

Development of a flow cytometric bead immunoassay as an aid to potency evaluation of enterotoxaemia vaccines

by

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DECLARATION

I declare that the dissertation, which I hereby submit for the degree M.Sc (Microbiology) at the University of Pretoria, is my own work, in my capacity as an employee of Onderstepoort Biological Products, and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signed:

Date:

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SUMMARY

Development of a flow cytometric bead immunoassay as an aid to potency evaluation of enterotoxaemia vaccines

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Enterotoxaemia is an economically important disease of sheep, goats and calves. The disease affects mainly young animals aged between four to six months. Enterotoxaemia is caused by systemic effects of the epsilon toxin produced by the anaerobic bacterium *Clostridium perfringens* Type D. Due to almost certain death of affected animals, there is no form of treatment. The only practical means of controlling the occurrence of enterotoxaemia is to immunize animals through vaccination. The vaccine is prepared by toxoiding the bacterial culture filtrate, and contains a range of proteins in addition to the epsilon toxin. Batches of the vaccine are thus required to be tested for safety, efficacy and potency. The potency of the vaccines is currently tested with the *in vivo* mouse neutralisation test (MNT). However, due to ethical, economic and technical reasons, alternative *in vitro* assays are needed. In this study, an indirect cytometric bead immunoassay (I-CBA) was developed for use in vaccine potency

testing and the results were compared with those obtained using an indirect ELISA (I-ELISA) and the MNT.

To investigate, three groups of eight guinea pigs were immunized with one of three different production batches of the enterotoxaemia vaccine. Guinea pig sera were collected prior to vaccination and at five weeks post-vaccination, and the sera of four guinea pigs per group were pooled to give six test sera. The levels of anti-epsilon toxin antibodies in the respective test sera were subsequently determined using MNT, I-ELISA and I-CBA. The I-CBA assay developed during the course of this study is based on coating of functional beads with purified epsilon toxin that serves to capture anti-epsilon toxin antibodies from the test sera. Following incubation with fluorescein isothiocyanate (FITC)-labelled anti-guinea pig IgG, the samples were analyzed by flow cytometry and the anti-epsilon toxin antibody concentrations were extrapolated from a standard curve using linear curve fitting. Although the intra- and inter-assay variability was satisfactory, epsilon antitoxin levels of the guinea pig test sera determined by both the I-CBA and I-ELISA tests were higher than that of the MNT assay which is accepted as the current “gold standard”. Moreover, in contrast to the MNT, all of the serum samples were identified as having antitoxin levels above the required minimum (i.e. not less than 5 U/ml). These results indicate that the respective *in vitro* tests in their current formats are not yet suitable alternatives to the *in vivo* MNT. The growing demand for a more humane, cost-effective and efficient method for testing the potency of enterotoxaemia vaccines, however, provides a strong impetus for further optimization and standardization of the I-CBA assay described in this study.

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ABBREVIATIONS

α	alpha
Å	Angstrom
ANOVA	analysis of variance
β	beta
BD	Becton Dickinson
BSA	bovine serum albumin
C	carboxy
°C	degrees Celsius
CBA	cytometric bead assay
CV	coefficient of variance
ddH ₂ O	double distilled water
DEAE	diethylaminoethyl
ϵ	epsilon
ELISA	enzyme-linked immunosorbent assay
<i>et al.</i>	<i>et alia</i> (and others)
FITC	fluorescein isothiocyanate
g	gram
x g	centrifugal force
i	iota
I-CBA	indirect cytometric bead assay
i.e.	<i>id est</i> (that is)
I-ELISA	indirect enzyme-linked immunosorbent assay
IgG	immunoglobulin G
IU	international units
kb	kilobase pairs
kDa	kilodalton
kg	kilogram
ℓ	litre
LAT	latex agglutination test
LD ₅₀	lethal dose 50
M	molar
mA	milliamperes
MDCK	Madin-Darby canine kidney
MFI	median fluorescence intensity

mg	milligram
ml	millilitre
MLD	minimum lethal doses
mM	millimolar
mm	millimetre
MNT	mouse neutralization test
MPB	meat pellet broth
N	amino
NaP	sodium phosphate
ng	nanogram
nm	nanometre
nM	nanomolar
OBP	Onderstepoort Biological Products
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
Ph. Eur.	European Pharmacopoeia
PKM	pulpy kidney medium
PS-2	parasporin-2
r^2	correlation coefficient
SDS	sodium dodecyl sulphate
TEMED	<i>N,N,N',N'</i> -tetramethylethylenediamine
TMB	Tetramethylbenzidine
TNT	toxin neutralization test
ToBI	toxin-binding inhibition
U	units
USDA	United States Department of Agriculture
v	volt
v/v	volume per volume
w/v	weight per volume
μg	microgram
μl	microlitre
μm	micrometre

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CHAPTER ONE

LITERATURE REVIEW

1.1. GENERAL INTRODUCTION

Livestock production and rearing is an important part of the economy of many countries and is considered to be the greatest contributor to the livelihood of the majority of the poor around the world (Perry *et al.*, 2002). The steady development in animal production and subsequent food security has been for centuries hampered by health conditions and diseases, of which infectious diseases have played a major role. To date, vaccination continues to be the most effective way of controlling animal diseases, either through preventing mortality or reducing morbidity (Rogan and Babiuk, 2005; Meeusen *et al.*, 2007). Moreover, vaccines also help to ensure a safe and efficient global food supply by reducing the transmission of zoonotic infections from animals to humans and by reducing the need for low-level antibiotics (Roth, 2011). Indeed, vaccines have been the only tools capable of eradicating infectious animal diseases, either globally as exemplified by the eradication of Rinderpest (Roeder *et al.*, 2004) or at a regional level as exemplified by the elimination of foot-and-mouth-disease in some South American countries (Rweyemamu and Astudillo, 2002).

Initial forms of vaccination are reported to have been practiced in China and India before 200 BC. The process consisted of inoculation of patients with a powdery substance prepared by picking off pieces from drying pustules of a person suffering from a mild case of smallpox, and then inserting these into the patient's nose in order to immunize them (Fenner *et al.*, 1997). However, it was only centuries later that the protective capacity of inoculations derived from pathogenic agents was demonstrated clearly. Vaccination was most likely popularized through the work of Edward Jenner towards the end of the 18th century (Stern and Markel, 2005). After inoculating an eight-year-old boy, James Phipps, with material from a human cowpox blister, Jenner exposed the boy to cowpox. Despite showing symptoms of cowpox infection, Phipps recovered fully and when re-infected with smallpox-infected material he did not show signs of

smallpox infection. By 1853, Louis Willems had provided a similar demonstration of protection from contagious bovine pleuropneumonia by inoculating animals with serous fluid from the lungs of infected cows (Lombard *et al.*, 2007). Notably, the approaches used by both Jenner and Willems are fundamentally similar to 'vaccination-challenge' tests that are used currently to establish the safety and efficacy of many animal and human vaccines.

As the value of vaccination gained credibility, medical and veterinary practitioners recognized the need to regulate the production and testing of vaccines in order to establish criteria indicative of safe and effective products. It was only in the early 20th century that government authorities began to develop strategies for assessing and controlling vaccines (Lilienfeld, 2008). The United States Congress passed the first piece of legislation mandating the control of vaccine quality in 1902, later known as the Biologics Control Act, and this was followed by the Virus-Serum-Toxin Act of 1913, which mandated similar controls over veterinary vaccines. Federal agencies were subsequently charged with ensuring vaccine safety through licensure processes that required scientific assessment of vaccine products before they could be sold (Milstien, 2004). In most countries, strict regulations and guidelines are in place to ensure that each veterinary vaccine product released for sale is pure, safe, potent and effective (Kulpa-Eddy *et al.*, 2011).

Many veterinary vaccines, including classical vaccines such as Clostridial toxoids, are tested for safety and potency in experiments that require the use of animals. Among the *Clostridium* spp., *C. perfringens* Type D is commonly encountered in veterinary medicine. It is the aetiological agent of enterotoxaemia in sheep, goats and calves (Niilo, 1980; Songer, 1996; Uzal, 2004). The disease is caused by the epsilon toxin, a major exotoxin produced by *C. perfringens* Type D (Songer, 1996; Rood, 1998). Despite its worldwide occurrence and potential for serious

economic loss, active immunization with an epsilon toxoid has proven to be effective in preventing enterotoxaemia (Titball, 2009). Potency testing of *C. perfringens*-containing vaccines requires vaccination of rabbits, followed by quantitative determination of the vaccination-induced antibodies in the rabbit sera using a toxin neutralization test in mice (USDA, 2007; European Pharmacopoeia, 2008). This test, using the lethality in mice as an indicator of non-neutralized toxin, leads to the death of a large number of animals. The severe distress and suffering experienced by the mice, in addition to the large number of animals used, necessitates replacement of the *in vivo* mouse neutralization test with an alternative *in vitro* method.

In this review of the literature, current knowledge regarding *C. perfringens* Type D will be presented and aspects relating to the production, structure and mode of action of the epsilon toxin will also be covered. This will be followed by a description of enterotoxaemia vaccines, with a specific emphasis on potency evaluation using *in vitro* methods. The section will be concluded with a description of the aims of this study.

1.2. ENTEROTOXAEMIA IN LIVESTOCK

1.2.1. Introduction to *Clostridium perfringens* Type D

The *Clostridium* genus encompasses more than 80 species that form a diverse group of Gram-positive, rod-shaped (3 to 10 µm in length) bacteria with the ability to form spores. These bacteria are obligate anaerobes, but some species can survive in an oxygen-rich environment for extended periods of time (Lyerly *et al.*, 2004). *Clostridium perfringens* is one of the most pathogenic species in the *Clostridium* genus (Petit *et al.*, 1999; Lyerly *et al.*, 2004). *Clostridium perfringens* is ubiquitous in the environment, being found in soil, water and sewage. Moreover,

they can also be found in the gastrointestinal tract of humans and animals where they form part of the common gastrointestinal flora (Lyerly *et al.*, 2004; Aitken, 2007).

Since the discovery of *C. perfringens* as the causative agent of gangrene at the end of the 19th century, the bacterium has been the object of intensive study (Rood, 1998). In addition to causing food poisoning, enteritis, gangrene and puerperal septicemia in humans, various forms of acute enteritis and fatal enterotoxaemias in animals have been attributed to *C. perfringens* (Niilo, 1980; Finnie, 2004) (Table 1.1). *C. perfringens* produces the largest number of toxins of any bacteria. As many as 17 exotoxins of *C. perfringens* have been described in the literature, but a definitive role in pathogenesis has been demonstrated for only a few (McDonel, 1980; Sakurai, 1995; Rood, 1998). For practical classification purposes, the species is classified into five toxinotypes (A, B, C, D and E) according to the production of four major toxins, namely alpha (α or CPA), beta (β or CPB), epsilon (ϵ or ETX) and iota (ι or ITX) (McDonel, 1980; Petit *et al.*, 1999). Type A is defined as strains producing α toxin, Type B as strains producing α , β and ϵ toxins, Type C as strains producing α and β toxins, Type D as strains producing α and ϵ toxins, and Type E as strains producing α and ι toxins. Since the respective toxins are antigenic, typing is achieved by neutralization of the toxins with type-specific antisera using mice or guinea pigs as test animals (Brooks *et al.*, 1957; McDonel, 1980).

Although both *C. perfringens* Types B and D produce the epsilon (ϵ) toxin, they cause different diseases in livestock animals. *C. perfringens* Type B, which also produces β -toxin, is the aetiological agent of dysentery in newborn lambs, whereas *C. perfringens* Type D causes enterotoxaemia mainly in sheep and lambs, but also in goats and calves (Niilo, 1980; Songer, 1998). The epsilon toxin is one of the most potent toxins known and its lethal activity ranges just below that of botulinum neurotoxins. Indeed, the lethal dose of epsilon toxin by

Table 1.1 Diseases associated with *C. perfringens* infections in animals (Table adapted from Coetzer and Tustin, 2004)

<i>C. perfringens</i> Type	Description of disease	Animal infected
A	<ul style="list-style-type: none"> • Gas gangrene • Myonecrosis • False blackleg • Enterotoxaemia • Sudden death • Haemorrhagic/necrotic enteritis 	<ul style="list-style-type: none"> • Sheep, goats and cattle • Sheep, goats and cattle • Cattle • Sheep and cattle • Cattle • Sheep
B	<ul style="list-style-type: none"> • Lamb dysentery • Haemorrhagic enterotoxaemia 	<ul style="list-style-type: none"> • Lambs • Sheep and goats
C	<ul style="list-style-type: none"> • Struck • Haemorrhagic necrotic enteritis 	<ul style="list-style-type: none"> • Sheep • Sheep and cattle
D	<ul style="list-style-type: none"> • Pulpy kidney disease • Enterotoxaemia 	<ul style="list-style-type: none"> • Sheep and goats • Cattle
E	<ul style="list-style-type: none"> • Haemorrhagic enterotoxaemia 	<ul style="list-style-type: none"> • Sheep and cattle

intraperitoneal injection in mice is 70 ng/kg (Gill, 1987). Based on structural data, the epsilon toxin has been grouped as a member of the family of aerolysin pore-forming toxins (Cole *et al.*, 2004). However, its precise mode of action, accounting for its high potency, remains to be defined (Knapp *et al.*, 2010).

1.2.2. *C. perfringens* Type D disease and pathogenesis

Of the different *C. perfringens* toxinotypes, Type D is possibly the best known pathogenic *C. perfringens* type. It is widely regarded as the causative organism of fatal enterotoxaemia of sheep and goats found worldwide (Uzal, 2004; Titball, 2009). The most important factor responsible for disease initiation is considered to be disruption of the microbial balance in the gastrointestinal tract of animals. Most cases of Type D enterotoxaemia in sheep relate to access to lush pastures after rains in spring and autumn (Bullen, 1963), as well as sudden changes in their diet, usually to feeds rich in highly fermentable carbohydrates (Uzal and Kelly, 1996) or continuous feeding of high levels of feed concentrates (Popoff, 1984). As a consequence, large amounts of undigested carbohydrates enter the small intestine from the rumen and are an excellent substrate for *C. perfringens* growth. The rapid multiplication of Type D bacteria results in the production of large amounts of the epsilon toxin, which, in turn, causes its absorption into the systemic circulation thereby leading to acute disease and death (Uzal and Kelly, 1996; Uzal *et al.*, 2004). Other factors that disturb the intestinal microbial balance, such as heavy parasite infestation (Thomas *et al.*, 1956) or deworming remedies (Bath and De Wet, 2000), may predispose the animals to Type D enterotoxaemia, but convincing evidence has not been published.

