Nucleic Acid Approaches To Toxin Detection

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ABSTRACT

PCR is commonly used for detecting contamination of foods by toxigenic bacteria. However, it is unknown whether it is suitable for detecting toxins in samples which are unlikely to contain bacterial cells, such as purified biological weapons. Quantitative real-time PCR assays were developed for amplification of the genes encoding *Clostridium botulinum* neurotoxins A to F, Staphylococcal enteroxin B (SEB), ricin, and *C. perfringens* alpha toxin. Botulinum neurotoxins, alpha toxin, ricin and V antigen from *Yersinia pestis* were purified at Dstl using methods including precipitation, ion exchange, FPLC, affinity chromatography and gel filtration. Additionally, toxin samples of unknown purity were purchased from a commercial supplier. Q-PCR analysis showed that DNA was present in crudely prepared toxin samples. However, the majority of purified or commercially produced toxins were not detectable by PCR. Therefore, it is unlikely that PCR will serve as a primary toxin detection method in future.

Immuno-PCR was investigated as an alternative, more direct method of toxin detection. Several iterations of the method were investigated, each using a different way of labelling the secondary antibody with DNA. It was discovered that the way in which antibodies are labelled with DNA is crucial to the success of the method, as the DNA concentration must be optimised in order to fully take advantage of signal amplification without causing excessive background noise. In general terms immuno-PCR was demonstrated to offer increased sensitivity over conventional ELISA, once fully optimised, making it particularly useful for biological weapons analysis.

Finally, genetic methods for the differentiation of toxigenic *Ricinus communis* strains were examined, including SSR, RAPD, RFLP and SNP analysis using next generation sequencing. The results showed that the species has low genetic diversity, making genotyping a particularly difficult task, however SSR analysis was able to provide a degree of differentiation and 454 Sequencing™ identified six SNP targets that warrant further investigation in future.

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1. INTRODUCTION

1.1 TOXINS AS BIOLOGICAL WEAPONS

Bioterrorism is the intentional use of microorganisms or toxins derived from living organisms to cause death or disease in humans, animals or plants on which we depend (Ashford, 2003). Biological warfare (BW) specifically refers to the use of such weapons in the battlefield (Carter, 2000). The Biological and Toxin Weapons convention (BTWC) prohibits the development, production and testing of biological weapons (United Nations report, 1971). A number of toxins are on the Centers for Disease Control and prevention (CDC) category A, B and C biological weapons threat list (http://www.bt.cdc.gov/agent/agentlist-category.asp), including *Clostridium botulinum* neurotoxin, ricin, Staphylococcal enterotoxin B (SEB) and *C. perfringens* alpha toxin. Toxins may be produced as agents either in liquid or powdered form (Carter, 2000), with the most potent method of delivery being via the aerosol route (Hawley *et al.,* 2001). Inhalation of the weapon may or may not be lethal. For example, SEB may evoke a quick and transient debilitation lasting less than 24 hours (Carter, 2000). Inhalation of botulinum neurotoxin is more likely to cause fatality (Madsen, 2001). Detection of a disease or intoxication outbreak is essential to aid the investigation of potential production facilities, environmental or clinical samples (Zilinskas, 1998). Antibody-based detection techniques such as ELISA were first described forty years ago (Engvall and Perlmann, 1971). The utility of ELISA for toxin detection has steadily increased since it was first used to detect cholera toxin (Holmgren and Svennerholm, 1973). However, identification of a toxin in a BTWC scenario would require confirmation using an orthologous technique. PCR is exquisitely sensitive and is routinely used for detection of trace amounts of DNA enabling specific identification of a source in humans (Jeffreys *et*

al., 1991) and similar methods exist for numerous other species including the BW agent anthrax (Hoffmaster *et al*., 2002). It is therefore conceivable that PCR may be suitable for detecting toxin DNA in samples where the quantity of DNA is limited. Currently, it is unknown whether DNA will be present in a toxin sample presented for BTWC analysis, since it may be subject to crude or stringent purification. To determine whether or not this is the case is the primary aim of this thesis. The secondary aim is to investigate the potential of alternative nucleic acid methods for detecting toxins.

1.2 TOXINS

Toxins are chemicals or proteins produced by living organisms such as bacteria, fungi, plants and animals ranging from dinoflagellates to vertebrates. Many have a deleterious effect on man. By definition, they have a toxic effect on the cell type they target, which in most cases leads to cell death. The result of this will be incapacitation or fatality of an intoxicated patient if untreated (Wassenaar, 2007).

Toxins can be classified depending on their mode of action. An exotoxin for example, is a toxin that is produced by a bacterium as part of its metabolism. It is normally released into the bacterium's surroundings. Additionally, they are often encoded by plasmids or prophages that have infected or integrated into a bacterial host (Abedon and LeJeune, 2007). They are highly immunogenic and stimulate the production of neutralising antibodies. They are usually categorised as neurotoxins, cytotoxins or enterotoxins (Wassenaar, 1997) according to their mode of action. For example, enterotoxins are exotoxins that specifically damage intestinal mucosa, causing vomiting and diarrhoea (Henkel *et al*., 2010). Generally, exotoxins conform

to an AB model, where the A subunit has enzymatic activity and the B subunit is involved in binding to its target (Gill, 1978).

Endotoxins are the heat stable lipopolysaccharides (LPS) in the cell walls of Gram-negative bacteria that are released when the bacterium lyses or during growth. The toxic part of LPS is the lipid A portion, a complex arrangement of lipid residues (Reeves and Wang, 2002). This thesis is concerned with protein exotoxins exclusively, as endotoxins are not on the CDC threat list independently of their parent organism.

It is thought that most agents that cause food borne intoxication are unable to survive *in vivo* except in infants. Consequently, food borne illness is often caused by pre-formed toxin (Balaban *et al.,* 2001). Similarly, toxins may be released as biological weapons independently of the organism that produced them. The presence of viable cells in a sample is not proof of the presence of toxin and presumably therefore, direct detection of toxins should be demonstrated where possible. Therefore, the method used for detecting a toxin should be carefully considered. For any toxin detection method to be successful, the nature of the causative organism must be understood.

1.3 GENETICS OF TOXIN PRODUCTION

1.3.1 *Clostridium botulinum*

Clostridium botulinum is an anaerobic, spore-forming rod that is widely distributed in soils and sediments of oceans and lakes. It is also the causative agent of botulism, a neuroparalytic disease of humans and other animals. Food borne botulism is the

most common occurrence of the disease, with 263 cases reported in the US in between 1990 and 2000 (Sobel *et al.,* 2004*)*. *C. botulinum* spores are commonly found in canned and vacuum packed foods (Fach *et al.,* 1995). If the spores are allowed to germinate, the neurotoxin is produced during vegetative cell growth. If contaminated food is then eaten without being heated thoroughly, the toxin remains active and causes botulism.

The classification of *Clostridium botulinum* is complex. Primarily, it is based on the type of neurotoxin it produces. There are seven *Clostridium botulinum* neurotoxin serotypes, A to G, each with different functional and antigenic attributes (Adekar *et al.,* 2008). However, several strains are able to produce more than one type of neurotoxin (Table 1.1). Species are also divided into four additional groups, based on cultural, biochemical and physiological characteristics. Furthermore, botulinum neurotoxins (BoNTs) are also produced by *C. baratii* and *C. butyricum* (BoNT/F and BoNT/E respectively; Collins and East, 1998).

BoNTs are synthesised as a single 150 kDa polypeptide chain (pro-toxin), which is post-translationally nicked to yield a di-chain composed of a light (50 kDa) and heavy (100 kDa) chain linked by a disulphide bridge in proteolytic strains. The Carboxy terminal (C) half of the heavy chain is involved in the binding to a surface nerve cell receptor whilst the amino (N) terminal half is implicated in the translocation of the toxin across the cell membrane. The light chain is involved in intracellular activity (Popoff and Marvaud, 1999).

Clinically, the most important strains are group I strains although group II strains also cause disease in humans. Generally, strains within groups I or II are closely

related to each other regardless of the toxin type they produce. In contrast, strains belonging to different phenotypic groups exhibit low relatedness even though some produce the same toxin type (such as type B strains of group I and II), (Minton, 1995). The optimum temperature for growth and toxin production of proteolytic strains is 35 ^{o}C, whereas non-proteolytic strains grow at 25 ^{o}C and can produce toxin in refrigeration (Peck, 2006). With this in mind, strict guidelines are given for the production of chilled foods (Peck *et al.,* 2008). Toxins produced by non-proteolytic strains only exert full toxicity once activated by trypsin whereas proteolytic strains initially produce fully active toxins.

There are three main types of human botulism, depending on the strain type and mode of infection (Brett *et al.,* 2004). Food borne botulism is caused by type A strains in America, type B in Europe and type E in the extreme Northern Hemisphere (Popoff and Marvaud, 1999). It is more rarely caused by types F and C (Brett *et al.,* 2004). This effect may be a result of the geographical distribution of strain types, although cultural differences in diet may also affect epidemiology. Strains belonging to group I cause a second category of disease, intestinal colonisation, resulting in botulism in infants and immunocompromised adults, as do *C. botulinum* C, *C. baratii* and *C. butyricum*. Wound infection by *C. botulinum* A or B from group I is the third cause of botulism (Popoff and Marvaud, 1999).

Table 1.1 Classification of *Clostridium botulinum* based on phenotypic characteristics compiled from Collins (1998) and Hill *et al.*, (2010);

Neurotoxin type	Group I Human disease High heat resistant. Proteolytic	Group II Human Disease Low heat resistant. Non-proteolytic	Group III Animal disease Non-proteolytic	Group IV Non toxigenic. Not proven to cause disease. Uncharacterised
A	A1, A2, A3, A4, A5			
B	B	B		
C			C	
D			D	
E		E		
F	F	F		
G				G

Botulinum neurotoxins inhibit the release of several neurotransmitters, resulting in flaccid paralysis (Davis, 2003). The toxin binds to the pre-synaptic receptor at neuromuscular junctions, which is followed by endocytosis directed by a domain next to the receptor-recognition element on the heavy chain of the toxin. (Figure 1.1). This is followed by zinc-dependent protease activity of the light chain culminating in prevention of fusion of the synaptic vesicle with the cell membrane and release of acetylcholine (Hambleton, 1992).

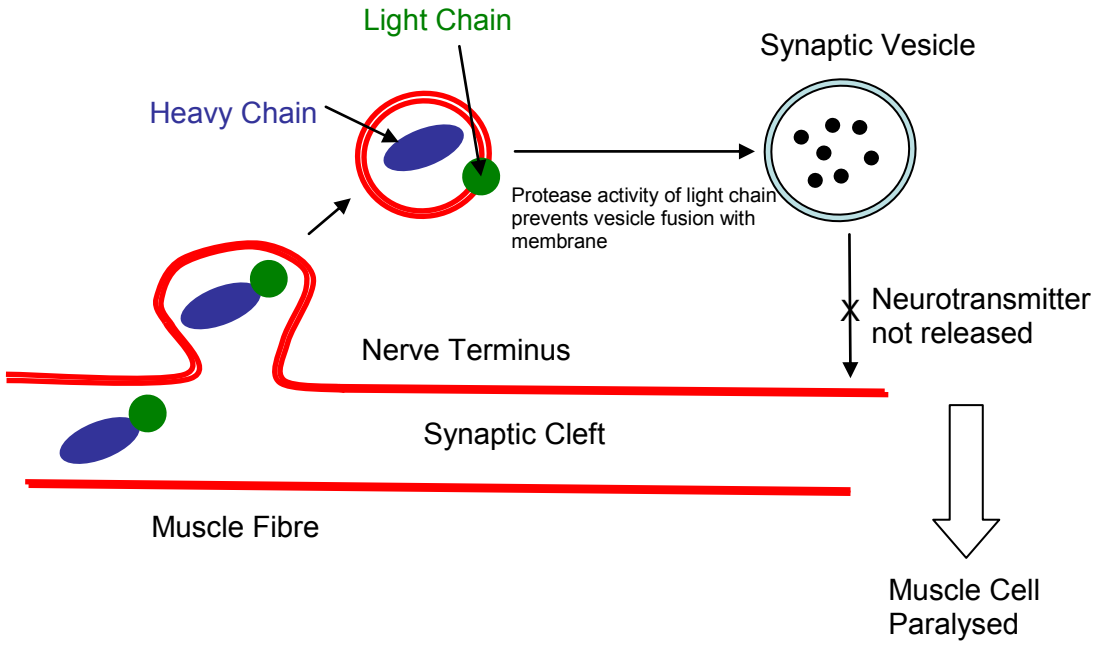


Figure 1.1 Endocytosis of botulinum neurotoxin to neuronal cells. The toxin binds to the nerve cell at the neuromuscular junction and enters by endocytocis. The light chain of botulinum toxin cleaves specific sites on the SNAP receptor proteins, preventing assembly of the synaptic fusion complex, thus blocking acetylcholine release (adapted from Shukla and Sharma, 2005).

There are different presynaptic receptors for each serotype, for example synaptobrevin is the SNAP receptor for neurotoxins of serotype B, D, F and G, whilst SNAP-25 is the receptor for type A (Shukla and Sharma, 2005). The physiological effects of flaccid paralysis caused by ingestion of the toxin are dry mouth, visual difficulty, speech difficulty, nausea, weakness, vomiting, dizziness, and progressive paralysis. Death is ultimately caused by respiratory or cardiac failure (Peck *et al.,* 2011). Documented cases of inhalational botulism are rare, but one reported case of accidental exposure in a laboratory in Germany in 1962 lead to symptoms similar to those described above (Arnon, 2001).

Terrorists have previously attempted to use botulinum toxin as a bioweapon. Aerosols were dispersed at various sites in Tokyo by the Japanese cult Aum Shinrikyo (Arnon, 2001). The toxin is extremely potent; as little as 1-2 µg of toxin can prove fatal (Shone *et al.,*1987). The toxin is unstable in the pure state, which is therefore unlikely to be used as a biological weapon (Gill, 1982). However, it is stable when partially purified and spray dried and it is also stable in cold water for 1 week which may be a more suitable way of disseminating the toxin (Gill, 1982).

BoNT is produced as part of a complex, which ranges in size and content depending on serotype, host strain and environmental factors (Peck, 2009). A non-toxin, non-haemaglutinin (NTNH) protein is common to each complex. The larger toxin complexes are more resistant to the extremes of pH and temperature found in the stomach than the purified toxin (Hambleton, 1992). There are several types of haemagglutinating (HA) proteins associated with BoNT, ranging in size from 147 to 625 amino acids, in two transcriptional units (Peck, 2009). The toxin (*bont*) and *ntnh* genes comprise one unit and the gene encoding HA proteins are transcribed in the

opposite direction. Between these units is a sigma factor which positively regulates gene expression (Dupuy and Matamouros, 2006). Types A1, A2, A3, A4, Proteolytic F, E and non-proteolytic F additionally contain a group of three open reading frames of, as yet, unknown function (Peck, 2009).

Until fairly recently, the accepted international standard for detection of *C. botulinum* in food samples involved enrichment for 5 days followed by microbiological tests and mouse bioassay which takes two weeks in total (Alsallami and Kotlowski, 2001). Immunological methods are quicker, but enrichment for five days may still be necessary to enable the production of enough toxin for detection by ELISA (Ferreira *et al.,* 1994). PCR is deemed sensitive enough by the food industry to side-step enrichment procedures and can deliver results in a few hours. Consequently, a lot of research has been published on the subject of PCR methods for detection of *C. botulinum* neurotoxin genes using extracted DNA. For example, traditional assays have been developed by Szabo *et al.,* (1992), Campbell *et al.,* (1993); Franciosa *et al.,* (1996) and Lindstrom *et al.,* (2001) amongst others, each with similar sensitivity and specificity. Real-time PCR assays have also been in existence for a number of years, for example an assay able to detect 10^2 CFU/ml of type E cells was developed by Kimura *et al.,* (2001), giving more sensitive detection than the comparative mouse lethality test.

Real time multiplex assays have been developed since the onset of this study by researchers including Fach *et al.,* (2009), Satterfield *et al.,* (2010), Kirchner *et al.,* 2010. Additionally, Macdonald *et al.,* (2008), successfully used VNTR analysis to type *C. botulinum* strains in crude preparations, indicating that PCR analysis of toxin

samples is feasible. However, the use of real time PCR to detect DNA in preparations of *C. botulinum* neurotoxin remains largely unstudied.

1.3.2 *Clostridium perfringens*

C. perfringens is a widely occurring spore-forming, non-motile, Gram-positive bacterium commonly found in soil, sewerage and the intestines of humans and other animals (Tsutsui *et al.,* 1995). It is one of the most common causative agents of gastrointestinal disease such as intoxication and antibiotic related diarrhoea (McClane, 2004). It is a fast growing obligate anaerobe whose virulence is largely attributed to the production of numerous exotoxins, with α, β, ε and ι being the major lethal toxins and θ and κ being implicated in some diseases (Titball, 1998). Individuals never produce all seventeen *C. perfringens* toxins identified (Ma *et al*., 2011), allowing strains to be classified as summarised in Table 1.2. Besides producing typing toxins, *C. perfringens* also produces sialidase, hyauronidase, perfringolysin O and an enterotoxin (Ma *et al*. 2011). Different toxinotypes produce different diseases, for example type A strains cause gas gangrene and type C strains cause necrotic enteritis in calves, lambs and pigs (Johnson *et al.,* 1997). Epsilon toxin, produced by type B and D isolates is an extremely potent pore-forming toxin which causes vascular endothelial damage (McClane, 2004), causing it to be a CDC category B biological weapon. As the exact nature of the toxin remains to be elucidated it was not included in this study. In contrast, alpha toxin, has been extensively studied (Vachieri *et al*. 2010).

C. perfringens alpha toxin is a 42.5 KDa protein, which can be expressed at levels up to 10 mg/l when growth conditions are optimal. If cloned into *E. coli*, production is three times greater (Popoff and Marvaud, 1999). It is a membrane-damaging toxin

with phospholipase C and sphingomyelinase activities. Phospholipases naturally hydrolyse phospholipid molecules, the location of the hydrolysed bond is used to characterise the enzyme as types A1, A2, C, or C2. *C. perfringens* alpha toxin is a phospholipase C (PLC) (Figure 1.2). Traumatic gas gangrene develops after a deep penetrating injury that compromises the blood supply, such as a gunshot or knife wound, that creates an anaerobic environment ideal for clostridial proliferation. Gas gangrene may also develop after a surgical procedure. It is caused by opportunistic infection of tissues by *C. perfringens* vegetative cells or spores in about 70% of cases with the remaining 30 % being caused by other Clostridial species (Stevens and Bryant, 1997). Anaerobic conditions resulting from vascular damage facilitate the germination of spores and the subsequent production of α toxin under the control of the VirR/VirS regulon (Ba-Thein *et al.,* 1996). Onset of the disease occurs 6-8 hours after colonisation by *C. perfringens*. This leads to gas production, increased vascular permeability, intravascular haemolysis, clot formation, necrosis and tissue damage which ultimately causes death by shock (Rood, 1998).

The genetic organisation of toxin production in *C. perfringens* is relatively uncomplicated compared to that of *C. botulinum*. Toxin types are genetically distinct from each other, which means that α and ε toxins, for example, are completely unrelated (McClane *et al*., 2006). The alpha toxins produced by *C. novyi* and *C. bifermentans* are also phospholipase Cs and are closely related to *C. perfringens* alpha toxin. Additionally, PLC from *Listeria monocytogenes* and *Bacillus cereus* may be closely related to that produced by *C. perfringens*.

PCR has been previously used for sensitive and specific detection of *C. perfringens* alpha toxin DNA in animals (Kadra *et al.,* 1999), autopsy tissue samples (50-500

genome copies per sample; Wu *et al.,* 2009) and faecal samples (30 genome equivalents; Rinttila *et al.,* 2011) amongst others. Whilst PCR is routinely used to detect purified DNA from *C. perfringens* samples, the potential for the assay to detect contaminating DNA in toxin preparations is unstudied.

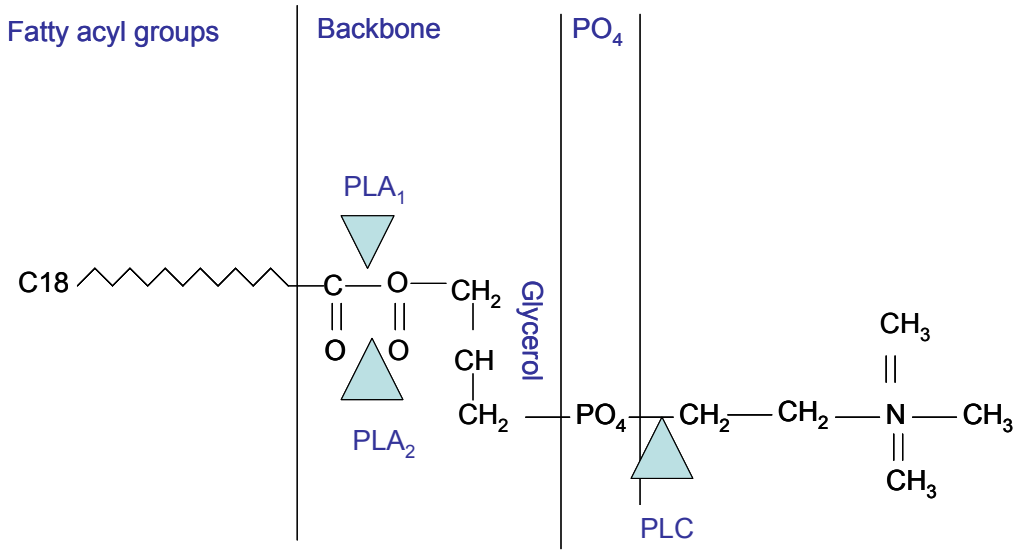


Figure 1.2 Site of phospholipid cleavage by *Clostridium perfringens* phospholipases PLA_1, PLA_2, or PLC (adapted from Titball, 1999).

Table 1.2. Classification of *Clostridium perfringens* toxinotypes, according to genotype, adapted from Tsutsui *et al*., (1995)

***C. perfringens* toxinotype**	**α**	**β**	**ε**	**ι**	**Strain**	**Genbank Accession Number**
A	+	-	-	-	NCTC 8237	M24904
A	+	-	-	-	8-6	X17300
A	+	-	-	-	PB6K	D32123
A	+	-	-	-	13	D32127
A	+	-	-	-	KZ21	D32124
B	+	+	+	-	NCIB 10691	D32128
C	+	+	-	-	NCIB 10662	D10248
D	+	-	+	-	L9	D40068
D	+	-	+	-	NCIB 10663	D49969
E	+	-	-	+	CL49	D32125
E	+	-	-	+	NCIB 10748	D32126

1.3.3 ***Staphylococcus aureus***

Staphylococcus aureus is a Gram-positive coccus that is found on the skin and in the nasal passages of humans and other mammals worldwide (Masiuk *et al.,* 2010). It is an important human pathogen as it causes several conditions such as boils, wound infections and pneumonia. It can readily enter food as it occurs so commonly and if allowed to multiply it will produce a range of exoproteins which enables it to colonise and persist within the host (Pinchuk, 2010). These include haemolysins, nucleases, proteases, leucocidins, lipases, collagenases, cell-surface proteins and various superantigens. Staphylococcal enterotoxins (SEs) belong to the pyrogenic toxin family because of their biological activities and structural relatedness (Balaban and Rasooly, 2001).

Staphylococcal enterotoxins are classified as superantigens that induce polyclonal proliferation of certain T cells bearing Vβ receptors (Fleischer *et al.,* 1991). Unlike conventional antigens, superantigens associate with the major histocompatability complex (MHC) class II and T cell receptor directly, without being processed by antigen presenting cells. The mechanism by which this occurs involves the formation of a tri-molecular complex of the superantigen with the Vβ receptor and MHC class II molecules outside the typical binding cleft (Figure 1.3). The consequence of such proliferation is a production of a cytokine bolus (Balaban and Rasooly, 2001), including interleukin 1 (IL-1), IL-2, Interferon-γ (IFN-γ) and tumour necrosis factor-α (TNFα) which ultimately induce symptoms in the host. The presence of these cytokines induces the expression of pro-inflammatory mediators IL-6 and IL-8, as well as platelet activating factor, leukotrienes, thromboxane A2 and

prostaglandins (Monday, 1999). TNF α acts on the hypothalamus to produce fever and also acts to produce extensive endothelial damage, capillary leakage and decrease in peripheral vascular resistance leading to hypotension and shock (Chesney, 1997).

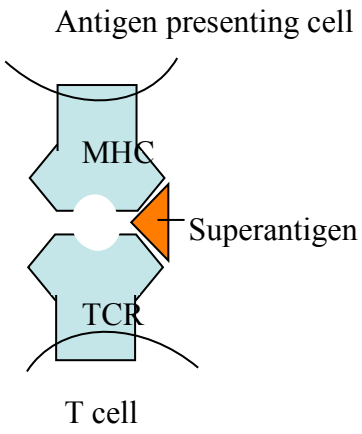


Figure 1.3 Schematic diagram of the mechanism of T cell activation by superantigens such as Staphylococcal enterotoxins.

Twenty types of SE have been identified (A to V; Pinchuk *et al.,* 2010). Although theoretically distinct, SEA, SED and SEE share 70 to 90 % sequence homology (Pinchuk *et al*., 2010) and antibodies to SEA cross react with SEE (Sharma *et al*. 2000). As with *C. botulinum* neurotoxin genes, SE genes are either carried by phages, plasmids, or on the chromosome. Chromosomal genes may be part of a pathogenicity island (PI) or in the *egc* cluster, an operon which contains five enterotoxin genes and two pseudogenes (Jarraud, 2001). Horizontal gene transfer is common, resulting in strains carrying between one and six enterotoxin genes (Varshney *et al.,* 2009), although the most common types are SEA and SEB (Pinchuk *et al.,* 2010).

Staphylococcal enterotoxin B (SEB) is incapacitating to a susceptible host (Hawley, 2001). The toxin is a 28 336 Da single chain protein (Jones and Khan 1986). It is heat stable, resistant to inactivation by proteases and is reasonably stable in aerosol form (Madsen, 2001). It is an incapacitating toxin, with an LD_{50} of 27 µg/kg intravenously. An ingested dose of 20-35 µg of purified protein is enough to cause vomiting in human volunteers within 4 hours (Merson, 1973). Symptoms caused by inhalational intoxication include fever, malaise, muscle and chest pains, headache, cough, loss of appetite but no vomiting or diarrhoea. Symptoms are rarely fatal (Madsen, 2001).

Detection of SEB has been reported in a variety of sample types including food, (Holeckova *et al.,* 2002; Rall *et al.,* 2008; Ertas *et al.,* 2010) biological fluids, (Mukhin and Chatterjee 1997), and the battlefield (Ulrich, 2000). Many research groups have developed sophisticated multiplex PCR reactions to enable the detection

of more than one staphyloccocal toxin at once, for example (Schmitz *et al.,* (1998); Mehrotra *et al.,* (2000); Sharma *et al.,* (2000); Nakayama *et al.,* (2006) and Manfredi *et al.,* (2010). Although SEB can be detected in food by PCR the application of the technique for the detection of purified SEB toxin remains to be studied.

1.3.4 ***Ricinus communis***

The plant toxin ricin is a protein component of *Ricinus communis* seeds, more commonly known as castor beans (Lord *et al.,* 1994). It is a 66 kDa lectin, a protein that binds carbohydrates, that belongs to the A-B family of toxins that are highly toxic to eukaryotic cells. Specifically, ricin is a Ribosome Inactivating Protein (RIP). Most RIPs are Type I, consisting of an A chain which on its own is harmless. Type II RIPs, such as ricin, are heterodimeric, with the toxin subunit bound to a lectin subunit (Sandvig *et al.,* 1996).

Although ricin is the best known type II RIP, several unrelated dicotyledonous plants also produce such toxins including abrin from *Abrus precatorius*, modeccin from *Adenia digitata*, volkensin from *Adenia volkensii*, ebulin from *Sambucus ebulis* and viscumin from *Viscum album* (mistletoe) (Olsnes *et al.,* 2001). Although the plants producing these toxins are from unrelated species, the protein sequence, structure and mode of action of each toxin is very similar. As ricin binds to both glycolipids and glycoproteins on the cell surface it is presumably transported inside the cell using all endocytic mechanisms in a given cell (Sandvig and van Deurs, 1996). One ricin molecule is sufficient to kill a cell and is reported to inactivate 1777 ribosomes per minute (Olsnes *et al.,* 1975). The A chain depurinates a universally conserved adenosine found in a GAGA tetraloop in eukaryotic 28S rRNA of the 60S ribosomal subunit. The depurinated RNA is susceptible to hydrolysis and unable to bind

elongation factor 2, which prevents protein synthesis. As ricin irreversibly inactivates ribosomes, its toxicity manifests in widespread cell death (Endo *et al.,* 1987).

Exposure to ricin toxin by inhalation will cause respiratory difficulty and pulmonary oedema after several hours. Ingestion of the toxin will cause nausea, vomiting, bloody diarrhoea, drowsiness, stupor, cyanosis, dehydration, myosis, convulsions and circulatory collapse within a few hours (Madsen, 2001). The lethal dose by inhalation is 15 µg and 3-7 µg/kg when injected intravenously (Gill, 1982). Ricin has previously been used as a biological weapon by terrorists. In 1972, the Bulgarian defector Georgi Markov was stabbed in the thigh with an umbrella impregnated with ricin in the tip. He died on the third day post-exposure (Madsen, 2001). Ricin is easily prepared with minimal knowledge or equipment; therefore, rapid detection and medical countermeasures remain priorities (Eitzen, 1998).

There are several isoforms of the toxin including ricin D and E and the closely related *R. communis* agglutinin (RCA) which is a tetramer, consisting of two ricin-like heterodimers (Olsnes *et al.,* 1974). RCA is only weakly toxic *in vivo*, but it is so structurally similar to ricin that antibodies raised to either ricin A or ricin B chains cross react with the corresponding chain of RCA (Lord *et al.,* 1994).

In contrast to the toxins that cause food poisoning described previously, few researchers have published research describing PCR detection of the ricin gene, as it is less relevant to public health. Nevertheless, He *et al.,* (2007) described an assay that was able to detect DNA in dairy products that equated to 5 ng of toxin per ml of sample. Melchior and Tolleson (2010) estimated their assay to be sensitive enough to detect 30 picograms of toxin which is well below the LD_{50}, demonstrating the

suitability of the technology to this area of research. As with the other toxins described however, analysis of weapons' grade ricin by PCR is yet to be carried out.

