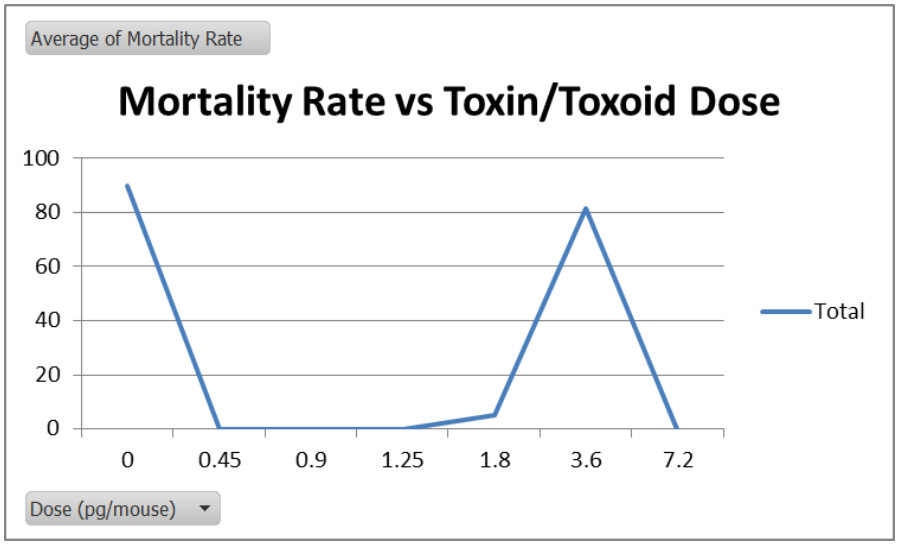
The varying mortality rates of different groups were predicted to give an indication of the Lethal Dose of the purified Protein Extract ("Toxin").

The actual relationship between "Toxin" dose and mortality is represented by the following graph

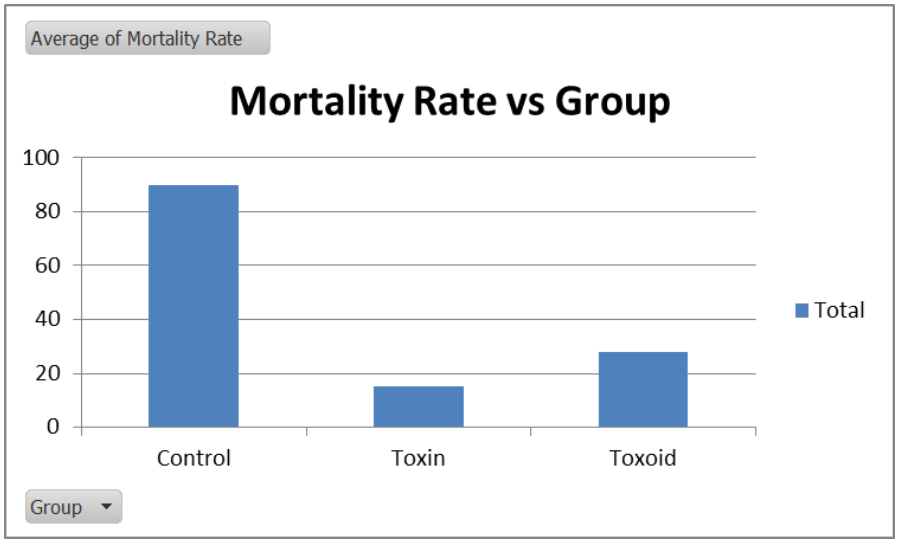


The relationship between "Toxin" dose and mortality rate was not consistent with the hypothesis that the toxin is the cause of the clinical signs. The timing of the clinical signs within 2 hours of injection was also not consistent with the premise that the "Toxin" was responsible.

The relationship looks similar when the "Toxoid" data is added to the "Toxin" data.

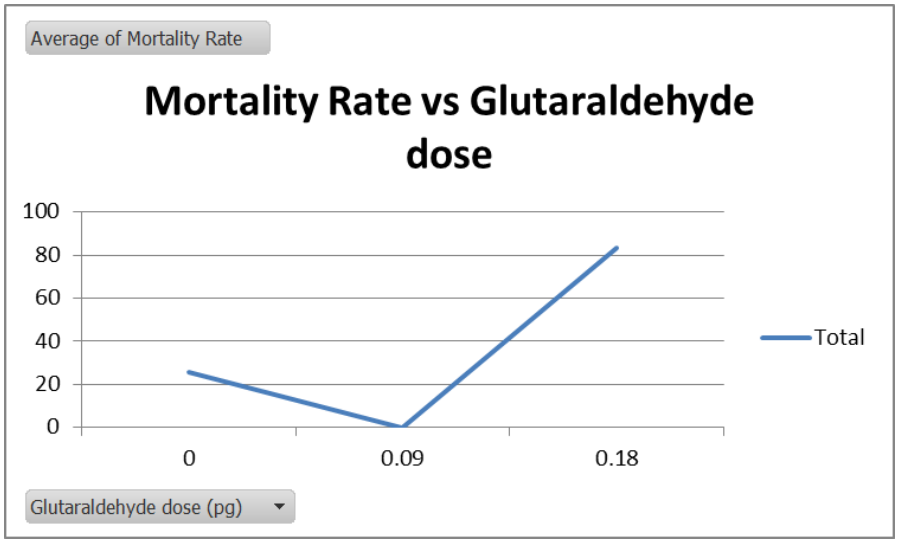


The pattern of Clinical sign in relation to whether the mice received “Toxin”, “Toxoid” or were in the control group is shown below.