C. perfringens Type D causes enterotoxaemia in sheep of all ages, except newborns (Uzal, 2004). Type D enterotoxaemia in lambs is rapidly fatal. Enterotoxaemia is the most prevalent

in lambs that are 3 to 10 weeks of age and is a predominant cause of death in weaned animals up to 10 months of age, usually those fed rich rations of grain in feedlots (Uzal, 2004; Aitken, 2007). *C. perfringens* Type D causes acute disease in sheep (Lewis, 2000). In the acute form, which is very rapid (few minutes to several hours, no more than 12 hours), the effects of increased epsilon toxin levels on the central nervous system and other tissues cause sudden death, with some animals surviving long enough to display clinical signs such as dullness, retraction of the head, opisthotonus, convulsions, frothing by the mouth and recumbency with paddling immediately before death (Lewis, 2000). Lambs and sheep that died from enterotoxaemia show characteristic modifications of the kidneys. Just after death, the kidneys are swollen or congestive, but they autolyse more rapidly than normal, with the cortical parenchyma being totally liquefied (Gardner, 1973a, 1973b). In addition to the kidneys, the epsilon toxin also accumulates massively in the brain, where it produces foci of liquefactive necrosis, perivascular edema and haemorrhage in especially the meninges (Buxton *et al.*, 1976). Focal encephalomalacia occurs sporadically in affected sheep and is characterized by haphazard roaming, blindness, head pressing and an inability to eat (Uzal *et al.*, 1997a).

In goats, *C. perfringens* Type D produces acute, subacute or chronic disease. The acute form occurs more frequently in young animals and is clinically similar to the acute disease in sheep (Blackwell *et al.*, 1991; Blackwell and Butler, 1992; Uzal and Kelly, 1996). The subacute form is more frequently seen in adult goats and is characterized by diarrhea, abdominal discomfort, severe shock, opisthotonos and convulsions. The disease may result in death 2-4 days after onset, but some animals recover (Blackwell *et al.*, 1991). Adult animals can also exhibit chronic disease, which is characterized by abdominal discomfort, weakness, anorexia and profuse watery diarrhea that often contains blood and mucus (Blackwell and Butler, 1992). This chronic

form may last for days or weeks and may culminate either in death or recovery (Uzal and Kelly, 1996).

In contrast to calves, it appears that Type D enterotoxaemia is relatively rare in cattle (Mumford, 1961; Niilo, 1980). More recently, a condition with brain lesions similar to those observed in sheep enterotoxaemia has been described in cattle (Fairley, 2005), but a causal relationship with *C. perfringens* Type D could not be established. Nevertheless, it is possible to produce Type D enterotoxaemia in cattle experimentally (Uzal *et al.*, 2002; Filho *et al.*, 2009). The results of these studies have shown distinctive pathological and bacteriological findings that were similar to those observed in sheep.

1.2.3. Diagnosis of enterotoxaemia

Large amounts of the epsilon toxin, as well as large numbers of *C. perfringens* can usually be observed in the intestinal contents of diseased or dead animals (Songer, 1996). Since *C. perfringens* Type D is a natural host of animal intestines, isolation of the bacterium is not by itself diagnostic. Consequently, the diagnosis of enterotoxaemia is based on clinical signs and postmortem findings, but identification of toxins in intestinal contents is required to confirm the diagnosis.

The most widely used method for toxin detection is the mouse neutralization test (MNT), but this *in vivo* assay has become undesirable because of the expense and the large number of laboratory animals required for the test. Moreover, if diagnosis is based solely on toxicological results, it is potentially possible to misdiagnose Type B (produces α , β and ϵ toxins) infections as Type D (produces α and ϵ toxins) if the β toxin is destroyed by the action of intestinal trypsin (Uzal and Songer, 2008). To address these concerns, a number of alternative *in vitro* tests

have been developed. DNA-based methods, such as nucleic acid hybridization and the polymerase chain reaction (PCR), have been developed for detection of the toxin encoding genes, and were reported to allow for more accurate and complete determination of *C. perfringens* toxinotypes than does testing in animals (Havard *et al.*, 1992; Daube *et al.*, 1994; Moller and Ahrens, 1996; Kalender *et al.*, 2005).

Most of the *in vitro* methods for detection of *C. perfringens* Type D are, however, based on immunoassays for detection of the epsilon toxin. These include immunodiffusion (Beh and Buttery, 1978), immunoelectrophoresis (Henderson, 1984; Tripathi *et al.*, 1992), the latex agglutination test (LAT) (Martin and Naylor, 1994; Marks *et al.*, 1999) and enzyme-linked immunosorbent assays (ELISA) (Weddel and Worthington, 1984; Sojka *et al.*, 1989; Wood *et al.*, 1991; Uzal *et al.*, 1997b; Uzal *et al.*, 2003). Among these tests, LAT and ELISA have been used the most frequently for laboratory diagnosis of enterotoxaemia, especially in cases of sudden death outbreaks in sheep. These tests are simple, cost-effective and provide quantitative results. Moreover, they can be used for both toxin-typing and for the differential diagnosis of *C. perfringens* Types A, B, C and D enterotoxaemias (Naylor *et al.*, 1987; El Idrissi and Ward, 1992; Martin and Naylor, 1994).

1.3. THE EPSILON TOXIN OF *C. perfringens* Type D

1.3.1. Epsilon toxin genetics and production

The gene encoding for the epsilon toxin, designated as *etx*, is located on extrachromosomal plasmid DNA in both *C. perfringens* Types B and D (Canard *et al.*, 1992; Hughes *et al.*, 2007). In *C. perfringens* Type B isolates the *etx* gene is located on a single plasmid of 65 kb (Sayeed

et al., 2010), whereas in Type D isolates five different plasmids, which range in size from 48 to 110 kb, have been identified that harbour the *etx* gene (Sayeed *et al.*, 2007). In addition to the *etx* gene, the larger plasmids from *C. perfringens* Type D were reported to also carry genes encoding for two other toxins, i.e. *C. perfringens* enterotoxin (CPE) and Beta2 toxin (Sayeed *et al.*, 2007). Interestingly, a *C. perfringens* strain can change from one toxinotype to another through the acquisition of a toxigenic plasmid. For example, Hughes *et al.* (2007) demonstrated conjugative transfer of an *etx* plasmid from a *C. perfringens* Type D isolate to a Type A isolate, essentially converting the Type A isolate to a Type D isolate, both genotypically and phenotypically. Additional evidence for genetic exchange among toxinotypes was provided by the finding that a *tcp* locus, which is required for conjugation, is present in some *etx* plasmids from both *C. perfringens* Types B and D isolates (Hughes *et al.*, 2007). It was thus concluded that the horizontal transfer of toxin plasmids may account for the large genetic diversity observed in *C. perfringens* strains.

The epsilon toxin is synthesized during the exponential growth phase of all *C. perfringens* Type D strains as a single protein containing an N-terminal signal sequence of 32 amino acids that directs the export of the prototoxin from the bacterium (Hunter *et al.*, 1992). The inactive secreted prototoxin is converted to the fully active mature toxin by proteolytic cleavage in the gut lumen, either by digestive proteases of the host, such as trypsin and α -chymotrypsin (Bhown and Habeeb, 1977), or by λ -protease, which is produced by *C. perfringens* (Minami *et al.*, 1997). However, differences in the toxicity of the mature epsilon toxin have been noted and are dependent on the protease used for activation of the prototoxin. The λ -protease removes 11 N-terminal and 29 C-terminal residues, whereas trypsin together with α -chymotrypsin cleaves 13 N-terminal residues and the same 29 C-terminal residues. Maximal activation of the epsilon toxin occurs with a combination of trypsin and α -chymotrypsin, producing a mature toxin with an

LD₅₀ of 70 ng/kg in mice (Gill, 1987). In contrast, if trypsin alone is used for activation, only the C-terminal 22 residues are removed, thus resulting in a lower toxicity in mice with an LD₅₀ of 320 ng/kg (Minami *et al.*, 1997). If *C. perfringens* λ-protease is used for activation, the C-terminus is cleaved at the same position as α-chymotrypsin but three extra residues are present in at the N-terminus. In this case, the processed epsilon toxin displays an activity closer to maximal with an LD₅₀ of 110 ng/kg (Minami *et al.*, 1997).

1.3.2. Structure of the epsilon toxin

The three-dimensional structure of the epsilon toxin has been determined (Cole *et al.*, 2004). The crystal structure revealed that the toxin is an elongated molecule (100Å × 20Å × 20Å) and contains three domains that are composed mainly of β sheets (Cole *et al.*, 2004) (Figure 1.1). Domain I of the epsilon toxin consists of a large α helix, followed by a loop and three short α helices. It has been suggested that a cluster of aromatic residues (Tyr₄₉, Tyr₄₃, Tyr₄₂, Tyr₂₀₉ and Phe₂₁₂) present in this domain could be involved in cell receptor binding (Cole *et al.*, 2004). Domain II is a β-sandwich composed of a two-stranded sheet with an amphipathic sequence (His₁₅₁-Ala₁₈₁), which contains alternate hydrophobic-hydrophilic residues characteristic of membrane-spanning β-hairpins (Cole *et al.*, 2004; Knapp *et al.*, 2009). Domain II is thus predicted to be a channel-forming domain. Supporting evidence for its importance in pore formation have been provided by Pelish and McClain (2009) who showed that mutagenesis of this domain prevented pore formation, but not receptor binding. Moreover, site-directed mutagenesis of the His₁₅₁-Ala₁₈₁ segment confirmed that it is involved in epsilon toxin channel activity in lipid bilayers (Knapp *et al.*, 2009). Domain III is also a β-sandwich and contains one four-stranded sheet and one three-stranded sheet. Domain III is proposed to be involved in the monomer-monomer interaction required for oligomerization of the epsilon toxin after the

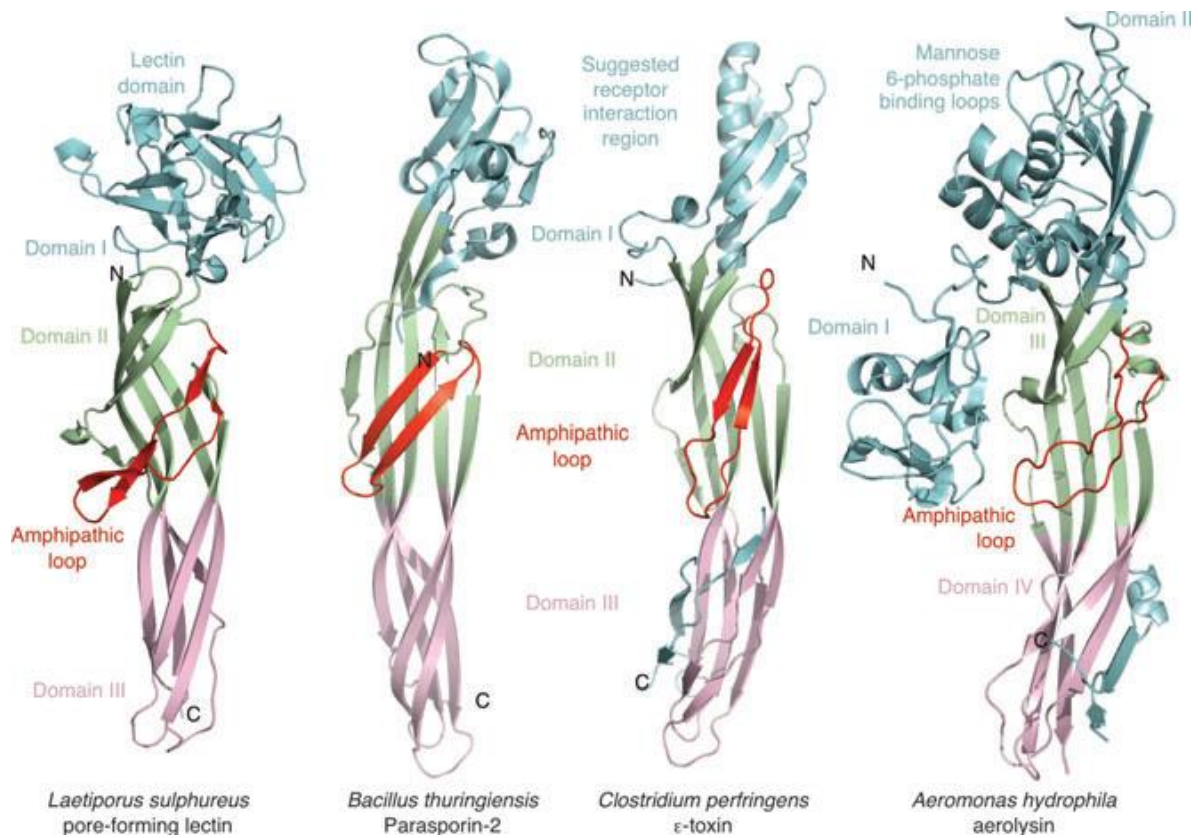


Figure 1.1 Structures of members of the aerolysin-like, β -pore-forming family, as solved by X-ray crystallography. Coloured cyan indicates the N-terminal membrane-interacting and other non-related regions, pale green and pink for domains important for oligomerization and membrane interaction, and red for the β -hairpin predicted to insert into the membrane (Adapted from Bokori-Brown *et al.*, 2011).

C-terminal amino acid residues have been removed by protease cleavage (Miyata *et al.*, 2001; Cole *et al.*, 2004).

Interestingly, the overall fold of the epsilon toxin structure shows similarity to aerolysin from the Gram-negative bacterium *Aeromonas hydrophila*, to parasporin-2 (PS-2) from *B. thuringiensis*, and to a pore-forming lectin, LSL, from *Laetiporus sulphureus* (Knapp *et al.*, 2010). Despite the low amino acid sequence identity (< 20%) between the above proteins, their structures show remarkably similar β -sheet arrangements (Figure 1.1) in their two C-terminal domains (Domains III and IV in aerolysin, and Domains II and III in the others). In addition to the structural similarity, the epsilon toxin also shares other properties with aerolysin and PS-2. Not only are they all secreted as protoxins and activated by proteolytic cleavage of N- and C-terminal sequences, but they are also all capable of forming pores (Knapp *et al.*, 2010). Consequently, it has been suggested that the epsilon toxin may have a similar mechanism of action to aerolysin and PS-2.

1.3.3. Mechanism of action of the epsilon toxin

Studies regarding the activity of the epsilon toxin have been facilitated by the observation that some cell lines are susceptible to the toxin (Knight *et al.*, 1990; Payne *et al.*, 1994; Shortt *et al.*, 2000). Although renal cell lines such as mpkCCDcl4 and human leiomyoblastoma (G-402) cells are sensitive to the toxin, most work have been carried out using Madin-Darby canine kidney (MDCK) cells. In MDCK cells, the dose of epsilon toxin needed to kill 50% of the cells is reported to be 15 ng/ml (Knight *et al.*, 1990). Cells treated with the epsilon toxin undergo morphological changes such as swelling and the formation of membrane blebs (Payne *et al.*, 1994; Petit *et al.*, 1997; Shortt *et al.*, 2000; Borrmann *et al.*, 2001). The loss of cell viability also correlates with a rapid efflux of K^+ and an influx of Cl^- and Na^+ ions (Petit *et al.*, 2001), indicating

that pore formation in the cell membrane is likely responsible for the rapid increase in permeability of cell monolayers (Petit *et al.*, 2003; Chassin *et al.*, 2007).