1.4 PROTEIN PURIFICATION

1.4.1 Biological weapons

Currently it is unknown whether toxins prepared as biological weapons would be crudely prepared or highly purified, since it would depend on the aggressor. Similarly, it is unknown whether toxins prepared as BW agents would contain residual DNA, as that too would depend on the nature of the threat. For example, botulinum neurotoxin is reported to have been prepared for weaponisation from culture supernatant during the Gulf War (Zilinskas, 1997). It is therefore important to understand the protein purification processes that may be used to prepare biological weapons.

1.4.2 Purification schemes

Protein purification strategies vary from simple one step precipitation procedures to large scale, validated production processes. The purification process employed is dependent on the properties of the protein, such as isoelectric point (pI) and hydrophobicity (Skopes, 1993). Often more than one step is necessary so the most appropriate techniques should be used to maximise yield with the least number of steps. By far the easiest method to produce large quantities of a protein is to clone the gene and over express the recombinant protein. The use of recombinant proteins also allows subtle changes to the protein to facilitate purification. A commonly used strategy is to capture the protein, follow that stage with an intermediate purification

step, followed by purification and, finally, polishing, commonly known as CIPP (Amersham Biosciences, 2001).

1.4.3 **Precipitation**

The oldest and simplest method for protein purification is precipitation, which alters the properties of a solute so that the proteins become insoluble. Only a limited amount of specialist equipment is required to concentrate large amounts of protein using this method. Indeed, UN weapons inspectors found 19 000 litres of acid precipitated botulinum neurotoxin in Iraq (Zilinskas, 1997). The proteins can then be concentrated by centrifugation and re-dissolved in the original solvent. Most proteins are soluble in cytoplasm. Once extracted, these conditions can be varied to precipitate the proteins of interest providing that the properties of the protein are understood, bearing in mind that most proteins have a minimum solubility around their isoelectric point.

1.4.4 **Purification using adsorbents**

Adsorbents used in protein chromatography have two essential features, the nature of the matrix and the type of functional group that carries out the adsorption (Skopes 1993). The purification matrix is normally made from an insoluble polymer which is cast into bead form. The size of the pore dictates the size of protein molecule that will be retained by the matrix. There are several types of material used as adsorbents:

1.4.4.1 **Ion Exchangers**

Ion exchangers (IEX) are the most widely used of all adsorbents. They exploit the different net charges on proteins at a given pH and interact with proteins by

electrostatic attraction. Anion exchangers such as DEAE, TEAE and QAE are positively charged whilst cation exchangers such as carboxymethyl and sulphonates are negatively charged. Cation exchangers may be weak carboxymethyl substituents, strong sulphonate groups or phosphates. The latter have certain affinity actions, in that they bind enzymes that interact with phosphorylated substrates such as nucleic acids.

1.4.4.2 **Hydrophobic and reverse phase adsorbents**

Hydrophobic and reverse phase adsorbents exploit the variability of external amino acid residues on different proteins. They interact with proteins as a result of their hydrophobic properties (Skopes, 1993). The most common type of hydrophobic adsorbent used contains short aliphatic chains, composed of four to ten carbons. Such hydrophobic interactions are weakened by detergents and strengthened by high salts and temperatures. Only a slight change in buffer condition is necessary to preferentially affect the binding properties of the purification matrix. Hydrophobic interation chromatography (HIC) is an ideal first step in a purification strategy (Amersham Biosciences 2001).

Reverse phase adsorbents are similar to hydrophobic adsorbents, but contain longer aliphatic chains between 8 and 18 carbons. They are used exclusively in High Performance Liquid Chromatography (HPLC). The operating conditions of the method are harsh, requiring the use of acids and organic solvents, which makes the method unsuitable for purification of some proteins. It utilises pumps, mixers, detectors and collectors to improve on traditional reverse phase chromatography. It operates under high pressure to increase the flow rate of chromatography columns and requires small beads that are strong enough to withstand such pressures.

Consequently, HPLC columns and equipment are costly to obtain and maintain and, as a result the procedure is usually restricted to specialised laboratories. Nevertheless, the high resolving power of this technique makes this a valuable and commonly used final step in protein purifications (Skopes, 1993). Fast Protein Liquid Chromatography (FPLC) is a variant of HPLC developed by Amersham Biosciences (2001). Its principles are based on those of ion exchange, but it uses highly refined Monobeads™ that are able to produce extremely high resolution.

1.4.4.3 **Affinity Chromatography**

Modern affinity chromatography was first used in 1967 (Axén 1967), using cyanogen bromide for the immobilisation of ligands on agarose supports. It is an effective method of purification that uses general or specific ligands to capture molecules. Specific ligands include antibodies, enzyme substrates, or cellular targets. General ligands are molecules which bind to certain groups of solutes, such as lectins or protein A which bind carbohydrates or various immunoglobulins, respectively (Walters, 1985). Naturally, the suitability of general ligands depends on what other components are in the sample.

If a specific ligand is not available, proteins can be cloned into a plasmid that has particular properties that allow affinity purification. For example, pGEX is designed for inducible, high level intracellular expression of genes with *Schistosoma japonicum* glutathione S transferase (GST). Expression is under the control of the *lac* promoter, which is induced using the lactose analogue isopropyl-β–D-thiogalactopyranoside (IPTG). Induced cultures are allowed to express GST fusion proteins for several hours and then cells are harvested. Protein purification can then be carried out in one step on a glutathione sepharose column. The protein of interest

is easily cleaved from its fusion protein by a site-specific protease and is readily eluted from the column to give a protein that is over 90% pure. Using this method 80 µg to 400 mg of protein can be purified (Amersham Biosciences, 2010).

His_6 tagging of proteins is an alternative to using large fusion tags such as GST. The purification strategy for His-Tag proteins is very similar to that for GST, in that the protein of interest is cloned into a vector that encodes a polyhistidine tag. Affinity separations are possible using a Ni^{2+} charged chelating sepharose. Weakly binding contaminants that have an affinity for nickel are washed away using a low concentration of imidazole. The strongly bound His-Tagged proteins are then eluted from the column by competitive elution with high concentrations of imidazole.

1.4.5 **Gel Filtration**

Gel filtration separates molecules according to differences in their sizes as they pass through a gel-packed column (Amersham Biosciences, 2010). The gel contains pores that have a controlled range of sizes which, in turn, allows for the controlled separation of molecules. The method is ideal for the final 'polishing' steps in a purification protocol. Samples are eluted in a single buffer as opposed to an elution gradient as used with ion exchange. High molecular weight molecules are eluted from the column first, followed by intermediate and low molecular weight molecules over one column volume. The type of gel filtration media chosen for an experiment depends on the molecule to be purified. For example, a Superdex 200 column is used for separating proteins or DNA fragments (Amersham Biosciences, 2001).

1.5 TOXIN DETECTION METHODS

Toxins can be detected directly using *in vivo* assays which demonstrate lethality in animals, such as the mouse bioassay for botulinum neurotoxins (Dowell *et al.,* 1977) or the guinea-pig skin test for SEB (Scheuber *et al*., 1983). However, they are used less commonly nowadays as they require the use of animals. *In vitro* testing has also been used for toxins lethal to culturable cells such as LT toxin from *Escherichia coli* (Guerrant, 1974). More recently, other methods have been used for antigen detection including mass spectrometry (Kawano *et al.,* 2000), surface plasmon resonance (Homola *et al.,* 2002), antibody microarrays (Panchal *et al.,* 2005), surface enhanced resonance scattering (Graham and Faulds, 2010) and nanotechnology using antibodies conjugated to gold or silver nanoparticles (Rosi and Mirkin, 2004; Schaller *et al.,* 2004; Jain, 2005;). However, the most commonly used method by far is ELISA.

1.5.1 Enzyme-linked immunosorbent assay (ELISA)

ELISA was first described by Engvall and Perlmann (1971). The simplest form of the assay, termed Direct ELISA, involves adsorbing the antigen to a solid support such as a polystyrene 96 well plate. Non-bound antigen is removed by washing, leaving an antigen-coated plate. An antibody solution is subsequently added to the plate and incubated. Unbound antibody is removed by washing and detection is performed using a substrate and chromogen which react with the enzyme label on the antibody to produce a colour change (Crowther, 2001). The colour change can be quantified on a spectrophotometer. An Indirect ELISA avoids the need to perform an enzyme labelling reaction with detection antibody. Instead, the detection antibody is in turn detected with a labelled species-specific antibody. For example, if the detection antibody was raised in mice, it would be possible to use labelled anti-

mouse antibodies to drive the colourimetric change, indirectly detecting the antigen of interest. A sandwich ELISA uses an antibody-coated plate to capture the antigen, which is consequently detected using the same direct or indirect method described above. The advantage of the sandwich assay is that it often has lower background signal, however it is more labour intensive as it involves an additional incubation step.

ELISA has a tendency to be subject to high background, since the polystyrene plate will passively adsorb proteins unless a blocking buffer is used. Numerous agents are suitable for use as blocking buffers, including detergents such as Tween-20, or proteins such as BSA or milk powder (Crowther, 2001). In spite of this, a colour change will most likely be observed in the negative control, limiting the sensitivity of the assay (Crowther, 2001). The sensitivity may also be limited by the colourimetric reaction, with enhanced chemiluminescence (ECL) being more suitable for ultrasensitive detection of toxin (Yang *et al*., 2011). Nevertheless, ELISA is a relatively simple technique that can be used for sensitive and specific detection of an antigen, once optimised. Immunoassay tickets, also known as lateral flow assays, are often a good alternative to ELISA. ELISA is sensitive and specific and can be performed in a basic laboratory. In contrast, immunoassay tickets, as epitomised by the home pregnancy test, are intended to be used by non-experts, making them suitable for use in countless scenarios (Tomaso *et al*., 2007).

The disadvantage of using ELISA or Immunoassay tickets for toxin detection is that specificity and sensitivity may be poor. It can be difficult to generate high quality antibodies to toxins, since the toxin often kills the animals before an efficient immune response is elicited. Formalin can be used to inactivate a toxin prior to

immunisation, to produce a toxoid. However this may alter the structure of the protein, generating antibodies with poor specificity to their target (Smith, 2009). PCR primers can be designed without such restrictions. Therefore, PCR was considered to be the most suitable alternative method for toxin detection, although it was unknown whether residual DNA would be present in toxins. Accordingly, immuno-PCR was also investigated with the aim of increasing the sensitivity of the assay in comparison to ELISA, whilst directly detecting the toxin.

1.5.2 **Polymerase Chain Reaction (PCR)**

PCR was the first method of nucleic acid amplification to be developed (Saiki, 1985). In those preliminary experiments, the Klenow fragment of DNA polymerase I from *Escherichia coli* was used to replicate DNA molecules in a manual series of heating to denature the template strand and cooling to allow primers to anneal to the target. Since then, the method has undergone significant improvements including the substitution of the Klenow fragment for a thermostable DNA polymerase and the advent of programmable thermocyclers. Typically, 30 to 40 temperature cycles are required to obtain maximum sensitivity of the assay. Beyond this, the sensitivity of the reaction is limited by depletion of reagents and accumulation of pyrophosphatase and PCR artefacts (Lee, 2001).

1.5.3 **End-point PCR**

End-point PCR reactions are carried out on a solid block thermocycler able to complete 40 cycles in approximately two hours. PCR products produced in this way must be stained with a DNA intercalator such as ethidium bromide and visualised by size separation on an agarose gel. This method is able to discriminate between

genuine products and PCR artefacts accurately based on differences in molecular weight.

1.5.4 **Real-Time PCR**

More recently, fluorescent probes have been used to detect and monitor *in vitro* DNA amplification, eliminating the need for post amplification handling of PCR products. This reduces the risk of carry-over contamination of subsequent reactions with amplicon and further automates the PCR process. Several manufacturers have developed thermal cyclers with built-in fluorimeters. Measuring the fluorescence generated during the reaction once per cycle enables the progress of the reaction to be monitored in real-time (Figure 1.4).

There are several types of fluorescent probe that can be used in real-time PCR. The simplest and cheapest type of probe is a non-specific double-stranded DNA intercalator such as ethidium bromide, YO-PRO-1 or Sybr Green™ I (Ririe *et al.,* 1997). Fluorescence is monitored once per cycle after product extension. Amplification of a PCR product results in an increase in fluorescence above background level. DNA intercalating dyes detect all double stranded DNAs including primer-dimers and secondary structures. Verification of product identity by melt curve analysis is therefore necessary (Figure 1.5).

At the end of the PCR cycles the reaction is heated at a rate of 0.2°C per second and the fluorescence is measured once per second. A rapid loss of fluorescence is seen near the denaturation temperature of the PCR product. The second derivative of this curve allows the melting point of the PCR product to be visualised as a peak, allowing differentiation of amplification products separated by less than 1 °C

(Wittwer *et al.,* 1997). High resolution melt analysis using a non-standard DNA intercalater (Wittwer, 2009) may allow discrimination between single nucleotide polymorphisms (SNPs), offering a rapid and simple method of strain typing.

Numerous specific real-time PCR chemistries also exist which overcome the disadvantages of DNA intercalaters, including TaqMan® (Livak, 1999), Scorpions™ (Whitcombe, 1999), Molecular Beacons™ (Tyagi *et al*., 1996) and dual Hybridisation Probes developed by Roche. TaqMan® assays were used exclusively in this study.

1.5.4.1 **TaqMan®**

The 5' exonuclease reaction (TaqMan®) uses the 5' to 3' exonuclease activity of *Taq* DNA polymerase. Holland *et al.,* (1991) created a substrate suitable for exonuclease activity in a PCR, an oligonucleotide probe designed to hybridise within the target sequence. The 5' end of the probe is labelled with a fluorophore such as fluorescein (FAM), which emits fluorescence at a certain wavelength when an excitatory light source of a different wavelength is provided by the thermal cycler. The 3' end is labelled with a quencher dye, such as tetramethylrhodamine (TAMRA). When the two dyes are in close proximity the fluorescence is quenched by fluorescence resonant energy transfer (FRET) (Livak, 1999). As the reaction proceeds, the 5' exonuclease activity of *Taq* polymerase cleaves the probe, separating the two dyes, causing an increase in fluorescence from FAM (Livak, 1999). As the number of amplicons increase, the fluorescence increases proportionally.

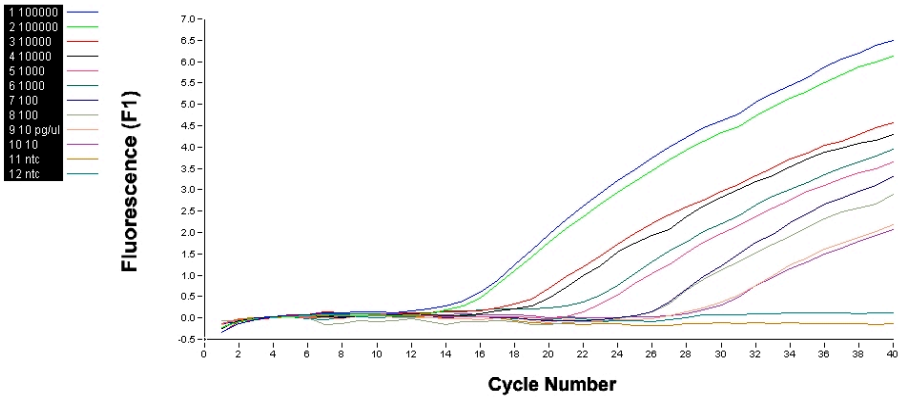


Figure 1.4 Typical real-time PCR fluorescence readings produced using a 10 fold dilution series of template DNA with Sybr Green™ I intercalator. An increase in fluorescence is observed with each thermal cycle as the quantity of PCR product increases exponentially. The threshold (or crossing point) at which the fluorescence exceeds a given value can be used to quantify DNA in an unknown sample, providing standards of a defined concentration are also amplified.

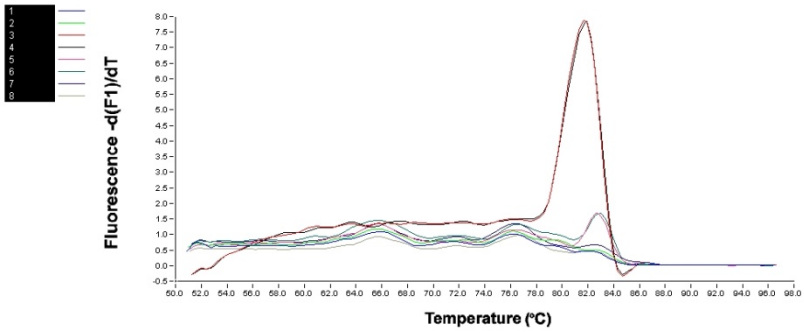


Figure 1.5 Example melt curve analysis graph. The apex of the curve represents the point at which an amplicon 'melts' and becomes single stranded, leading to a rapid loss of fluorescence. This melting temperature (Tm) can be used to differentiate between PCR products.

1.5.4.2 **Quantitative PCR**

Quantitative PCR is a fast, accurate and reliable means of measuring the distribution or expression of target nucleic acids. Real-time PCR can be quantitative if samples containing unknown amounts of DNA are analysed against data generated from amplification of samples of known concentration. Many machines have PCR capabilities that generate quantitative data based on the PCR at early cycles when the fidelity of the reaction is highest. Data generated outside of the log-linear phase of the reaction is disregarded, as the amplification efficiency is poor (Rasmussen *et al.,* 1998). For accurate quantification it is necessary to verify that PCR efficiency is independent of the initial concentration of target DNA (Ponchel *et al.,* 2003). Testing primer pairs against a dilution series of target DNA across several logs serves to do this as well as a standard curve.

1.5.4.3 **Multiplex PCR**

Multiplex PCR uses more than one primer set in a reaction with the aim of detecting two or more genetic targets simultaneously. Visualisation of multiple products relies on differences in fluorescent probe signature, size or melting temperature, depending on whether traditional or real-time PCR is used. Many research groups have developed such systems for detection of bacterial virulence markers using traditional PCR (Mehtrota *et al.,* 2000; Sharma *et al.,* 2000; Luo, 2002). Normally four or five targets are amplified at once using this method (Mehrotra *et al.,* 2000). For example, quadruplex real-time PCR has successfully been used for the differentiation of *C. botulinum* strains A, B, E and F (Satterfield *et al.,* 2009). However, it is possible to amplify as many as twenty different targets simultaneously (Butler, 2002) using traditional PCR.

Probes such as TaqMan® or Molecular Beacons™ that fluoresce at different wavelengths for each target have successfully been used to identify amplification of two bacterial virulence markers in one tube (Belanger, 2002; Palladino, 2003; Ryu, 2003). Currently, the degree to which real-time PCR assays can be multiplexed is limited to between four and six targets by the range of detection channels available in machines such as the I-Cycler™ (Biorad, UK), Smartcycler™ (Cepheid, USA) or the Rotorgene™ (Qiagen, UK). This range may be extended if alternative fluorophores can be coupled to oligonucleotides, providing that the instrumentation to detect a wider range of wavelengths is available.

1.5.4.4 **Internal Controls**

Co-amplified internal positive controls are portions of DNA that are genetically modified to contain the same specific PCR primer sequences as the specific gene (Cone *et al.,* 1992). They can be constructed by amplification of a suitable portion of stuffer DNA using chimeric primers flanked with the target specific primers. For example, Hoorfar *et al.,* (2000) developed an internal control for a *Salmonella enterica* assay using *Drosophilla melanogaster* specific primers flanked by *Salmonella* specific sequences to amplify *Drosophilla* DNA.

Internal controls can be used in both traditional and real-time PCR. They should be constructed to be larger than the target amplicon to ensure that they are not amplified preferentially over the target DNA. This also allows the internal control and the target amplicon to be differentiated using gel electrophoresis. Alternatively, a probe that hybridises to the stuffer DNA may be used to report amplification of the internal control in real-time PCR.

1.5.5 **Immuno-PCR**

Immuno-PCR was first described by Sano *et al.,* (1992). It is based on a standard sandwich ELISA format, able to detect antigen directly, combined with the sensitivity of PCR. The secondary or tertiary antibody in a sandwich ELISA is labelled with a reporter DNA sequence, which can then be amplified by PCR, as a potentially more sensitive alternative to colorimetric detection. A number of strategies have been reported for linking the DNA to the antibodies. Sano *et al.,* (1992) used streptavidin to bind to biotin labelled DNA, an approach which is simple and easy to adopt in any laboratory. Other teams have used covalent linkage of DNA to antibodies, which although more difficult to perform than utilising the avidin-biotin interaction, allows the assay time to be reduced significantly (Niemeyer *et al.,* 2007). Other approaches include using phage displayed antibodies (Guo *et al.,* 2006) although theoretically any ligand can be labelled with DNA and amplified using any nucleic acid detection method (Niemeyer *et al.,* 2007).

The advantages of I-PCR over conventional antibody-based assays are well established (Niemeyer *et al.,* 2007). For example, unlike ELISA, immuno-PCR theoretically allows the detection of multiple analytes simultaneously if the reporter DNA used to label an antibody is different for each analyte. If real-time PCR is used, a different fluorescent probe is needed for each analyte, therefore up to four targets could be detected simultaneously using the most commonly used instruments.

Immuno-PCR has been successfully used to detect bacterial toxins by a number of research groups. Wu *et al.,* (2001) developed an immuno-PCR system for the detection of *Clostridium botulinum* neurotoxin A using a standard immunoassay

format with monoclonal antibodies that were covalantly coupled to reporter double stranded DNA. The I-PCR assay was 1000 fold more sensitive than the ELISA, with the detection limit of the assay being 50 picograms. Chao *et al.,* (2004) independently developed an I-PCR assay which reduced the detection limit of Bont/A to 50 femtograms by optimising streptavidin and reporter DNA concentrations in both indirect immuno-PCR and indirect sandwich immuno-PCR formats. Fischer *et al.,* (2007) also observed a 1000 fold increase in sensitivity with an assay developed for detection of SEB. They used real-time immuno-PCR to detect antibodies covalently labelled with reporter DNA to detect 600 femtograms of SEB. Yang *et al.,* (2009) used a gold nanoparticle based immuno-PCR assay to detect SEB spiked into meat and baby food, detecting 0.01 ng of toxin per ml. It can be seen that immuno-PCR is particularly well suited to toxin detection as it is generally more sensitive than ELISA. It is not used as routinely as PCR, with approximately 100 papers being published on the subject in the past twenty years. However, it was included in this study to determine whether the increase in sensitivity offered by the technique would facilitate detection of the limited quantities of toxin that may be present in BTWC samples.

As previously mentioned almost any ligand can be used for immuno-PCR. For this reason, aptamers were also studied as they themselves are nucleic acid molecules with the potential to detect toxins.

1.5.5.1 **APTAMERS**

An aptamer is a small oligonucleotide with high specificity for their target due to their specific three-dimensional conformations (Fan *et al*., 2008). They are 'evolved' to detect a specific target by a process known as SELEX (**S**ystematic **E**volution of

Ligands by **EX**ponential enrichment; Tuerk and Gold, 1990). It has been used to isolate high affinity nucleic acid ligands, known as aptamers, to a variety of protein and carbohydrates as well as organic dyes, amino acids and antibiotics (Jayasena, 1999). They have the potential to replace antibodies in detection assays in general, but may be particularly suitable for use with toxins as they can be generated *in vitro*. This is advantageous as it avoids the use of animals in experiments and circumvents the requirement for formaldehyde inactivation of the toxin, with the native toxin being used instead. Theoretically, aptamers have the potential to have higher specificity for a toxin than an antibody raised in an animal using inactivated toxin (Bruno and Kiel, 2002).

Aptamers have many uses both in *in vitro* and clinical settings, with their therapeutic uses being reviewed recently in Ni *et al.,* (2011). Aptamer based detection of targets can be carried out in numerous ways, being as easy to conjugate to reporter molecules as antibodies. Aside from eliminating the use for animals in their generation, one of the main advantages of aptamers over antibodies is that they can be chemically synthesised with numerous modifications at any oligonucleotide synthesis house. For this reason they make incredibly diverse biosensors utilising beacons (Nutiu and Li, 2003) and quantum dots (Ellingham *et al*., 2000; Fan *et al*., 2008) amongst other things (Juskowiak, 2011).

The selection protocol used depends on the target molecule. For example, aptamers to small compounds such as peptides can be obtained by selection on an affinity column containing the immobilised ligand (Jayasena, 1999). SELEX experiments that target proteins and utilize RNA pools can be carried out by isolating protein-aptamer complexes on modified cellulose filters or magnetic beads. To avoid

selection of matrix-binding molecules, the pool should be pre-treated with the matrix and matrix molecules should subsequently be eliminated from any further rounds of selection with the target protein (Vivekanenda and Kiel, 2006). The specificity of aptamers can be increased by using a close relative of the antigen of interest in negative selection experiments before (Chen, 2007) or after (Shangguan *et al.,* 2007) the SELEX experiment.

SELEX is a versatile process. The success of an experiment reportedly depends on the properties of the target ligand (Jayasena, 1999). Generally, nucleic acid binding proteins will be better aptamer targets than other proteins. Positively charged ligands will be easier to work with than negatively charged ligands owing to the negative charge on DNA molecules (Jayasena, 1999). Controlling the concentration of target and magnesium can vary the stringency of selection (Osborne, 1997). The inclusion of molecules that compete with nucleic acids for binding to a target will encourage the isolation of high-affinity species from a random sequence population. Non-specific competitors such as tRNA can help eliminate most weakly binding species from the population. Therefore the stringency of a selection can also be controlled by varying the ratio of pool to competitor.

SELEX has previously been used to develop aptamers to toxins including pepocin (Hirao *et al.,* 1997), ricin (Hesselberth *et al.,* 2000), SEB (Bruno and Keil, 2002; Purschke, 2003), botulinum neurotoxin (Wei *et al*., 2009; Tok and Fischer, 2008; Chang *et al.,* 2010) and Shiga toxin (Fan *et al.,* 2008). The selection processes used by each research group differed depending on whether DNA or RNA was used, however the most popular methods involved semi-automation using magnetic bead bound targets (Bruno and Keil, 2002; Chang *et al*., 2010).

1.6 NEXT GENERATION SEQUENCING

The final aim of this study was to investigate the use of DNA sequencing as a strain typing tool, a possible requirement following positive identification of a BTWC sample. DNA sequencing was first described in 1977 by Maxam and Gilbert. The method involved cleavage of radioactively tagged oligonucleotides at different points followed by size separation by agarose gel electrophoresis. In the same year an alternative method was developed by Sanger *et al*., (1977). The method developed by Sanger *et al*., (1977), termed dideoxy sequencing, employed novel nucleoside analogues that would act as chain terminators when incorporated by DNA polymerase. It was used extensively to sequence the complete genome from a large number of species, including humans (Venter *et al.,* 2001) and the biological agents *Francisella tularensis* (Larsson *et al.,* 2005) and *Yersinia pestis* (Parkhill *et al.,* 2001). The gold standard for molecular strain differentiation is DNA sequencing. It can be used to characterise fragments of genomes (such as amplicons) or entire genomes. Although extremely powerful, 'Sanger' sequencing was labour intensive, taking years to generate the data to assemble a draft genome.

Ronaghi *et al.,* (1996) developed the concept of detecting the pyrophosphatase released during DNA amplification to sequence-by-synthesis, using enzyme-coupled reactions and bioluminescence to report nucleotide incorporation. This allows simultaneous sequencing of many fragments of a genome in a very short space of time. Next generation sequencing platforms began to emerge nearly ten years after the concept was first reported, including Roche 454™ Sequencing, Illumina GA2 and the ABI SOLiD, and are reviewed in Hall (2007). Each utilises a different

reaction chemistry, resulting in different read lengths. 454™ Sequencing offers the greatest read length (500 basepairs) of any currently used system, capable of generating one million random reads which equates to 500 megabases of sequence per run (Roche, 2009). The technology therefore has the potential to rapidly sequence prokaryotic genomes and larger eukaryotic species can theoretically be sequenced within a few runs.

1.7 PROJECT AIMS

The aim of this thesis was to determine the utility of nucleic acids for analysis of weaponised toxin samples. Principally PCR was investigated, analysing toxin samples of varying purifications thereby mimicking samples that may be encountered in a BTWC environment. To meet the requirements of sensitive and unequivocal detection, it was necessary to investigate alternative nucleic acid approaches to the problem. The aim of the final Chapter in this thesis was to investigate genetic methods for strain differentiation of toxin-producing organisms, with a specific focus on 454 Sequencing™.

2 MATERIALS AND METHODS

2.1 BACTERIAL STRAINS

2.1.1 *Clostridium botulinum* strains

Clostridium botulinum strains listed in Table 2.1 were obtained from NCTC (National Collection of Type Cultures, HPA Collindale, London, UK) or ATCC (American Type Culture Collection, LGC, London, UK). Proteolytic *C. botulinum* strains were inoculated into 10 ml cooked meat carbohydrate broth (Oxoid) in a Don Whitley Mark III Anaerobic cabinet and grown statically at 34 ^{o}C overnight. Saccharolytic strains were inoculated into cooked meat broth supplemented with 1 % sucrose (w/v) and grown in the same way. The number of colony forming units was subsequently determined by spreading triplicate 100 μl samples of overnight culture, serially diluted in PBS from 10^{-1} to 10^{-8}, onto anaerobic blood agar plates (Oxoid). Resulting colonies were counted after overnight static incubation at 34 ^{o}C in the anaerobic chamber. The number of colony forming units per ml (CFU/ml) was then calculated by multiplying the number of colonies counted by 10 and then by the dilution factor. The mean CFU/ml were calculated as one third of the sum of the values obtained.

Table 2.1. List of *Clostridium botulinum* strains used. Cultures were obtained from the National Type Culture Collection (NCTC; HPA London) or American Type Culture Collection (ATCC, LGC London). " –" indicates that the culture source was unknown.