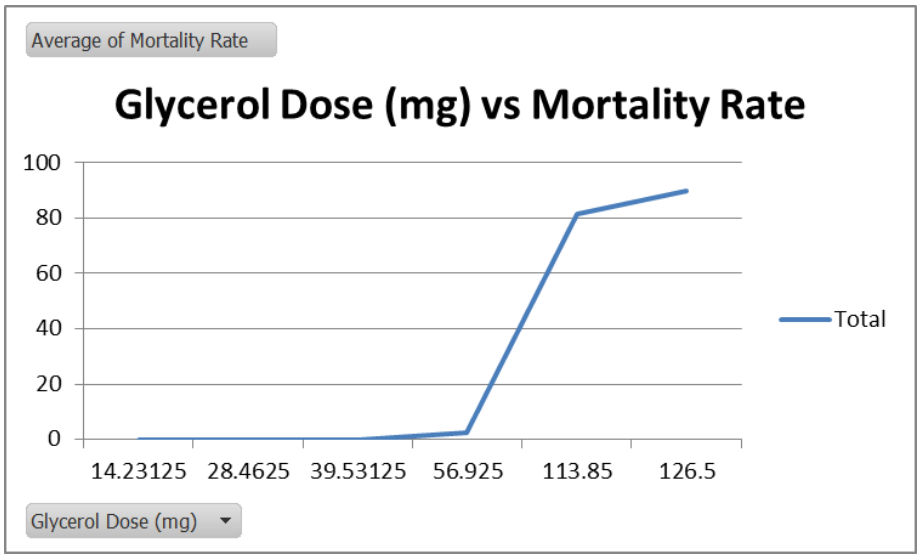
There was a higher mortality rate in control group than in the groups receiving “Toxin” or Toxoid”.

Glutaraldehyde is another potential cause of mortality in the mice. However the dose was known to be approximately 10% of LD50. The relationship between Glutaraldehyde dose and Clinical Signs is represented below.



This pattern is not consistent with the premise that Glutaraldehyde is the cause of the deaths.

The Glycerol content of the injections correlates with the mortality rate.



It is believed that the glycerol was the toxic agent. There was no evidence that the purified Protein Extract, whether in the form of "Toxin" or "Toxoid" has any toxic effect at all.

5. Application of Research

The project did not achieve its ultimate objective of producing and inactivating STX2e.

Some spin off benefits were achieved. The optimization of the PCR for detection of toxin genes at Biosecurity Sciences Laboratory has added to the capacity of veterinarians in Australia to accurately diagnose disease.

The screening of large numbers of isolates against this PCR has generated a sound knowledge of the prevalence of STX2e genes in pigs in QLD.

The development of the Vero cell Assay may prove useful in a diagnostic or research capacity.

The successful application of STX2e toxoids, either to generate active or passive immunity in weaner pigs, is closer now than before as some specific technical hurdles have been overcome.

If this technology is required by the Australian Pig Industry, further research has some foundations on which to stand.

One tangential finding was that some cases previously diagnosed as Oedema disease, due to the sudden death it causes piglets and the age at which it strikes, might not have been caused by STX2e toxin, as the *E. coli* strains isolated from one farm in this category repeatedly tested negative to *stx2e* genes but did have other ETEC genes.

In order to proceed further with this line of investigation we believe one of both of the following prerequisites needs to be found:

1. Methods for producing STX2e in higher concentrations. GMOs have been shown to be able to achieve this and are an avenue to pursue.
2. Methods for isolating the toxin from the supernatants with both a higher sensitivity (so not so much is lost) and higher specificity (to decrease the amount of background protein). Affinity columns utilizing antibodies are one suggested method to achieve this.
3.

Some other novel ways include:

1. Research has progressed to producing transgenic lettuce plants expressing 2 x STX2eB with a suitable linker peptide that accumulate as much as 80 mg per 100 g fresh weight. These lettuce plants may in future be used to generate vaccine material.
2. The neutralization of STX2e via recombinant bacteria expressing mimics of Globotriose and globotetraose, which are the receptors for the toxin. These recombinant bacteria which display Globotriose on their surface adsorb and neutralize the Shiga toxins with very high efficiency according to Paton *et al*. (2001). DNA vaccines have been produced as well and Ren *et al*. (2013) have published their experience with a DNA vaccine encoding the major virulence factors.
3. The intranasal immunization of F4 fimbriae and heat label enterotoxin giving protection against enteric colibacillosis (Lin *et al*. 2013). This opens the possibility to not only protect against enteric colibacillosis disease but also against Oedema disease. The findings of the survey undertaken to choose the strains highlights the diversity of strains, some having the F18 fimbriae but not the STX2e toxin. This opens the possibility to inject a variety of antigens targeting more than one disease.

6. Conclusion

The production and purification of STX2e from E.coli isolates from the field appears to be inefficient and results in low toxin concentrations that cannot be purified effectively.

Our experimental method failed and we could not determine if the toxin was toxic or the toxoid inactivated because many of the controls died; but the high concentrations of toxin did not kill the mice so it looks safe.

The *stx2e* gene sequence is present in a significant number of isolates from Queensland farms (26% of isolates tested positive for the *stx2e* gene).

Further research into the area of E.coli toxoids should focus on more efficient production and filtration methods and ways of measuring a lethal toxin dose.

7. Limitations/Risks

There is a risk that the technology we were attempting to produce, notably the use of passive immunity to STX2e will be discredited or fall from favour as a result of this work, we need to increase the amount of STX2e with or without GMOs before this approach will bear fruit.

8. Recommendations

It is recommended that further work in this area focus on increasing toxin production levels in vitro and/or work to create more efficient toxin filtration techniques.

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