With regard to its mode of action, the epsilon toxin has been observed to bind preferentially to the apical site of MDCK cells and results in the formation of large membrane complexes (Petit *et al.*, 1997). Endocytosis and internalization of the toxin into the cell have not been observed, and the toxin remains associated with the cell membrane throughout the intoxication process (Petit *et al.*, 1997). It is likely that the epsilon toxin may recognize a specific cell receptor, which is absent in insensitive cells. Although the identity of the receptor is not yet known, it has been suggested that the epsilon toxin receptor might be related to a 34 or 46 kDa protein or glycoprotein in MDCK cells (Petit *et al.*, 1997), and to a 26-kDa sialoglycoprotein in rat brain (Nagahama and Sakurai, 1992). The lipid environment of the epsilon toxin receptor also appears to be critical for binding of the toxin to the cell surface, because binding is prevented by pre-treatment of the cells with detergent (Petit *et al.*, 1997). Since the epsilon toxin is capable of forming channels in lipid bilayers in the absence of a receptor (Petit *et al.*, 2001), albeit with less efficiency (Nagahama *et al.*, 2006), it has been suggested that the receptors may serve to concentrate the epsilon toxins, thereby permitting interaction between toxin monomers and subsequent oligomerization (Petit *et al.*, 1997; Abrami and Van Der Groot, 1999). In contrast to the activated epsilon toxin, a recombinant toxin possessing the C-terminal sequence was unable to form large complexes despite being able to bind to MDCK cells (Miyata *et al.*, 2001). These results therefore indicate that the C-terminal amino acid residues of the epsilon prototoxin are responsible for controlling the toxin activity by preventing oligomerization.

As mentioned in the preceding section, Domain II of the epsilon toxin is composed of a five-stranded β -sheet with an amphipathic hairpin. The latter has been predicted to form the

membrane insertion domain due to its alternating hydrophilic-hydrophobic character (Cole *et al.*, 2004), and its importance in pore formation has been established in both lipid bilayer membranes (Petit *et al.*, 2001; Knapp *et al.*, 2009) and MDCK cells (Pelish and McClain, 2009). The size of the pore formed by epsilon toxin has also been investigated. Petit *et al.* (1997) suggested a pore size in the 2-nm range, whereas a more recent study suggested that the pores may be asymmetrical (Nestorovich *et al.*, 2010). The pore size was estimated to be 0.4 nm on the side of toxin insertion and 1.0 nm on the opposing side.

Despite evidence for the epsilon toxin being capable of forming pores, the exact mechanism through which this occurs is not yet known. Nevertheless, a mechanism of pore formation has recently been proposed by Bokori-Brown *et al.* (2011). According to this prediction, the prototoxin is secreted by the bacterium and activated by *C. perfringens* λ -protease or by host proteases such as trypsin and/or α -chymotrypsin. Following receptor binding, the activated epsilon toxin oligomerizes on the cell surface, which may lead to formation of a pre-pore complex. The final step of pore formation might involve unfolding of the amphipathic hairpin and its insertion into the cell membrane to form the walls of the pore.

1.4. PREVENTION OF ENTEROTOXAEMIA DISEASE

Since eradication of *C. perfringens* Type D enterotoxaemia appears to be almost impossible, control and prophylaxis are based on systematic vaccination of herds with epsilon toxoided vaccines. Indeed, these vaccines have been used extensively over the past decades to prevent disease in domesticated livestock (Titball, 2009). Classically, toxin-based vaccines are prepared by treating *C. perfringens* Type D culture filtrates with formaldehyde to toxoid components (Habeeb, 1969). Since relatively crude culture filtrates are used, the vaccines are

likely to contain additional proteins to the epsilon toxoid. Although culture filtrates may contain approximately 15-300 MLD (minimum lethal doses)/ml of the secreted epsilon toxin (Sterne and Batty, 1975), research has been performed to optimize continuous production of the toxins by varying the culture medium composition (Pivinick *et al.*, 1964, Walker, 1992, 1997). In addition, screening of different *C. perfringens* Type D strains in order to identify strains capable of producing more potent epsilon toxins has also been undertaken with some success (Goncalves *et al.*, 2009). Typical immunization regimens involve vaccination of lambs at the age of 3 months for the first time and then with an alum-based vaccine 4 to 6 weeks later. Sheep are boosted every 6-12 months, whereas goats are boosted every 3-4 months (Bath and De Wet, 2000). Although the toxoid vaccines are effective in preventing enterotoxaemia in animals, there are reports of variable immune responses following vaccination (Uzal *et al.*, 1998) and inflammatory responses following vaccination have been reported to lead to reduced feed consumption (Stokka *et al.*, 1994).

As a consequence of bacterial cultivation, varying amounts of the epsilon toxin is produced and secreted. Consequently, alternative approaches have been investigated whereby greater amounts of the epsilon toxin can be produced reliably. These approaches involve using genetic engineering to produce the toxin and then using the recombinant protein for toxoiding (Chandran *et al.*, 2010; Lobato *et al.*, 2010; Souza *et al.*, 2010). A promising approach is the production of recombinant genetically detoxified epsilon toxin mutants that are biologically inactive but still retain the toxin immunogenicity. In this regard, Oyston *et al.* (1998) reported production of site-directed mutants of the epsilon toxin of which one of the variant toxins was shown to be non-toxic to mice. Following immunization of mice with the variant toxin, it was subsequently shown that the mice were protected against a challenge with 1000 MLD doses of the wild-type epsilon toxin.

In addition to inactivated and recombinant vaccines, there has also been a growing interest in the use of anti-epsilon toxin polyclonal and monoclonal antibodies as a means to prevent the toxic effects of the toxin. The passive transfer of polyclonal antibodies against the toxin into newborn lambs has been achieved by injection (Odendaal *et al.*, 1989) and by feeding the animals colostrum that contained antibodies (Clarkson *et al.*, 1985). More recent reports have described the generation of monoclonal antibodies that are able to protect cultured cells (Percival *et al.*, 1990; McClain and Cover, 2007) and mice (Percival *et al.*, 1990) from intoxication. The finding that a single monoclonal antibody is able to provide good protection indicates that a single epitope is required for the induction of protection. McClain and Cover (2007) mapped the location of the epitope recognized by the protective monoclonal antibody to amino acids 134-145 (peptide sequence: SFANTNTNTNSK), which overlaps the putative membrane inserting loop of the epsilon toxin. Although these antibodies may find application in the prevention or treatment of enterotoxaemia, note should be taken that the rapidity of onset of symptoms in animals would make treatment of epsilon intoxication difficult.

1.5. VETERINARY VACCINE POTENCY TESTING

1.5.1. General principles

Many vaccines are derived from living organisms in a batch-wise procedure and therefore it is possible that their characteristics may vary from batch to batch. Consequently, strict controls have to be in place in order to ensure production consistency so that the purity, safety, potency and efficacy of each vaccine batch can be assured (Hendriksen *et al.*, 1998). Potency is defined as a measure of the relative strength of a biological product as compared to a reference preparation and must be established for each batch of vaccine (McVey and Shi, 2010). The

potency test must be capable of detecting batches which possess an activity different to that of a batch, or batches, for which efficacy has been demonstrated. The test should therefore be capable of detecting batches that have either a lower or higher activity, and should normally be capable of quantifying that difference. However, for routine batch release purposes, it might be sufficient that the test indicates only that the test batch has a potency at least equal to the reference preparation or to an efficacious batch produced previously (Hill, 2011; Woodland, 2011).

Potency tests for most veterinary vaccines are performed by means of vaccination and subsequent challenge of the vaccinated animals with the live pathogenic agent (Lombard *et al.*, 2007; McVey and Shi, 2010). The challenge tests typically rely on the use of laboratory animals (including rabbits, mice and guinea pigs), but the host animal is also often used. Potency tests can require that large numbers of animals be used in challenge tests. In this regard, it has been estimated that about 10% of the total number of laboratory animals used in Europe are required for the safety and potency testing of vaccines for veterinary and human application (Woodland, 2011).

1.5.2. Refinement of animal use in veterinary vaccine potency testing

Animal welfare considerations, cost, assay duration and assay variability have all been drivers for veterinary vaccine producers to find alternative assays to vaccination-challenge tests (Milstien, 2004). Global regulatory agencies are also actively encouraging the evaluation, development and implementation of novel approaches that reduce, refine and replace (3Rs) the use of animals in vaccine safety and potency testing (Russel and Burch, 1959; Hendriksen, 2007; Hendriksen, 2008). Subsequently, a number of alternative methods have been developed and proposed as replacements to current challenge-based potency tests

(Hendriksen *et al.*, 1998; Kulpa-Eddy *et al.*, 2011). Many of these methods incorporate either direct antigen quantitation in vaccine batches or quantify the amount of protective antibody produced in vaccinated animals, which is compared to the reference antibody value known to provide protection in the challenge test. Such assays therefore quantify the amount of protective antibody produced in response to the vaccination dose. The above measurements have been made possible through the development of diverse *in vitro* techniques, including ELISA, toxin-binding assays, and other serological and analytical methods for the detection of specific antigens or antibodies (Draayer, 2011; Kulpa-Eddy *et al.*, 2011). Where cost comparisons are available, *in vitro* techniques are generally less costly than *in vivo* methods owing to the significant expense associated with purchasing, housing and managing animals in the laboratory (Langley *et al.*, 2007).

Although serological assays can potentially provide for significant refinement in terms of animal use, for some vaccines, however, the serum collected from vaccinated animals may still require additional animals in a test to determine the presence of protective antibodies to toxin. This *in vivo* toxin neutralization test (TNT) involves combining the protective antibody collected from vaccinated animals with known amounts of toxin produced by the causative agent (e.g. *Clostridium* spp. vaccines). This mixture is then administered to laboratory animals to assess whether the level of protective antibody fully neutralizes the toxin. If sufficient protective antibody is present to fully neutralize the toxin, the test animals are not exposed to any free toxin and survive. However, if there is residual free toxin, the test animals develop clinical signs or die (USDA, 2007; European Pharmacopoeia, 2008). It is worth noting that *in vitro* methods to quantify antibodies for some Clostridial toxins are available. Examples include indirect ELISAs for *C. tetani* (Lensing *et al.*, 2002) and for *C. chauvoei* (Crichton *et al.*, 1990), whereas toxin

neutralization tests using cell cultures are available for potency testing of *C. septicum* (Lucken *et al.*, 2002) and *C. novyi* Type B vaccines (Borrmann and Schulze, 1998).

1.6. POTENCY EVALUATION OF *C. perfringens* Type D VACCINES

As mentioned previously, enterotoxaemia can be prevented through vaccination of herds with epsilon toxoids (Titball, 2009). These vaccines are produced either from culture filtrates (Habeeb, 1969) or through the production of recombinant toxins (Oyston *et al.*, 1998; Lobato *et al.*, 2010) and are used in inactivated form as toxoids. As highlighted in the preceding sections, veterinary vaccine producers are obliged to prove both the safety and potency of each production batch. Animal experiments still play a central role in potency testing of enterotoxaemia vaccines. The potency testing of *C. perfringens* vaccines, as prescribed by the European Pharmacopoeia (Ph. Eur. 7.0, Monograph 01/2008:0363), requires vaccination of laboratory rabbits followed by quantitative determination of the vaccination-induced antibodies in the rabbit sera using the toxin neutralization test in mice. These epsilon toxoid vaccines confer protection in animals only if they induce antibody titres equivalent to five International Units (IU) of antitoxin (Rosskopf-Streicher *et al.*, 2003). Although highly sensitive, the test is costly, time-consuming and requires specialized technicians and a large number of test animals. It has been estimated that a single potency test on a batch of *C. perfringens* vaccine requires 10 rabbits and 100 mice (Cussler, 1997). Consequently, *C. perfringens* vaccines have been identified as a high priority for animal reduction studies (Kulpa-Eddy *et al.*, 2011). According to the European Pharmacopoeia, alternative validated serological methods may be used, provided that the criteria for acceptance are in accordance with a reference batch of vaccine that has given satisfactory results using the prescribed tests (Ph. Eur. 7.0, Monograph 01/2008:20724). In the following sections, alternative *in vitro* methods that have been proposed to measure

vaccination-induced antibodies in rabbit sera against *C. perfringens* Type D epsilon toxin will be discussed.

One of the first investigations regarding the replacement of the mouse neutralization test (MNT) with an alternative *in vitro* immunoassay was performed by Pfahler *et al.* (1998). In their study, an indirect ELISA was developed and evaluated for potency testing of *C. perfringens* Type D epsilon toxoid vaccines. Individual and pooled sera of vaccinated rabbits were tested at a single dilution level, the optical density readings were converted to IU/ml and then compared with results obtained by the conventional toxin neutralization test in mice. The ELISA was reported to give reproducible estimates of antitoxin levels and showed good correlation (correlation coefficient of 0.97) with the conventional *in vivo* test in mice.

In a different approach, Ebert *et al.* (1999) developed a competitive ELISA, based on the use of a monoclonal antibody, to quantify neutralizing antibodies against the *C. perfringens* Type D epsilon toxin in the sera of rabbits immunized with different epsilon toxoid vaccines. The ELISA was used in addition to the MNT to determine epsilon-antitoxin levels in the rabbit serum samples. Not only was there good agreement between the rank order of sera determined *in vivo* by the MNT and the rank order determined *in vitro* by the ELISA, but linear regression analysis yielded a correlation coefficient of 0.41 for the competitive ELISA. Moreover, a pre-validation study was performed in four different laboratories to evaluate the transferability of the competitive ELISA procedure and the inter-laboratory reproducibility of the results. Three coded serum samples were tested between three and six times in the respective laboratories. Despite two of the participating laboratories having had only limited experience with ELISA procedures at the start of the pre-validation study, the results indicated that the inter-laboratory variation was only 9.4-17.9%. This therefore indicated that the competitive ELISA was easy to implement

and readily transferable to all of the laboratories. These results were subsequently confirmed in a second collaborative study that involved seven international laboratories (Roskopf-Streicher *et al.*, 2003).

As an alternative to the ELISA test systems, two other approaches for potency testing of *C. perfringens* Type D epsilon toxoid vaccines have been described. Borrmann *et al.* (2006) developed a cell culture assay for the quantitative determination of the antibody titres against *C. perfringens* Type D epsilon toxin in rabbit sera as a possible alternative to the MNT in mice. The cell culture assay was based on the use of MDCK cells, which have previously been shown to be sensitive to the *C. perfringens* Type D epsilon toxin (Knight *et al.*, 1990; Petit *et al.*, 2003). In essence, the toxin solution was mixed with serum dilutions, incubated and then a MDCK cell suspension was added to the toxin-antitoxin mixture. The mixtures were plated in 96-well plates and cell suspensions were prepared after three days of incubation. The cells were examined for morphological changes by microscopic examination and evaluation of viable cells was performed with a 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) test (Mossmann, 1983). Using the MTT assay, neutralization curves for each rabbit antiserum were estimated. The correlation coefficients of 0.6 and 0.72 that were subsequently determined for the two test serum samples indicated that there was moderate to good correlation between the results of the cell culture assay and the MNT in mice. Although the cell culture assay does not require the use of monoclonal antibodies, which is a prerequisite for some ELISA systems (Ebert *et al.*, 1999; Roskopf-Streicher *et al.*, 2003), a concern that has been raised is that the cell cultures may not be sufficiently specific and may only indicate cytopathic effects (Pfahler *et al.*, 1998). Nevertheless, it was concluded that the cell culture assay might be suitable as an *in vitro* alternative to the *in vivo* mouse neutralization experiments required for potency tests of Clostridial vaccines, but further validation studies are necessary.