Organism	Strain	Genotype/ designation	Origin
Clostridium botulinum A	NCTC 7272	A1	Unknown
Clostridium botulinum A	NCTC 887	A1	Crop of chicken with botulism, 1920
Clostridium botulinum A	NCTC 2916	A1 (B)	Home made canned corn 1926
Clostridium botulinum A	NCTC 3806	A	Canned peas, 1932
Clostridium botulinum A	NCTC 4587	A	-
Clostridium botulinum A	NCTC 9837	A2	Fish from Mauritius, 1955
Clostridium botulinum A	NCTC 11199	A1 (B)	Faeces, 1978
Clostridium botulinum A	NCTC 2012	A2	Unknown
Clostridium botulinum B	NCTC 751	B Group I	California, 1920
Clostridium botulinum B	NCTC 3807	B Group I	Soil, Blue Ridge Mountains, 1021
Clostridium botulinum B	NCTC 3815	B Group I	Cheese, 1932
Clostridium botulinum B	NCTC 7273	B Group I	Beans, 1947
Clostridium botulinum B	NCTC 12265	B	Hazelnut yoghurt, 1989
Clostridium botulinum B	9581	-	-
Clostridium botulinum B	ATCC 17841	B Group I	-
Clostridium botulinum B	ATCC 17844	B Group II (2217)	N/D
Clostridium botulinum B	ATCC 17845	B (2254)	-
Clostridium botulinum B	Eklund 17B (ATCC 25765)	B Group II	Marine Sediment, USA

Clostridium botulinum B	ATCC 17780	B (3137)	-
Clostridium botulinum B	ATCC 17783?	B	-
Clostridium botulinum B	ATCC 439	Group I	-
Clostridium botulinum B	ATCC 17843	B (2017)	-
Clostridium botulinum E	NCTC 8266	E Nanaimo Group II	MRE Porton 1952
Clostridium botulinum E	NCTC 8550	E Group II	Institut Pasteur 1952, possess OS variants.
Clostridium botulinum E	ATCC 17854	E Group II	-
Clostridium botulinum E	ATCC 8550	E Group II	-
Clostridium botulinum E	ATCC 17854	E Group II	-
Clostridium botulinum E	ATCC 17786	E Group II	-
Clostridium botulinum E	ATCC 11219	E Group II	-
Clostridium botulinum E	ATCC 9564	E Group II (Hazen)	Smoked Salmon, Canada
Clostridium botulinum F	NCTC 10281	Langeland Group I	Liver paste, Vancouver 1962.
Clostridium botulinum F	ATCC 23387	Group II Eklund (202F)	-
Clostridium botulinum F	ATCC 25764	Group I	Crab
Clostridium botulinum F	ATCC 27321	VPI 2382	-

Table 2.2. List of *Staphylococcus aureus* strains used. Cultures were obtained from the National Type Culture Collection (NCTC; HPA London) or American Type Culture Collection (ATCC, LGC London). " –" indicates that the culture source was unknown.

Organism	Strain	Genotype/ designation	Origin
Staphylococcus aureus Rosenbach	ATCC 6538 (Oxoid)	FDA 209	Human lesion
Staphylococcus aureus Rosenbach	ATCC 25923 (Oxoid)	Seattle 1945	Clinical isolate
Staphylococcus aureus Rosenbach	ATCC 33862 (Oxoid)	SS697	-
Staphylococcus aureus Rosenbach	ATCC 9144 (Oxoid)	3R7089 strain Oxford	-
Staphylococcus aureus	ATCC 23144 (held at Dstl)	-	-
Staphylococcus aureus	NCTC 10656 (Dstl)	-	Turkey salad, 1964
Staphylococcus aureus	NCTC 10657 (Dstl)	-	-
Staphylococcus aureus	NCTC 10652 (Dstl)	-	Ham involved in food poisoning

2.1.2 *Staphylococcus aureus* strains

S. aureus strains detailed in Table 2.2 were cultured from glycerol stocks held at Dstl or obtained as Culti-loops® from Oxoid. Culti-loops® were inoculated into Nutrient broth (Oxoid) and grown in a shaking incubator at 200 rpm (Stuart, Jencons, UK) at 37 ^{o}C overnight. A streak plate was made by plating 10 µl of liquid culture or glycerol stock onto a Nutrient agar plate (Oxoid), followed by growth in a static 37 ^{o}C incubator (Hettich, Jencons, UK).

2.1.3 *Bacillus cereus* strains

B. cereus spores were produced from glycerol stocks of vegetative cells held at Dstl. Sporulation broth (100 ml of Schaeffer's sporulation medium: 0.8% nutrient broth (Difco), 27 mM KCl, 2 mM $MgSO_4 \cdot 7H_2O$, 1 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.1 mM $MnCl_2 \cdot 4H_2O$, and 1 μM $FeSO_4 \cdot 7H_2O$) was inoculated with a single colony of *B. cereus* NCTC 2566 from a L-agar streak plate made by plating 10 μl of glycerol stock onto a L-agar plate. Broths were incubated with shaking (200 rpm) at 37 °C for two days. Cells were heat shocked at 65 °C for 1 hour, followed by incubation with lysozyme (Roche, UK) at a final concentration of 200 μg/ml for 30 minutes at 37 °C. Percentage sporulation was assessed by microscopic examination. Where required, sporulation was continued for a further two days and the culture was re-examined by microscopy. Culture broth (100 ml) was transferred to sterile centrifuge pots (Sorvall, Thermo Fisher, UK) and centrifuged at 800 x *g* for 30 minutes in order to collect spores.

2.1.4 Growth of recombinant *E. coli*

Liquid cultures of recombinant *E. coli* cells were obtained using grid plates, glycerol stocks or cryobeads as starter cultures, as detailed in Section 2.2.3, 2.3.3 or 2.7. Small or large volumes (5 or 100 ml) of inoculated L-ampicillin broth (containing 12.5 mg of ampicillin; Roche) were grown at 37 °C overnight in a shaking incubator at 200 rpm (Stuart, Jencons, UK).

2.1.5 *Ricinus communis* strains

Twenty cultivars of *R. communis* (Table 2.3) were grown on a large scale by a commercial grower in France, (Sandeman Seeds).

Table 2.3 List of cultivars of *Ricinus communis* used in this study.

R. communis cultivar	Origin
A007, Black Diamond 1, Black Diamond 2, Dwarf Australia, Imp A Ethiopia, JanXiang 1, JanXiang 2, Noori Jalab Pakistan, Noori Pakistan, TCO 202, Sara Hybrid Brazil, VC007/9 Paraguay, VCR Colombia, Zanzibar, Zanzibar Kenya, Zibo 1, Zibo 2, Zibo 3, Zibo 101, Zibo 108, Zibo 305.	Sandeman seeds, France
Black Diamond, Jin Castor 2, Kunming 202, Sanguinis, Shang Dong 108, Zanzibar, Zibimas.	Private garden, U.K.

2.2 DNA EXTRACTION PROCEDURES

2.2.1 *C. botulinum* genomic DNA extraction

Clostridium botulinum samples were handled in a designated laboratory, with particular safety restrictions. To comply with Dstl safety rules in the toxin laboratory a DNA extraction kit that used magnetic particle collection was used. Toxin or culture broth samples (200 µl) were centrifuged at 11 000 x *g*, resuspended in 200 µl bead lysis buffer and incubated at 65 ºC for ten minutes to aid cell lysis, followed by DNA binding to silica coated Dynabeads DNA Direct 1 (Dynal, UK). Magnetic particles were then collected and washed in 500 µl wash buffer three times. Finally, DNA was eluted from the beads in 50 µl TE (10 mM Tris HCl, 1 mM EDTA) buffer.

2.2.2 **Plasmid DNA extraction**

Plasmid DNA was extracted using a QIAGEN Plasmid mini kit (Qiagen, UK), according to the manufacturers' instructions. Bacterial cells grown as described in Section 2.1 were centrifuged at 5000 x *g*. The supernatant broth was removed and the cell pellet was gently resuspended with 1 ml of a chilled resuspension buffer (P1, containing 50 mM TE, 10 mM EDTA and 100 μg/ml RNase A), to release plasmids without disrupting genomic DNA. Proteins were then precipitated gently in 250 μl of lysis buffer P2 and 350 μl N3 on ice for 15 minutes and removed by centrifugation at 10 000 x *g* for 1 minute. DNA was subsequently bound to a silica column by centrifugation as above, washed in 500 μl buffer PB and re-centrifuged. Further washes were performed using 750 μl of buffer PE and centrifugation as before. Traces of ethanol were removed during a further centrifugation step at 10 000 x *g* for 2 minutes and DNA was subsequently eluted in buffer 50 μl EB and stored at – 20 ^{o}C until needed.

2.2.3 **Genomic DNA extraction**

Genomic DNA was extracted from all other microbial species and sample types using a QIAamp DNA mini-prep kit (QIAgen, UK), according to the manufacturers' instructions, unless stated otherwise. Bacterial or fungal cells (1 ml of an overnight culture) grown as detailed in Section 2.1 were centrifuged at 10 000 x *g* for 3 minutes. The cell pellet was resuspended in 200 μl of lysis buffer AL and 20 μl of proteinase K (20 mg/ml). The cells were lysed further by incubation at 56 °C for 10 minutes in a Thermomixer (Eppendorf, Fisher, UK) at 1400 rpm. Ethanol (96%; 500 μl, VWR, UK) was then added to the cell lysate which was spun through a silica spin column at 15 700 x *g* for 1 minute. DNA bound to the column was washed in wash

buffers AW1 and AW2 and finally eluted in 50 µl of TE buffer. DNA concentration was determined by using a Nanodrop spectrophotometer (Labtech International, UK).

2.2.3.1 **Masterpure Plant DNA extraction**

Small volumes of plant leaf samples (35 mg) were processed using the Masterpure Plant leaf DNA purification kit® from Epicentre (Cambio, UK). The plant leaf was ground in 300 µl of extraction buffer using a pestle and mortar and then heated to 70 °C for 30 minutes to aid cell lysis. Samples were then precipitated on ice for 10 minutes. Cell debris was collected by centrifugation at 10 000 x *g* for 5 minutes. The supernatant was then re-centrifuged to remove remaining debris and the resulting supernatant was precipitated in an equal volume of isopropanol (Sigma, UK). DNA was collected by centrifugation at 10 000 x *g* for 5 minutes and resuspended in 100 µl of Cleanup Solution. DNA was then precipitated again in 100 µl isopropanol and collected as previously described. The pellet was washed in 70 % ethanol and the resulting DNA was resuspended in 50 µl TE buffer and stored at – 20 ^{o}C until needed.

2.2.3.2 **Powerplant® DNA extraction of *R. communis* DNA**

DNA was extracted for 454™ sequencing from 50 mg of *R. communis* leaf using the MoBio PowerPlant® DNA Isolation Kit (Cambio Ltd, UK). Plant tissue was added to a bead tube containing stainless steel balls. Bead solution (550 µl) was added to the tube which was then vortexed. PB1 lysis buffer (60 µl) was added to the tube and then plant tissue was degraded at 65 ^{o}C for 10 minutes in a waterbath (Grant, Fisher, UK). The tube was allowed to cool briefly and then plant tissue underwent mechanical lysis with the stainless steel beads at 2000 rpm for 30 seconds in a

Fastprep 24 (MPBio, UK). Following centrifugation for 30 seconds at 10 000 x *g* the supernatant was transferred to a clean tube. PB2 buffer was added to the tube (250 µl) to precipitate proteins at 4 ^{o}C for 5 minutes. Particles were collected by centrifugation at 10 000 x *g* for 1 minute. The supernatant was transferred to a clean tube and 1 ml of PB3 was added. The tube was inverted 5 times to mix the contents and then incubated at room temperature for 10 minutes. The tube was centrifuged at 13 000 x *g* for 15 minutes and the resulting supernant was discarded. The pellet was resuspended in 100 µl PB6 (TE) and 500 µl PB4 (isopropanol). The entire contents were then captured on a silica spin column which was centrifuged at 10 000 x *g* for 1 minute. The flow through was discarded and the filter was washed in 500 µl PB5 (ethanol). The tube was centrifuged at 10 000 x *g* for 30 seconds. The flow through was discarded and the tube re-centrifuged to remove residual alcohol. The purified DNA was subsequently eluted from the column in 50 µl PB6 at 10 000 x *g* for 30 seconds.

2.2.3.3 **Gel purification of amplicon (QIAquick®)**

Agarose gels (Type II, Sigma, UK) were made with 1 X TAE buffer (Biorad Laboratories Ltd, Herts, UK) and stained with 1.5 mg/ml crystal violet (Sigma). Gel slices were excised from the gel using a scalpel blade whilst the DNA band was illuminated by white light on a light box (UVITEC, Cambridge, UK) and transferred to a 1.5 ml Eppendorf tube. The gel slice was weighed then three volumes (w/v) of buffer QG (QIAquick®, Qiagen, UK) were added to the tube, which was subsequently incubated at 60 ^{o}C until the gel had dissolved. Isopropanol (one volume) was then added to the mix to facilitate binding of the DNA to the silica in a QIAquick Spin Column. The DNA was bound to the column following centrifugation at 10 000 x *g* for one minute and then washed in 0.75 ml Buffer PE by

centrifugation as before. Residual ethanol contained in buffer PE was removed by a further centrifugation step as before. The DNA was eluted into a clean 1.5 ml microcentrifuge tube with 50 µl Buffer EB (10 mM Tris HCl, pH 8.5) by centrifugation at 10 000 x *g* for 1 minute.

2.3 PCR METHODOLOGY

Simple sequence repeat primers were obtained by searching the castor bean genome using the FASTA Simple Sequence Repeat finder software http://abajian.net/sputnik/ (Jewell *et al.,* 2006). All other PCR assays were designed using sequences obtained from the public Genbank database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) or the castor bean EST database (http://plantta.tigr.org). DNA sequence analysis was subsequently performed using DNASTAR (DNASTAR Inc, Madison, Wisconsin, USA). PCR primer and probe sets were designed using Primer Express 1.0 software (Applied Biosystems, CA, USA). Oligonucleotide PCR primers were synthesised by various companies including Thermo Hybaid (Thermofisher, UK), ATDBio (Southampton, UK), Sigma Genosis (UK), and MWG, (Germany). The sequences of the designed primers are included in Chapter 3.

2.3.1 Traditional PCR

Traditional PCR was carried out on a 9700 thermal cycling machine (Applied Biosystems, Warrington, UK). Reactions contained 1 x reaction buffer (Roche), 0.04U/ µl *Taq* polymerase (Roche), 500 nM of each primer, DNA and dH_2O in a total volume of 20 µl. Thermal cycling conditions are detailed in chapter 3.

2.3.2 Real-time PCR

Real-time PCR was initially carried out on the Roche Lightcycler™. Later work was performed on the Cepheid Smartcycler™, or the Corbett Rotor-Gene™. In all cases, each 20 µl reaction contained 0.5 µM of each primer, 1x PCR buffer composed of 50 mM Tris pH 8.8 (Sigma, UK), 3 or 4 mM $MgCl_2$ (Sigma, UK), 0.2 mM each dNTP (Roche, UK), 0.04 U/µl *Taq* polymerase (Roche, UK), 250ng/µl BSA (Sigma, UK), and 8% glyercol (w/v) (Sigma), plus either 1 x SYBR® Green I (Sigma) or a specific TaqMan® probe (Qiagen, UK). Thermal cycling conditions were optimised for each primer set; the results of this are detailed in Chapter 3.

2.3.3 Internal controls

Internal PCR controls were constructed for each assay used in this study. Composite primers were designed so that toxin-specific primers flanked primers specific for a region of the human β actin gene (Figure 2.1).

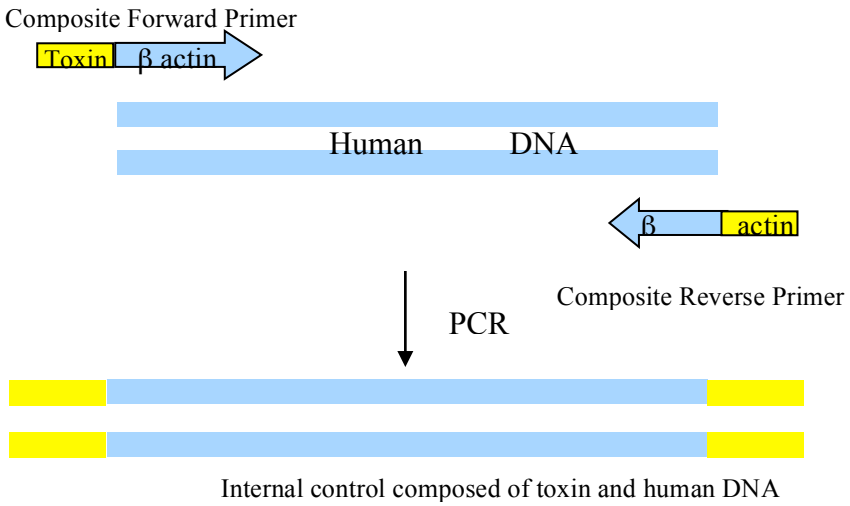


Figure 2.1 Construction of internal controls using chimeric primers. Chimeric primers were synthesised to contain the sequence of interest followed at the 3' end by primers specific for the human β actin gene. The forward and reverse primer sequences used were AGTACTCCGTGTGGATCGGA and GCTGATCCACATCTGCTGGA respectively.

2.4 CLONING

2.4.1 Microconcentration

Amplicon or plasmid DNA was purified by microconcentration to remove PCR reagents or reduce the volume. Preservative was removed from a Microcon YM 100 centrifugation cup (Millipore, Fisher, UK) by washing through with 500 µl of water using centrifugation at 6000 x *g* for 5 minutes. This was followed by inversion of the cup at 4000 x *g* for 1 minute to remove traces of water. Plasmid DNA or completed PCR reaction mixtures were recovered, pooled and added to the cup and the volume made to 500 µl with dH_2O. Waste products were removed by centrifugation at 6000 x *g* for 5 minutes. The retained DNA was washed in a further 500 µl dH_2O. Purified DNA was recovered by inverting the centrifuge cup into a clean tube and centrifuging at 4000 x *g* for 8 minutes.

Centricon purification of larger volumes was occasionally carried out, as detailed in the Results chapters and described in Section 2.7.5.

2.4.2 Restriction enzyme digestion

Reactions were composed of 1 X NEB # 2 reaction buffer (New England Biolabs, UK), 10 X BSA, *Sfi* I (10 units), 1 µg of DNA to be digested and dH_2O to make a total volume of 50 µl. Reactions were then incubated at 50 ºC for 4 - 16 hours on a Perkin Elmer 9700 PCR thermocycler. Digested products were purified by agarose gel electrophoresis as described in Section 2.2.3.2.

2.4.3 Ligation

Internal control PCR amplicons were ligated into a TA cloning vector (pT7 Blue, Novagen, Cambridge Bioscience, UK) and single chain antibody amplicons were

ligated into pAK100 (as detailed in Krebber *et al*. 1997). Ligation reactions comprised 200 ng of linearised plasmid, 20 ng of purified single chain antibody amplicon, 1 unit of T4 DNA ligase (NEB, UK), 1 x ligase buffer and water in a volume of 20 μl. The contents were mixed well and then incubated for 16 hours at 16 ºC in a Perkin Elmer 9700 thermal cycler.

2.4.4 **Transformation of chemical competent cells (internal control cloning)**

Recombinant plasmid DNA (between 1 and 50 ng) was added to 100 μl of *E. coli* JM109 competent cells in a 15 ml polypropylene tube (Falcon®, Fisher, UK). The cells were then shocked at 40 ^{o}C for 40 seconds and immediately chilled on ice for 20 minutes. SOC broth (900 μl, Promega, UK) was added to the cells which were then incubated at 37 ^{o}C with shaking in a Stuart Orbital S150 incubator at 200 rpm for 1 hour. The resulting culture (100 μl) was plated out onto LB agar plates supplemented with 100 mg/ml of ampicillin. Plates were incubated in a static 37 ^{o}C incubator (Hereus, Jencons, UK) overnight.

2.4.5 **Transformation by electroporation (single chain antibody production)**

Electrocompetent *E. coli* cells (100 μl; Stratagene, UK) were thawed slowly on ice. Recombinant single chain antibody cDNA (prepared as described in Section 2.8.6.2) was then added to the cells at a concentration of 10 pg/μl. The contents of the tube were then quickly added to an electroporation cuvette and pulsed at 2500 V, 200 ohm, 25 μF, with a time constant of ~4.5 - 5.0 milliseconds. Room temperature 2 X YT broth (900 μl; 16g Bacto® Tryptone, 10g Bacto® yeast, 5g NaCl per litre) was added to the cells which were then incubated at 37 ^{o}C with shaking at 150 rpm for one hour. Transformed cells were subsequently plated onto 10 cm 2 X YT plates (made as 2 X YT broth, plus 15g Difco agar) supplemented with 30 μg/ml of

chloramphenicol and incubated for 16 hours at 37 ^{o}C. Cells were then recovered from the plates by gently scraping them into a 50 ml tube (Falcon, VWR, UK), concentrated by centrifugation at 5000 x *g* at 4 ^{o}C for 10 minutes and the concentration adjusted to OD_{600}=1.

2.5 ANALYSIS OF DNA BY GEL ELECTROPHORESIS

2.5.1 Agarose gel electrophoresis

DNA amplicons were analysed by agarose gel electrophoresis. Between 10 μl and 25 μl of each sample, with a final concentration of 1 X tartrazine loading buffer (50% v/v glycerol, Sigma, UK, 50 % dH_2O, plus 0.1 g tartrazine, Sigma) was loaded onto a 2 % (w/v) agarose gel (Type II, Sigma). Gels were made with 1 X TAE buffer (Tris Acetate EDTA; Biorad Labs Inc, UK) and contained a final concentration of 0.05 % ethidium bromide (Biorad, UK) or 1.5 mg crystal violet (Sigma, UK). Molecular weight standards XIV (100 bp ladder), or III (both obtained from Roche, UK) were loaded into adjacent lanes. Each gel was run in 1 X TAE buffer at 90 volts until the amplicons were separated (30 to 60 minutes). Agarose gels were visualised using a Gel Doc 2000 (Biorad, UK). E-gels from Invitrogen were run using the automatic settings on the iBase powerpack appropriate for the size of gel. Sample was added to the wells in the absense of loading buffer.

2.5.2 Polyacrylamide gel electrophoresis (PAGE)

PAGE analysis of SSR PCR products was carried out. Pre-poured Novex® Tris Borate EDTA (TBE) PAGE gels (10%; Invitrogen) were placed into an XCell *Surelock*™ Mini-Cell tank (Invitrogen) which was then filled with 1 X Novex® TBE running buffer. The wells were rinsed three times in running buffer by gently

running and discharging a 1 ml pipette across the tops of the wells (Invitrogen). Samples were prepared in 1 X Novex® TBE High-Density sample buffer. The gels were electrophoresed 175 V per gel for 45 minutes. The cassettes were then carefully split apart with a palette knife so that the gel remained on one half of the casing. The gels were stained in ethidium bromide (0.025 mg/ml in dH_2O) for 20 min and de-stained for 20 min in three changes of dH_2O. The gels were carefully peeled from the cassette casing onto the transilluminator for visualisation.

2.6 DNA QUANTIFICATION

2.6.1 UV densitometry

Molecular weight markers III or XIV (Roche) were loaded in triplicate. The DNA samples were loaded alongside the markers in two concentrations (undiluted and 1/10 dilution) in triplicate. Gels were electrophoresed and visualised as described in Section 2.5. The saturation function was then employed to ensure DNA band intensity was proportional to the DNA concentration. DNA concentrations were computed using a comparison of values for known (markers) and unknown samples.

2.6.2 UV spectrophotometery

DNA samples were analysed on the Nanodrop® (Labsystems, UK), which measured the absorbance at A260 nm and A280 nm of 1 µl and calculated the DNA concentration accurately, provided it was between 1 and 1000 ng/µl. It also compared the ratio of A260/A280. A value of 1.8 to 2 was indicative of a good quality DNA preparation with little or no protein contamination.

2.6.3 Picogreen/Ribogreen quantification of DNA (for 454™ sequencing)

Double- and single-stranded DNA quantification was carried out for 454™ sequencing using the Qubit™ fluorometer. Picogreen® or Ribogreen® Quant-iT™ intercalater was diluted 1:200 in Quant-iT™ buffer immediately before use to make a working solution. The Qubit was calibrated before use with 10 µl of lambda phage standard provided in the kit diluted with 190 µl of working solution. DNA samples (1-10 µl) were diluted to 200 µl in working solution and incubated at room temperature for 2 minutes. Samples were subsequently analysed on the fluorometer which automatically calculated the concentration.

2.7 PROTEIN PURIFICATION

2.7.1 GST fusion protein purification of recombinant V antigen.

2.7.1.1 Preparation of cell lysate

Recombinant V antigen from *Yersinia pestis* was prepared as a Glutathione S-transferase (GST) fusion protein in *E. coli* by Sarah Hayward (Dstl) using the pGEX recombinant expression system from GE Healthcare (UK), as described in Carr *et al.,* (1999). The pGEX 6-P-1 vector containing the *lcrV* gene from *Yersinia pestis* was grown in *E. coli* BL21. Cultures were grown in 4 litres of L broth supplemented with 100 mg/ml of ampicillin at 37 ^{o}C overnight with shaking at 200 rpm in a Stuart Orbital Incubator S150. Cells were harvested by decanting into 400 ml centrifuge pots (Beckman Coulter, UK) followed by centrifugation at 8000 x *g* for 15 minutes. The pellets were resuspended in 50 ml PBS supplemented with DNase I and RNase A (2 mg of each; Roche, UK). Cell lysis was carried out in aliquots of 15 ml using four bursts of sonication at 20 000 Hz for 30 seconds on ice to prevent excess heat build up. Cell debris was removed by centrifugation at 8000 x *g* for 15 minutes.

2.7.1.2 **Purification using glutathione sepharose 4B**

A pre-packed 5 ml glutathione Sepharose-4B column (GE Healthcare, UK) was prepared on an AKTA™ *prime* automated liquid chromatography system by washing in 20 ml of PBS to remove all traces of ethanol then pre-equilibrated in 6 ml PBS plus 1% Triton X-100 (Sigma, UK). On completion of centrifugation, the supernatant was injected onto the pre-equilibrated Glutathione Sepharose-4B HiTrap™ affinity column through a syringe in the presence of protease inhibitor (GE Healthcare, 10 μl/ml) at a flow rate of 1 ml per minute. Proteins were eluted from the column by the addition of 10 column volumes of elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0) at a rate of 4 ml per minute and 7.5 ml fractions were collected, in accordance with the manufacturer's instructions.

2.7.1.3 **PreScission Protease cleavage of eluted GST-tagged LcrV protein**

To remove low molecular weight contaminants, such as salt, a quick buffer exchange step was carried out. The eluate was loaded onto a 5 ml HiPrep™ 26/10 Desalting column (GE Healthcare), containing Sephadex G25 gel filtration medium. The eluate was added to the column in 15 ml volumes through a syringe. Proteins were then eluted in PreScission cleavage buffer (GE Healthcare; 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), pH 7.5). The GST tag was subsequently cleaved from the LcrV protein with the addition of 80 μl (160 U) PreScission Protease cleavage buffer per ml of eluate followed by incubation for 4 hours at 4 °C. Following digestion, the GST moiety of the tagged protein was bound to a pre-equilibrated GSTrap 4B column (as described in Section 2.7.1.2) during a 30 minute incubation at room temperature. The flow-through containing the purified LcrV protein was collected in 2 ml fractions. Fractions containing the most protein, as determined by spectrophotometry at A_{280}, nm were pooled.

2.7.2 **Acid precipitation of *Clostridium botulinum* neurotoxins**

An overnight culture of *Clostridium botulinum* (50 ml) was prepared as described in Section 2.1.1 and centrifuged at 10 000 x *g* for 30 minutes. The supernatant was transferred to a clean centrifuge tube (Falcon®, Beckton Dickinson, UK) and neurotoxins were precipitated in house by Dr Beverley Roberts using one of two methods, ammonium sulphate precipitation or acid precipitation.

2.7.2.1 **Ammonium sulphate precipitation**

Bacterial culture supernatants were precipitated with the addition of ammonium sulphate to 60 % (w/v) saturation. The suspension was centrifuged at 10 000 x *g* for 20 minutes. The pellet was resuspended in 50 mM Tris buffer, pH 6.5 and transferred into a 10 KDa molecular weight cut off Slide-A-Lyzer (Pierce, Fisher, UK) dialysis cassette using the syringe and needle supplied with the cassette. Air was withdrawn from the membrane using the syringe and dialysis was carried out overnight in 10 litres of 50 mM Tris buffer, pH 6.5 at 4 ^{o}C with constant stirring using a sterile magnetic flea, in order to remove traces of ammonium sulphate.

2.7.2.2 **Acid Precipitation**

The pH of the culture supernatant was subsequently adjusted to 3.5 with 3M sulphuric acid. The acid precipitate was formed during an overnight incubation at 4 ºC. Precipitate was concentrated by centrifugation at 10 000 x *g* for 20 minutes. The majority of the supernatant was removed by carefully decanting, and the precipitate was resuspended in the 2 ml of the residual supernatant.

2.7.3 **Anion exchange chromatography purification of alpha toxin using the AKTA™Prime module**

Recombinant *C. perfringens* alpha toxin was extracted at Dstl by Dr Helen Jones using anion exchange chromatography. Genetically modified *E. coli* JM109 cells containing the cloned *plc* gene in pBluescript were grown as described in Section 2.1.5. The overnight culture was subsequently divided between four 400 ml centrifuge pots with O-ring sealed lids (Beckman Coulter, UK) and centrifuged at 10 000 x *g* for 10 minutes at 4 °C. Cell pellets were resuspended in 50 ml of ice cold 20 mM Tris-HCl pH 8, containing 10 µl/ml protease inhibitor (GE Healthcare, UK). Aliquots of 15 ml were sonicated for four 30 second bursts on ice with a 30 second rest period between each and then centrifuged at 15 000 x *g* for 20 minutes. The ionic strength of the supernatant was adjusted to 20 mM Tris-HCl (from 16 to 4.5) with distilled water and then filtered through a 0.2 µM membrane (Minisart™, Sartorius UK). The clarified extract (150 ml) was loaded onto the Superloop™ (GE Healthcare), which was then connected to the FPLC valve on an AKTA™*Prime* (GE Healthcare, UK). The extract was loaded onto a 5 ml HiTrap™ Q ion exchange column previously equilibrated in 40 ml 40mM Tris-HCl pH 8 (GE Healthcare, UK). The column was then washed in 40 ml 40 mM Tris-HCl pH 8 to remove unbound proteins. Adsorbed toxin was eluted in a continuous ionic strength NaCl gradient ranging from 0 to 100% over 10 column volumes. Fractions containing the most α toxin, as determined by phospholipase activity assay as described in Section 2.7.3.1 were pooled. Samples were then dialysed as described in Section 2.7.2.1 against 4 litres of 20 mM Tris-HCl, pH 8 overnight. Samples were then filtered through a 0.2 µM membrane (Minisart™, Sartorius) and then loaded onto a 10 ml Mono Q HR anion exchange superloop (GE Healthcare, UK). The column was washed in a NaCl

gradient as described above, eluted and concentrated from 40 to 4 ml in a YM 100 Centricon column (Millipore UK) as described below in Section 2.4.2. The sample was filtered again and then a 10 ml Superdex 200 prep HiLoad 16/60 gel filtration column (GE Healthcare, UK) was loaded at a rate of 1 ml per minute. Fractions were eluted from the column with a continuous flow of PBS. The eluates were filtered as described above one final time and then pooled.

2.7.3.1 **Phospholipase activity assay**

Toxin samples (200 µl) were double diluted across a 96 well ELISA plate (Immulon 2, Corning) in DMG buffer pH 7.2 (0.2 M 3,3 dimethylglutaric acid, 5 mM calcium lactate, 0.2 mM zinc sulphate, 1 mg/ml BSA). Egg yolk emulsion (100 µl; Oxoid) diluted 1:10 in PBS was added to each well and incubated at 37 ^{o}C for one hour. The absorbance at 540 nm was then read on a microtitre plate reader (Ascent scan, Thermo Fisher, UK).

2.7.3.2 **FPLC purification of ricin**

Ricin was extracted from castor beans at Dstl by Nicki Walker using the following procedure. Beans (100 g in 200 ml 0.5 M NaCl) were disrupted using a Waring blender using four bursts at low speed for 15 seconds. The homogenate was then acidified to pH 4.0 using glacial acetic acid and stirred overnight at 4 °C. The homogenate was centrifuged at 18 600 x *g* for 30 minutes to remove large plant seed debris and castor oil. The turbid supernatant was then poured into a bottle (Duran, Sigma UK) and clarified using 5 volumes of petroleum ether. The organic and lipid phases were discarded and the aqueous phase was drained and filtered through a Whatman Number One filter paper. The clarified supernatant (30 ml) was subjected to precipitation with 60 % ammonium sulphate (v/v) overnight. Following

centrifugation and dialysis as described in Section 2.7.2.1, the extract was centrifuged at 13 000 x *g* for 30 minutes. The dialysed extract containing the toxin was applied to a 0.2 μM filter (Minisart, Sartorius) through a syringe. It was then loaded via the FPLC superloop onto a sepharose-4B affinity chromatography column, using the same method as described in Section 2.7.1.2. The column was then washed with 25 ml 0.5 M NaCl to remove unbound proteins, and the toxin (and agglutinin) eluted with 25 ml of 0.5 M NaCl with 0.15 M galactose. Fractions containing ricin toxins, as determined by SDS-PAGE (see Section 2.7.5) were pooled and concentrated. The ricin and agglutinin in the concentrated pool were then separated by gel filtration chromatography again using a Sephadex 200 column. The sample was loaded via the superloop onto the column and eluted in 25 ml with a continuous flow of 0.5 M NaCl and 0.2 M galactose. Fractions containing agglutinin were discarded and those containing ricin were pooled.

2.7.4 **Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)**

The inner core of a Mini-PROTEAN 3 Module (Biorad) was filled with 1 X Tris-Glycine SDS running buffer (Biorad; 25mM Tris, 192 mM Glycine, SDS 0.1%, pH 8.3). A pre-made 12% Tris-HCl Ready 15 lane gel was loaded into the tank and then loaded with 5 μl of marker (SeeBlue Plus 2; Invitrogen, UK). Samples were diluted in 2 X Laemmli sample buffer (62.5mM Tris-HCl pH6.8, 2% SDS, 25% glycerol, 0.01% Bromophenol Blue freshly prepared to contain 5 % β-mercaptoethanol; Biorad, UK) and denatured by heating to 95 ^{o}C for 5 minutes. Samples were loaded onto the gel and then the outside core was filled with 1 X running buffer. Gels were run for 30 minutes at 200 volts and then stained with SimplyBlue SafeStain (Invitrogen, UK).

2.7.5 Centricon™ concentration of proteins

Proteins were concentrated using a 50 ml YM-100 Centricon™ column from Millipore (UK). Preservative was removed from the centrifugal cup by washing in 50 ml of water at 5000 x g for 5 minutes. The sample was then loaded onto the cup (50 ml) and centrifuged for 45 minutes at 5000 x *g*. The sample was recovered by inverting the cup into a clean tube and centrifuging at 3000 x *g* for 5 minutes.

2.8 IMMUNO-PCR

2.8.1 Biotinylation of antibodies

Rabbit anti-ovalbumin polyclonal antibodies were obtained from Dr Steven Lonsdale (Dstl). A Biotinylation kit was obtained from Sigma and used in accordance with the manufacturer's instructions. The antibody was dissolved in phosphate buffer to a final concentration of 10 mg/ml. The biotinylation reagent was dissolved in dimethylformamide (DMF) to a concentration of 25 mg/ml and added slowly to the antibody solution while agitating, to a molar excess of 15 times. The solution was then mixed for one hour at room temperature. Following incubation, the biotinylated antibody was purified using a Sephadex G25 column, pre-equilibrated with three times 10 ml volumes of phosphate buffered saline (PBS). The biotinylated antibody was eluted with 13 ml of PBS and 650 μl fractions collected. Fractions containing protein were pooled, as determined by measuring the absorbance at 280 nm on a spectrophotometer (Helios Omega UV/Vis spectrophotometer, Thermo Fisher, UK).

2.8.2 Enzyme-Linked Immunosorbent Assay

Immulon 2 plates (Corning, UK) were coated in 5 μg of capture antibody in 100 μl PBS at 4 °C overnight. Plates were then washed three times in PBS with 0.01 %

Tween (PBS-T) using a plate washer (Titertek, UK) and then blocked in 2 % (w/v) skimmed milk powder (Marvel) solution in 150 µl PBS (colloquially known as Blotto) for 1.5 hours at 37 °C. The plate was washed three times in PBS-T and then incubated with a 150 µl of a dilution series of ovalbumin in 2 % Blotto, ranging in concentration between 1 µg/ml and 11 pg/ml for 1 hour at 37 °C. Plates were washed again and 150 µl of biotin labelled detection antibody, labelled as described in Section 2.8.1, was added at a concentration of 2.5 µg/ml in Blotto, unless detailed otherwise. Plates were incubated for 1 hour at 37 °C and then washed three times in PBS-T. Extravidin-HRP (1:2000 in Blotto) (150 µl; Sigma, UK) was added to each well to a final concentration of 1 µg/ml and incubated in the plate for 1 hour at 37 °C. A stock solution of ABTS chromogenic substrate (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) was prepared with 5 µl of hydrogen peroxide and 2 ABTS tablets (both Sigma UK) in 100 ml of ABTS solution. The plate was washed five times and 100 µl of ABTS was added to each well. Colorimetric changes were read following fifteen minutes incubation at room temperature using an Ascent scan plate reader at 414 nm (Thermo Fisher, UK).

2.8.3 **Direct Antibody Labelling (Alchemy UK)**

A 72 bp amino-modified oligonuceotide (an *Aspergillus flavus ord* PCR product) was synthesised by Thermo Hybaid (UK). Antibody labelling was then carried out under an extra-mural research contract by Alchemy (BBI, Dundee, UK). The 5' terminus was derivatised to incorporate a primary aliphatic amino group (Qiagen UK). The antibody modification was then performed 75 minutes after the oligo modification to minimise the deactiviation of maleamide groups.

2.8.3.1 **Preparation of acetylthioacetyl derivatised DNA (from Hendrickson, 1995)**

Amino modified oligonucleotide (50 to 60 nmol) was added to 667 µl of reaction mixture containing 100 mM sodium bicarbonate buffer (pH 9; Sigma), 13.3 mg/ml N-succinimidyl-S-acetylthioacetate (SATA; Pierce) and 50% (v/v) Dimethylformamide (DMF; Sigma). After 30 minutes at 25 °C, the reaction mixture was immediately applied to a 1 x 20 cm Sephadex® G 25 column (GE Healthcare, UK) and eluted at room temperature with 100 mM sodium phosphate buffer, pH 6.5, at a flow rate of 1 ml/minute. The absorbance at 280/260 nm was monitored and fractions containing the modified oligonucleotides were pooled. Fractions were concentrated using Centricon™ columns (Millipore, UK), centrifuged at 7500 x *g* for 45 minutes at room temperature. The modified oligonucleotides were stored at room temperature in the dark until needed.

2.8.3.2 **Preparation of maleimide-modified antibodies**

The secondary ovalbumin antibodies were derivatized with maleimide groups using sulfo-SMCC. An aliquot containing 25 nmol of antibody was added to a reaction mixture (2.2ml) containing 100mM sodium phosphate buffer (pH7.0), 1.2 mg/ml sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate (sulfo-SMCC, Pierce), 1.5% (v/v) DMF. After the reaction mixture had reacted for 30 minutes at 25 °C, it was immediately applied to a 1 x 20 cm Sephadex® G 25 column and eluted at room temperature with 100 mM sodium phosphate buffer pH 6.5 at a flow rate of 1 ml/min. Fractions containing the modified protein were pooled into one tube.

2.8.3.3 **DNA oligonucleotide-antibody conjugations**

The pooled antibody (4-6 ml) was immediately added to a 15 ml Falcon® tube (Fisher, UK). The concentrated modified oligos were added to the same tube and mixed well. The coupling reaction was initiated by adding 75 µl of 1 M hydroxylamine hydrochloride (Pierce Chemical Co, UK), pH 7, 50 mM EDTA (Sigma) and mixing well. The reaction mixture was then transferred to an Amicon Model 3 mini-ultrafiltration stirred cell fitted with a YM5 membrane filter (Millipore, UK). The cell was connected to a helium source adjusted to 60 psi. The coupling reaction proceeded with stirring at room temperature while the entire vessel was covered with foil to reduce exposure to light. The reaction mixture was concentrated to 1 ml, removed from the MiniCell apparatus and transferred to a 4.0 ml amber vial. This vial was incubated in the dark at room temperature on a tube roller until the reaction time totalled 2 hours. The reaction was terminated by the addition of 10 µl of 10 mM *N*-ethylmaleimide in DMF (Pierce, UK).

2.8.3.4 **Purification of the oligo-antibody conjugates**

The reaction mixture was incubated with 1.5 mg of Protein A beads (Dynabeads, Dynal UK) for 1 hour to remove unconjugated DNA. The antibodies were eluted from the beads by the addition of 20 µl elution buffer (Dynal, UK). Next, ds DNA-antibody conjugates were bound to an ion exchange column (Qiagen tip-20) and the unconjugated antibodies were washed off. The conjugates were eluted from the resin with 50 mM 3-(N-morpholino) propanesulphonic (MOPS, Sigma) acid plus 1.25 M NaCl, pH 8. Conjugate-containing fractions were pooled and dialysed against 100 mM sodium phosphate plus 1 mM EDTA, pH 7 in a Centricon 100 column (Millipore, UK) treated with acetylated BSA (Promega). The final conjugate

solution was stored at 4 °C in a solution of 100 mM sodium phosphate, 1 mM EDTA and 100 mg/l BSA, pH 7.

2.8.4 **Immuno-Phage PCR**

2.8.4.1 **Treatment of *B. cereus* spores**

Bacillus cereus spores were grown as described in Section 2.2.1. Spores (2 ml of 1 x 10^{10} spores/ml) were incubated with 5% chloros solution for one hour at room temperature to remove extracellular DNA. Spores were centrifuged at 5000 x *g* for 10 minutes. The supernatant was removed and the spores were resuspended in 2 ml dH_2O. This process was repeated until three washes had been carried out and the spores were suspended in dH_2O at their original concentration of 1 x 10^{10} spores/ml.

2.8.4.2 **Single chain antibody production**

Single chain antibodies for the detection of *B. cereus* were engineered by Dr Carl Mayers at Dstl, according to the protocol described in Krebber *et al.,* (1997).

2.8.4.3 **Messenger RNA extraction**

Messenger RNA (mRNA) was extracted from the spleens of five mice that had been immunised with *Bacillus* spores using a Qiagen RNeasy mini kit according to the manufacturer's instructions. Spleens were ground in a pestle and mortar once they had rapidly been frozen in liquid nitrogen (BOC gases, UK). The tissue powder and liquid nitrogen were decanted into an RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube (Ambion, Fisher, UK). The liquid nitrogen was evaporated and 600 μl of buffer RLT was added to the tissue whilst still frozen. The lysate was added to a QIAShredder tube and centrifuged at 10 000 x *g* for two minutes. The supernatant was transferred to a clean RNase-free tube and one volume of 70 %

ethanol was added. The sample was then transferred to an RNeasy spin column and centrifuged at 8000 x *g* for 15 seconds. RW1 buffer (700 µl) was added to the spin column which was then centrifuged at 8000 x *g* for 15 seconds. The spin column was washed in 500 µl of buffer RPE at 8000 x *g* for 2 minutes. The tube was then placed in a new collection tube and re-centrifuged at 8000 x *g* for 1 minute to remove residual alcohol. The spin column was transferred to a new collection tube and the RNA was eluted in 50 µl RNase free water by centrifugation at 8000 x *g* for 1 minute. The resulting RNA was used immediately for cDNA synthesis.

2.8.4.4 **cDNA synthesis**

Reverse transcription of RNA was carried out using a SuperScript™ II Reverse Transcriptase kit (Invitrogen, UK). Reaction mixes were prepared to contain 5 µg of total RNA, 10 mM dNTPs and 50 ng of random hexamers in RNase free water. Samples were incubated at 65 ^{o}C for 5 minutes and then snap cooled on ice for 1 minute. Following heat denaturation, reverse transcription reactions were prepared using 10 X RT buffer (2 µl), 4 µl 25mM $MgCl_2$, 0.1 M Dithiothreitol (DTT; 2 µl) and 2 µl of RNase Out™ Ribnuclease inhibitor (Invitrogen). This was then added to 9 µl of RNA/primer mixture and incubated at 25 ^{o}C for 2 minutes. SuperScript™ II Reverse Transcriptase (1 unit; Invitrogen, UK) enzyme was then added to the mixture. Reverse transcription was then carried out at 42 ^{o}C for 50 minutes. The reaction was terminated at 70 ^{o}C for 15 minutes and then chilled on ice. cDNA hybrids were removed using 1 µl of RNase H for 20 minutes at 37 ^{o}C.

2.8.4.5 **Amplification of antibody light and heavy chains (V_L and V_H)**

Heavy and light chain antibody fragments were amplified using the method described Krebber *et al*. 1997. PCR reactions were performed in 50 µl volumes

containing 0.005 units pfx 50 *Taq* polymerase (Invitrogen), 2-5 μl of cDNA, 2 μM of primers (see Table 2.4), 2 mM $MgCl_2$ and 1 x reaction buffer (Invitrogen).

Table 2.4 Primer sequences used for amplification of single chain antibodies (Krebber *et al*., 1997); see Section 2.8.4.6 for amplification conditions.

Primer	Sequence (5' to 3')
VHF	GAGGAGACGGTGACTGAGGT
VHR	GAGGTGAAGGTCATCGAGTC
VLF	ACGTTTGATTTCCAGCTTGG
VLR	ATCCAGCTGACTCAGCC
SCFOR	GGATTCGGCCCCCGAG
SCBACK	TTACTCGCGGCCCAGCCGGCCATGGCGGACTACAAAG

2.8.4.6 **Joining ScFv heavy and light chains by overlap extension PCR**

Primers SCFOR and SCBACK (50 nM each; Table 2.4; Sigma Genosys, UK), as detailed in Krebber (1997), were used in 50 μl reactions with 10 ng of purified heavy and light chain DNA, 1 mM $MgSO_4$, 0.3 nM dNTPs, 1 x *Pfx*50 buffer and 1 unit of Platinum *Pfx* 50 *Taq* polymerase (all reagents from Invitrogen, Fisher, UK). The reactions were thermal cycled for 3 minutes at 94ºC to denature the amplicons, followed by seven cycles of:

15 seconds at 94 ºC, 30 seconds at 63 ºC, 30 seconds at 58 ºC, 60 seconds at 68 ºC.

This was immediately followed by 23 cycles of:

15 seconds at 94 ºC, 30 seconds at 63 ºC and 60 seconds at 68 ºC. Samples of the resulting amplicon were visualised on a 2 % crystal violet stained agarose gel to confirm the product size. Amplicon was ligated into pAK100 (Krebber *et al*. 1997) as described in Section 2.4.3 and transformed as described in Section 2.4.5.

2.8.5 **Panning for single chain antibodies**

2.8.5.1 **Production of phage particles**

Broth (50 ml of 2 X YT) supplemented with 1% (V/V) glucose (Sigma, UK) and 30 μg/ml chloramphenicol (Roche, UK) was inoculated with sufficient volume of transformed ScFV library (prepared as described in Section 2.8.4.3) to give a starting OD_{600} of 0.05, in a 250 ml sterile conical flask with vented cap (Corning, VWR, UK). The flask was incubated with shaking at 200 rpm in a Stuart Orbital Incubator S150 incubator at 37 ºC until an OD_{600} of 0.4-0.5 was reached. A ten-fold excess (10^{11} transforming units) of helper phage (M13KO7; Sigma) was added to the cells and the mixture was incubated for a further 30 minutes. Expression of the single chain fragments, under the control of the *lac* operon, was induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG; Roche, UK) at 30 ºC with shaking (180 rpm) for 16 hours.

2.8.5.2 **Phage purification**

Phage particles were separated from *E. coli* cells by centrifugation at 4000 x *g* for 15 minutes at 4 ºC. The supernatant containing phage particles (40 ml) was added to 10 ml 20 % (v/v) polyethylene glycol (PEG 8000; Sigma, UK) and 2.5 M NaCl in order to facilitate precipitation. Phage particles were collected by centrifugation at 4000 x *g* at 4 ºC for ten minutes and resuspended in 1 ml PBS.

2.8.5.3 **Estimation of phage titre**

A dilution series of phage in 2 X YT broth (225 μl) with 25 μl log phase *E. coli* XL1-Blue was made. Antibiotic resistance was allowed to occur during growth at 37 ºC for 15 minutes. The resulting cultures (100 μl) were grown on 2 X YT plates supplemented with 1 % (v/v) glucose and 30 μg/ml chloramphenicol at 37 °C for 16

hours and the phage concentration was estimated by plaque counts, as indicated by clear regions on the plate.

2.8.5.4 **Panning**

Immunotubes (4 ml; Nunc, Fisher UK) were coated in 2 ml *Bacillus* spores at a concentration of 1 x 10^7/ml overnight at 4 °C. The tubes were then washed three times in 4 ml of PBST, then blocked in 4 ml 1% blotto at room temperature for 2 hours. During this time, pre-blocked phage was produced by incubating 100 μl of phage particles (prepared as described in Section 2.8.7.2) with 900 μl of blotto. The spore coated tubes were then washed three times in 4 ml of blotto to remove excess blocking agent. Binding of phage particles (1 ml) was then carried out at room temperature for one hour with mixing on a Dynal windmill. The tubes were subsequently washed with 5 to 20 washes (depending on the stringency of the panning round) of PBS, 0.1% (v/v) Tween 20. A further 5 washes were carried out using PBS without Tween 20, to further increase the panning stringency.

2.8.5.5 **Phage recovery**

The antibody-spore bond was broken using 1 ml of freshly made 100 mM triethylamine during an 8 minute incubation period at room temperature. The contents of the immunotube were rapidly transferred to a tube containing 500 μl 1M Tris HCl pH 7.5 to neutralise the solution. The phage with an affinity for *B. cereus* spores were then incubated with 10 ml log-phase *E. coli* XL-1 Blue statically for 30 minutes, followed by shaking 30 minutes at 37 ºC. Infected *E. coli* cells were then spread and grown on 24 cm 2 X YT plates supplemented with 1 % (v/v) glucose and 30 μg/ml chloramphenicol at 37 °C for 16 hours. Phage titre was estimated by plaque counts as described in Section 2.8.5.3. The panning protocol was repeated

until phage particles showed sufficient affinity for *B. cereus* spores, as demonstrated by ELISA, at panning rounds three onwards.

2.8.6 **I-ϕ-PCR**

Bacillus cereus spores (50 µl of a suspension containing 10^7 to 10^0 spores/ml) were dried onto an Immulon 2 ELISA plate at 37 °C overnight. The plate was then washed three times in PBS-T in a Titertek plate washer and blocked in 2% Blotto for 1.5 hours at 37 °C. The plate was washed a further three times in PBS-T and 50 µl of phage suspension diluted in Blotto were added per well. Following incubation at 37 °C for one hour, the plate was washed three times in PBS-T then three times in dH_2O to remove unbound phage.

2.9 **SYSTEMATIC EVOLUTION OF LIGANDS EXPONENTIALLY (SELEX)**

2.9.1 **Aptamer Library**

SELEX DNA templates were composed of a single stranded template obtained from Cruachem UK, with randomised bases (N) in the midsection, flanked by a 5' and 3' primer site as follows: CCGGATCCGTTGATATATAAAATTC $(N)_{50}$ TTGAATGTGGTGTTGGCTCCC. AP F and R PCR primers (Table 4.4) were obtained from Qiagen UK. PCR was carried out to form a double stranded SELEX library.

A 25 µl or 100 µl PCR master mixture consisting of 1ng of DNA, 1 µM each primer, 0.2mM each dNTP, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl and 50 U *Taq* polymerase was used. PCR (100 µl) was carried out on an ABI 9700 thermal cycler

with 40 cycles of 95 °C for 1 minute, 55 °C for 1 minute and 72 °C for 1 minute. 25 µl reactions additionally contained Sybr Green ® I at a final concentration of 2 X and were carried out on the Roche Lightcycler.

2.9.2 **Coating tosyl activated Dynabeads with ovalbumin (Bruno and Kiel 1999)**

Ovalbumin was captured by Tosyl activated Dynabeads (Dynal, UK) by incubating 1 ml of 0.1 mg/ml ovalbumin in PBS with 1 ml bead solution (1.3 x 10^8/ml Tosyl activated beads) for 16 hours at 37 °C in a Thermomixer (Eppendorf). The beads were collected with a Dynal magnetic particle collector (MPC), then washed in three times in 1 x coating buffer (0.5M NaCl, 10 mM Tris HCl pH 7.5, 1 mM MgCl and 0.1 % Triton ® X-100).

2.9.3 **Elimination of aptamers with affinity to Tosyl activated Dynabeads**

A negative pre-selection round was performed by incubating 100 µl of the beads prepared in Section 2.9.2 with 5 µg of DNA aptamer pool in 1 x binding buffer (20 mM Tris (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$) for 1 hour on a rolling platform (Spiramixer, VWR). The beads were isolated using an MPC and unbound species were collected, PCR amplified as described in Section 2.9.1 and used in the rest of the SELEX experiment.

2.9.4 **SELEX Procedure**

In the first round of selection 5 µg of the aptamer pool was added to 40 µl of ovalbumin bound Dynabeads in 1 X binding buffer. The aptamers were allowed to bind to the beads during a 1 hour incubation at room temperature on a rolling

platform. Beads were isolated using a magnetic rack then washed three times in 1 ml of 2 X binding buffer. Beads were resuspended in 100 ml of 1 X binding buffer and then heated in a boiling waterbath for 30 seconds. The beads were isolated rapidly using the MPC and the supernatant was transferred immediately to a clean 1.5 ml tube. The isolated DNA was then amplified by PCR as described in Section 2.9.1 and then used directly as the DNA pool for SELEX round 2. Subsequent rounds of SELEX were carried out using the same procedure, as discussed in Chapter 4.

2.9.5 **Enzyme-linked oligonucleotide assay (ELONA)**

An ELISA type assay was performed, similar to that reported by Drolet *et al*. (1998). Anti ovalbumin aptamers (100 µl) were diluted 1 in 500 in Reacti-Bind® (Pierce, UK) and added a 96 well Immulon 2 plate. DNA was allowed to bind to the plate overnight. DNA was then made single stranded by placing the plate in a boiling waterbath for 30 seconds. Free DNA was decanted and the plate was then manually washed three times in 300 µl 1 x binding buffer. The plate was then coated in a blocking solution (2 % skimmed milk powder, 10 ng salmon testes DNA) at 37 °C for 1 hour. The plate was washed three times in 1 x binding buffer. Ovalbumin was added to the plate at concentrations ranging from 100ng to 0.1 fg/ml and was incubated at 37 °C for 1 hour. The plate was then washed and 100 µl of anti ovalbumin biotinylated antibody was added to the plate at a concentration of 2.5 µg/ml. Following washing, streptavidin peroxidase (1 µg/ml) was added to each well. Colour was developed by the addition of 100 µl 2,2'-azino-di(3-ethyl-benzthiazoline-6-sulphonic acid) (ABTS) plus 50 µl H_2O_2. Colourimetric changes were measured on a plate reader at 440 nm.

2.9.6 **SELEX using biotin labelled aptamers (Bruno and Keil 2002)**

Tosyl activated beads were used as described in Section 2.9.2 to produce ovalbumin coated magnetic particles. SELEX library (23 μg) was amplified by PCR as described in Section 2.9.1 using biotin labelled APF and APR primers as described in Chapter 4. A negative pre-selection was carried out to eliminate bioinylated aptamers that bind to Tosyl activated magnetic beads as described in Section 2.9.2. The unbound species were then heated to 96 °C for 5 minutes and then added to 200 μl of ovalbumin coated beads (12.6×10^7 beads coated with 20 ng ovalbumin). The beads and aptamer pool were incubated together for 1 hour at room temperature on a rolling platform, and then washed three times in 1 ml of binding buffer. The aptamer bound beads were then added directly to a 100 μl PCR reaction containing quadrupled quantities of reagents detailed in Section 2.9.1. The supernatant containing amplified bioinylated aptamers was recovered by magnetic bead separation in a Dynal MPC. A further 100 μl of dH_2O was added to the beads which were then heated to 96 °C in a Perkin Elmer 9700 machine for 5 minutes to release any remaining DNA bound to the beads. Both aptamer solutions were combined with 100 μl 2x binding buffer, heated to 96 °C as before to denature the DNA and then added to 200 μl of fresh ovalbumin coated beads. This process was repeated until 5 rounds of SELEX had been carried out.

2.10 DIFFERENTIATION OF *RICINUS COMMUNIS* STRAINS USING DNA ANALYSIS

2.10.1 Random Amplified Polymorphic DNA (RAPD) analysis

RAPD primer sequences were obtained from Eurofins MWG (Germany; Table 2.5). RAPD PCR was carried out on a Perkin Elmer 9700 with 200 μM dNTP (Roche, UK), 10 pmol each primer; approximately 5ng template DNA, 1x Roche PCR buffer,

2.5 U *Taq* polymerase (Roche, UK) and 2.5 mM Mg^{2+}, using the thermal cycling parameters outlined in Table 2.6).

Table 2.5 DNA sequences of the six RAPD primers used.

Oligo name	DNA sequence Sequence (5' – 3')
OPAA1	AGACGGCTCC
OPAB5	CCCGAAGCGA
OPT05	GGGTTTGGCA
OPAB18	CTGGCGTGTC
OPAA10	TGGTCGGGTG
OPC20	ACTTCGCCAC

Table 2.6 Thermal cycling profile for RAPD reactions.

Temperature	Duration	Number of cycles
94 ^{o}C	2 min	1 cycle
94 ^{o}C	30s	45 cycles
42 ^{o}C	45s	
72 ^{o}C	30s	
72 ^{o}C	7 min	1 cycle

2.10.2 **Restriction Fragment Length Polymorphism (RFLP) analysis**

Amplicon was restriction enzyme digested as described in Section 2.4.2. The enzymes shown in Table 2.7 were used.

Table 2.7 Restriction enzymes and their conditions of use.

Enzyme	Supplier	Buffer	Incubation temperature
*Hae*III	Sigma (U.K.)	SM buffer	37 °C
*Taq*I	Sigma (U.K.)	SB buffer	37 °C
*Hae*III *Taq*I		SB Buffer	37 °C
*Hind*III	Sigma (U.K.)	SB buffer	37 °C
*Eco*RI	Sigma (U.K.)	SH buffer	37 °C
*Hind*III *Eco*RI		SB buffer	37 °C
*Bst*NI	New England Biolabs (U.S.A.)	Buffer 2	60 °C

2.11 454™ SEQUENCING

454 sequencing was carried out according to the manufacturers instructions issued in October 2008, with updates provided in August 2009 (Issue number April 2009), December 2009 (issue October 2009) and January 2010 (October 2009 Jan 2010 revision). All components were obtained from Roche unless stated otherwise.

2.11.1 Shotgun library preparation

2.11.1.1 Nebulisation

Library preparation was carried out using 5 µg of DNA from *R. communis* Jianxiang 1 or Zibo 1. Whole genomic DNA was prepared using PowerPlant® extraction as described in Section 2.2.3.2. DNA was quantified as described in Section 2.6.3. Nebulisation was then carried out to shear the DNA fragments using nitrogen gas at 30 psi for one minute in 500 µl nebulisation buffer (Roche). The sheared DNA was subsequently purified using a MinElute PCR purification kit (Qiagen, UK).

2.11.1.2 MinElute purification

Buffer QBI (2.5 ml) was added to the nebuliser and the contents were mixed. The DNA was bound to a MinElute column in 750 µl aliquots by centrifugation at 10 000 x *g* for 1 minute. The flow though was discarded and the column was re-centrifuged as before. DNA was then eluted from the column in 10 µl of buffer EB and collected following centrifugation at 10 000 x *g* for 1 minute.

2.11.1.3 AMPure bead removal of small DNA fragments

The volume of eluate was measured with a pipette. The volume was then made to 100 µl with buffer EB (Qiagen). AMPure size exclusion beads (125 µl) were added to the DNA. DNA was bound to the beads during a 5 minute incubation at room temperature. The beads were washed twice in 500 µl of ethanol. DNA was eluted from the beads in 24 µl buffer EB.

2.11.1.4 DNA quality analysis

The quality of the resulting DNA (1 µl) was assessed on the Agilent Bioanalyser using an HS DNA chip (Agilent, UK). The gel dye mix was prepared by adding 15 µl of high sensitivity dye to a vial of gel matrix. Gel dye matrix (9 µl) was added to the chip in well G. The chip was primed in the priming station by releasing the syringe. Marker (5 µl) was added to each well and 5 µl of sample or water was added to each unmarked lane. DNA ladder (1 µl) was added to the yellow well and the chip was vortexed using an adapter at 2400 rpm for 1 minute (Agilent, UK). The chip was then loaded into the Bioanalyser and run for 30 minutes.