More recently, an *in vitro* toxin-inhibition binding (ToBI) assay was developed and evaluated for quality control of *C. perfringens* Type D epsilon toxoid vaccines (Sobrinho *et al.*, 2010). The ToBI test uses antibodies obtained from immunized animals to inhibit toxin binding to antitoxins adsorbed onto a preincubated microplate. The ToBI test was used to evaluate serum samples of known antibody content, obtained from rabbits immunized against enterotoxaemia with different vaccines. The correlation coefficients between the results obtained from the ToBI test and toxin neutralization in mice ranged from 94.23% to 97.75%. Inter-plate coefficients of variation were low (0.350-0.400%), indicating that the ToBI test is reproducible and reliable. Although the ToBI test may be useful as an alternative to the MNT for assessing vaccine potency, it is sensitive to the presence of impurities that can interfere with antigen-antibody binding and therefore requires the use of highly purified reagents. Moreover, the method involves a series of detailed procedures that must be rigorously followed to ensure reliable results (Sobrinho *et al.*, 2010).

1.7. CYTOMETRIC BEAD-BASED FLUORESCENT IMMUNOASSAYS

As indicated above, the ELISA is a specific and reproducible assay that is well suited to the screening of many samples for a single analyte (Ellington *et al.*, 2010). However, with the increased demand for multiplexing capability, shorter analysis time, smaller sample volume and higher sensitivity, other techniques have been explored to perform immunoassays. One such technique that has gained popularity is the cytometric bead assay (CBA), which relies on the use of microbeads and a flow cytometer to carry out the immunoassays. Indeed, as early as 1977, flow cytometry was applied to the detection of antigens captured by antibodies coated on the surface of microbeads (Horan and Wheeless, 1977). The technology has since expanded to such an extent that microbeads are commonly used as a solid support for numerous molecular

reactions quantitated by flow cytometry. Antibodies, proteins or small peptides have all been adsorbed or chemically coupled to the surface of microbeads to capture analytes that are subsequently measured by a fluorochrome-conjugated detection molecule (Vignali, 2000; Fitzgerald, 2001; Kellar and Iannone, 2002; Edwards *et al.*, 2004; Ellington *et al.*, 2010). In these assays, the reporter intensity relates to the quantity of analyte bound and therefore establishes the relative concentration of the analyte (Morgan *et al.*, 2004).

There are several advantages associated with the use of microbeads for immunoassays. Not only do they provide an increased surface to volume ratio and thus a reduction in the reagent volumes required, but they also move freely in solution so that the reaction kinetics are faster. Moreover, the immobilization of biomolecules on the particle surface localizes the chemistry to a defined point in order to facilitate rapid analysis (Vignali, 2000; Holmes *et al.*, 2007). A wide range of surface functional beads are available commercially that allow for binding of antibodies, proteins, peptides and other biomolecules. In addition, fluorescent microbeads with different emission spectra and intensities are also available that permit the simultaneous detection of several to potentially 64 different analytes in the same sample volume (Kettman *et al.*, 1998; Carson and Vignali, 1999; Whitelegg *et al.*, 2012).

Numerous studies have reported the use of cytometric bead-based assays to perform immunoassays for detecting antibodies to various antigens in serum samples, as well as to monitor immune responses to vaccination (Best *et al.*, 1992; Pickering *et al.*, 2002a; Lal *et al.*, 2004; Ferbas *et al.*, 2007; Elberse *et al.*, 2010; Whitelegg *et al.*, 2012). Typically, the antigens are covalently conjugated onto the solid surface of the microbeads and the antigen-coated beads are then incubated with the serum sample of interest. This is followed by incubation with a species-specific anti-immunoglobulin reagent labeled with a fluorochrome, such as fluorescein

isothiocyanate (FITC), to allow fluorescent detection in the flow cytometer. The shift in the mean fluorescence intensity (MFI) in the channel reading the detector fluorochrome reflects the amount of antibody bound to the antigen on the bead and can be quantified by comparison with a calibration curve. In a variation of the above assay, antigen was immobilized on the beads through a bridging intermediate. For this purpose, an epitope-specific antibody was first conjugated to the bead surface and used to bind the antigen on the bead. The subsequent steps remained essentially the same as those described above (Figure 1.2).

A number of studies have compared cytometric bead-based immunoassays to ELISAs with regards to sensitivity, specificity and dynamic range of measurement. Not only were the cytometric bead-based immunoassays superior to ELISAs in these aspects, but the assays were also reported to be more reproducible and took considerably less preparation time compared to the ELISA assays (Carson and Vignali, 1999; Pickering *et al.*, 2002b; Du Pont *et al.*, 2005). According to Morgan *et al.* (2004), the major advantages of this technology is its ability to allow for the measurement of single analyte or multiple analytes in a quantitative fashion, a reduction in the sample volume required for analysis and a reduction in the time spent in generating data. However, a major limitation of cytometric bead-based assays is the complexity and expense of flow cytometers.

1.8. AIMS OF THIS STUDY

Onderstepoort Biological Products (OBP) produces approximately 15 batches (200 000 doses) of enterotoxaemia vaccine containing the epsilon toxoid every year in South Africa. Since the enterotoxaemia vaccine is sold nationally and internationally, OBP is obliged to prove the purity, safety, efficacy and potency of each production batch. The potency test currently in use is that

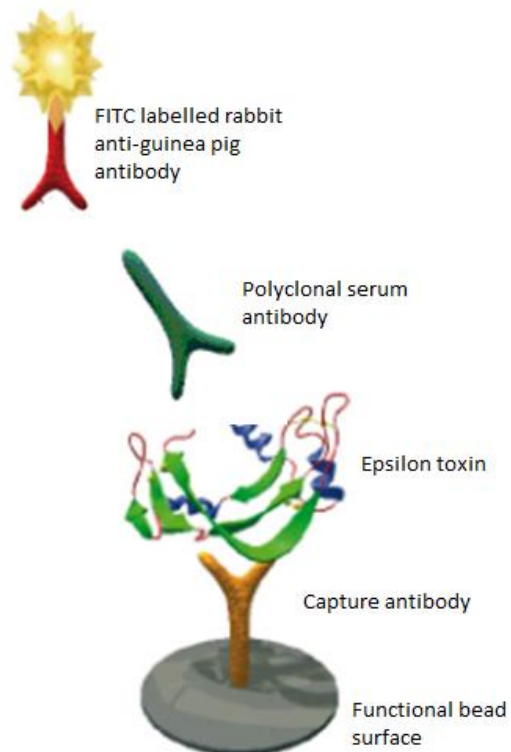


Figure 1.2 A schematic representation of the reagents used in a microbead-based flow cytometric immunoassay. In this example, the antigen is bound to microbead via bridging intermediate (capture antibody). The assay involves incubation with serum, followed by addition of a fluorescent detection reagent (FITC-conjugated anti-guinea pig IgG) and analysis on a flow cytometer. The intensity of fluorescence is therefore proportional to the amount of serum antibody captured by the bead (Figure adapted from Kellar and Iannone, 2002).

prescribed by the European Pharmacopoeia (Ph. Eur. 7.0, Monograph 01/2008:0363), namely the *in vivo* toxin neutralization test which is also referred to as the mouse neutralization test (MNT). Each batch potency test with this *in vivo* test requires the use of 8 guinea pigs and 100 mice. This, together with the use of additional laboratory animals for batch safety testing, contributes to the high cost of the vaccine. In lieu of animal welfare and the large number of animals used in these tests, there is a clear need for more humane and cost-effective methods whereby the potency of the enterotoxaemia vaccines can be determined accurately and reliably. The European Pharmacopoeia indicates that alternative *in vitro* methods such as validated ELISAs and immunofluorescent techniques may be used for potency testing (Ph. Eur. 7.0, Monograph 01/2008:20724). Consequently, the aims of this study were therefore:

- To develop and evaluate an indirect cytometric bead immunoassay (I-CBA) whereby the anti-epsilon toxin antibody levels in the sera of guinea pigs vaccinated with the OBP Enterotoxaemia alum vaccine[®] can be determined.
- To compare the results obtained with the newly developed I-CBA assay to those obtained using an indirect ELISA and the MNT.

CHAPTER TWO

MATERIALS AND METHODS

2.1. SERUM SAMPLES

Sera were obtained from guinea pigs that had been vaccinated with three independently produced batches of OBP Enterotoxaemia alum vaccine[®] (Onderstepoort Biological Products Ltd.). For each vaccine batch, eight naïve guinea pigs were injected subcutaneously with 2 ml of the vaccine and booster injections were given three weeks following the initial vaccination. Sera were collected in serum vacu-tubes (Lasec) prior to immunization of the guinea pigs and at five weeks post-vaccination. The guinea pig sera were prepared into two serum samples, designated A and B, where A comprised the pooled sera of four randomly selected guinea pigs and B comprised the pooled sera of the remaining four guinea pigs. The serum samples were stored at -22°C until their use in vaccine potency evaluations by means of the mouse neutralization test (MNT), an indirect cytometric bead immunoassay (I-CBA) and an indirect enzyme-linked immunosorbent assay (I-ELISA). The guinea pigs and mice used to perform the MNT formed part of routine vaccine batch testing at OBP and the information obtained from these batches were incorporated in this study. The same sera were used to perform the two *in vitro* assays after the MNT indicated that the three vaccine batches passed their quality evaluation.

2.2. EPSILON TOXIN PURIFICATION

2.2.1. *Bacterial strain and culturing conditions*

A vaccine production strain of *Clostridium perfringens* Type D was cultured anaerobically at 37°C in a seed flask containing meat-pellet broth (MPB) for 24 hours. Culturing was done in a controlled anaerobic environment in an anaerobic chamber (Forma Anaerobic Chamber Model 1025; Thermo Scientific). Anaerobic conditions were maintained by making use of a hydrogen,

nitrogen and carbon dioxide gas mixture with palladium as catalyst. The seed flask was used as the inoculum for a pilot fermenter containing a sterile in-house developed pulpy kidney medium (PKM). Following anaerobic growth at 37°C until a marked change in pH was observed, the inoculum was pumped from the pilot fermenter into the production fermenter containing sterile PKM medium under the same environmental conditions. Anaerobic growth was allowed until the pH stabilized at 6.8, which was typically observed after 18 hours of incubation. During this culturing process the epsilon toxin, amongst others, is secreted into the culture supernatant.

2.2.2. Precipitation of the epsilon toxin

The epsilon toxin was precipitated with ammonium sulphate from the culture supernatant according to the procedures described by Whitaker and Granum (1980), Thomson (1962) and Watson *et al.* (1927). The bacterial cells from a 5-ℓ culture were collected by centrifugation at 3000 x g for 10 minutes at 4°C in an Eppendorf 5804 R centrifuge. The cell-free culture supernatant was recovered and transferred into a sterile 5-ℓ flask. For each 1 ℓ of cell-free culture supernatant, 390 g of ammonium sulphate (Sigma-Aldrich) was added and allowed to dissolve by stirring slowly on a magnetic stirrer. The solution was subsequently incubated at 4°C for 24 hours (without stirring). The precipitated proteins were collected by centrifugation at 10 000 x g for 30 minutes at 4°C and the protein pellet was suspended in 50 ml of 0.02 M phosphate buffer (pH 7.2). Residual salts were removed from the protein sample by dialysis against sterile ddH₂O at 4°C for 24 hours. The dialysis was performed in a snake skin dialysis tube with a 3-kDa molecular weight cut-off limit (Pierce). To confirm the removal of sulphide ions (S²⁻), 1 ml of the ddH₂O was mixed with 1 mg of lead acetate (Merck) and the appearance of a clear solution, as opposed to a milky solution, confirmed that no sulphide ions were present

(US Pharmacopoeia, 2005). The dialysate was removed from the dialysis tube and the epsilon toxin purified by ion exchange chromatography, as described below.

2.2.3. Ion exchange chromatography

To purify the epsilon toxin, the dialysate was subjected to ion exchange chromatography using DEAE Sepharose resin (GE Healthcare) according to the procedures described by Habeeb (1969). For this purpose, a glass column (26 mm x 200 mm) was packed with 50 ml of DEAE Sepharose and the resin was equilibrated with 50 mM NaP buffer (pH 6.5) prior to use. Subsequently, 50 ml of the sample was loaded onto a 50-ml bed volume. The sample was pumped through the matrix at a flow rate of 60 ml/h using 50 mM NaP buffer (pH 6.5). Fractions were collected every 5 minutes from the bottom of the column with an automated fraction collector (Frac-920; GE Healthcare) and the optical density (OD) of each fraction was determined at 280 nm with a spectrophotometer (SpectroQuant Pharo 300; Eppendorf). Fractions containing the epsilon toxin were pooled for use in subsequent assays.

2.3. CHARACTERIZATION OF PURIFIED EPSILON TOXIN

2.3.1. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

To assess the purity of the epsilon toxin preparation, samples were analyzed by electrophoresis in a discontinuous gel system as described by Laemmli *et al.* (1970). Prior to electrophoresis, 5 μ l of the sample was mixed with an equal volume of 2-mercaptoethanol (Sigma-Aldrich) and the toxin was denatured by heating to 100°C for 5 minutes in a boiling water bath. The 5% stacking gel (5% [w/v] acrylamide, 0.17% [w/v] bis-acrylamide, 125 mM Tris-HCl, 0.1% [w/v] SDS; pH 6.8) and the 10% separating gel (10% [w/v] acrylamide, 0.34% [w/v] bis-acrylamide, 0.375 mM

Tris-HCl, 0.1% [w/v] SDS; pH 8.8) were each polymerized by addition of 150 μ l of 10% (w/v) ammonium persulphate and 15 μ l of TEMED. Electrophoresis was performed in a vertical electrophoresis unit (Cleaver Scientific Ltd.) at 120 V for 90 minutes in 1 x TGS buffer (25 mM Tris, 192 mM glycine, 0.1% [w/v] SDS; pH 6.8). Following electrophoresis, the gel was stained for 20 minutes with 0.025% (w/v) Coomassie Brilliant Blue R-250 prepared in 10% (v/v) glacial acetic acid, and then destained in a solution containing 10% (v/v) glacial acetic acid until the proteins were visible. The sizes of the resolved proteins were estimated by comparison to reference molecular marker proteins (PageRuler pre-stained protein ladder; Fermentas).

2.3.2. Determination of the toxin concentration

The concentration of the purified epsilon toxin was determined by the method of Bradford (1976) using a commercial kit (Quick Start Bradford Protein Assay; BioRad) with bovine serum albumin (BSA) as standard. Aliquots (5 μ l) of each sample were added to 250 μ l of Bradford reagent, mixed well and the optical density (OD) at 595 nm was determined following incubation at room temperature for 5 minutes. Bradford reagent was used to zero the readings and the toxin concentration (mg/ml) was then determined from the prepared standard curve.