2.11.1.5 Fragment end polishing

Sticky ends were removed from the nebulized, size selected DNA (23 µl in total), in a reaction containing 5 µl 10 × polishing buffer, 5 µl BSA, 5 µl ATP, 2 µl dNTP Mix, 5 µl T4 polynucleotide kinase, 5 µl T4 DNA polymerase in a total volume of 50 µl. The reaction was carried out at 12 ^{o}C for 10 minutes in a 9700 PCR machine, followed by 25 ^{o}C for 15 minutes. Polished DNA was then purified using a MinElute kit (as in Section 2.11.1.2).

2.11.1.6 Sequence capture adapters

Sequence capture adapters were then ligated to the polished DNA (10 µl) in a reaction containing 20 µl 2 × Ligase Buffer, 5 µl Adaptors, and 5 µl Ligase at 25 ^{o}C for 15 minutes in a 9700 PCR machine. DNA was purified using a MinElute kit as in Section 2.11.1.2, however 200 µl QBI was used and DNA was eluted in 50 µl EB. Small DNA fragments were removed using the AMPure purification described in Section 2.11.1.3.

2.11.1.7 Library immobilisation (Roche)

The DNA library (25 µl) was then captured onto 50 µl immobilisation beads (prepared by washing twice in 2 x library binding buffer and eluted in 25 µl) at room temperature on a rolling platform (Spiramixer; Jencons, UK) for 20 minutes. The beads were washed twice in 100 µl 2 x library wash buffer and eluted in 50 µl of fill in reaction, prepared as described below.

2.11.1.8 Fill in reaction (Roche)

A fill in reaction was prepared to fill in gaps in the immobilised DNA using 40 µl dH_2O, 5 µl 10× fill-in polymerase buffer, 2 µl dNTP Mix and 3 µl fill-in polymerase.

The reaction mix was added to the beads prepared in Section 2.12.1.7 and incubated at 37 ^{o}C for 20 minutes. The beads were were washed twice in 100 µl 2 x library wash buffer and eluted in 50 µl of melt solution (0.1 N NaOH, Sigma, UK) prepared as described in Section 2.11.1.9. A MinElute column was used to purify the resulting ssDNA as described in Section 2.11.12. Quality of the ssDNA was assessed using an RNA Pico 6000 chip on the Bioanalyser (as Section 2.11.1.4). DNA was quantified using Ribogreen (Invitrogen). The number of molecules per bead was calculated (molecules/µl = (Sample concentration; ng/µl) × (6.022 × 10^{23}) / (328.3 × 10^{9}) × (average fragment length; nt). Library DNA at a concentration of 1x10^{8} molecules/bead was then stored in frozen aliquots until needed.

2.11.2 **Emulsion PCR**

2.11.2.1 **Preparation of emulsion reactions**

Roche emulsion oil was shaken in a Qiagen TissueLyser at 28 Hz for 2 minutes. Mock amplification mix (5ml) was then added to the emulsion oil which was shaken at 28 Hz for 5 minutes. Live amplification mix was made with 2400 µl dH_2O, 3000 µl emPCR additive, 5 x amplification mix, 560 µl amplification primer, 400 µl enzyme mix and 10 µl PPiase (Roche). DNA capture beads were washed twice in 1 ml 1 x capture wash buffer (Roche), using centrifugation at 10 000 x g for 10 seconds to collect the beads each time. DNA (at 10^{7} molecules/bead), were vortexed and then added to the live amplification mix tubes. The live PCR mix was added to the emulsion oil prepared above and was then shaken at 12 Hz for 5 minutes. The reaction mixes (100 µl) were dispensed into four 96 well plates using a 5 ml digital Eppendorf multistepper pipette. The plates were sealed with Epppendorf caps and were cycled on a Perkin Elmer 9700 machine for 4 minutes at 94°C followed by 50

cycles of 30 seconds at 94°C, 4.5 minutes at 58°C, 30 seconds at 68°C and then held at 10°C for ten hours.

2.11.2.2 **emPCR recovery**

The caps were carefully removed from the PCR plates and emulsions were aspirated and collected into two 50 ml Falcon® tubes (Fisher, UK) using the LV vacuum assisted breaking equipment supplied by Roche. The plates were washed twice in 100 µl isopropanol (VWR, UK) and collected using the transpipette. The transpipette was rinsed in a further 5 ml of isopropanol. The volume of the tubes was then supplemented with isopropanol to total 40 ml and centrifuged at 930 x *g* for 5 minutes. The supernatant was removed and the pellet was resuspended in 35 ml isopropanol. Following centrifugation as above the pellets were washed in 35 ml ethanol (96 %; VWR, UK). The beads were then washed in 35 ml enhancing fluid (Roche, UK). The majority of the supernatant was discarded and the pellets were resuspended in 2ml of residual supernatant. The concentrated bead solutions were transferred to 1.7 ml eppendorfs using centrifugation at 10 000 x g for 30 seconds to reduce the volume. The 50 ml tubes were washed in 600 µl enhancing fluid to collect any remaining beads which were then transferred to the 1.7 ml tubes. The tubes were centrifuged for 30 seconds as before and the supernatant discarded. The beads were washed three times in 1 ml enhancing fluid (Roche, UK) and then resuspended in 1 ml of 0.1 N NaOH. The beads were incubated at room temperature for 2 minutes. The tubes were centrifuged and a futher 1 ml of NaOH was added. The beads were washed twice in 1 ml of annealing fluid and resuspended in 45 µl annealing buffer and 25 µl enrichment primer (Roche, UK). The tubes were incubated at 65 ^{o}C for 5 minutes and then snap cooled on ice for 2 minutes. The

beads were resuspended in 800 µl enhancing fluid, centrifuged and washed twice more in a further 1000 and then finally resuspended in 800 µl.

The magnetic enrichment beads were prepared by washing three times in 1 ml enhancing fluid, using a magnetic particle collector (Dynal, UK) to concentrate the beads each time. The beads were resuspended in 320 µl enhancing fluid. The beads were then divided between the four dolphin nosed tubes and incubated on a Labquake roller for 5 minutes at room temperature. The beads were then washed at least ten times in 1 ml enhancing fluid, or until no beads were visible in the discarded supernatant. Clonally amplified DNA-bead complexes were eluted from the magnetic beads twice in 1 ml 1N NaOH. The DNA-beads were collected by centrifugation and washed three times in 1 ml annealing fluid and resuspended in 200 µl. The sequencing primer (50 µl; Roche, UK) was added to each tube which were then incubated at 65 ^{o}C for 5 minutes and then cooled on ice. The beads were resuspended in 800 µl of annealing buffer and washed twice in 1 ml. The beads were finally resuspended in 1 ml and stored at 4 ^{o}C until sequenced (within two weeks).

2.11.3 **Sequencing and data analysis (Roche)**

The reagents were taken from the foil packaging and defrosted in the refrigerator overnight. Bead buffer 2 (BB2) was made by adding 34 µl apyrase and 1.2 ml of titanium supplement CB to a 200 ml bottle of titanium bead buffer. The PicoTiterPlate™ (PTP) was submerged in BB2 for 10 minutes. The packing beads were washed three times in 1 ml BB2 by centrifuging at 10 000 x g for 5 minutes. The packing beads were resuspended in 550 µl BB2. The entire contents of both DNA (sample) beads were vortexed, centrifuged to remove supernatant and transferred to a new 1.7 ml tube. The control beads (10 µl) were added to each tube

and the volume in the tube adjusted to 30 µl by removing supernatant. DNA bead incubation mix (DBIM) was prepared by adding 150 µl DNA polymerase cofactor to 1570 µl BB2 and 300 µl DNA polymerase. DBIM (320 µl) was added to each tube of DNA beads and these were incubated on the Spiramixer (Jencons, UK) for 15 minutes. The enzyme and PPiase beads were washed three times in 1 ml BB2, resuspended in 1ml or 500 µl BB2 respectively then diluted as shown in Table 2.8.

Table 2.8: Dilution of the Enzyme and PPiase Beads for bead layers 1, 3, and 4.

Bead Layer	BB2 (µl)	Enzyme Beads (µl)	PPiase Beads (µl)	Total Volume (µl)
1	3250	550		3800
2	2500	1300		3800
3	3340		460	3800

The DNA beads (350 µl) were combined with 100 µl packing beads and 210 µl BB2 to form the final bead layer (layer 2). The bead layers were deposited into the PTP in turn by pipetting 660 µl into the bead deposition device and centrifuging at 1620 x *g* for 5 minutes. The enzyme beads prelayer was loaded first, followed by the DNA and packing beads, then the enzyme beads post layer, followed by the PPiase layer. The sequencing reagents tray was then prepared by adding 6.6 ml CB supplement and 1 ml Dithiothreitol (DTT) to each bottle of Titanium buffer CB. PPiase (5 µl) was diluted in 45 µl Inhibitor TW. A sodium chlorite tablet was added to tube 1, 13.5 µl diluted PPiase was added to tube 9, 260 µl apyrase was added to tube 11 and 3 ml of ATP was added to tube 10. The tube tray was inverted 20 times to mix the contents and was then transferred to the sequencer. The Genome Sequencer FLX instrument monitored each well simultaneously overnight. Data analysis was subsequently carried out on an 8 node IT cluster. The Roche GS reference mapper software was used to identify high confidence differences between the reference Hale strain and either Jianxiang or Zibo 1.

3 PCR DETECTION OF PROTEIN TOXINS

3.1 INTRODUCTION

3.1.1 PCR as a tool for toxin detection

PCR is an exquisitely sensitive DNA analysis technique, used frequently to identify genes for many purposes including genetic fingerprinting (Jeffreys *et al*., 1991), diagnosis of infectious disease (Arthur *et al*., 1989), phylogenetics (Littlewood, 1994), diagnosis of hereditary disease (Wong, 1987) and cloning. However analysis of proteins lacks an analogous technique. Instead, proteins are detected using antibody based methods, most commonly ELISA and its derivatives. Detection of proteins using PCR is not intuitive; however it may be possible for the presence or absence of a protein toxin to be confirmed by PCR amplification of residual or contaminating DNA in a sample. PCR is commonly used successfully to infer the presence of toxin in food samples (for example De Medici *et al.,* 2009; Rall *et al.,* 2008) as it is rapid, sensitive and is able to detect non-viable organisms (Masters *et al.,* 1994). However, it is unknown whether PCR is capable of detecting toxins that have been purified, as may be the case with a toxin used as a biological weapon.

3.1.2 Toxin purification

Protein purification methods vary greatly depending on the properties of the protein and its intended end use. The simplest form of purification is to precipitate the protein by altering the solvent so that the protein becomes insoluble, often by adding acid or alcohol. Other methods of purification may be more labour intensive and require more equipment and expertise. Affinity chromatography simplifies a purification strategy to an extent as the properties of the protein need not be

determined in fine detail (Axen, 1967), however the protein is often cloned into a plasmid vector bearing a tag and then transformed into an organism such as *E. coli* or *Pichia pastoris* (Skopes, 1993). The recombinant protein is subsequently expressed and purified using a column with affinity for the cloned tag, for example a $(histidine)_6$ tag binding to a nickel column. Ion exchange requires numerous stages for capture, intermediate purification and polishing (CIPP) and therefore must be optimised using a protocol adapted to the properties of the protein (Skopes, 1993).

3.1.3 **Aims**

The aims of this section were firstly to develop real time quantitative PCR assays for detection of the toxin genes shown in Table 3.1 and secondly, to use the resulting PCR assays to detect toxin samples taken during the purification. Several toxins were purified at Dstl for this research using protocols that have been previously optimised by the protein biochemistry team, as summarised in Table 3.1.

Table 3.1 Toxins and purification strategies used in this study.

Organism	Toxin/protein	Purification method
Clostridium botulinum	Botulinum neurotoxin	Acid precipitation
Yersinia pestis	V antigen	Affinity chromatography (Carr *et al*. 1999) (GST fusion protein)
Ricinus communis	Ricin	Affinity chromatography (sepharose)
Clostridium perfringens	Phospholipase C	Ion exchange (CIPP)

3.2 ASSAY DESIGN AND OPTIMISATION

3.2.1 Design

The DNA sequences of each toxin gene were obtained from the Genbank database. Sequences were also obtained from homologues where available. The genes were aligned using SeqMan software (DNAstar) and PCR primers were designed in regions of DNA sequence identity. The details, including accession numbers, are summarised in Table 3.2.

3.2.2 PCR thermal cycler settings

Each toxin assay was designed using Primer Express software (Applied Biosystems) using the default thermal cycling parameters. This would allow a number of different assays to be run simultaneously on the same machine. PCRs were optimised using a 2 step reaction, with a denaturation time of less than one second at 95 ^{o}C and a combined annealing and extension phase at 60 ^{o}C for 30 seconds. However a three step PCR was more suited to a number of assays as discussed further in the following sections and summarised in Table 3.12.

3.2.3 Reagents

PCR was carried out with the minimum concentration of reagents required for amplification to increase the specificity of the PCR (as detailed in Table 3.12). Primers were used at a concentration of 500 nM to limit the formation of primer dimer. Assays were initially tested using Sybr® Green I, a double stranded DNA intercalator, to report PCR amplification on the Roche Lightcycler™. Once a primer set was demonstrated to be suitable in terms of specificity and sensitivity, the assay was converted to a TaqMan® assay. This was performed by replacing Sybr® Green I with a specific probe labelled with Fluorescein (FAM) at the five prime end and

either Tetramethylrhodamine (TAMRA) or Black Hole Quencher at the three prime end. The concentration of TaqMan® probe was commonly optimised at 0.3 or 0.4 μM. The final magnesium concentration was also optimised for each assay with 3 or 4 mM being used for the majority. The results are discussed in detail in the following sections and are summarised in Table 3.12.

Table 3.2. Accession numbers of the target genes used in this study.

Gene	Locus Name	GenBank Accession #	Gene	Locus name	Accession #
bont/a (C. baratii)	CB43256	Y12091	*bont/f; ntnh/f (nonprot)*	CLOBONT	L35496
bont/a	CBANB	X92973	*bont/b*	CLOBOTB	M81186
bont/b	CBB2129	X70814	*bont/a (62A)*	CLONEUR	M30196
bont/b	CBBEKLUND	X70819	*bont/f (202F)*	CLONEUTOXF	M92906
C.butyricum bont/e	CBBNTTE	X62088	*bont/c and d*	CLOTNCO	D38442
bont/b	CBBONTB	X71343	*bont/c*	CSTC1TOX	D90210
bont/f	CBBONTFG	X81714	*bont/f (202F)*	S73676	S73676
bont/g	CBBONTG	X74162	*bont/f; ntnh/f (nonprot)*	CLOBONT	L35496
bont/a	CBBOTAG	X52066	*bont/b*	CLOBOTB	M81186
bont/b	CBBOTNTBG	Z11934	*bont/e (C. butyricum)*	CBTOXE	X53180
bont/b	CBBPROT	X70817	*ntnh/b*	CBU63808	U63808
bont/c	CBCBONT	X72793	*bont/b ntnh*	CBY13630	Y13630
bont/c	CBCNIS	X68204	*bont/f*	CBY13631	Y13631
bont/c	CBCPHGC1	X53751	*bont/a*	CBY14238	Y14238
bont/c	CBCTLE	X68205	*bont/b*	CBY14239	Y14239
bont/c	CBCTOX	X62389	*bont/c*	CLOBN	D49440
	CBDNABNG	X87974	*plc*	CLOCPA	L43545
	CBDNAPTCG	X87972		CLOCPAA	L43546
bont/d	CBDTLE	X68206		CLOCPAB	L43547

bont/e	CBEDOLMAN	X70818		CLOCPAC	L43548
bont/e	CBEHAZEN	X70815		CLOOLC1	D32123
bont/f	CBFCRAIG	X70816		CLOPLC	M24904
bont/f	CBFHOBBS	X70820		CLOPLC05	D32127
bont/f	CBFLANGE	X70821		CLOPLC2	D32124
bont/b	CBHA3317	X79103	*novyi*	CLOPLC3	D32125
bont/a (7272)	CBHA3372	X79104	*subtype e*	CLOPLC5	D32126
bont/a	CBHA3373	X79102		CLOPLC6	D32128
bont/e	CBNEUTOXE	X62089		CLOPLC7	D49968
ntnh/f	CBNTNH	X99064		CLOPLC8	D49969
ntnh/f	CBNTNHF	X71086		CLOPLCA	D10248
ntnh/f	CBNTNHGEN	X87850		CPA	X13608
bont/a ntnh/a	CBNTNHNTA	X87848		CPPLCG	X17300
bont/b ntnh/b	CBNTNHNTB	X87849			D63911
bont/a	CBNTOXA	X73423			AF204209
bont/d	CBNTTD	X54254	*C. biferment ans plc*		AF072123
bont/e	CBNTTE	X62683			
ntnh	CBP47NTNH	Y10770			
seg		AB016487			
seg		AF053140			
seg		AF064773			
sei		AF064774			
sed	M94872	IB4ENTTOXD			
sec	SAENTC1	X05815			
sec	SAENTC3A	X51661			
seh	SAU11702	U11702			
sea	STATOXAA	M18970			
sec	SAENTC1	X05815			
sec	SAENTC3A	X51661			
see	STAENTE	M21319			
seg	AB016487	AB01648			

		7
seh		AB060537
seg		AB060535
S. intermedius C entrotoxin		SIU91526
sea	STAENTAA	L22565
sea	STAENTAB	L22566
seb	STAENTB	M11118
sed	STAENTD	M28521
sea	STAENTE	M21319
sec	STAENTEROA	L13374
sec	STAENTEROB	L13375
sec	STAENTEROC	L13376
sec	STAENTEROD	L13377
sec	STAENTEROE	L13378
sec	STAENTEROF	L13379
sec3	STAENTTXC	M28364
seb	STASEB	M33833
sea	STATOXAA	M18970
abrin	APRICA72	X54872
abrin a chain	APRICA73	X54873
ricin E A chain		E01355
ricin D		E01356
agglutinin	RCCAGG	M12089
ricin E beta chain	RCCRICA	M17631
ricin cDNA	RCPPRICR	X02388
ricin	RCRICIN2	X52908
ricin	RCRICIN	X03179
ricin		S40366
agglutinin		S40368
Aflatoxin ord	AFU81807	U81807

3.3 *CLOSTRIDIUM BOTULINUM* NEUROTOXIN DETECTION

3.3.1 Generic detection

There are seven antigenically distinct botulinum neurotoxins, serotypes A to G. Thirty six *C. botulinum* sequences were obtained from Genbank and imported to SeqMan (Table 3.2). As expected the homology between serotypes was calculated by the software to be as little as 40 %. SeqMan was unable to align sequences with such low homology, so an alignment was performed in MegAlign using the Clustal V algorithm (Higgins and Sharp 1989), which examines sequence distances between all the pairs. The consensus sequence produced by the software was not representative of all seven strains. Several regions appeared to share enough sequence homology to permit binding of generic primers; however none was identified by Primer Express as suitable. This was attributed to the low GC content of the *C. botulinum* genome resulting in the software discarding potential primers with a low melting temperature. Additionally, distance between the putative primer sites was too great to facilitate rapid real time PCR. Therefore it was not possible to design a generic *bont* PCR assay that was suitable for adding to the suite of real time gene probe assays available to Dstl. The *ntnh* gene was investigated as an alternative target.

3.3.1.1 *ntnh* PCR assay

The *ntnh* gene, also part of the neurotoxin gene complex, is more conserved between strains than the *bont* gene. All available sequences were entered into an alignment but distinct groups were formed. One alignment comprised the A and B genes, one of type F, one D and one type G (Figure 3.1). The contigs were forced to align by increasing the number of mismatches allowed and removing some of the longer sequences, resulting in a consensus sequence representative of *ntnh/A, ntnh/B, ntnh/F*

and *ntnh/G.* This permitted the design of a primer and probe set in Primer Express that was predicted to amplify a region of the consensus sequence.

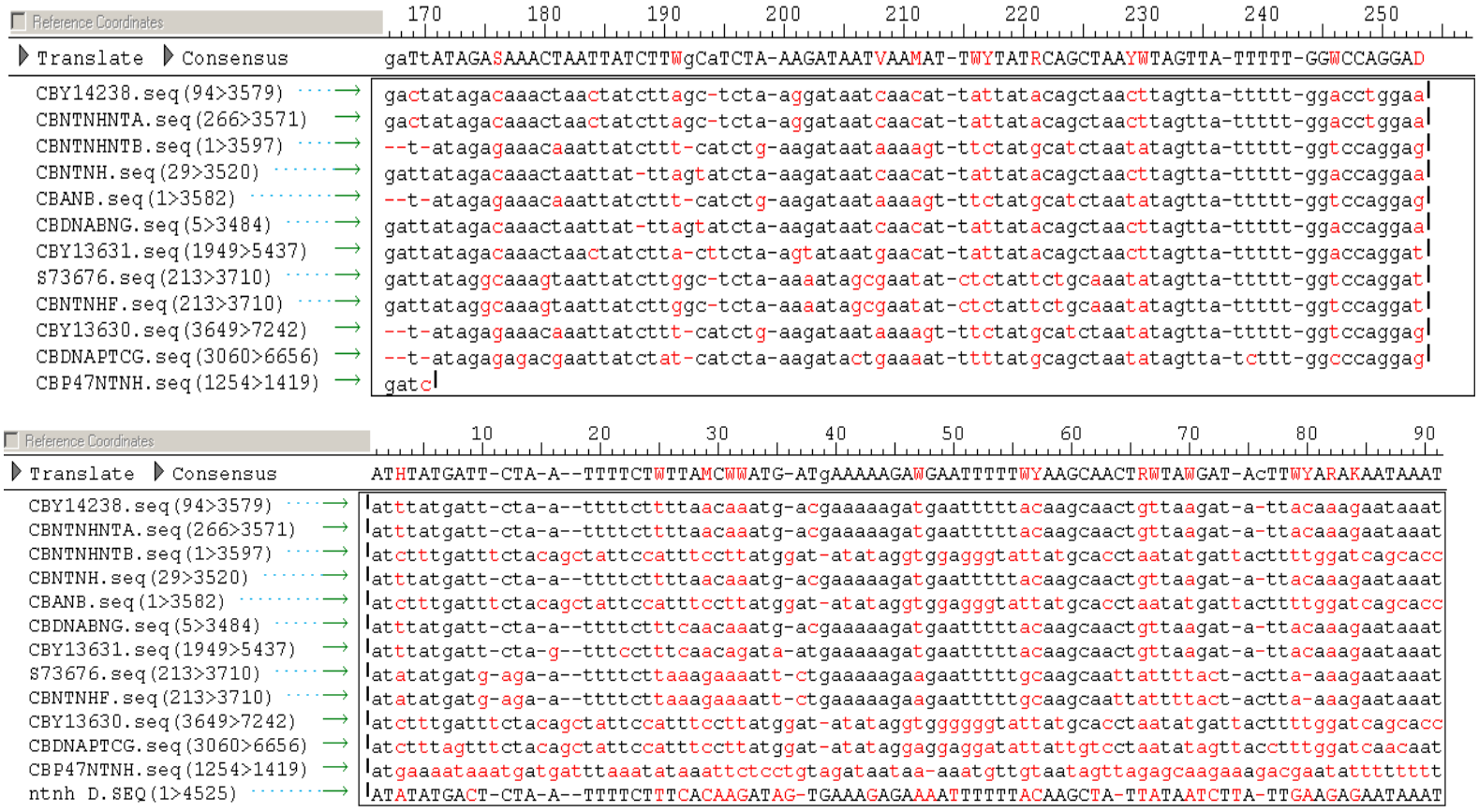


Figure 3.1 Alignment data used to design PCR primers for the detection of *Clostridium botulinum ntnh* gene. DNA sequences from the *ntnh* gene of various strains were obtained from Genbank and aligned in SeqMan (DNAstar, Lasergene 6). The resulting consensus sequence was exported into Primer Express v 1.0 (Applied Biosystems) for primer design. The *ntnh* amplicon designed for this study is shown. The *ntnh* F primer used was CTCCTGGACCAAAAATAAC (position 253-232 of the top alignment). The reverse primer sequence was ATCTTTCATTCTACAGCTATTC (position 1-24).

Several DNA samples were tested with the *ntnh* assay, as shown in Figures 3.2 and 3.3. Melt curve analysis of the amplicons highlighted in Figure 3.2 showed that ampification had failed, as there was no difference in melting temperature between the samples and the no template control (NTC). In contrast, the data presented in Figure 3.2 demonstrates that the assay was suitable for detection of the *ntnh* gene in certain samples, as products with a different melting temperature from the NTC were obtained. These results are summarised in Table 3.3.

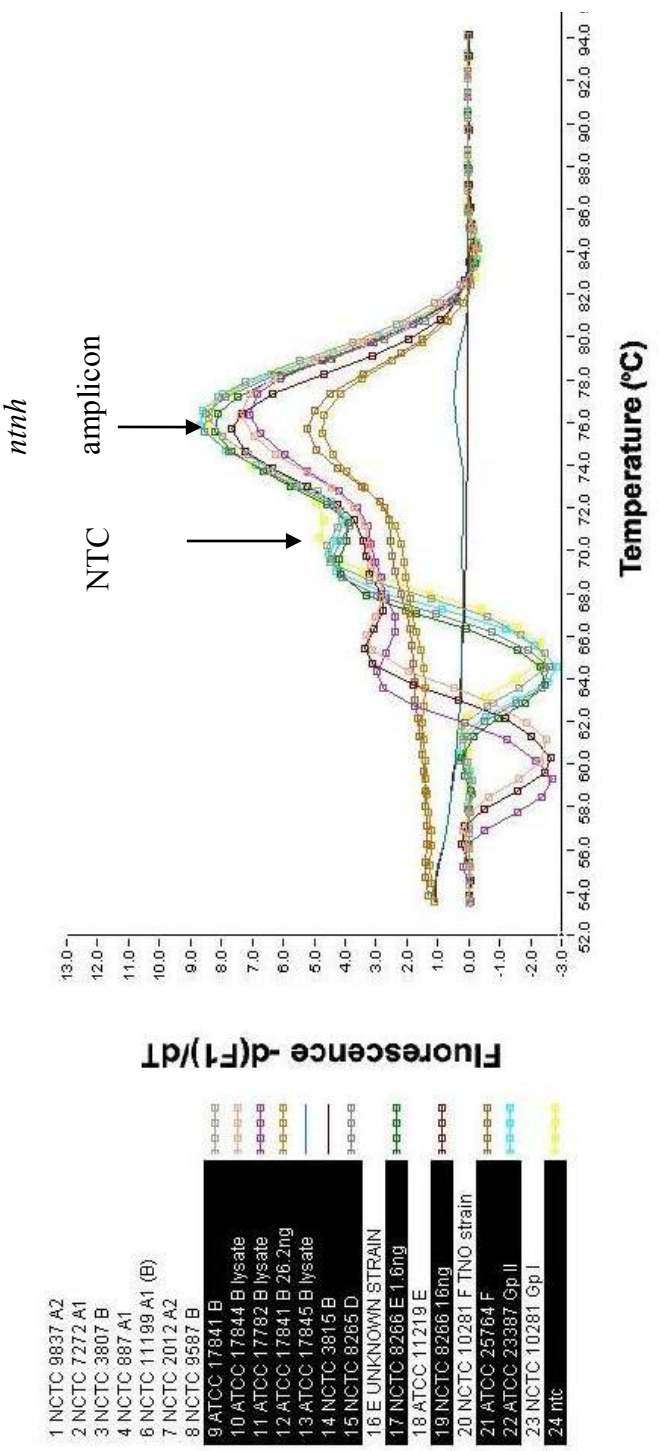


Figure 3.2 Melt curve analysis of *C. botulinum* amplification products obtained with DNA extracted from strains B to F using the *ntnh* PCR primer set (refer to Table 3.3 for details of ATCC/NCTC strain numbers). A non specific product with a Tm of 75 °C was obtained with the no template control. All other amplification products shown above also had a Tm of 75 °C.

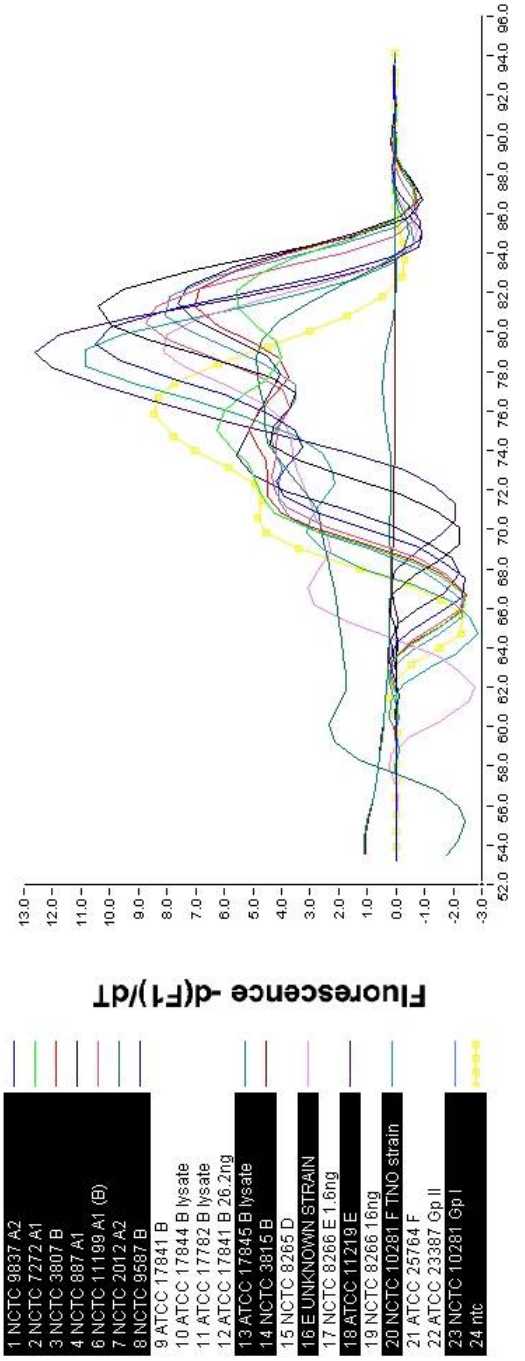


Figure 3.3. Melt curve analysis of *C. botulinum* amplification products obtained with DNA extracted from strains A to F using the *ntnh* PCR primer set (refer to Table 3.3 for details of ATCC/NCTC strain numbers). A non specific product with a Tm of 75 °C was obtained with the no template control. All other amplification products shown above had a Tm of between 77 and 83 °C.

Table 3.3 Summary of *ntnh* PCR results with *C. botulinum* DNA obtained from strains A to F (+ indicated amplification of the *ntnh* gene).

Strain	*ntnh* result
A NCTC 9837	+
A NCTC 7272	+
A NCTC 3807	+
A NCTC 887	+
A NCTC 11199	+
A NCTC 2012	+
B 9587	+
B ATCC 17841	inconclusive
B ATCC 17844	-
B ATCC 17782	-
B ATCC 17845	-
B NCTC 3815	-
D NCTC 8265	-
E unknown	+
E NCTC 8266	-
E ATCC 11219	+
F NCTC 10281	+
F ATCC25764	-
F ATCC 23387	-

3.3.2 *C. botulinum* A PCR assay

3.3.2.1 Assay development

Eight sequences described as encoding *C. botulinum* neurotoxin A were obtained from Genbank. Of these, three encoded the entire toxin gene, with the rest encoding a partial gene or a region upstream of the toxin gene encoding the *ntnh* gene. The sequences overlapped sufficiently to allow each one to be aligned into a *bont/A* contig in SeqMan. Overall homology was high between strains, at 94 to 98 %, facilitating the design of primer and probe sequences using a representative consensus sequence.

Detection of *Clostridium botulinum* type A strains was achieved using the primer and probe set described in Table 3.12, designed using Primer Express 1.0 software (Applied Biosystems) without having to alter the pre-set parameters. PCR reactions were optimised using 0.2 ng of DNA from strain NCTC 2916. The PCR assay was demonstrated to be robust and efficient in comparison to the other *C. botulinum* PCR assays developed for this study. For example, amplification occurred equally well with either a two or three stage PCR. Similarly between 0.2 and 0.4 μM of Taqman probe allowed efficient amplification. Consequently each *C. botulinum* A DNA tested was amplified successfully as summarised in Table 3.4. The assay was demonstrated to be specific for the *bont/A* gene, with no cross reactivity being observed with other *C. botulinum* strains. All available strains of *C. botulinum* A were detectable using the primer set designed for this study.

Table 3.4 Detection of *C. botulinum* A strains using the *bont* A PCR primer and probe set. A '+' symbol refers to a positive PCR signal. Type A strains are either of type M, L or LL complex.

Strain	Genotype	Origin/Alternative collection name	PCR type
NCTC 7272	A1	Unknown	+
NCTC 887	A1	Crop of chicken with botulism, 1920	+
NCTC 2916	A1 (B)	Home made canned corn 1926 (2447)	+
NCTC 3806	A	Canned peas, 1932 (97)	+
NCTC 4587	A		
NCTC 9837	A2	Fish from Mauritius, 1955	+
NCTC 11199	A1 (B)	Faeces, 1978 (F199-200/78)	+
NCTC 2012	A3	Loch Maree	+

3.3.3 **Detection of BoNT/A by PCR**

Clostridium botulinum neurotoxin samples were prepared by acid precipitation, with sulphuric acid or ammonium sulphate as outlined in Sections 2.4.2.1 and 2.4.2.2. Briefly, *C. botulinum* neurotoxin was prepared from a 50 ml overnight culture. The toxin is exported into the culture supernatant when grown *in vitro* and therefore the first stage in the purification was to centrifuge the culture. The cell debris was discarded and the toxin within the culture supernatant was precipitated by 'salting out' with 60 % ammonium sulphate or sulphuric acid during an overnight incubation at 4 ^{o}C. The toxin was then concentrated by centrifugation, resuspended and dialysed in 50 mM Tris buffer, pH 6.5 overnight.

DNA was extracted from the purified toxin using a silica-coated magnetic bead kit (Nuclyx™), in order to eliminate the toxin prior to import into the PCR analysis laboratory. Analysis of the extracted DNA showed that a low concentration was present in the toxin sample. It was not possible to quantitate the amount of DNA in the toxin sample as the control DNA obtained for this project was not of sufficient concentration to facilitate real time PCR amplification in the log-linear phase. It was therefore not possible to generate a standard curve. The real time PCR graph in Figure 3.5 shows that acid precipitated toxins contained DNA. Although not quantitative, different crossing point values were obtained from amplification of the two toxin samples (NCTC 2916 and NCTC 2012). The difference in DNA concentration obtained from the two samples is not explained, but may be attributed to differences in DNA binding to the protein through electrostatic forces or differences in DNA extraction efficiency or quality. However, it seems most likely

that the initial cell culture concentrations simply differed as it was not possible to measure the OD_{600} of the cultures grown in the toxin lab due to safety restrictions.

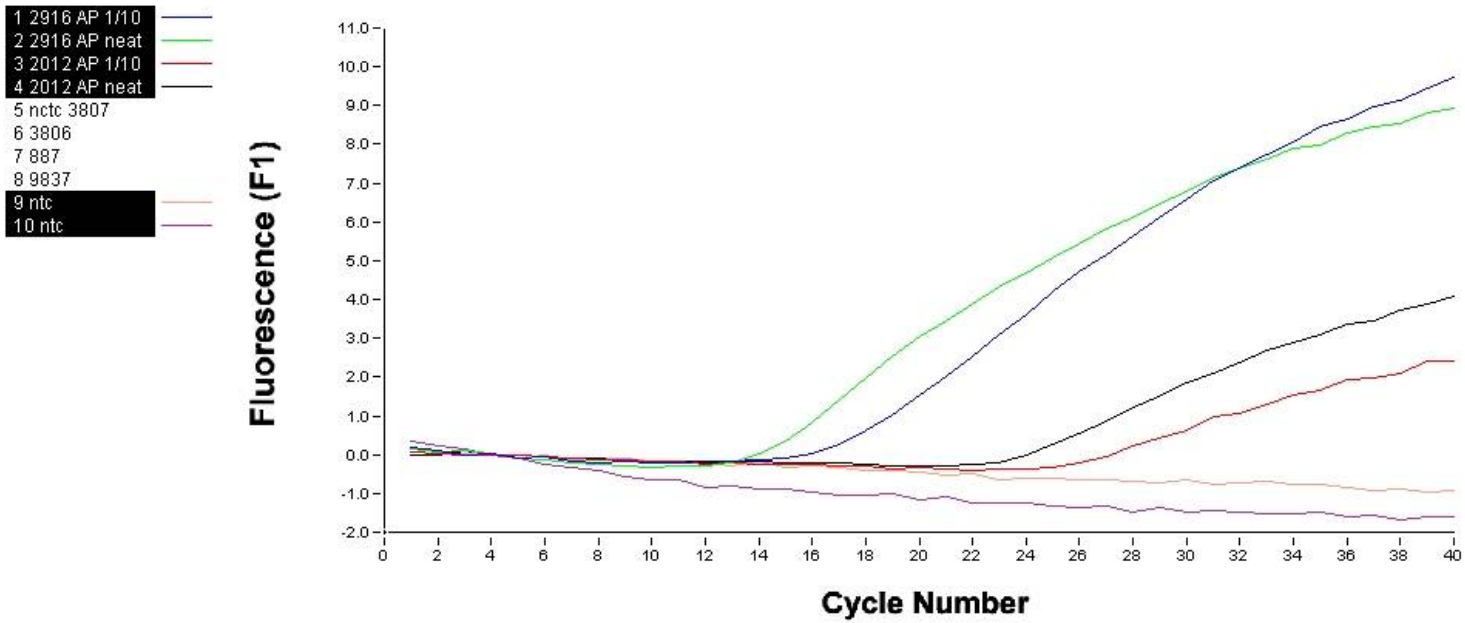


Figure 3.4. Analysis of *C. botulinum* A acid precipitated neurotoxin samples using the *bont*/A primer set. Toxin samples were extracted from NCTC strains 2916 and 2012 by acid precipitation. DNA was extracted from toxin samples using a Nuclyx™ DNA extraction kit and eluted into 50 µl of dH_2O. Analysis was carried out using 2 µl per PCR. An increase in fluorescence was observed in all samples apart from the no template control (NTC).

3.3.4 *Clostridium botulinum* B PCR assay development

Six *C. botulinum* B gene sequences were obtained from Genbank, from both proteolytic and non-proteolytic phenotypic groups. The primary sequence used was 3875 bp (accession number M81186, Table 3.2). When aligned in SeqMan they exhibited homology of 95 to 99 %. A primer and probe set was identified using Primer Express, although it was necessary to increase the permitted amplicon length from 150 bp to 220 bp and lower the annealing temperature by 5 °C to compensate for the low GC content of the gene.

C. botulinum B DNA was initially obtained from the test panel described in Appendix 1 rather than from known cultures so, strain typing information was unavailable. The assay was shown to require a lower annealing temperature than normally used for TaqMan® assays designed using Primer Express. Amplification of 0.1 ng DNA occurred at a cycle threshold of 20 with an annealing temperaure of 55 ^{o}C. This was reduced to a cycle threshold value of 18 when the annealing temperature was reduced 50 ^{o}C; data not shown). Therefore, reducing the annealing temperature allowed the DNA to be amplified earlier, thereby increasing the sensitivity of the assay but reducing its compatability with other assays used in this study. The specificity of the assay was investigated using a Sybr® Green I assay with DNA from the *C. botulinum* A to F, *C. perfringens* and *C. novyi*. As expected, the assay was specific for *C. botulinum* B (Figures 3.5 and 3.6). The assay was then optimised using the specific TaqMan® probe (data not shown), again monitoring the cycle threshold value for each different concentration of probe. An optimal probe concentration of 0.3 μM was established, as summarised in Table 3.12.

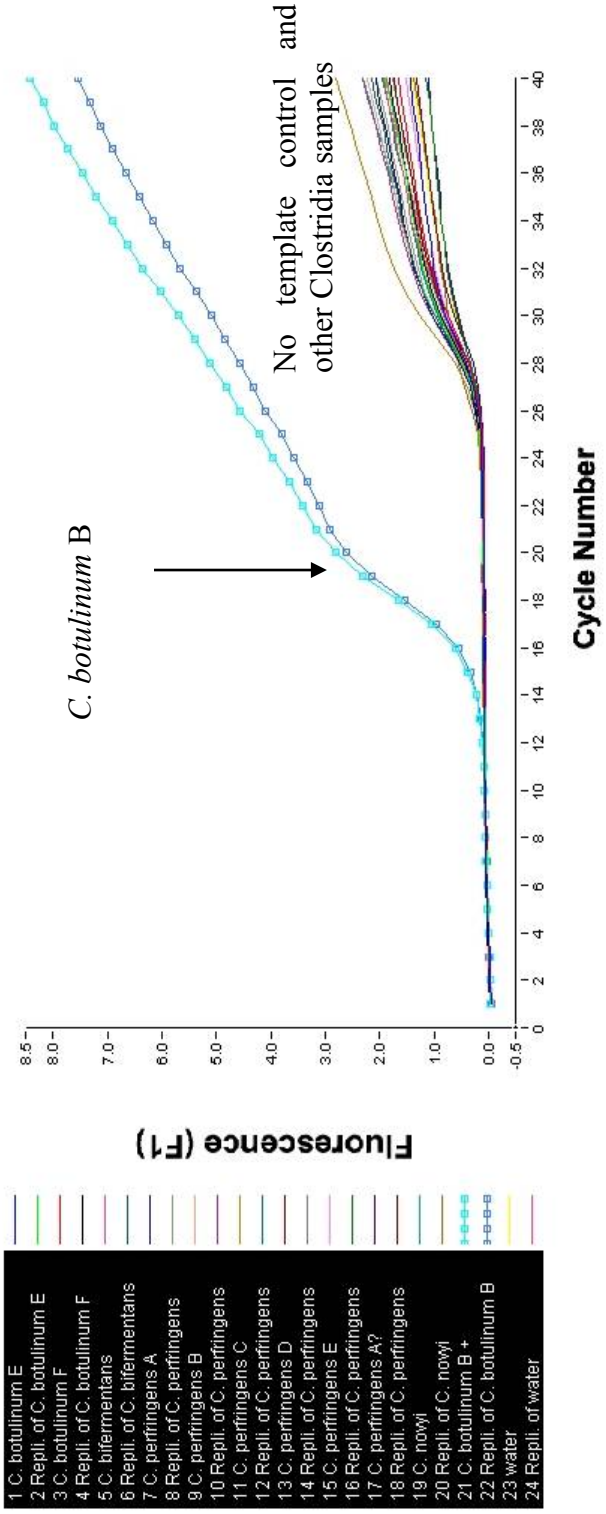


Figure 3.5 Dependence of relative fluorescence on PCR cycle number for *C. botulinum* B sybr Green I PCR assay containing the bont B1 primers, in the presence of various Clostridial DNAs.

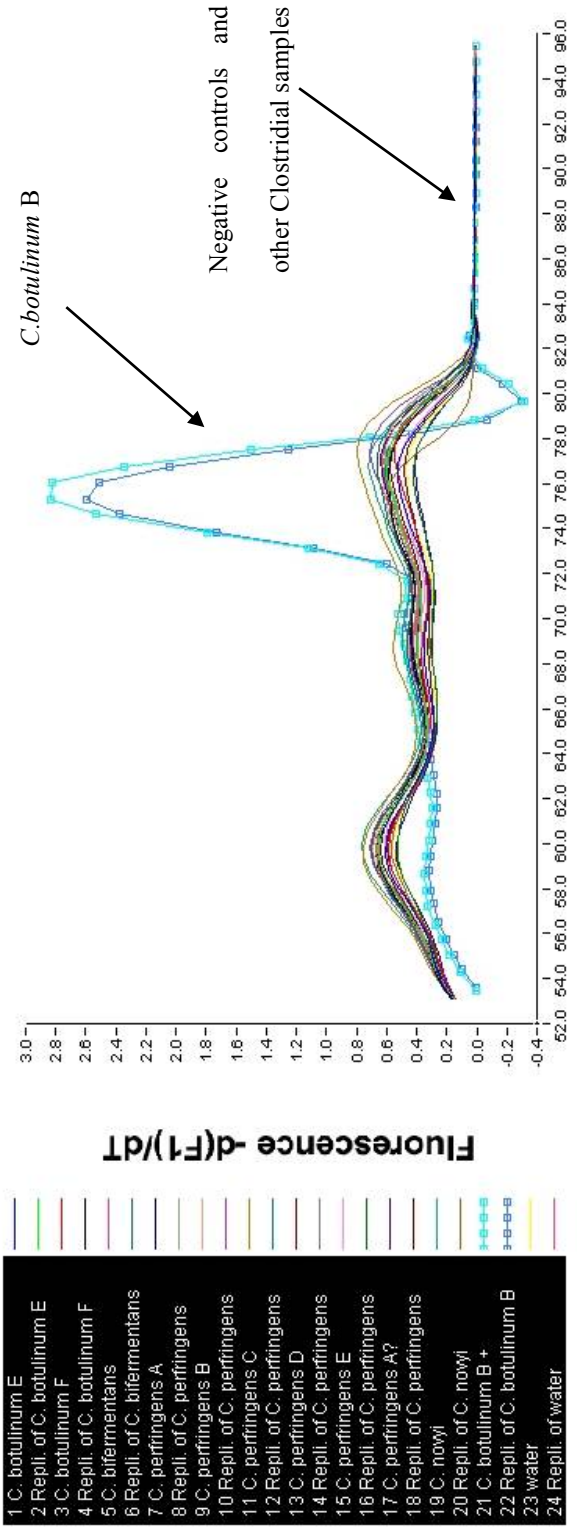


Figure 3.6 First derivative of the fluorescence curves shown in Figure 3.5, obtained from amplification of various Clostridial DNA with the *bont /b* primer set. A product with a melting temperature of 75 ^{o}C was obtained with *C. botulinum* B DNA. Non specific products with a melting temperature of 78 ^{o}C were obtained with the no template controls (samples 23 and 24) and other clostridial DNA samples.

Fourteen type B strains (see Table 3.5) had been stored in refrigeration at Dstl for a number of years. Cultures were grown and DNA extracted as described previously. However, PCR results using the extracted DNA were variable. ATCC strains 17780, 17783, 17841 and 25765 were detectable using primers targeting the type B toxin gene (Figures 3.7 to 3.9). Other strains were not detectable using this assay, as summarised in Table 3.6.

To determine whether false negative results were being obtained using the original primer set DNA was analysed with primers obtained from the open literature (Campbell *et al.,* 1993; Szabo *et al.,* 1994). These results confirmed those obtained using the primers designed for this study, indicating that the gene was not present in the DNA extracts tested.

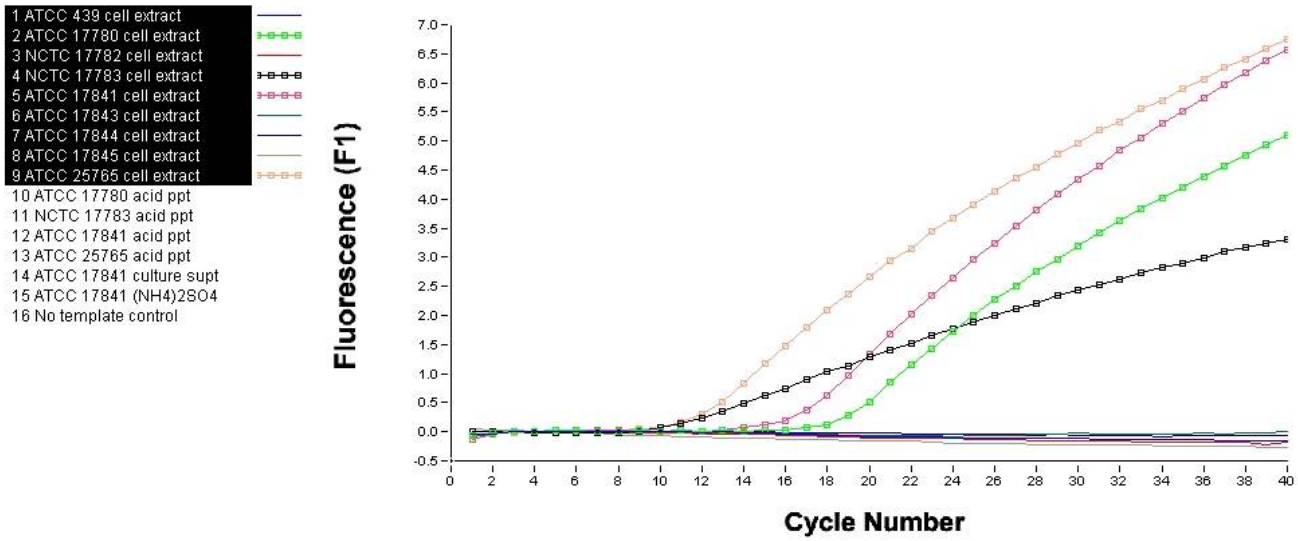


Figure 3.7 Real-timeTaqMan® PCR detection of *Clostridium botulinum* type B gene in 9 different strains using the *bont* B primer set. An increase in fluorescence was seen with strains 17841, 17780, 17783 and 25765

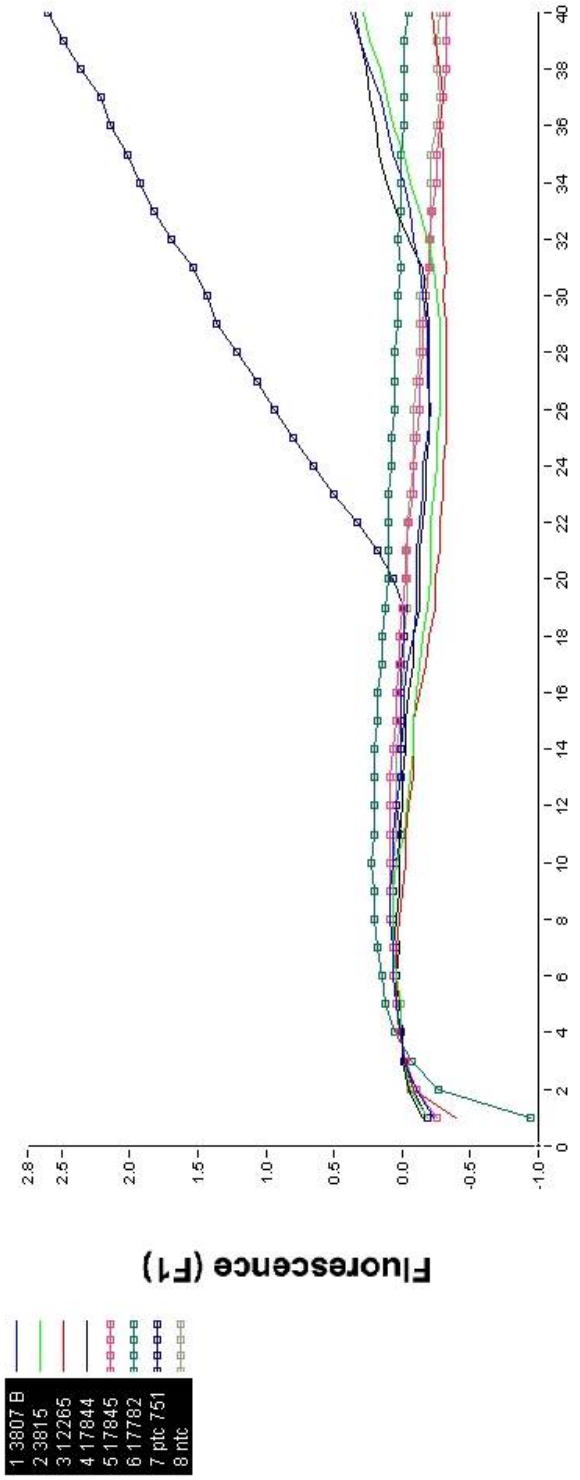


Figure 3.8 Analysis of *C. botulinum* B DNA using the *bont* B PCR primer and probe set. An increase in fluorescence was seen with DNA obtained from strain 751 (positive template control). A weak increase in fluorescence was also seen with strains 3807, 3815 and 17844. No increase in fluorescence was observed with strains 17845 and 17782,

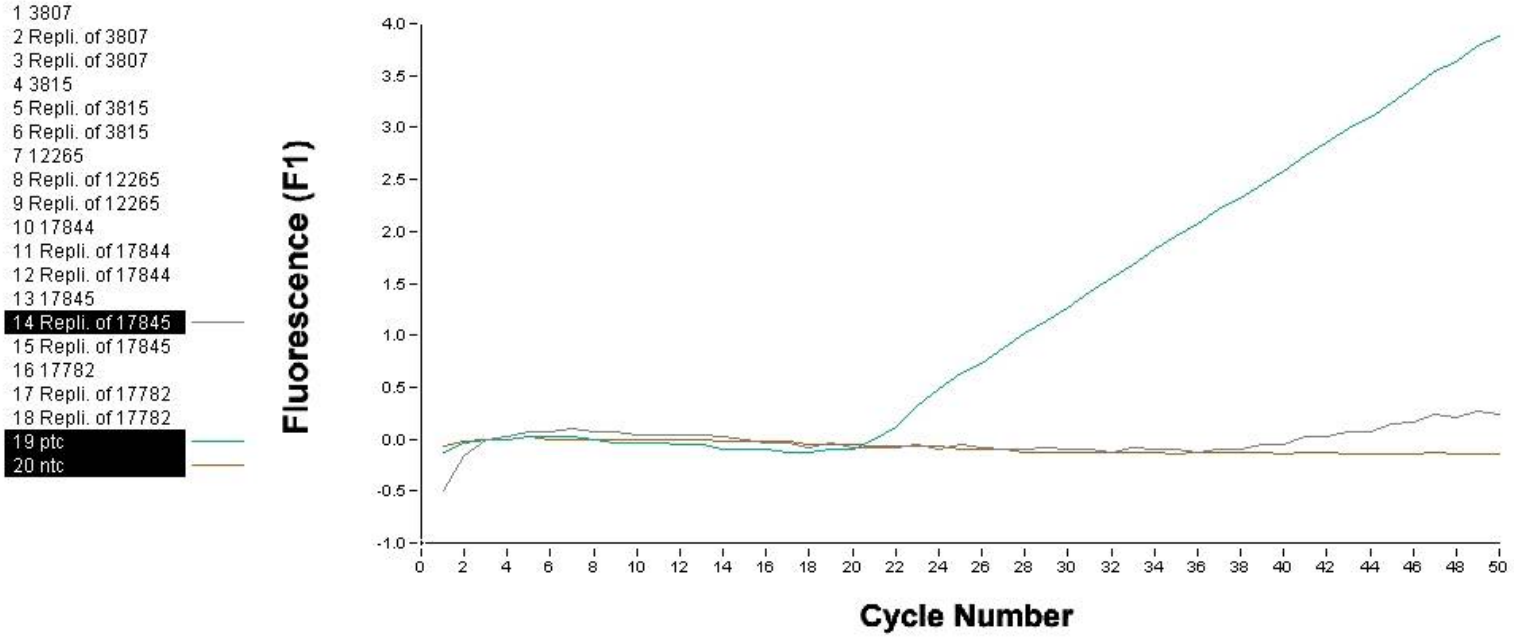


Figure 3.9 Analysis of *C. botulinum* B DNA using the *bont* B PCR primer and probe set. An increase in fluorescence was seen with DNA obtained from strain 751 (positive template control). A very weak increase in fluorescence was also seen with strain 17845 (in contrast to the results shown in Figure 3.11).

Table 3.5 Summary of *C. botulinum* B PCR results using four different assays. Positive amplification" is indicated by a '+' symbol. DNA samples marked with a '*' were negative initially. DNA was then extracted from stock cultures and analysed as positive by PCR. '-' samples were not amplified. Results marked N/D were not determined, those marked '?' were unclear.

Strain	Genotype	Origin	BoNT PCR			*Ntnh* PCR
			Set1	Cambpell	Szabo	
NCTC 751	B GpI	California, 1920	+	N/D	N/D	
NCTC 3807	B Gp I	Soil, Blue Ridge Mountains, 1021	*	N/D	N/D	+ ?
NCTC 3815	B Gp I	Cheese, 1932	*	N/D	N/D	
NCTC 7273	B Gp I	Beans, 1947	+	N/D	N/D	
NCTC 12265	B	Hazelnut yoghurt, 1989	*	N/D	N/D	
9581				N/D	N/D	+
ATCC 17841	B Gp I		+	+	+	+

ATCC 17844	B Group II (2217)	N/D	*	-	-	-
ATCC 17845	B (2254)		*	-	-	-
Eklund 17B (ATCC 25765)	B Gp II	Marine Sediment, USA	+	+	+	
ATCC 17780	B (3137)		+	+	+	
ATCC 17783?	B		+	+	+	+
ATCC 439	Gp I		+		-	+?
ATCC 17843	B (2017)		+	N/D	+	+

3.3.5 **Purification of Botulinum Neurotoxins B by acid precipitation**

Neurotoxin samples were produced from the culture supernatant of strain ATCC 17841, known to be detectable using the PCR assay designed for this study. Toxins were expressed during an overnight culture step and then precipitated using ammonium sulphate or sulphuric acid according to the method described in Section 2.4.2.1 and 2.4.2.2. DNA was then extracted from the toxin samples using a Nuclyx™ magnetic bead kit.

All toxin samples produced from ATCC 17841, a group I strain, were detectable using the *bont*/*b* PCR (Figure 3.10). The PCR was not quantitative, however the crossing point (Cp) of the amplification curve (not shown in Figure 3.10 as it is not depicted in the primary fluorescence graph) from ammonium sulphate and acid precipitated samples indicated that a low concentration of DNA was present in the toxin samples. A sample of the culture supernatant was also extracted using the Nuclyx kit. The crossing point value obtained with this sample (Cp 24) was slightly higher than the ammonium sulphate precipitated toxin sample (Cp 25) and lower than the acid precipitate (Cp 20). This again indicates that DNA was co-precipitated and concentrated with the toxins.

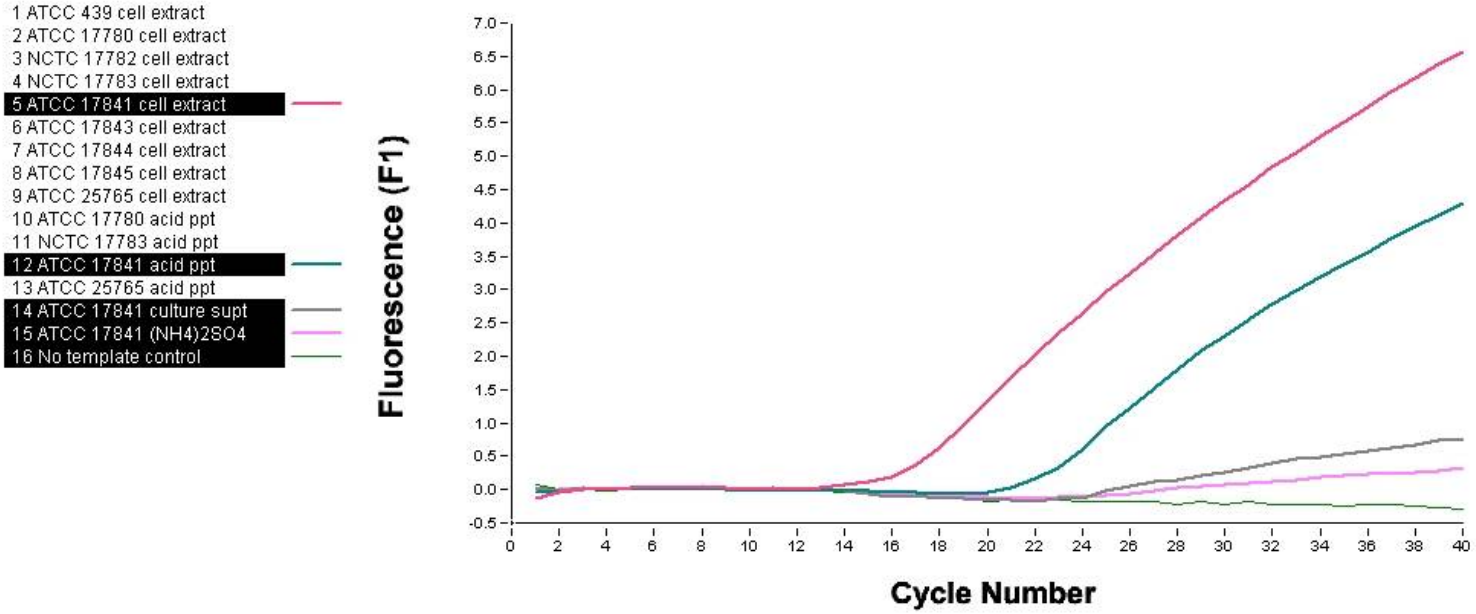
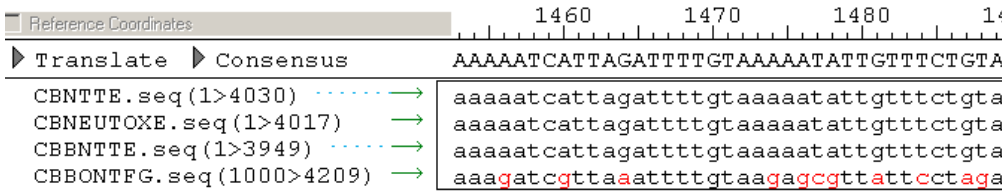


Figure 3.10 Detection of *C. botulinum* neurotoxin B gene in toxin preparations. Toxin samples were purified from *C. botulinum* B strain ATCC 17841 culture supernatants using ammonium sulphate or sulphuric acid. DNA within the toxin sample was extracted using a Nuclyx extraction kit and then tested for the presence of the toxin gene using the bont B primer and probe set.