2.3.3. Confirmation of the identity of the purified toxin

To confirm the identity of the purified epsilon toxin, an Enterotoxaemia ELISA was performed using the BioK 270 kit according to the instructions of the manufacturer (Bio-X Diagnostics). Briefly, 100 μ l of the purified toxin preparation was added to the wells of the supplied microtiter plate pre-coated with monoclonal anti-alpha toxin antibodies, monoclonal anti-beta toxin antibodies and monoclonal anti-epsilon toxin antibodies, respectively. After incubation at room temperature for 1 hour, the plate was washed with kit washing buffer and 100 μ l of peroxidase-

labelled conjugates (colour-coded for each antigen) was added to the respective wells. The plate was incubated at room temperature for 1 hour and 100 µl of the tetramethylbenzidine (TMB) peroxidase substrate was added to each well. After incubation at room temperature for 10 minutes, 50 µl of the supplied stop solution was added to each well and the optical densities (OD) were measured at 450 nm with a BIO-TEK ELISA plate reader. The purified epsilon toxin sample was subsequently dispensed as 200-µl aliquots into Eppendorf tubes and stored at -22°C until required.

2.4. MOUSE NEUTRALIZATION TEST (MNT)

The mouse neutralization test (MNT) was used to evaluate anti-epsilon toxin serum antibodies from guinea pigs for epsilon toxin neutralization. In order to confirm the dilution accuracy of a positive epsilon toxin control, the toxin was serially diluted in filtered saline (0.45 µm, pH 7.2) and the LD₅₀ was established (Table 2.1). A second confirmation for accuracy was performed against standardized positive control guinea pig serum (Table 2.2). Subsequently, the guinea pig test sera were serially diluted in filtered saline from a 1/2 to a 1/16 dilution to a final volume of 1 ml. Each dilution tube was mixed with 1 ml of purified epsilon toxin previously standardized so that a 1/180 dilution of a 1 mg/ml toxin neutralizes 10 U/ml of standardized positive control guinea pig serum (Table 2.3). The prepared test samples were incubated at 37°C for 30 minutes and 0.2 ml of the samples were injected intravenously into four mice per dilution. The mice were observed for 24 hours and the results were noted. Mice that died were noted with (+) and mice that survived were noted with (L). The lowest unit/ml-value where the mice died was noted as the protective value of the serum sample (European Pharmacopoeia, 2008).

Table 2.1 Serial dilution range in the MNT for evaluating the 1/180 dilution accuracy of a standardized epsilon toxin with a 1 mg/ml concentration

	Tube number	1	2	3	4
Step 1	Dilution value	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$
	Saline (ml)	0	1	1	1
	Toxin (ml)	1 (standardized toxin, diluted 1/180)	1 (standardized toxin, diluted 1/180)	1 (of a $\frac{1}{4}$ dilution of the toxin)	1 (of a $\frac{1}{8}$ dilution of the toxin)
Step 3	After steps 1 and 2, add saline (ml)	1	1	1	1

⇒ Remove 1ml ($\frac{1}{16}$)

Table 2.2 Serial dilution range in the MNT for evaluating the 1/180 dilution accuracy of a standardized epsilon toxin (1 mg/ml) using a standardized positive serum control (10 U/ml)

5-ml glass tubes	Tube number	1	2	3	4	5
	Units/ml	33.3	20	10	5	2.5
	Saline (ml)	0.4	0.9	0.8	0.6	0.2
	Serum sample (ml)	0.6 (of a $\frac{1}{100}$ dilution of the serum)	0.1 (of a $\frac{1}{10}$ dilution of the serum)	0.2 (of a $\frac{1}{10}$ dilution of the serum)	0.4 (of a $\frac{1}{10}$ dilution of the serum)	0.8 (of a $\frac{1}{10}$ dilution of the serum)
	Prepared standardized toxin, diluted 1/180 (ml)	1	1	1	1	1

Table 2.3 Serial dilution range in the MNT for evaluating the guinea pig test serum samples using a standardized epsilon toxin (1 mg/ml)

5-ml glass tubes	Tube number	1	2	3	4	5	6	7
	Units/ml	50	33.3	20	10	5	2.5	1
	Saline (ml)	0.6	0.4	0.9	0.8	0.6	0.2	0.8
	Serum sample (ml)	0.4 (of a $\frac{1}{100}$ dilution of the serum)	0.6 (of a $\frac{1}{100}$ dilution of the serum)	0.1 (of a $\frac{1}{10}$ dilution of the serum)	0.2 (of a $\frac{1}{10}$ dilution of the serum)	0.4 (of a $\frac{1}{10}$ dilution of the serum)	0.8 (of a $\frac{1}{10}$ dilution of the serum)	0.2
	Prepared standardized toxin, diluted 1/180 (ml)	1	1	1	1	1	1	1

2.5. INDIRECT CYTOMETRIC BEAD IMMUNOASSAY (I-CBA)

2.5.1. Assay procedure

The Cytometric Bead Array (CBA) kit from Becton Dickinson was used to develop a flow cytometric-based immunoassay in order to semi-quantify anti-epsilon toxin antibodies in the sera from vaccinated guinea pigs. The optimal antigen concentration for coating the beads was determined by a standard checkerboard titration method (Owens *et al.*, 2000) prior to performing the indirect cytometric bead immunoassay. In order to optimize the assay, purified epsilon toxin was diluted across the rows of a 96-well Nunc microtiter plate in a 10-fold fashion from a 1 mg/ml to a 1 µg/ml concentration to a total volume of 50 µl per well. Functional beads (supplied in the CBA kit) were diluted to a 1 x working concentration and 50 µl were added to each well. Following incubation of the microtiter plate at room temperature for 1 hour, 150 µl of the kit washing buffer was added to each well and the plate was centrifuged at 200 x g for 5 minutes in an Eppendorf 5804 R centrifuge. Unbound epsilon toxin was aspirated using a multichannel pipette. On a separate microtiter plate, positive control guinea pig serum at 50 U/ml (as determined by the MNT) was diluted in a 2-fold fashion from a 1/100 to 1/1600 dilution using kit serum diluent across the columns of the plate. Fifty (50) µl of the diluted positive control guinea pig serum was added to respective wells of the plate containing the epsilon toxin-coated beads and incubated at room temperature for 1 hour. After incubation the plate was washed and the beads pelleted as described above. The supernatant from each well was aspirated using a multichannel pipette and 50 µl of fluorescein isothiocyanate (FITC)-labelled anti-guinea pig IgG (Sigma-Aldrich), diluted 1/128 in the kit sample diluent, was added to each well, mixed and then incubated at room temperature for 2 hours. Samples were washed once, as described above, then re-suspended in 300 µl of wash buffer and analyzed on a FACSCalibur™ flow cytometer (Becton Dickinson). Guinea pig test serum samples were diluted

1/400 in PBS (pH 7.2) prior to analysis according to the methodology described above. These assays were performed in duplicate on three different occasions.

2.5.2. Data analysis and interpretation

On the day of the assay, the positive control guinea pig serum used in the standard checkerboard titration was diluted with serum diluent (supplied in the CBA kit) from 50 U/ml to 40 U/ml, 30 U/ml, 20 U/ml, 10 U/ml and 5 U/ml, respectively, in order to construct a standard curve. The reactivity threshold was calculated using negative sera from the guinea pigs obtained pre-vaccination. The mean + 1 standard deviations were used to predict an analytical cut-off. Antibody concentrations in each test serum were extrapolated from the standard curve using linear curve fitting.

Before sample acquisition, the flow cytometer instrument setup was performed according to the instructions and template provided by the manufacturer (BD FACSCalibur™ Flow Cytometer BD™ CBA Flex Sets: Instrument Setup, Data Acquisition, and Analysis Instruction Manual). The median fluorescence intensity (MFI) of each bead cluster was quantified using CellQuest Pro™ acquisition and analysis software (Becton Dickinson).

2.6. INDIRECT ELISA (I-ELISA)

2.6.1. Assay procedure

An antibody-capture immunoassay, using purified epsilon toxin antigen and an indirect detection format, was utilized to semi-quantify antibody levels to the epsilon toxin in guinea pig test sera. Optimal concentrations of ELISA reagents were determined by a standard checkerboard

titration method (Owens *et al.*, 2000), following the same procedure as described below, but the positive control guinea pig serum (50 U/ml as indicated by the MNT) was diluted in a 2-fold fashion from a 1/100 to 1/1600 dilution using blocking buffer that comprised 1% (w/v) fat-free milk powder in TST buffer. The previously purified epsilon toxin was diluted in a 10-fold fashion from 1 mg/ml to 1 µg/ml using filtered saline (pH 7.2).

In the optimized assay, 100 µl of the purified epsilon toxin (diluted to 100 µg/ml in 20 M carbonate buffer) was passively adsorbed onto Nunc MaxiSorp ELISA plates overnight at room temperature. The plates were then washed three times with 300 µl per well of TST buffer (0.8 M Tris-HCl, 0.15 M NaCl, 0.05% [v/v] Tween-20; pH 8) to remove unbound epsilon toxin. The coated plates were blocked by the addition of 300 µl of blocking buffer at 37°C for 1 hour. After washing three times with TST buffer, 100 µl of each test serum, diluted 1/400 in blocking buffer, was added in triplicate into the coated wells. The positive control guinea pig serum (previously estimated using the MNT to be 50 U/ml) was diluted to 40 U/ml, 30 U/ml, 20 U/ml, 10 U/ml and 5 U/ml, respectively, and 100 µl from each dilution was added in triplicate into coated wells. Following incubation at 37°C for 1 hour, plates were washed and a volume of 100 µl of recombinant protein G conjugated with horseradish peroxidase (Sigma-Aldrich), diluted 1/8000 in blocking buffer, was added to each well. After incubation at 37°C for 1 hour, the plates were washed three times in TST buffer and 100 µl of tetramethylbenzidine (TMB) peroxidase substrate (Invitrogen) was added to each well. The plates were incubated in the dark at room temperature for 10 minutes. The reactions were stopped by addition of 50 µl per well of 2 N H₂SO₄ and the optical densities (OD) were measured at 450 nm with a BIO-TEK ELISA plate reader. These assays were performed in duplicate on three different occasions.

2.6.2. Data analysis and interpretation

A standard curve was prepared that ranged from 50 U/ml to the minimum requirement of 5 U/ml using a positive control guinea pig serum. The reactivity threshold was calculated using the six pooled groups of pre-vaccination guinea pig serum samples. The mean + 1 standard deviations were used to predict an analytical cut-off. The antitoxin content of the sera was determined using the equation for a straight line in order to extrapolate the antibody concentration in each test serum.

CHAPTER THREE

RESULTS

3.1. PURIFICATION OF THE *C. perfringens* TYPE D EPSILON TOXIN

Purified epsilon toxin was required for the quantification of anti-epsilon toxin antibodies in post-vaccination guinea pig serum using the mouse neutralization test (MNT), indirect cytometric bead immunoassay (I-CBA) and indirect ELISA (I-ELISA). To purify the epsilon toxin, the extracellular proteins from the cell-free culture supernatant of a *C. perfringens* Type D culture was precipitated with ammonium sulphate, the residual salts were removed by dialysis and the dialysate was subjected to ion exchange chromatography. The purified toxin preparation was subsequently characterized to verify its purity and identity.

During ion exchange chromatography, elution of the toxin from the DEAE Sepharose matrix was monitored by determining the OD_{280} values of the collected fractions. The result (Figure 3.1) indicated a gradual increase in the OD_{280} values of the first 13 fractions, after which the values gradually decreased before reaching a plateau from fractions 29 to 33. To ensure maximal recovery of purified epsilon toxin, fractions displaying OD_{280} values of 0.4 or higher were pooled (i.e. fractions 1 to 28). To verify that the epsilon toxin had been recovered and to assess its purity, samples of the pooled fractions were analyzed by SDS-PAGE. Analysis of the Coomassie Blue-stained gel indicated the presence of a 33-kDa protein of which the molecular mass is in agreement with that reported for the epsilon toxin. Moreover, the toxin was purified to near homogeneity as was evident by the lack of contaminating proteins (Figure 3.2). The concentration of the purified epsilon toxin was determined to be 1.126 mg/ml using a Bradford protein assay. To verify the identity of the purified 33-kDa protein, an Enterotoxaemia ELISA kit was used that is capable of distinguishing between *C. perfringens* alpha, beta and epsilon toxins. The result, presented in Table 3.1, indicated that the toxin preparation tested negative for both the alpha and beta toxins but positive for epsilon toxin. The purified epsilon toxin was subsequently used as the antigen component in the three assays involved in this study.

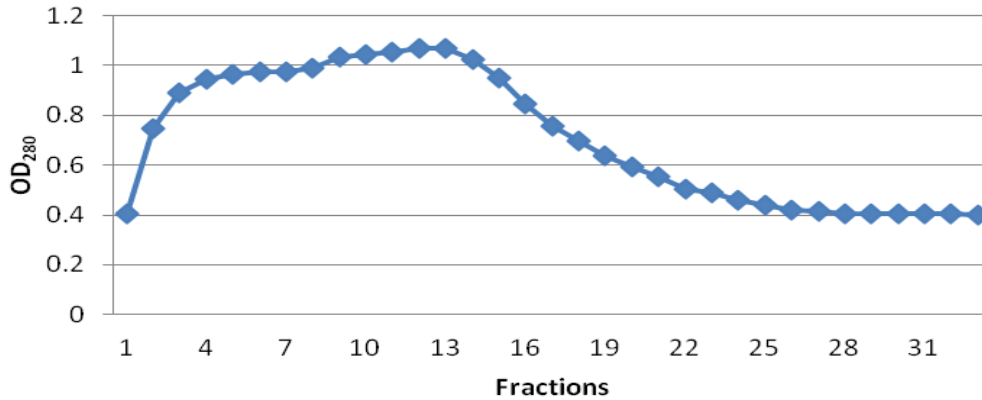


Figure 3.1 Optical density (OD) measurements of fractions eluted from a DEAE Sepharose column during ion exchange chromatography. The fractions were collected from the bottom of the column and the OD₂₈₀ was determined spectrophotometrically. A total of 33 fractions were collected; fraction 1 was the first fraction collected and fraction 33 the last. Fractions 1 through 28 were pooled and subsequently analyzed by SDS-PAGE.

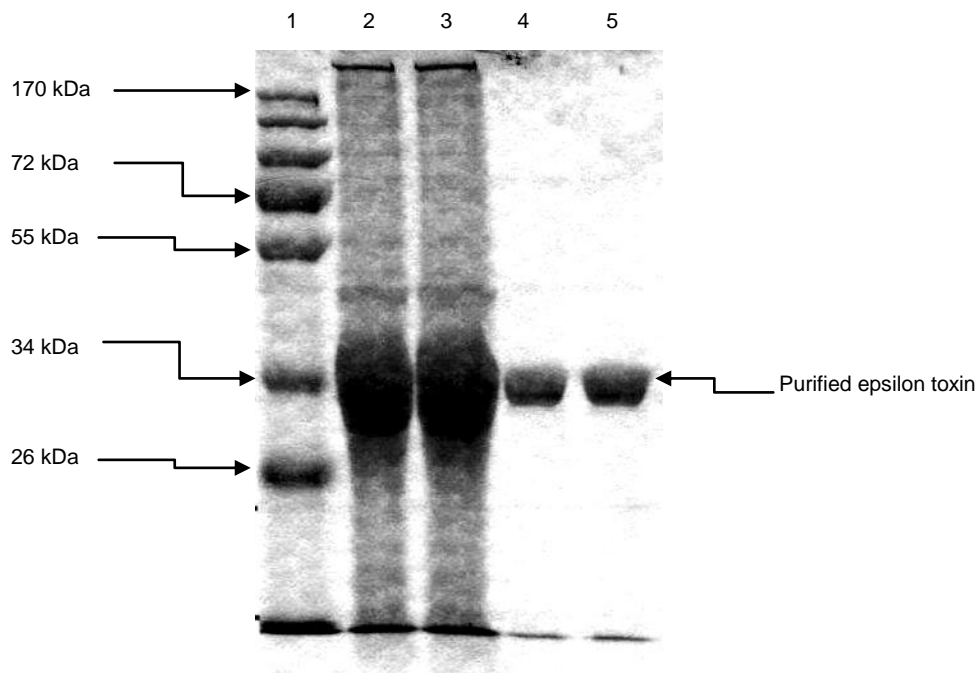


Figure 3.2 SDS-PAGE analysis of the purified toxin preparation. The proteins were resolved by 10% SDS-PAGE and visualized by staining the gel with Coomassie Brilliant Blue R-250. Lane 1, Protein marker; lanes 2 and 3, samples of proteins precipitated with ammonium sulphate from the cell-free *C. perfringens* Type D culture supernatant; lanes 4 and 5, samples of the purified toxin preparation following ion exchange chromatography. The position of the purified epsilon toxin is indicated by an arrow to the right of the figure and the sizes of the protein molecular weight markers (lane 1) are indicated to the left of the figure.

Table 3.1 Enterotoxaemia ELISA results to determine the identity of the purified toxin

Description	Positive Control (provided in the kit)	Purified toxin preparation
^a Alpha toxin	2.033	0.011
Result (Percentage positivity)		0.54% Negative
^b Beta toxin	2.703	-0.1
Result (Percentage positivity)		-3.69% Negative
^c Epsilon toxin	3.062	1.828
^d Result (Percentage positivity)		59.69% Positive

- ^a The assay is valid when the absorbance value for the positive control alpha toxin is more than 1.261, and the sample is accepted as positive for alpha toxin if the percentage positivity is more than 5.95%.
- ^b The assay is valid when the absorbance value for the positive control beta toxin is more than 1.138, and the sample is accepted as positive for beta toxin if the percentage positivity is more than 6.59%.
- ^c The assay is valid when the absorbance value for the positive control epsilon toxin is more than 1.175, and the sample is accepted as positive for epsilon toxin if the percentage positivity is more than 6.39%.
- ^d Prior to calculation of the percentage positivity of the purified toxin preparation, the absorbance values of the control and sample were corrected by subtracting the absorbance value of a negative control supplied in the kit.

3.2. MOUSE NEUTRALIZATION TEST (MNT)

The mouse neutralization test (MNT) is a quantitative *in vivo* toxin neutralization test to assess antitoxin potency (Jansen, 1967; Cameron, 1982). A toxin dilution of known potency is reacted with a series of antitoxin dilutions of unknown potency, obtained from, for example, vaccinated guinea pigs. The dilution series is then injected into mice and mouse mortality over a period of 24 hours is recorded. The largest dilution at which there is still sufficient antitoxin to protect the mice indicates the potency of the vaccine.

In this study, three groups of eight guinea pigs were immunized with one of three different production batches of the OBP Enterotoxaemia vaccine[®] (i.e. batches 198, 199 and 200). Guinea pig sera were collected before vaccination and at five weeks post-vaccination, and the sera of four guinea pigs per group were pooled to give six test sera that were designated 198A and 198B, 199A and 199B, and 200A and 200B.

The dose of toxin used in the MNT was standardized by determining the LD₅₀ in mice (Table A.1, Annexure A), and further confirmation of the toxin dose was established by MNT using a standardized positive control guinea pig serum (Table A.2, Annexure A). All pre-bleed test sera were negative for anti-epsilon toxin antibodies (Table 3.2). Five of the six groups of guinea pig test serum samples collected five weeks post-vaccination had antitoxin levels above the required minimum (i.e. not less than 5 U/ml), whereas group 200A did not meet the requirement (Table 3.3). Based on these results, it was corroborated that the robust MNT was a good benchmarking assay in order to predict the quantity of anti-epsilon toxin antibodies and compare the results with the two *in vitro* assays (I-ELISA and the I-CBA) involved in this study.

Table 3.2 Mouse neutralization test (MNT) of guinea pig sera obtained prior to immunization

		Serum dilution								Result (U/ml)
		50 U/ml	40 U/ml	30 U/ml	20 U/ml	10 U/ml	5 U/ml	2.5 U/ml	1 U/ml	
Test serum	198A	++++	++++	++++	++++	++++	++++	++++	++++	<1 U/ml
	198B	++++	++++	++++	++++	++++	++++	++++	++++	<1 U/ml
	199A	++++	++++	++++	++++	++++	++++	++++	++++	<1 U/ml
	199B	++++	++++	++++	++++	++++	++++	++++	++++	<1 U/ml
	200A	++++	++++	++++	++++	++++	++++	++++	++++	<1 U/ml
	200B	++++	++++	++++	++++	++++	++++	++++	++++	<1 U/ml

Table 3.3 Mouse neutralization test (MNT) of guinea pig sera obtained five weeks post-vaccination

		Serum dilution								^a Result (U/ml)
		50 U/ml	40 U/ml	30 U/ml	20 U/ml	10 U/ml	5 U/ml	2.5 U/ml	1 U/ml	
Test serum	198A	++++	++++	++++	++++	LLLL	LLLL	LLLL	LLLL	20 U/ml
	198B	++++	++++	++++	++++	++++	LLLL	LLLL	LLLL	10 U/ml
	199A	++++	++++	++++	++++	++++	LLLL	LLLL	LLLL	10 U/ml
	199B	++++	++++	++++	++++	++++	LLLL	LLLL	LLLL	10 U/ml
	200A	++++	++++	++++	++++	++++	++++	++++	LLLL	2.5 U/ml
	200B	++++	++++	++++	++++	++++	LLLL	LLLL	LLLL	10 U/ml

^a The minimum potency requirement is 5 U/ml according to the European Pharmacopeia (2008).

3.3. INDIRECT CYTOMETRIC BEAD IMMUNOASSAY (I-CBA)

A Cytometric Bead Array (CBA) kit, distributed by Becton Dickinson, was used to develop a flow cytometer-based immunoassay for the semi-quantification of post-vaccination anti-epsilon toxin antibodies in guinea pig sera. The CBA system combines a conventional ELISA immunoassay with the sensitivity of fluorescence detection via flow cytometry (Morgan *et al.*, 2004; Ferbas *et al.*, 2007). In this study, the purified epsilon toxin was immobilized onto microbeads, thus providing a matrix for the capturing of anti-epsilon toxin antibodies in the test serum samples. Following addition of a FITC-labelled anti-guinea pig IgG antibody, fluorescence was recorded with a FACSCalibur™ flow cytometer (Becton Dickinson) using the fluorescein channel.

The first step in the development of the assay was to determine the optimum concentration of antigen for coating of the beads. This was done with a standard checkerboard titration (Table B.1, Annexure B), the results of which indicated that the optimal antigen concentration to use was 100 µg/ml. The second step involved the setting up of a standard curve using a standard internal positive control serum.

The optimized methodology, described in Section 3.5.1, was followed to test the serum samples (i.e. 198A and 198B, 199A and 199B, and 200A and 200B). Sample acquisition using CellQuest Pro™ software of the flow cytometer generated dot plots for every sample, which generated median fluorescence intensity (MFI) values for every population of dots (representing the sample) (Figure B1, Annexure B). Unconjugated negative control beads were acquired using a dot plot with every assay (Figure B2, Annexure B), thus verifying the instrument setup performed as described previously. The average MFI values for the pre-vaccination serum samples plus 1 standard deviation were used to calculate the cut-off value with 95% confidence. An MFI value of 33.3 was estimated to be the lower level of detection (Table 3.4).

Table 3.4 Median fluorescence intensity (MFI) values determined with the I-CBA for the different guinea pig pre-vaccination serum samples

Test serum	^a Average MFI value
198A	46.2
198B	24.9
199A	53.3
199B	43.7
200A	22.7
200B	12.2
Average MFI	22.9
Standard deviation	10.4
^b Negative cut-off MFI value	33.3

^a Average MFI values are from three independent experiments, each performed in duplicate.

^b The negative cut-off MFI value was calculated using the average MFI + one standard deviation.

The average MFI values obtained for each dilution of the standard positive control guinea pig serum (Table B.2, Annexure B) were plotted onto a XY scatter plot (Figure 3.3). With the addition of a linear trend line and the use of the equation for a straight line, $y = bx + a$, it was possible to determine the amount of antibodies raised against the epsilon toxoid antigen in each of the serum samples. The x-value, which is the value for the unknown test sera in U/ml, is calculated by substituting the y-value representing the average MFI value for each serum sample into the generated equation; $y = 5.3724x + 17.426$. The results for the guinea pig test serum samples, presented in Table 3.5, indicated that serum sample 200A had the lowest level of anti-epsilon toxin antibodies, similarly indicated by the results from the MNT.

For statistical validation purposes, it was necessary to prove linearity of the regression curve. This was achieved by calculating the r^2 value, which is the correlation coefficient indicating the correlation between observed and expected values. This value, which was calculated to be 0.9986, should be as close to 1.00 as possible in order to assume that the regression linearity is significant. Various validation criteria were taken into account; the slope is an indication of the calibration sensitivity of the test and should not be equal to zero for the sensitivity to be satisfactory. The statistical results are summarized in Table B.4 (Annexure B).

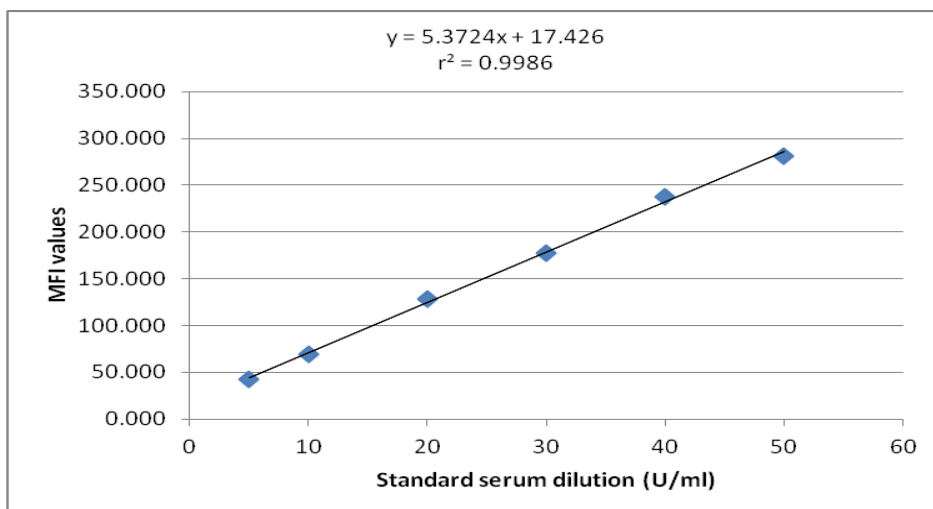


Figure 3.3 Standard curve for the positive control serum in order to calculate the unknown x-value for each serum sample. The results of the six repeats from the positive control was plotted against the known serum concentration and a standard curve was generated using a straight line equation. This equation assisted in calculating the unknown serum value by substituting the MFI value of each serum into the equation.

Table 3.5 Vaccine potency values determined with the I-CBA for the different guinea pig post-vaccination test serum samples

Test serum	^a Average MFI value	^b Corrected MFI value	^c Result (U/ml)
198A	241.1	207.8	35.4
198B	239.2	205.9	35.0
199A	209.3	176.0	29.5
199B	198.1	164.8	27.4
200A	179.4	146.1	23.9
200B	249.9	216.6	37.0

^a Average MFI values are from three independent experiments, each performed in duplicate (also see Table B.3, Annexure B).

^b MFI values corrected with the average MFI + one standard deviation obtained for guinea pig pre-bleed serum (MFI = 33.300, Table 3.4).

^c Vaccine potency values were calculated using the equation $y = 5.3724x + 17.426$, obtained from the standard curve presented in Figure 3.3.

3.4. INDIRECT ELISA (I-ELISA)

An indirect ELISA was used as a second *in vitro* test to semi-quantify anti-epsilon toxin antibodies in guinea pig serum samples five weeks post-vaccination. The OD₄₅₀ values for the pre-vaccination serum samples plus 1 standard deviation were used to calculate the cut-off value with 95% confidence. An OD₄₅₀ value of 0.046 was estimated to be the lower level of detection (Table 3.6).

The checkerboard titration results indicated that the optimal epsilon toxin concentration for coating the plates was approximately 100 µg/ml (Table C.1, Annexure C). The positive control serum was tested at 50 U/ml, 40 U/ml, 30 U/ml, 20 U/ml, 10 U/ml and 5 U/ml, respectively (Table C.2, Annexure C), in order to generate a standard curve (Figure 3.4). I-ELISA results for the test sera were calculated through linear curve fitting. The results for the guinea pig serum samples obtained five weeks post-vaccination are presented in Table 3.7. The results indicated that all six groups of guinea pig test serum samples had antitoxin levels above the required minimum antitoxin level (i.e. not less than 5 U/ml). However, the value obtained for group 200A was the lowest as was also indicated by the results from both the MNT and I-CBA.

For statistical validation purposes, it was necessary to prove linearity of the regression curve. This was achieved by calculating the r^2 value, which is the correlation coefficient indicating the correlation between observed and expected values. This value, which was calculated to be 0.9987, should be as close to 1.00 as possible in order to assume that the regression linearity is significant. Various validation criteria were taken into account; the slope is an indication of the calibration sensitivity of the test and should not be equal to zero for the sensitivity to be satisfactory. The statistical results are summarized in Table C.4 (Annexure C).

Table 3.6 OD₄₅₀ values determined with the I-ELISA for the different guinea pig pre-vaccination serum samples

Test serum	^a Average OD ₄₅₀ value
198A	0.025
198B	0.024
199A	0.026
199B	0.043
200A	0.050
200B	0.044
Average OD ₄₅₀	0.035
Standard deviation	0.011
^b Negative cut-off OD ₄₅₀ value	0.046

^a Average OD₄₅₀ values are from three independent experiments, each performed in triplicate.

^b The negative cut-off OD₄₅₀ value was calculated using the average OD₄₅₀ + one standard deviation obtained for guinea pig pre-bleed serum.