3.3.6 *Clostridium botulinum* E PCR assay devlopment

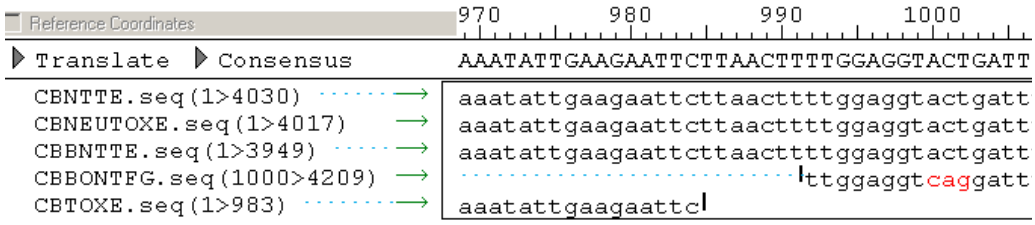
BoNT/E is produced by *C. botulinum*, *C. butyricum* and *C. baratii.* Six *bont/e* sequences were available at the start of this study, four *C. botulinum* and two *C. butyricum* (Table 3.2). The sequences from both species aligned with 98 % homology, indicating that the assay would be generic for the type E neurotoxin rather than specific for *C. botulinum*. Type E DNA was initially obtained from the DNA test panel described in Appendix 1. The *bont*/*e* assay amplified DNA from the unknown type E strain successfully. The assay was then optimised, using this DNA, for annealing temperature and magnesium concentration (Table 3.12) and tested for cross reaction with near-neighbour strains of Clostridia using the process previously described in Section 3.3.3. The primers were demonstrated to cross react with *C. botulinum* type F DNA (Figures 3.12 and 3.13). Sequence analysis had not previously indicated the type E assay should cross react with type F DNA, but the assay was redesigned to target more differences between the two strains (Figure 3.11).

The re-designed primers and probe did not cross react with DNA from the Clostridia test panel (data not shown) therefore the specificity of the assay against the three type F DNA samples available was determined. The type E primers did not produce an amplicon with DNA from *C. botulinum* F (Figure 3.14). The new assay was tested with DNA extracted from eight subcultured type E strains as summarised in Table 3.6. The assay was verified to work well with all but one, NCTC 8550. PCR analysis of the original stock culture was successful, again demonstrating the deleterious effect of subculture on genetic integrity.

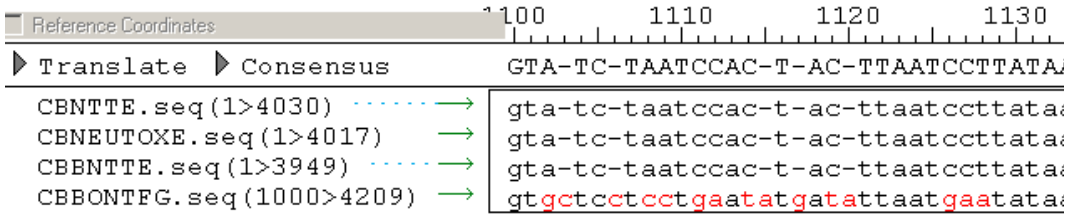


3.11a *bont/E* Forward sequence alignment

3.11b *bont/E* Reverse sequence alignment



3.11c *bont/E2* Forward sequence alignment
5' AACTTTTGGAGGTACTGAAT 3'



3.11d *bont/E2* Reverse sequence alignment
5' TATAAGGATTAAGTAGTGGATTAGATAC 3'

Figure 3.11. Sequence alignment data for *bont/E* and *bont/E2* primers. Primer *bont/E1* F and R was demonstrated to cross react with *bont/F* DNA, therefore it was disregarded. A second set (*bont/E2*) was designed targeting more mismatches between the type E and F sequences.

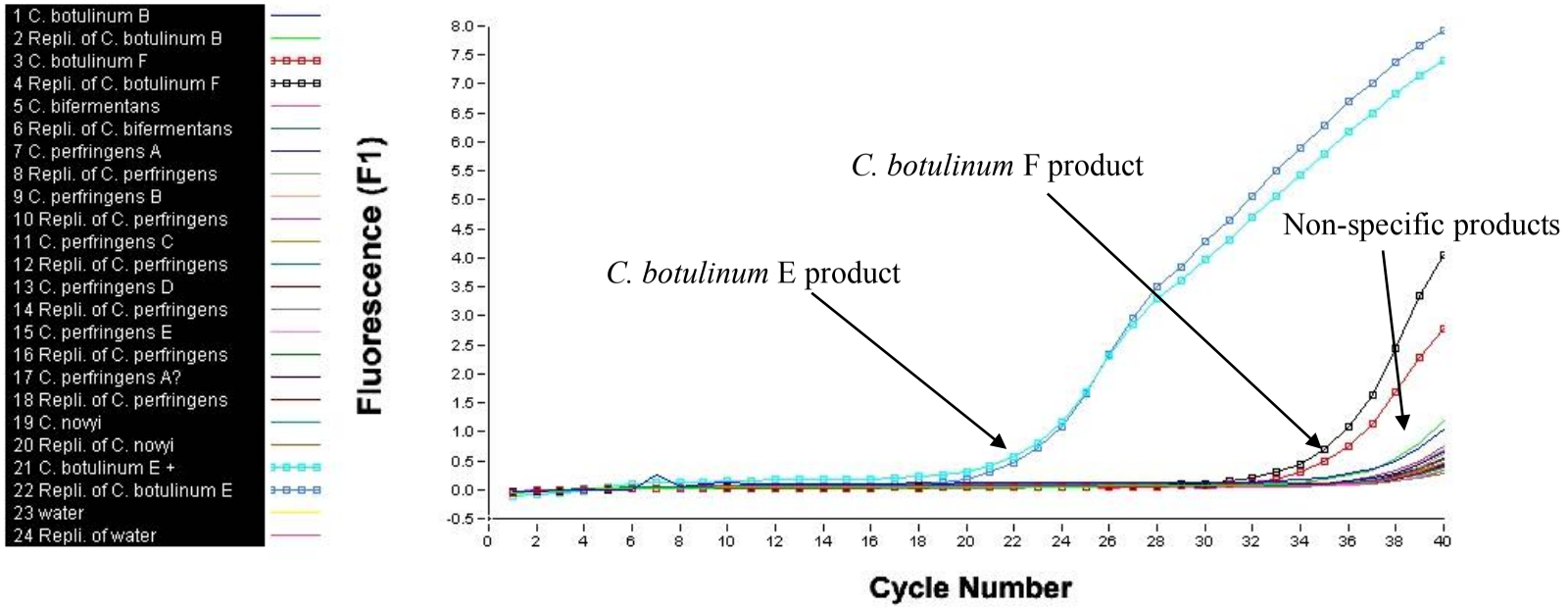


Figure 3.12 Analysis of the specificity of the *C. botulinum* type E PCR assay. Sybr Green I real time PCR was carried out using the *bont/e* primer set with DNA extracted from type E and F strains. A large increase in fluorescence was observed with type E DNA. A smaller increase in fluorescence was observed resulting from amplification of type F DNA. A weak increase in fluorescence was also observed after cycle 36 in the negative control (water) and other clostridial DNAs. This weak increase in fluorescence was caused by incorporation of Sybr Green I into artefacts of the PCR (primer-dimer).

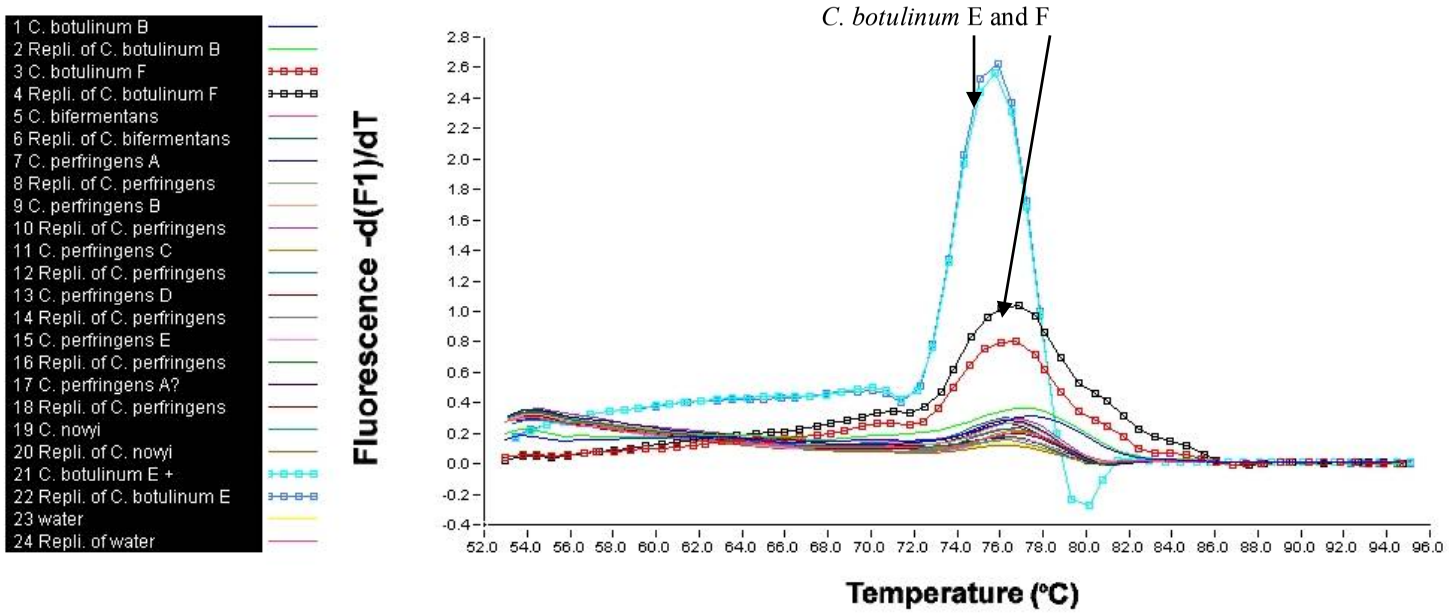


Figure 3.13 Melt curve analysis of products obtained with *bont* E assay and near-neighbour strains. A product with a melting temperature of 76 °C was obtained from *C. botulinum* E DNA. A product with a melting temperature of 77 °C was obtained with *C. botulinum* F DNA. PCR artefacts were also observed with a melting temperature of 76.5 °C.

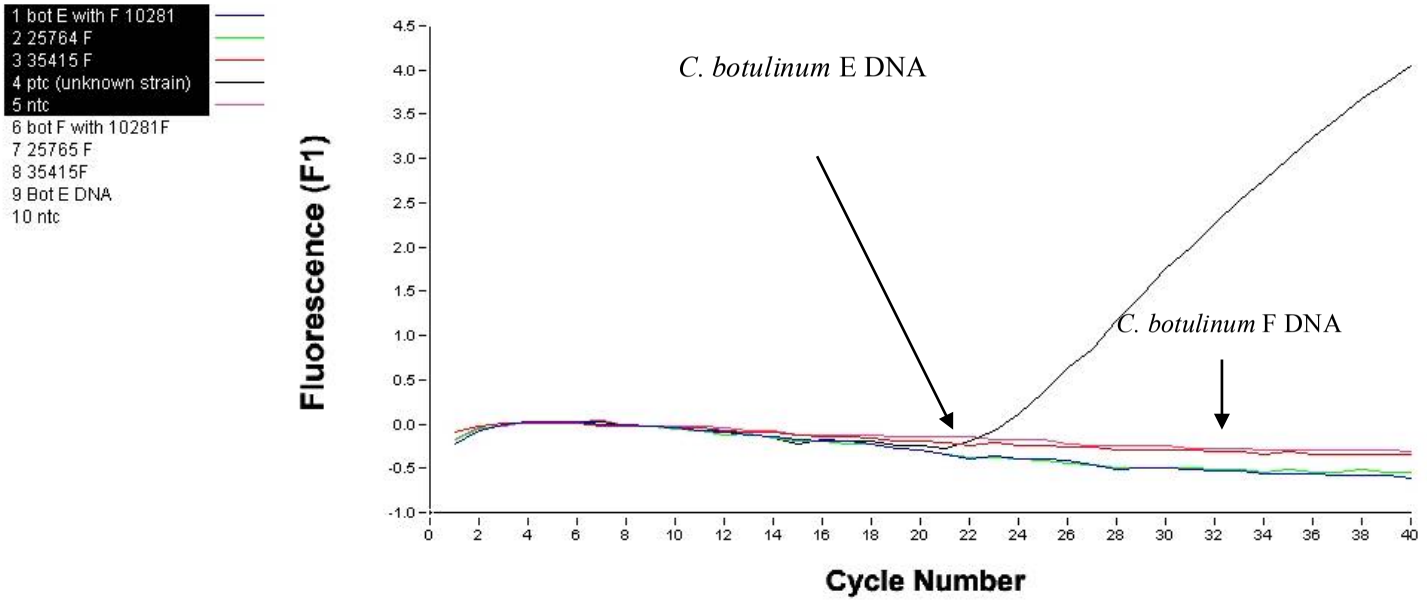


Figure 3.14 Analysis of the specificity of the *C. botulinum* E TaqMan® PCR assay. DNA was extracted from type F strains NCTC 10281, ATCC 25764 and ATCC 35415 using a Qiagen DNA mini prep kit and analysed using the *bont* E primer set. An increase in fluorescence was observed with the positive control *C. botulinum* E DNA.

Table 3.6 Detection of *C. botulinum* E species using the *bont*/E assay designed for this study. Positive amplification is indicated by a '+' symbol. DNA samples marked with a '*' were negative initially. DNA was then extracted from stock cultures and analysed as positive by PCR.

Strain	Genotype	Origin	PCR result
NCTC 8266	E Nanaimo Gp II	MRE Porton 1952	*
NCTC 8550	E Gp II	Institut Pasteur 1952, possess OS variants.	*
ATCC 17854	E Gp II		+
ATCC 8550	E Gp II		+
ATCC 17854	E Gp II		+
ATCC 17786	E Gp II		+
ATCC 11219	E Gp II		+
ATCC 9564	E Gp II (Hazen)	Smoked Salmon, Canada	+

3.3.7 **Detection of acid precipitated toxins from *C. botulinum* type E.**

Acid precipitated toxin samples were obtained from several type E neurotoxin producing strains grown in cooked meat broth in the presence of sucrose. DNA was extracted from the four samples using the same acid precipitation method described for BoNT/A and BoNT/B. The concentration of DNA carried over into the toxin preparation differed between samples as observed with the extractions taken from BoNT/A and BoNT/B. One sample was estimated to contain 1000 times more DNA than the other positive samples and one sample did not contain detectable DNA (Figure 3.15). A negative mouse lethality or ELISA test performed with fresh culture supernatant would determine whether the PCR negative sample contained toxin. However, it was not possible to test this theory during this study as funding for antibody or animal work was unavailable. Although the results were variable, overall BoNT/E was successfully detected by PCR.

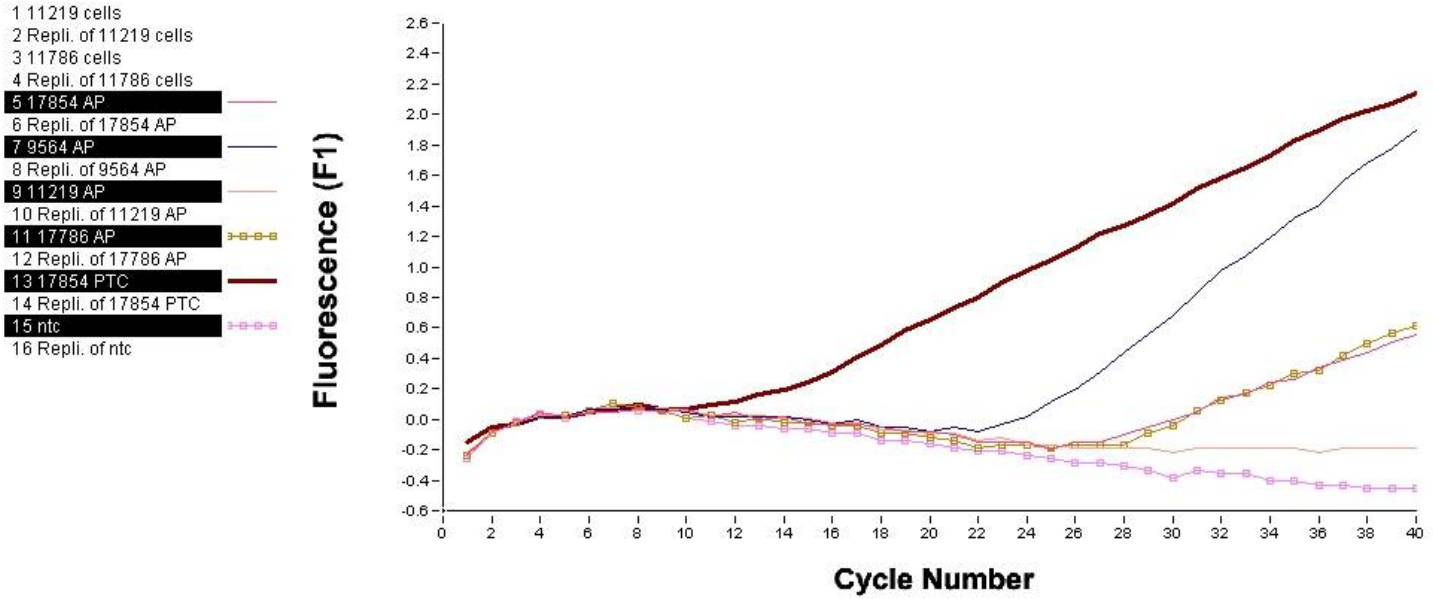


Figure 3.15 Detection of *C. botulinum* E DNA in acid precipitated neurotoxin samples from strains ATCC 17854, ATCC 9564, ATCC 11219 and ATCC 17786. An increase in fluorescence was observed in all samples apart from the no template control and the toxin sample prepared from strain ATCC 11219.

3.3.8 *Clostridium botulinum* F PCR assay development

Alignment of the seven *C. botulinum* type F *bont* gene sequences obtained from NCBI showed homology was lower than with the other neurotoxin genes at 89 to 99 %. This may be a result of the species falling into two distinct physiological and genetic groups (I and II). Although data relating to the physiological group of the Genbank sequences was unavailable, assumptions have been made based on the sequence name (Table 3.7). In spite of low homology, a representative consensus sequence was obtained and used to design TaqMan® primer and probes with no changes to the parameters within Primer Express. The assay was therefore predicted to work well.

Table 3.7. Homology of *C. botulinum* neurotoxin F genes obtained from Genbank.

Accession name	Assumed physiological group	Percentage match to CBY13161.seq
CLONEUTOXF	?	95
CBBONTFG	?	89
CBFCRAIG	II	98
CBFHOBBS	II	99
CBFLANGE	I	90
CBNTNH	?	92

The primer set chosen was tested using a range of magnesium concentrations. Once optimised at 3 mM it was challenged with the DNA test panel described in Appendix

1, as well as the clostridial test panel. The results showed that the assay was specific for *C. botulinum* F DNA (Figure 3.16).

The primer set was then tested with DNA extracted from the four strains that had been subcultured and stored in refrigeration. Only two out of four strains were detectable initially (Figure 3.17). Further PCR analysis using DNA extracted from stock cultures using a Qiagen protocol was performed, however ATCC strains 23387 and 27231 were not detected (Table 3.8). The reason for this is postulated to be due to poor culture maintenance as with the type B strains. However it was not possible to rule out the possibility of poor assay design as reliable DNA samples were unavailable.

No toxin samples were available from *C. botulinum* type F strains, however it is assumed that detection would have been possible using PCR, in line with results obtained with types A, B and E. In general terms, PCR detection of botulinum neurotoxins prepared by acid precipitation was possible. This finding is significant as it is likely that this would be the method of choice for toxin production by terrorists (Zilinskas 1997). Therefore, PCR was useful for detecting residual DNA contained in *Clostridium botulinum* neurotoxin samples.

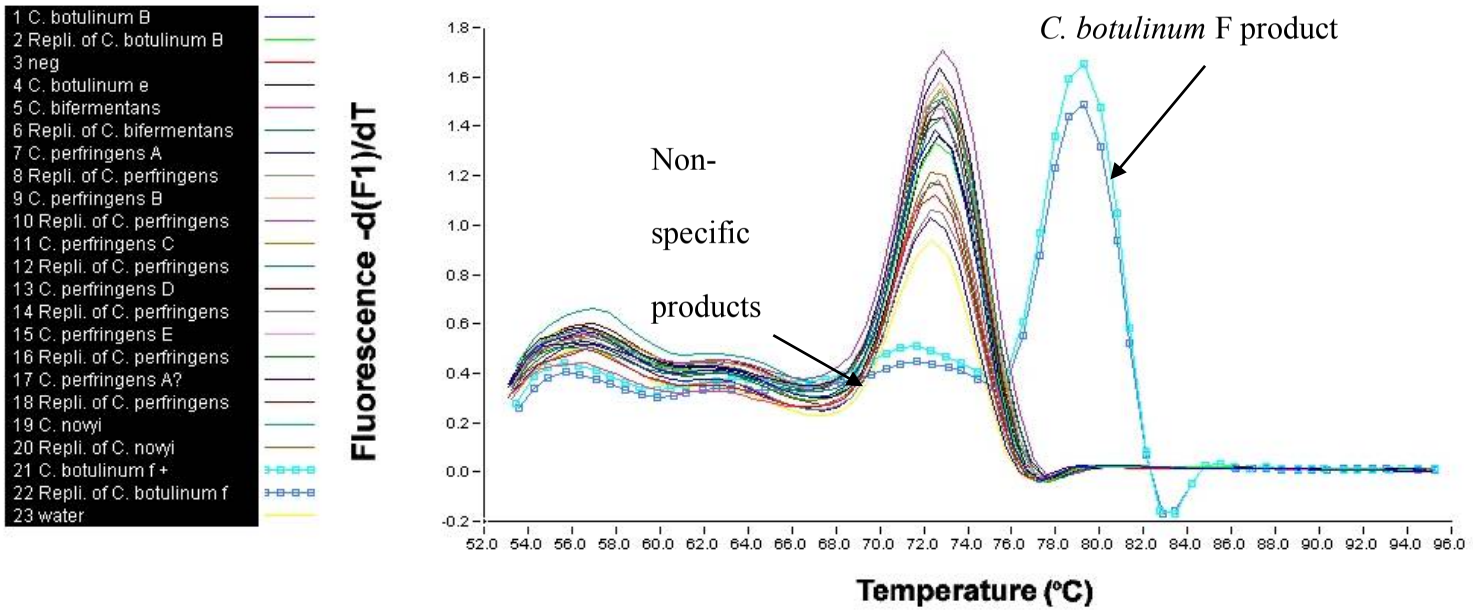


Figure 3.16 Melt curve analysis of PCR products obtained using a clostridial test panel of DNAs with *C. botulinum* F specific primers. A PCR product with a Tm of 79 °C was obtained with *C. botulinum* F DNA (samples 21 and 22). Products with a Tm of 72 °C were obtained with all other clostridial DNA samples and the no template control (water, sample 23).

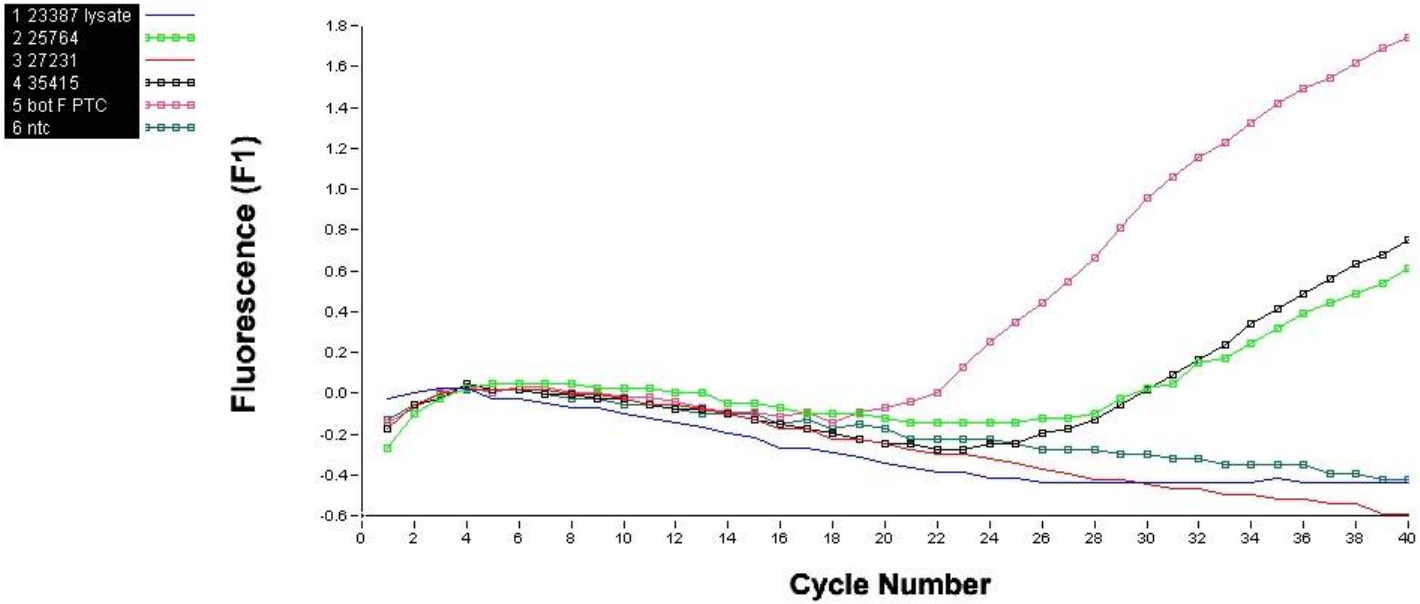


Figure 3.17. Real-time TaqMan® PCR detection of *Clostridium botulinum* F DNA samples extracted from in house culture stocks using the bont F primer and probe set. An increase in fluorescence was observed in reactions containing DNA extracted from ATCC 35415 (black line), ATCC 25764 (green line) and the positive template control (PTC; pink line). No increase in fluorescence was observed in reactions containing ATCC 23387 (blue line), ATCC 27231 (red line) or the no template control (teal line).

Table 3.8 *C. botulinum* F PCR assay results using the bont F real-time PCR assay designed for this study. Positive amplification is indicated by a '+' symbol. DNA was then extracted from stock cultures and analysed as positive by PCR. '-' samples were not amplified. N/K denotes Not Known.

Strain Name	Alternative designation	Origin	PCR result
NCTC 10281	Langeland Gp I	Liver paste, Vancouver 1962.	+
ATCC 23387	Gp II Eklund (202F)	N/K	-
ATCC 25764	Gp I	Crab	+
ATCC 27321	VPI 2382	N/K	-

3.4 RECOMBINANT V ANTIGEN PCR ASSAY

The *Yersinia pestis lcrV* gene sequence, encoding V antigen, was obtained from GenBank (detailed in Price *et al*., 1989) and then imported into Primer Express. Primer and probe sites were identified for amplification of a 68 bp portion of the gene. The assay was optimised with DNA obtained from *Y. pestis* strain C092, and was shown to work well using standard real-time PCR conditions. Although designed to work as a TaqMan® assay, the probe was not deemed a requirement of the project and was therefore not purchased. Similarly, the specificity was not determined as part of this research.

An in house preparation of the 37 K Da recombinant V antigen (rV) from *Yersinia pestis* was available for PCR analysis. The protein has been studied extensively as a potential vaccine target during research carried out at Dstl (Carr *et al.,* 1999). The concentration of DNA present in protein vaccines is regulated by the Medicines and Healthcare products Regulatory Agency (MHRA) in the UK and the Food and Drug Administration (FDA) in the US and should be low or negligible (Ng and Mitra 1994). Therefore PCR analysis of highly purified rV protein, as a model for a highly purified toxin, was considered a interesting digression.

3.4.1 Purification of Recombinant V Antigen by Affinity Chromatography

Recombinant V antigen was prepared as a glutathione S transferase (GST) fusion protein and was purified by Sarah Hayward at Dstl, described in detail in Section 2.7.1. Several samples were taken during the process as summarised in Table 3.9. Briefly, a four litre recombinant *E. coli* culture was grown overnight and then centrifuged to harvest cells. The pellets were resuspended in PBS supplemented with

DNase I and RNase to reduce the viscosity of the sample prior to further treatments. The cells were then lysed by sonication (sample A), releasing both proteins and DNA. Cell debris was removed by centrifugation and the lysate was loaded onto a glutathione sepharose 4 B affinity purification column. Protein was eluted from the column in buffer containing glutathione (samples B to D), along with picogram levels of DNA. Low molecular weight contaminants were removed from the toxin solution by gel filtration using a sephadex G25 size exclusion column and the high molecular weight proteins and DNA were eluted. The GST tag was removed by protease cleavage which was retained on a GS 4B column and the flow through containing the protein and DNA was collected in 2 ml fractions (E to K). The fractions containing the most LcrV were pooled (L). DNA was then extracted from sample L using a Qiagen miniprep kit. Figure 3.18 shows the real time PCR results obtained from amplification of samples taken during the purification of recombinant V antigen by affinity chromatography. Most of the samples tested contained small but detectable amounts of DNA. More importantly the purified protein sample (L) contained a mean concentration of 18.25 ng/μl (n=2), as determined by quantitative PCR. In this instance DNA was co-purified with a protein purified by affinity chromatography as a GST fusion protein. Therefore PCR was suitable for detecting recombinant V protein prepared by affinity chromatography.

Table 3.9 Recombinant V Q-PCR data.