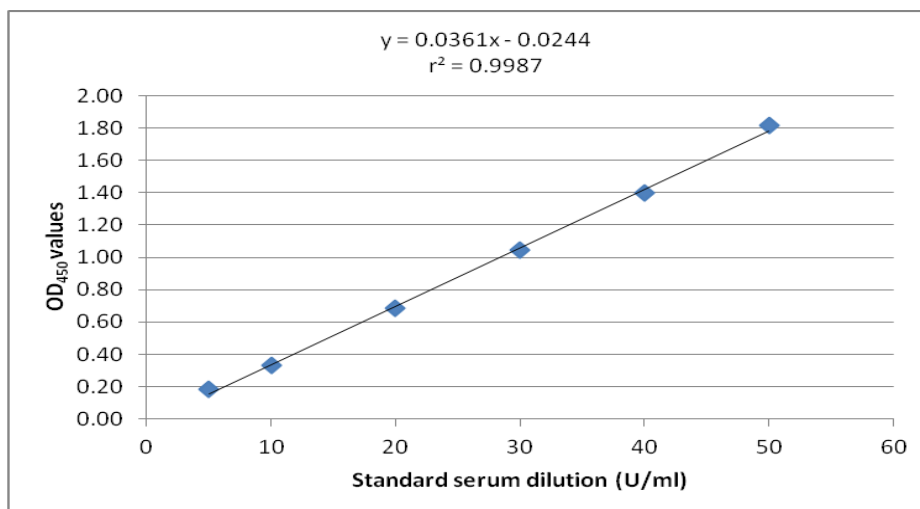


Figure 3.4 Standard curve for the positive control serum in order to calculate the unknown x-value for each serum sample. The results of the six repeats from the positive control was plotted against the known serum concentration and a standard curve was generated using a straight line equation. This equation assisted in calculating the unknown serum value by substituting the OD value of each serum into the equation.

Table 3.7 Vaccine potency values determined with the I-ELISA for the different guinea pig post-vaccination test serum samples

Test serum	^a Average OD ₄₅₀	^b Corrected OD ₄₅₀	^c Result (U/ml)
198A	1.177	1.131	32.0
198B	0.981	0.935	26.6
199A	1.041	0.995	28.2
199B	1.030	0.984	27.9
200A	0.243	0.197	6.1
200B	0.925	0.879	25.0

^a Average OD₄₅₀ values are from three independent experiments, each performed in duplicate (also see Table C.3, Annexure C).

^b OD₄₅₀ values corrected with the average OD₄₅₀ + one standard deviation obtained for guinea pig pre-bleed serum (OD₄₅₀ = 0.046, Table 3.6).

^c Vaccine potency values were calculated using the equation $y = 0.0361x - 0.0244$, obtained from the standard curve presented in Figure 3.4.

CHAPTER FOUR

DISCUSSION

Enterotoxaemia caused by *Clostridium perfringens* Type D is a fatal disease of sheep, goats and calves (Niilo, 1980; Uzal, 2004). The major pathological agent is the epsilon toxin (Songer, 1996), which is produced as an inactive prototoxin and activated by proteolytic enzymes (Bhown and Habeeb, 1979; Minami *et al.*, 1997). Since enterotoxaemia in young animals often evolves to a superacute stage, the disease is difficult to treat. Economic losses may, however, be prevented by immunization through vaccination (Oyston *et al.*, 1998; Titball, 2009). In South Africa, a monovalent vaccine containing the epsilon toxoid is manufactured and licensed by Onderstepoort Biological Products (OBP Ltd.). The requirements for assessing the quality, safety, efficacy and potency of these vaccines are contained in Monograph 01/2008:0363 of the European Pharmacopoeia.

The potency of *C. perfringens* Type D vaccines is measured in terms of their ability to induce antitoxins against the epsilon toxoid, and the *in vivo* mouse neutralization test (MNT) is the statutory method for determining the level of anti-epsilon toxin antibodies in the sera of vaccinated rabbits or guinea pigs. To be approved, a vaccine must produce an antibody response of at least 5 U/ml of anti-epsilon toxin antibodies (Roskopf-Streicher *et al.*, 2003). Although the MNT is known to be sensitive, it is cumbersome, expensive and slow, and it can be relatively imprecise as often a ranged result is determined (Uzal *et al.*, 1997b; Pfahler *et al.*, 1998). Moreover, apart from ethical considerations due to the use of large numbers of animals, the MNT may also suffer disadvantages inherent to biological tests such as variation in animal sensitivity (Kozaki *et al.*, 1979; Henderson, 1984; Levings *et al.*, 1993) and precision of the inoculation technique (Pfahler *et al.*, 1998). The European Pharmacopoeia test protocol for vaccine potency testing does not take into account the immune status of laboratory animals at the start of the test, nor does it define exactly the time of serum collection and the period between the first and the second vaccination. These factors may also contribute to the

variability of the *in vivo* results (Ebert *et al.*, 1999). Although differences of about 20% are accepted by the European Pharmacopoeia Monograph 01/2008:0363 for repetition of the *in vivo* test, such repeat testing is not performed due to the large numbers of animals required and the extreme distress involved in the MNT.

Various reports have expressed the need for a reduction in the use of animals for vaccine potency testing (Milstien, 2004; Hendriksen, 2008; Kulpa-Eddy *et al.*, 2011). While there is no alternative to immunizing test animals when assessing the potency of vaccines, the quantification of protective anti-epsilon toxin antibodies may, however, be determined by *in vitro* methods. Previous studies have reported on the development and evaluation of an indirect ELISA (Pfahler *et al.*, 1998) and competitive ELISA (Ebert *et al.*, 1999; Rosskopf-Streicher *et al.*, 2003) to measure epsilon antitoxin in the sera of vaccinated rabbits. ELISAs have been reported to offer several advantages over the MNT test. In addition to precluding the use of laboratory animals, the ELISA technique allows for greater numbers of samples to be processed at one time and includes fewer complex standardization processes compared to the MNT (Levings *et al.*, 1993; Uzal *et al.*, 1997b).

As an alternative *in vitro* test to quantify anti-epsilon toxin antibodies in the sera of vaccinated guinea pigs, a flow cytometric microbead-based indirect immunoassay was developed and evaluated during the course of this study. In contrast to the medical field where cytometric bead-based immunoassays have been used extensively for diagnostics and research (Vignali, 2000; Morgan *et al.*, 2004; Sanchez-Carbayo, 2006), it has not yet been widely applied in the veterinary field nor in veterinary vaccine potency testing. This technique, which is based on the melding of ELISA-based technology with flow cytometry, uses microbeads as the solid support for a conventional immunosorbent assay with a green-fluorescent reporter dye (Kellar and

Iannone, 2002). Although this technique incorporates all the advantages of the ELISA, the dynamic range of the cytometric bead-based immunoassay is greater than that of ELISA. Such an increase in the dynamic range reportedly makes these assays more sensitive and accurate than ELISA systems, and significantly reduces the sample volume required (Lal *et al.*, 2004; Morgan *et al.*, 2004; Du Pont *et al.*, 2005).

The epsilon toxoid vaccine produced by OBP is prepared from the culture filtrate of a *C. perfringens* Type D isolate and therefore the corresponding vaccine induces antibodies against numerous cellular and extracellular proteins of the bacterial culture. Since the specificity of the indirect cytometric bead assay (I-CBA) and indirect ELISA (I-ELISA) is mainly a function of the purified antigen used in these tests, only epsilon toxin purified by ion exchange chromatography was used in this study. The results demonstrated that with the purification technique used, the antigen obtained was indeed epsilon toxin since it reacted specifically with an anti-epsilon toxin monoclonal antibody (Table 3.1). The purified epsilon toxin was subsequently used as the antigen component in the *in vivo* MNT and the *in vitro* I-CBA and I-ELISA tests. Moreover, for quantitative immunoassays, standard curves were obtained for the I-CBA and I-ELISA after six assays at different concentrations of a standardized positive control guinea pig serum. Note should be taken that although sensitivity in serology usually expresses the lowest detectable amounts of specific antibodies, for potency testing of vaccines the detection of extremely low antibody activities is of minor interest. Consequently, in this study, the positive control serum was diluted from 50 U/ml to 5 U/ml, of which the latter is the minimum requirement for a vaccine to be approved (European Pharmacopoeia, 2008). The correlation coefficient of $r^2 = 0.998$ obtained for both standard curves (Figures 3.3 and 3.4) serves to confirm the reliability of the results (Naute, 2011). The standard curves were subsequently used to estimate the anti-epsilon toxin antibody levels contained in guinea pig test sera.

The epsilon antitoxin levels of six guinea pig test serum samples determined *in vitro* using the I-ELISA and the I-CBA were calculated as mean values of three replicate assays performed independently. The intra-assay coefficient of variation was 7.2-15.6% and the inter-assay coefficient of variation was 10-18.6% for the I-CBA (Table B.3), whereas the intra- and inter-assay coefficients of variation were respectively 4.8-13.9% and 2.7-19.5% for the I-ELISA (Table C.3). The intra- and inter-assay variability of the respective assays is within the normal range and should not exceed 20-30% (Tijssen, 1988; Ray *et al.*, 2009), and is also comparable to that reported in the literature (Ebert *et al.*, 1999; Lal *et al.*, 2004). Therefore, the reproducibility of the I-CBA and I-ELISA utilized in this study was considered to be satisfactory.

The epsilon antitoxin levels of each guinea pig serum determined by the I-ELISA and I-CBA was subsequently compared with the corresponding result of the MNT. For all serum samples tested, the I-CBA (Table 3.5) and I-ELISA (Table 3.7) tests obtained higher antitoxin levels than that of the MNT assay (Table 3.3). The degree of correlation between the MNT and the I-ELISA or I-CBA was not directly assessed in this study as the small sample size used in this proof-of-concept study ($n = 6$) precluded such statistical analyses. However, the quantitative correlation between the MNT and the respective *in vitro* tests is an important consideration and needs to be examined with a significant number of serum samples in further studies. Nevertheless, the higher antitoxin levels determined in the I-CBA and I-ELISA compared to the MNT may be ascribed to the binding of both neutralizing and non-neutralizing anti-epsilon toxin antibodies present in the sera of vaccinated guinea pigs to the epsilon antigen in the respective *in vitro* tests. In contrast, the MNT allows for quantification of only neutralizing anti-epsilon toxin antibodies that are capable of protecting the mice against the lethal effects of the epsilon toxin and therefore does not take non-neutralizing antibodies into account (Notermans *et al.*, 1982; Levings *et al.*, 1993; Pfahler *et al.*, 1998).

Despite use of the same antigen and the same serum samples in the I-ELISA and I-CBA tests, the values obtained with the I-CBA was noticeably higher than those obtained in the I-ELISA (Figure 4.1). It is tempting to speculate that the differences may be due to technical differences between the two test systems. For example, the fluorescent readout of the I-CBA is more stable and sensitive than the calorimetric readout of the ELISA, which is dynamic. As the ELISA requires enzyme amplification, it is prone to errors in the amount of amplification (Ellington *et al.*, 2010). Moreover, an additional factor that may affect the readings is attachment of the capture antigen. In the I-CBA the epsilon toxin is attached firmly to the surface-functional microbeads by covalent bonds via standard amine chemistry (Hashida *et al.*, 1984). In contrast, the antigen is attached to the wells of an ELISA plate primarily by hydrophobic interaction, albeit that some electrostatic forces may also contribute (Engvall and Perlmann, 1972). Consequently, it is possible that detachment of the bound antigen and immune complexes during subsequent washing steps can give rise to poorer sensitivity (Howell *et al.*, 1981).

The post-vaccination guinea pig serum samples used in this study had varying levels of potency as assessed in the *in vivo* MNT. The serum samples showed potencies mostly in the range of 10 U/ml, but serum sample 198A had potency in the range of 20 U/ml and serum sample 200A had potency less than the required 5 U/ml (Table 3.3). Of the two *in vitro* assays, the I-ELISA could discriminate differences in the potency of the vaccines to a greater extent than the I-CBA. The highest epsilon antitoxin content was determined for serum 198A (32 U/ml) and the lowest for serum 198A (6 U/ml), whereas the remainder of the serum samples had epsilon antitoxin levels ranging between 25-28 U/ml. In contrast, epsilon antitoxin levels determined in the I-CBA did not differ significantly from each other. Nevertheless, all of the serum samples were unequivocally identified in both *in vitro* tests as being positive, with more than 5 U/ml epsilon

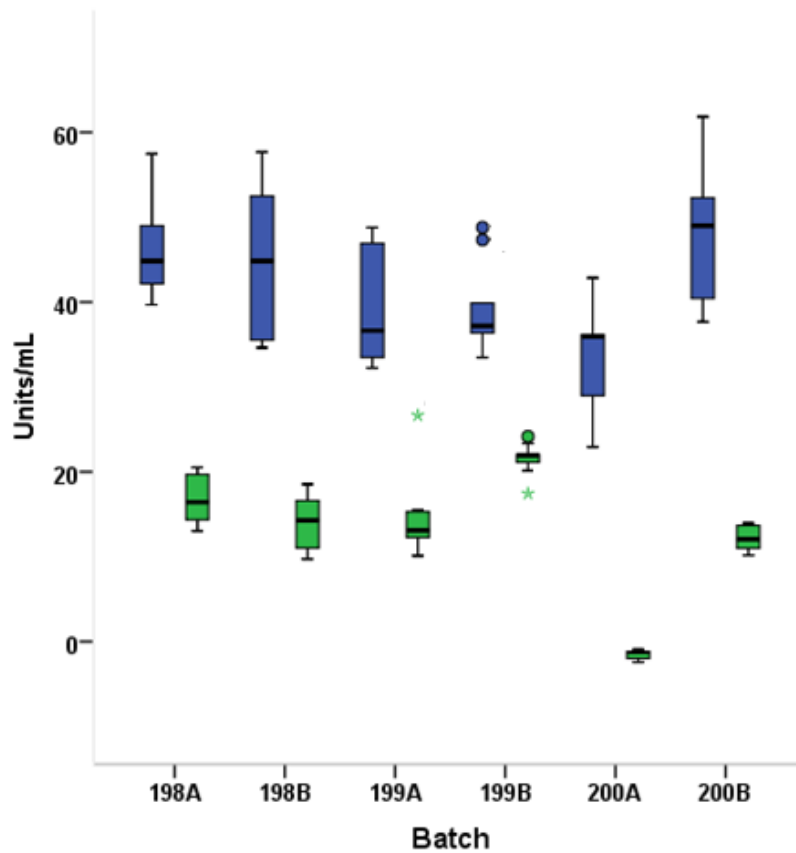


Figure 4.1 Whisker-box plot depicting the variation in the anti-epsilon toxin antibody concentrations, as determined in the I-CBA (blue boxes) and I-ELISA (green boxes) in duplicate on three different days. Boxes represent the interquartile range and the solid line represents the median value. Whiskers represent the minimum and maximum values. Circles and stars represent outliers that fell between 1.5 to 3 box-lengths from the edge and more than 3 box-lengths from the edge, respectively.

antitoxin content. In contrast, the MNT was the only assay that indicated that serum 200A did not meet the required value (i.e. not less than 5 U/ml). This therefore means that neither the I-CBA nor I-ELISA in their current format can be used as possible alternatives to the MNT for estimating the potency of epsilon toxoid-containing *C. perfringens* Type D vaccines for veterinary use. However, further optimization of the respective *in vitro* tests may enable their use in routine potency testing of these vaccines.

In this study, the results of a standard checkerboard titration indicated that the optimum antigen concentration and serum dilution to be used in the I-ELISA was 100 µg/ml and 1/400, respectively. However, in contrast to previous studies, the antitoxin levels determined in the I-ELISA did not compare favourably to those obtained in the MNT. Notably, in the only other study to have used an I-ELISA for potency testing of epsilon toxoid veterinary vaccines, Pfahler *et al.* (1998) reported that best correlation with the MNT results was obtained for plates coated with high amounts of purified antigen (15 g/ml) and low serum sample dilutions (1/25). Indeed, it was reported that serum dilutions of 1/100 and higher gave recurrently higher results than the MNT (Pfahler *et al.*, 1998). Since only high avidity antibodies are neutralizing *in vivo* (Lehtonen and Eerolo, 1982), it was suggested that high antigen density and low sample dilution are most likely to enhance the selection of such high affinity antibodies (Pfahler *et al.*, 1998). Moreover, it was also demonstrated that Protein A peroxidase as the detecting conjugate gave better values than the use of an anti-species IgG peroxidase conjugate, which produced higher background readings (Pfahler *et al.*, 1998). As an alternative to optimizing the current I-ELISA with regards to the above-mentioned parameters, consideration may also be given to the use of a competitive ELISA in future studies. Several reports have demonstrated a high correlation (at least 0.93) in epsilon antitoxin levels determined by competitive ELISA, based on the use of a

monoclonal antibody, and the MNT (Uzal *et al.*, 1997b; Ebert *et al.*, 1997, 1999; Roskopf-Streicher *et al.*, 2003).

Despite the apparent better assay performance of the I-ELISA compared to the I-CBA (Figure 4.1), there are, however, a number of features of the I-CBA that makes it attractive for further development and use in routine potency testing of enterotoxaemia vaccines. As highlighted above, the fluorescent readout of the I-CBA is more direct, stable and sensitive than the calorimetric readout of ELISA test systems (Ellington *et al.*, 2010). Moreover, the I-CBA assay is also theoretically more accurate than the ELISA because the data are calculated from the mean of at least 100 beads, each of which functions as an individual replicate and triplicate tests are therefore unnecessary (Vignali, 2000). In contrast, the ELISA data are derived from a single reading of an individual well and samples are tested, at most, in duplicates or triplicates. It is also conceivable that the larger surface area of an ELISA plate well can result in an increase in non-specific binding and therefore reduce the sensitivity of the ELISA (Carson and Vignali, 1999). Furthermore, use of the I-CBA in place of the I-ELISA could reduce the cost and amount of time expended. The cost of developing and optimizing the I-CBA described in this study was high initially, but once established the running cost was low and more cost-efficient compared to the I-ELISA. The time required to perform the I-CBA was also less than that required for the I-ELISA since the assays were simpler to setup, and took considerably less time for antigen coupling (60 minutes versus overnight incubation for the I-ELISA) and it comprises of fewer washing steps. In addition, the software is simple and data acquisition is performed in real time so that the rapidity of the assay was enhanced even further.

In order to improve the I-CBA assay for use in measuring vaccine potency, further optimization of the assay is required. Dasso *et al.* (2002) tested how the density of mouse monoclonal

capture antibodies on the beads varied during repeated coupling procedures. The authors observed that minor variations in the coupling procedures can markedly affect the density of coupled capture antibodies, which, in turn, can influence the sensitivity of the assays. Therefore, the variability introduced by using bead sets coupled independently with the purified epsilon antigen needs to be determined. Another issue that may require further attention is the choice of the fluorochrome conjugate. It has been reported that in a comparative analysis of different conjugates, conjugates incorporating streptavidin gave low background staining, whereas Alexa488 conjugates were significantly brighter than FITC conjugates and thus increased the sensitivity of the assay (Carson and Vignali, 1999). The sensitivity of the flow cytometric system may also be enhanced further by reducing the number of beads per test. It has been reported that such reduction results in an increase in the ratio of antigen to antibody in each test without reducing the potential signal strength of the assays (the number of capturing antigen per bead) (Vignali, 2000; Dasso *et al.*, 2002). It should be noted that although the I-CBA assay incorporates washing steps, a number of studies have reported rapid “no wash” assays that can be performed in less than two hours (Tripp *et al.*, 2000; Cook *et al.*, 2001). Although this may reduce the time required to perform the assays, the effect of omitting washing steps on the sensitivity of the assay would need to be determined first.

In conclusion, the results indicated that the *in vitro* I-CBA and I-ELISA tests in their current formats are not yet suitable as alternatives to the *in vivo* MNT for assessing the potency of *C. perfringens* Type D epsilon-toxoided vaccines. However, the I-CBA assay that was developed and evaluated during the course of this study has the potential to become a viable alternative to the MNT for measuring vaccine potency. Besides the advantage of avoiding the use of large numbers of animals as indicators for lethal and sublethal intoxication, the low costs and the simple performance of the I-CBA may allow, on a routine scale, the investigation of serum

samples from vaccinated guinea pigs. However, for the I-CBA to be considered as a viable platform to the MNT, further optimization of the assay is required as highlighted above. An optimized I-CBA assay will consequently offer the possibility of assay standardization and validation, which are a precursor for replacement of the MNT.

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ANNEXURE A

DATA GENERATED BY THE MOUSE NEUTRALIZATION TEST (MNT)

Table A.1 Results of the mouse neutralization test (MNT) using purified epsilon toxin to verify the toxin working dilution of 1/180

	1/1	1/2	1/4	1/8	^a Result
Toxin dilution of 1/180	++++	++++	++LL	LLLL	$\frac{1}{2}$ - $\frac{1}{4}$

^a The minimum lethal dose (MLD) requirement is between a dilution of $\frac{1}{2}$ and $\frac{1}{4}$.

Table A.2 Results of the MNT using standardized guinea pig control serum to verify the toxin working dilution of 1/180

	33.3 U/ml	20 U/ml	10 U/ml	5 U/ml	2.5 U/ml	^a Result
Standardized guinea pig serum control	++++	++++	++LL	LLLL	LLLL	10-20 U/ml

^a The requirement for the control serum is 10-20 U/ml.

ANNEXURE B

DATA GENERATED USING THE INDIRECT CYTOMETRIC BEAD IMMUNOASSAY (I-CBA)

Table B.1 Mean fluorescence intensity (MFI) values for checkerboard titrations of the purified epsilon toxin

Purified epsilon toxin	Serum dilutions				
	1/100	1/200	1/400	1/800	1/1600
1 mg/ml	*ND	107	59	48	50
100 µg/ml	1301	459	361	52	32
10 µg/ml	1207	502	233	25	19
1 µg/ml	376	273	82	82	15

* ND, Not Determined. The entire sample volume was acquired without any dot plot generation because of blockage.

Table B.2 Median fluorescence intensity (MFI) values for positive control guinea pig serum used in the construction of a standard curve (Figure 3.3)

^a Positive control serum	^b Average MFI values				^c Average	Intra-assay ^d (%CV)	Inter-assay (%CV)
	Day 1	Day 2	Day 3	Day 4			
50 U/ml	261.500	213.000	351.000	301.200	281.700	7.629	20.404
40 U/ml	239.500	201.500	262.500	249.700	237.300	2.386	11.230
30 U/ml	191.000	154.500	175.500	190.000	177.800	10.096	13.044
20 U/ml	131.500	117.000	142.100	124.200	128.700	11.434	13.720
10 U/ml	^e ND	80.800	67.800	60.000	69.500	7.811	15.726
5 U/ml	40.800	37.900	49.100	42.400	42.500	8.754	15.302

^a The concentration of the positive control serum (as obtained from the MNT) was diluted from 50 U/ml to 5 U/ml.

^b The assay was repeated over four days, and the average value of two replicates per day is shown.

^c The average value of the assays performed on the individual control serum samples.

^d CV, Coefficient of Variation.

^e ND, Not Determined. The entire sample volume was acquired without any dot plot generation because of blockage.

Table B.3 Median fluorescence intensity (MFI) values of the six guinea pig post-vaccination test serum samples

Test serum	^a Average MFI values			^b Average	Intra-assay ^c (%CV)	Inter-assay (%CV)
	Day 1	Day 2	Day 3			
198A	264.000	243.333	215.889	241.074	7.377	10.011
198B	263.000	266.000	188.667	239.222	8.895	18.313
199A	250.333	191.667	186.000	209.333	5.074	17.016
199B	238.333	190.333	165.700	198.122	15.56	18.644
200A	197.667	147.333	193.222	179.407	11.210	15.532
200B	250.333	293.667	205.778	249.926	7.246	17.584

^a The assay was repeated over three days, and the average value of two replicates per day is shown.

^b The average value of the six assays performed on the individual test serum sample.

^c CV, Coefficient of Variation.

Table B.4 Statistical analysis of the regression curve and six guinea pig test sera of the I-CBA

Data analysis of the regression curve generated regression statistics and using ANOVA it was possible to predict significant linearity of regression

Regression Statistics	
Multiple r	0.999
r ²	0.998
Adjusted r ²	0.998
Std. error	4.026
No. of observations	6

ANOVA					
	Degrees of freedom	SS	MS	F	Significance F
Regression	1	43929.987	43929.987	2709.734	8.151E-07
Residual	4	64.847	16.211		
Total	5	43994.835			

	Coefficients	Std. error	t Stat	p-value	Lower 95%	Upper 95%
Intercept	17.408	3.133	5.556	0.005	8.709	26.106
Slope (b)	5.374	0.103	52.055	8.151E-07	5.087	5.661

Figure B1 CellQuest Pro™ dot plot generated for one repeat of the test serum samples

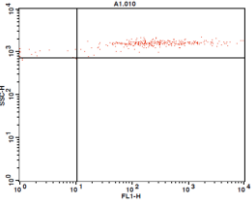
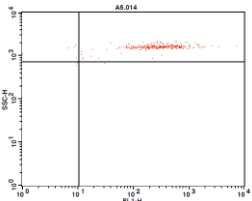
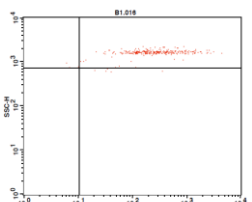
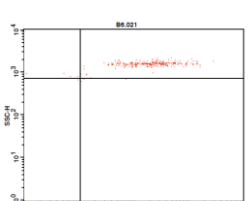

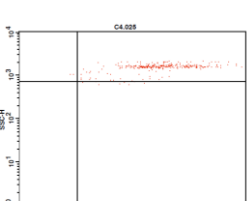
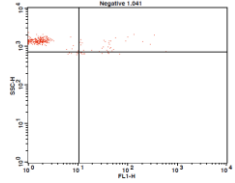
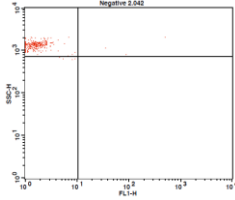
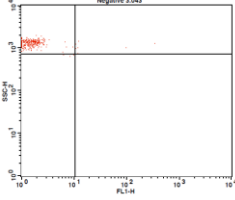
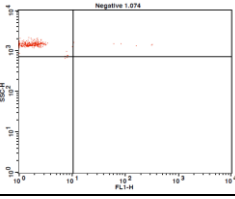
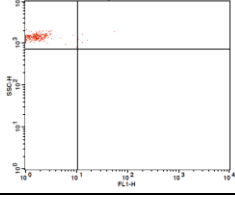
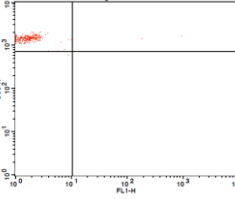
Test serum samples	CellQuest Pro™ dot plot output
❖ Test serum sample 198A	
❖ Test serum sample 198B	
❖ Test serum sample 199A	
❖ Test serum sample 199B	
❖ Test serum sample 200A	
❖ Test serum sample 200B	

Figure B2 CellQuest Pro™ dot plot generated for unconjugated negative control beads to verify the instrument setup

Test serum samples	CellQuest Pro™ dot plot output
❖ Average MFI: 43.7	
❖ Average MFI: 22.7	
❖ Average MFI: 12.2	
❖ Average MFI: 11.7	
❖ Average MFI: 13.0	
❖ Average MFI: 18.6	

ANNEXURE C

DATA GENERATED USING THE INDIRECT ELISA (I-ELISA)

Table C.1 Mean optical density (OD₄₅₀) values for checkerboard titrations of the purified epsilon toxin

Epsilon toxin dilutions	Serum dilutions					
	1/100	1/200	1/400	1/800	1/1600	1/3200
1 mg/ml	2.087	1.784	1.233	0.782	0.517	0.355
333.3 µg/ml	2.342	1.173	0.994	0.67	0.568	0.34
111.1 µg/ml	2.07	1.409	* 1.009	0.828	0.576	0.321
37 µg/ml	1.947	1.41	1.015	0.745	0.475	0.379
12.3 µg/ml	2.24	1.653	1.015	0.78	0.542	0.453
4 µg/ml	2.495	1.803	1.472	0.93	0.539	0.405

*The results indicated that a 1/400 dilution of the serum, together with approximately 100 µg/ml of the antigen, gave an absorbance value closest to 1.00 and were therefore chosen for use in subsequent assays.

Table C.2 Optical density (OD₄₅₀) values for the positive control guinea pig serum used in the construction of a standard curve (Figure 3.4)

^a Positive control serum	^b Average OD ₄₅₀ values			^c Average	Intra-assay ^d (%CV)	Inter-assay (%CV)
	Day 1	Day 2	Day 3			
50 U/ml	2.810	2.997	2.165	2.657	3.162	4.760
40 U/ml	2.592	2.583	1.895	2.357	4.986	4.634
30 U/ml	2.091	1.992	1.631	1.904	1.870	2.616
20 U/ml	1.501	1.511	1.187	1.399	3.188	3.000
10 U/ml	0.954	0.822	0.741	0.839	3.241	7.410
5 U/ml	0.498	0.573	0.489	0.520	7.682	11.306

^a The concentration of the positive control serum (as obtained from the MNT) was diluted from 50 U/ml to 5 U/ml.

^b The assay was repeated over three days, and the average value of two replicates per day is shown

^c The average value of six assays performed on the individual test serum sample.

^d CV, Coefficient of Variation.

Table C.3 Optical density (OD₄₅₀) values of the six guinea pig post-vaccination test serum samples

Test serum	^a Average OD ₄₅₀ values			^b Average	Intra-assay ^c (%CV)	Inter-assay (%CV)
	Day 1	Day 2	Day 3			
198A	1.278	1.212	1.040	1.177	13.896	2.678
198B	0.894	1.187	0.863	0.981	7.809	16.630
199A	1.176	0.976	0.970	1.041	13.929	19.527
199B	1.014	0.994	1.081	1.030	5.806	5.402
200A	0.280	0.219	0.229	0.243	4.774	12.447
200B	1.028	0.962	0.785	0.925	8.865	3.393

^a The assay was repeated over three days, and the average value of two replicates per day is shown.

^b The average value of six assays performed on the individual test serum sample.

^c CV, Coefficient of Variation.

Table C.4 Statistical analysis of the regression curve and six guinea pig test sera of the I-ELISA

Data analysis of the regression curve generated regression statistics and using ANOVA it was possible to predict significant linearity of regression

Regression Statistics	
Multiple r	0.994
r ²	0.989
Adjusted r ²	0.986
Std. error	0.098
No. of observations	6

ANOVA					
	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>F</i>	<i>Significance F</i>
Regression	1	3.528	3.528	362.008	4.495E-05
Residual	4	0.038	0.009		
Total	5	3.567			

	<i>Coefficients</i>	<i>Std. error</i>	<i>t Stat</i>	<i>p-value</i>	<i>Lower 95%</i>	<i>Upper 95%</i>
Intercept	0.368	0.076	4.794	0.008	0.155	0.581
Slope (b)	0.048	0.002	19.026	4.495E-05	0.041	0.055