Sample Type/method N.B. 4 l overnight culture pelleted, sonicated and treated with DNase and RNase prior to first sample being taken.	Lightcycler calculated conc (pg/µl) (mean of duplicate samples)	
	Preparation 1	Preparation 2
A – sonicated cells run on GST affinity column	90	150
B 7.5 ml GST fraction	Inhibited	Inhibited
C 7.5 ml GST fraction	10	10
D 30 ml GST fraction	70	75
E – protease cleaved fraction 1	Inhibited	Inhibited
F – protease cleaved fraction 2	121	130
G protease cleaved fraction 3	50	40
H protease cleaved fraction 4	17	18
I protease cleaved fraction 5	13	10
J protease cleaved fraction	29	8
K protease cleaved fraction	10	4
L pooled protein sample	Inhibited	Inhibited
L DNA extracted sample	4.7 ng/µl	32 ng/µl

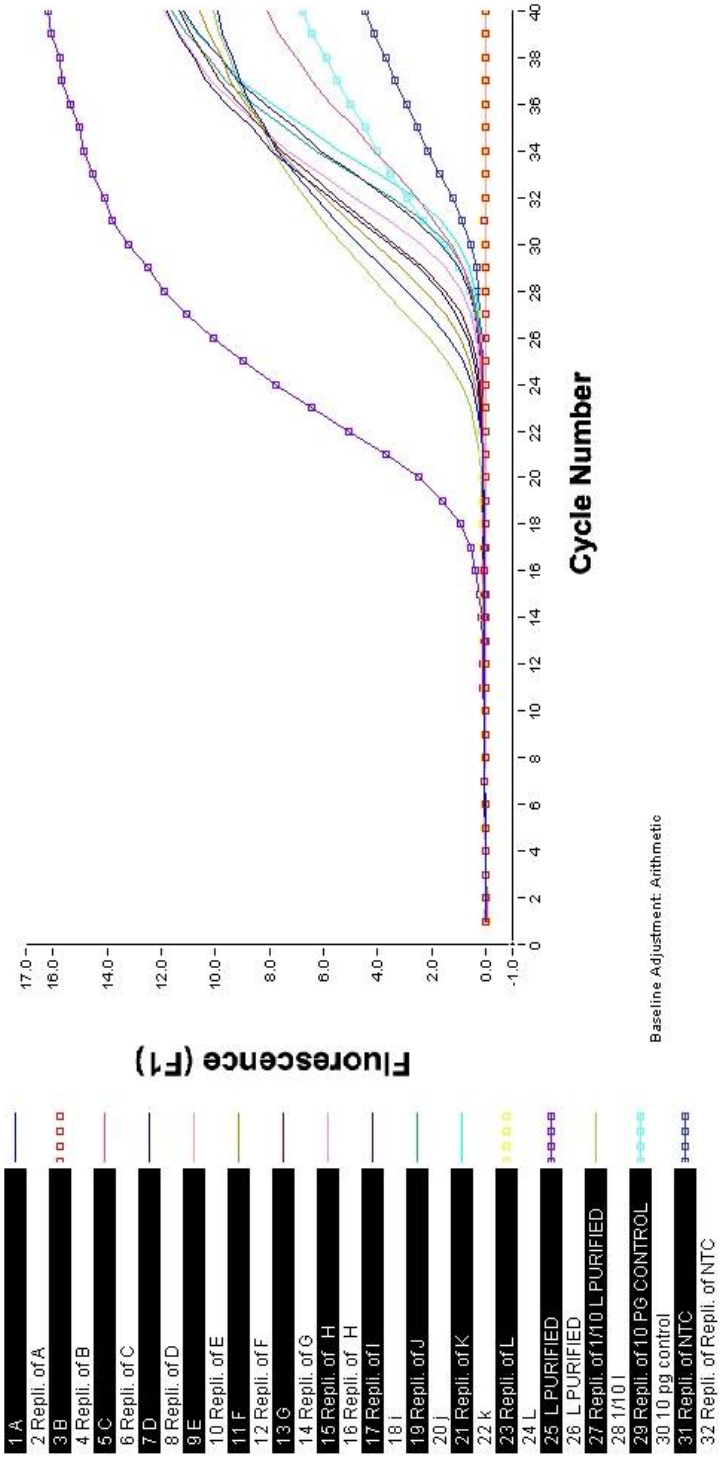


Figure 3.18. Analysis of samples taken throughout the 12 stage recombinant V antigen purification process using the *Y. pestis* rV PCR assay designed for this study. An increase in fluorescence was observed in all of the above samples apart from the no template control (NTC, samples 31 and 32). rV antigen was extracted by sonication and purified using a GST affinity column (A-D), protease cleaved (1-5, samples E-K respectively) and pooled. The pooled product was processed using a QIAamp DNA mini kit.

3.5 *RICINUS COMMUNIS* RICIN DETECTION

3.5.1 *R. communis* assay development

Sixteen *Ricinus communis* ricin gene sequences were obtained from GenBank. Sequence identity was high between those deposited into the database at the onset of this study (prior to the *R. communis* genome being sequenced at JCVI), at 96 – 100 %. Ribosome inactivating proteins (RIP) have a similar mode of action but are produced by several unrelated plant species. Sequence analysis of RIP gene sequences from species such as *Abrus precatoris* (abrin) and *Viscum album* (viscumin) did not align with the ricin gene. Furthermore, BLAST analysis of primer and probe sequences identified by Primer Express did not identify cross reactions with non target species. Therefore, the assay was predicted to be specific for ricin. The complete sequence of the gene was reported as between 2362 and 1695 bp and a consensus of 2344 bp was obtained. The sequence alignment included ricin isoforms D and E as well as the agglutinin gene which aligned with 93 % homology. The resulting consensus sequence was exported to Primer Express which identified an optimal primer and probe set and was calculated to have very little secondary structure or primer dimer formation.

The assay amplified target DNA with high efficiency using standard TaqMan® PCR conditions (Table 3.12) as predicted by the software. Cross reactivity tests were performed with plant DNAs obtained from two alternative type II RIP producing species (Figure 3.19). The results confirm that although the plant toxins have similar mode of action, the gene sequences are completely unrelated. However, DNA was unavailable from other type II RIP producing plants so confirmatory analysis of a positive result would be desirable.

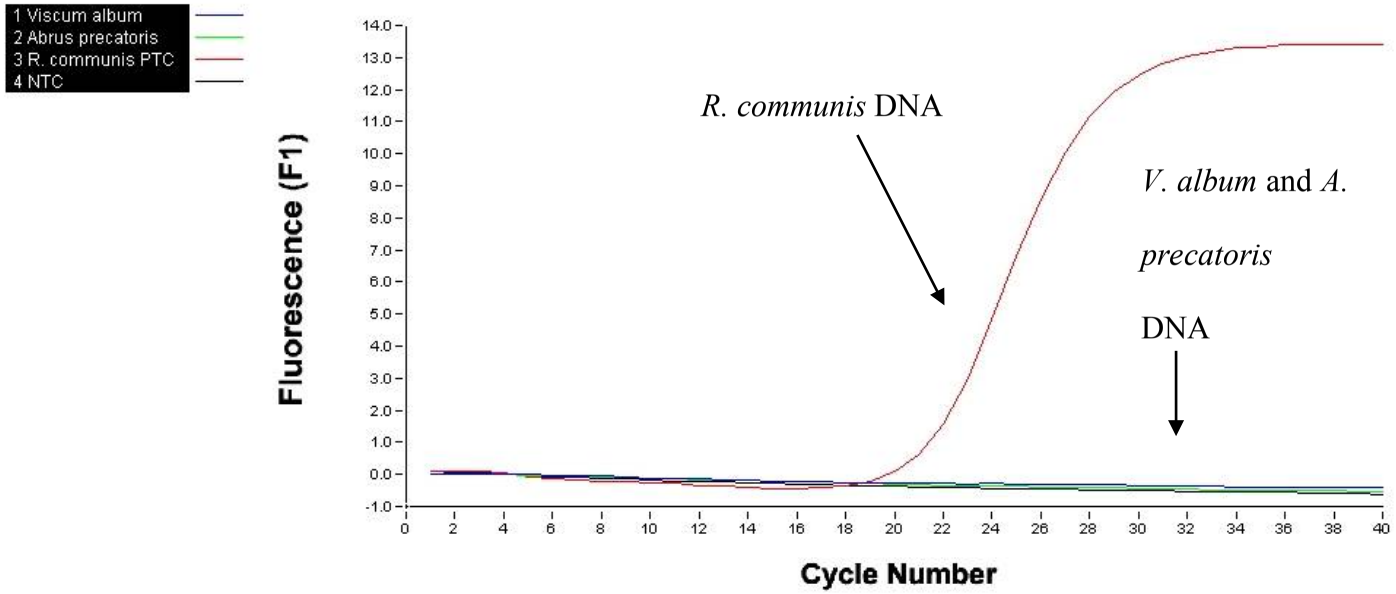


Figure 3.19 Real-time PCR analysis of DNA extracted from the ribosome inactivating protein producing plants *R. communis, V. album* and *A. precatoris*, using ricin specific PCR primers and probes. An increase in fluorescence was observed with the *R. communis* sample.

3.5.2 **Purification of Ricin by solvent extraction and Gel Filtration**

Ricin was extracted from castor beans by a colleague at Dstl using solvent extraction and gel filtration. Briefly, castor beans soaked in buffer were homogenised in a Waring blender and then acidified using glacial acetic acid. Plant seed debris and castor oil were removed by centrifugation and the supernatant was clarified using petroleum ether. The aqueous phase was drained, filtered and then acid precipitated overnight. The precipitate was collected by centrifugation and residual ammonium sulphate was removed by dialysis. The dialysed extract containing the toxin was filtered and purified on a sepharose-4B affinity chromatography column. Unbound proteins were washed from the column in a salt solution and the toxin (and agglutinin) were eluted with salt and galactose. The ricin and agglutinin in the concentrated pool were then separated by gel filtration chromatography again using a Sephadex 200 column.

A standard curve was constructed for Q-PCR analysis using preproricin cDNA kindly donated by a colleague at Dstl, as genomic DNA was not available at the start of the project. The amount of DNA present in each sample is therefore better presented in this instance as copies/µl. The gene is approximately 2362 basepairs in length (RCRICIN2 locus, Genbank accession number X52908) therefore the quantity of DNA calculated to be present in sample 1 (Table 3.10) is equivalent to 11.5 copies [(0.00000003 ng * 10^{-9})/2362 bp x 650) * 6.022 x 10^{23}]. Accordingly, 1.15 copies/µl would weigh 3 attograms. The results in Figure 3.20 show that a low concentration of DNA was present in the initial stages of the purification process. Quantitative-PCR results are given in Table 3.10. The bean soak (sample 1) contained only 11.5 copies of the gene per microlitre of sample. The bean homogenate pellet (sample 3) that was discarded was calculated to contain 61 copies per microlitre, indicating that

homogenisation released DNA. The majority of the DNA within the acidified homogenate was removed with the pellet (sample 5) and DNA was detected in the solvent (sample 6) but not aqueous phase (sample 7) of the petroleum ether extracts. DNA was not detected in further samples of the purification process.

Table 3.10 Mean DNA concentrations observed in samples taken during the 13 step ricin prurification process, as determined by quantitative PCR analysis of duplicate samples.

Sample	Sample Type	Buffer	Q-PCR estimated DNA concentration
1 Bean soak	Bean Soak	0.5 M NaCl	0.03 fg/µl
2 Bean homogenate	Supernatant	0.5 M NaCl	0.175 fg/µl
3 Bean homogenate	Pellet	0.5 M NaCl + acetic acid pH 4	0.185 fg/µl
4 Acidified bean homogenate	Post incubation supernatant	0.5 M NaCl+ acetic acid pH 4	0.0002 fg/µl
5 Acidified bean homogenate after incubation	Post incubation supernatant pellet	0.5 M NaCl+ acetic acid pH 4	0.4 fg/µl
6 Lipid extract	Solvent phase	Petroleum ether	0.375 fg/µl
7 Lipid extraction	Aqueous phase	0.5 M NaCl + acetic acid pH 4	Not detectable
8 Post dialysis		0.5 M NaCl	Not detectable
9 Affinity sepharose column	Flow	0.5 M NaCl	Not detectable
10 Affinity sepharose column	Wash	0.5 M NaCl + 0.2 M galactose	Not detectable
11 Affinity sepharose column	Eluate	0.5 M NaCl + 0.2 M galactose	Not detectable
12 Gel filtration	Ricin peak	0.5 M NaCl + 0.2 M galactose	Not detectable
13 Gel filtration	Agglutinin peak.	0.5 M NaCl	Not detectable

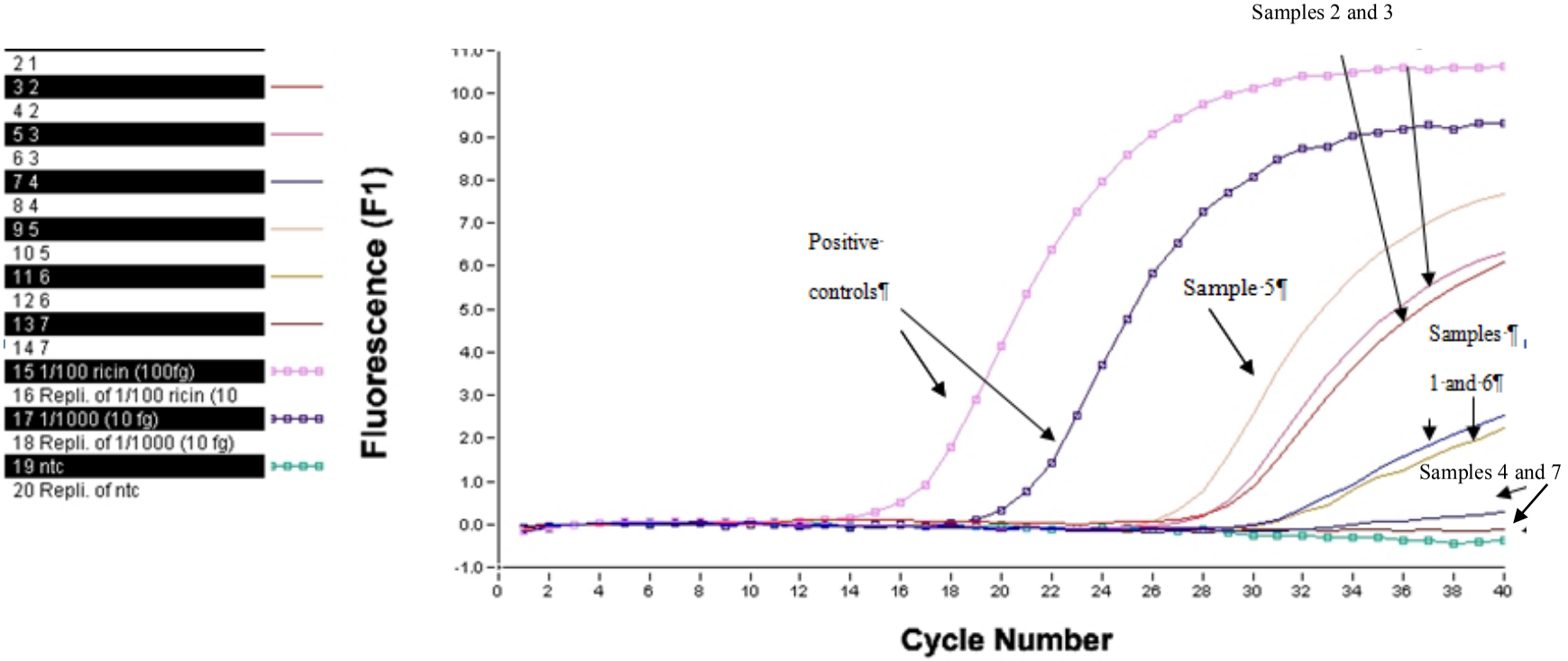


Figure 3.20 Amplification of the ricin gene from samples taken during the ricin purification process. Samples 1 to 6 taken throughout the ricin purification process were amplified. Sample 7 and onwards, purified by dialysis, affinity separation and gel purification were not amplified

3.6 *CLOSTRIDIUM PERFRINGENS* PHOSPHOLIPASE C

3.6.1 *plc* assay design

Eighteen *Clostridium perfringens* alpha toxin gene sequences were obtained from Genbank and aligned using SeqMan software. Sequence identity was high (98 - 99 %) between the sequences indicating that the gene is highly conserved between strains of the same species. Several other bacteria produce phospholipases C, including other clostridial species and *L. monocytogenes* (Titball, 1993), consequently, the potential for cross-reaction with non-target species was high. Therefore, sequences of *C. bifermentans* and C. *novyi plc* homologues were also obtained from Genbank. The inclusion of homologous *plc* genes introduced a number of gaps into the consensus sequence, with the overall homology reduced to 40% and the highest number of mismatches found towards the 3' end. The consensus sequence was exported to Primer Express, however it was not possible to identify suitable primer sequences in the region of divergence due to the low GC content of the Clostridium genome. Increasing the desirable amplicon length from 100 to 250 bp permitted the software to identify regions of the gene suitable for use as primer and probe sites.

The PCR assay was tested and optimised using DNA from NCTC 8237 extracted at Dstl prior to the onset of this study. The reaction did not proceed well using a standard two-stage real time PCR thermal cycling profile (40 cycles of 95 ^{o}C for 1 second and 60 ^{o}C 30 seconds) and required a long extension phase, possibly due to the assay requiring further optimisation due to low GC content, although this

possibility was not investigated at the time of study. The optimal thermal cycling profile and reaction components are summarised in Table 3.12.

Cross reactivity analysis was carried out using the *plc* primers and DNA from near neighbours of *C. perfringens* (Figure 3.21). In spite of the PCR primers and probe targeting a region of poor homology to *C. novyi* and *C. bifermentans* the assay was demonstrated to cross react with *C. novyi* and *C. histolyticum*, with low levels of amplification being observed (Figure 3.21). At the time of primer design, the sequence of the phospholipase C gene from *C. histolyticum* was unavailable, therefore it was not possible to redesign the assay to a region of lower homology. Additionally, the cross reaction with the *C. novyi plc* gene was undesirable. It was not possible to eliminate the cross reaction, however amplification of *C. novyi* DNA was not efficient. Consequently, the assay could be redesigned to amplify the *C. novyi* and C. *histolyticum plc* gene with higher efficiency, intentionally making the assay more generic in future work.

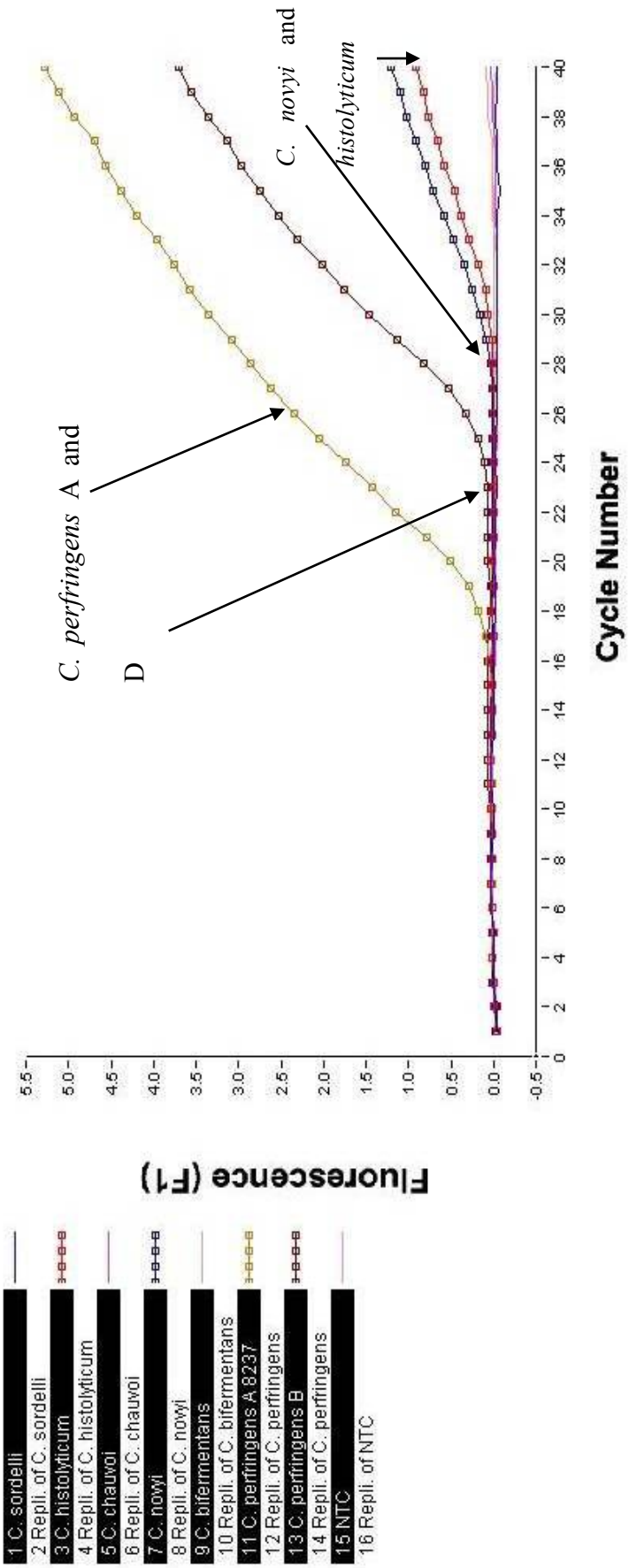


Figure 3.21. Real time PCR analysis of Clostridial DNA samples using primers specific for the *plc* gene. The increase in fluorescence is indicative of amplification of the *plc* gene product in *C. perfringens A* and *D*, plus *C. novyi* and *C. histolyticum*.

3.6.2 **Purification of Alpha Toxin using Ion Exchange Chromatography and gel filtration**

Alpha toxin was purified as a recombinant protein from *E. coli* cell lysates during the 22-step process outlined in Table 3.11 by Dr Helen Jones at Dstl. Q-PCR analysis was carried out on samples taken at each stage in the process, also detailed in Table 3.11. Briefly, cells were harvested by centrifugation and resuspended in Tris buffer containing a protease. The cells were lysed by sonication and removed by centrifugation. The supernatant was filtered through a 0.2 μM membrane to remove particulates; alpha toxin was bound to a HiTrap™ Q ion exchange exchange column (sample 1). Adsorbed toxin and DNA were eluted in a continuous ionic strength NaCl gradient ranging from 0 to 100% (samples 2 to 5). Sample 2 was discarded and samples 3 to 5 were pooled. Sample 2 contained 1 μg of DNA whilst samples 3 to 5 contained picogram levels. Therefore a high concentration of DNA was removed from the toxin preparation at an early stage in the purification process. The toxin was subsequently loaded onto a Mono Q HR anion exchange column and eluted using a NaCl gradient (samples 9 to 20). Each of these samples contained picogram or negligible quantities of DNA. The toxin was then concentrated using a YM 100 centricon column, filtered again and further purified by gel filtration (samples 21 and 22). Neither of the gel filtered samples contained detectable toxin DNA, therefore they were subjected to a Qiagen DNA extraction procedure (samples 23 and 24). Q-PCR analysis was carried out on sample 23 using DNA standards of defined concentrations (Figure 3.22). The software calculated 23 pg/μl of DNA was present in the Qiagen extracted DNA from the first toxin sample, however the second gel filtration eluate did not contain amplifiable DNA.

Table 3.11 Mean DNA concentrations present in duplicate samples taken during the alpha toxin purification process, as determined by quantitative PCR using a standard curve (Figure 3.22).

Sample	Sample Type	DNA concentration
1 Ion Exchange (IEX); strong anion	Sonicated supernatant loaded on Mono Q column	1.008 ng/µl
2 IEX Hi trap Q	fraction 32	1000 ng/µl
3 IEX Hi trap Q	fraction 33	167 pg/µl
4 IEX Hi trap Q	fraction34	172 pg/µl
5 IEX Hi trap Q	fraction 35	162 pg/µl
6 IEX Hi trap Q	fraction 33, 34,35 pooled before dialysis	-
7	Fraction 32 dialysed in 20 mM Tris	140 pg/µl
8	Pooled fractions dialysed	130 pg/µl
9 IEX Mono Q	IEX run 1, 13	0
10 IEX Mono Q	IEX run 2, 21	0.6 pg/µl
11 IEX Mono Q	IEX run 3, 18	0.45 pg/µl
12 IEX Mono Q	IEX run 4, 12	0.5 pg/µl
13 IEX Mono Q	IEX run 4, 11	0
14 IEX Mono Q	IEX run 2, 20	0
15 IEX Mono Q	IEX run 2, 19	0
16 IEX Mono Q	IEX run 3, 15	0
17 IEX Mono Q	IEX run 1, 14	0
18 IEX Mono Q	IEX run 3, 17	0.45 pg/µl
19 IEX Mono Q	IEX pool 1	0.8pg/µl
20 IEX Mono Q	IEX pool 2	0
21: Gel filtration Superdex 200 column	fraction 1 (Pure α toxin 2.8 mg/ml)	0
22: Gel filtration Superdex 200 column	fraction 2 (Pure α toxin 1.1 mg/ml)	0
23:Qiagen DNA extraction kit	Extracted α toxin 1.1 mg/ml	23 pg/µl (10 µl sample)
24:Qiagen DNA extraction kit	Extracted α toxin 2.8 mg/ml	0

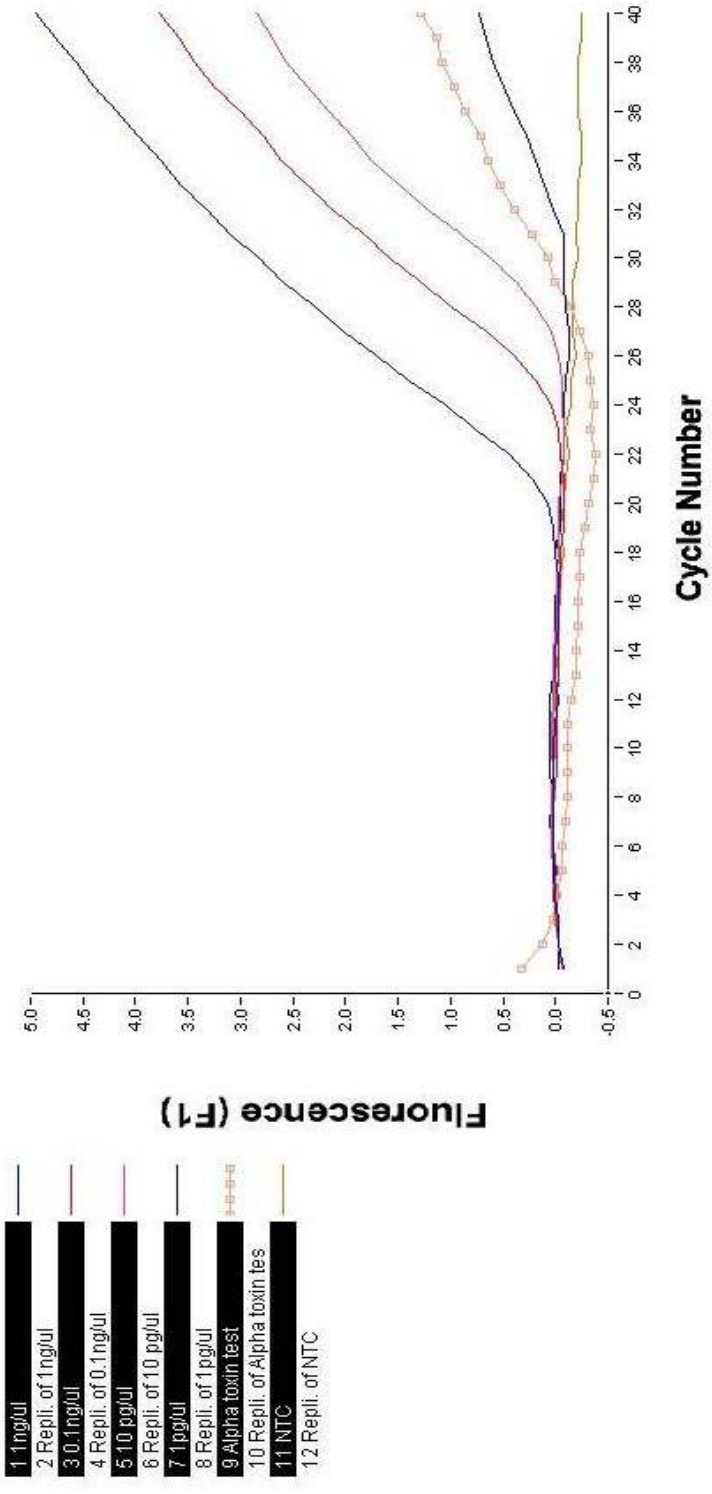


Figure 3.22. Quantitative PCR analysis of purified alpha toxin with the *plc* primer and probe set. DNA standards are shown in unbroken lines, the alpha toxin sample is shown in the hatched line. Samples were run in duplicate, however only single samples are shown for illustrative purposes.

3.7 DETECTION OF SIGMA TOXINS

It was not possible to produce and purify Staphylococcal enterotoxin B for analysis at Dstl during this study. However, commercially produced toxins were available. Therefore, SEB, ricin and four types of phospholipase C were obtained from Sigma and analysed by Q-PCR.

3.7.1 *Staphylococcus aureus* assay development

Numerous *Staphylococcus aureus* enterotoxin gene sequences were obtained from NCBI of which two encoded SEB. Distinct contigs were successfully formed for each toxin type with homology between toxin types reportedly 57 % or less. Consequently, the PCR primer and probe set designed using the derived SEB consensus sequence was predicted to be specific for SEB.

The SEB assay was demonstrated to work with standard TaqMan® thermal cycling conditions once optimised for magnesium ion concentration (Table 3.12). The results in Figure 3.23 show that the *seb* PCR assay was specific and did not detect the non-toxigenic strains obtained from Oxoid (ATCC strains 6538, 25923, 33862, 9144 and 23144). More importantly, it did not detect strains that produce other staphylococcal enterotoxins (NCTC 10652 and 10656 producing SEA and SED respectively), although the test panel used in this study was limited.

3.7.2 Detection of Sigma SEB

SEB was obtained from Sigma as a lyophilysed powder, purified using a proprietory process. The toxin was reconstituted to a concentration of 3.5 mg/ml. The purification process used to extract SEB was proprietory to Sigma. A small sample

(2 μl) was analysed by PCR but no amplification was observed (Figure 3.23), indicating that the concentration of DNA in the preparation was low. A 200 μl sample of toxin was subjected to a Qiagen DNA extraction to concentrate and purify the sample. Again, no amplification was observed.

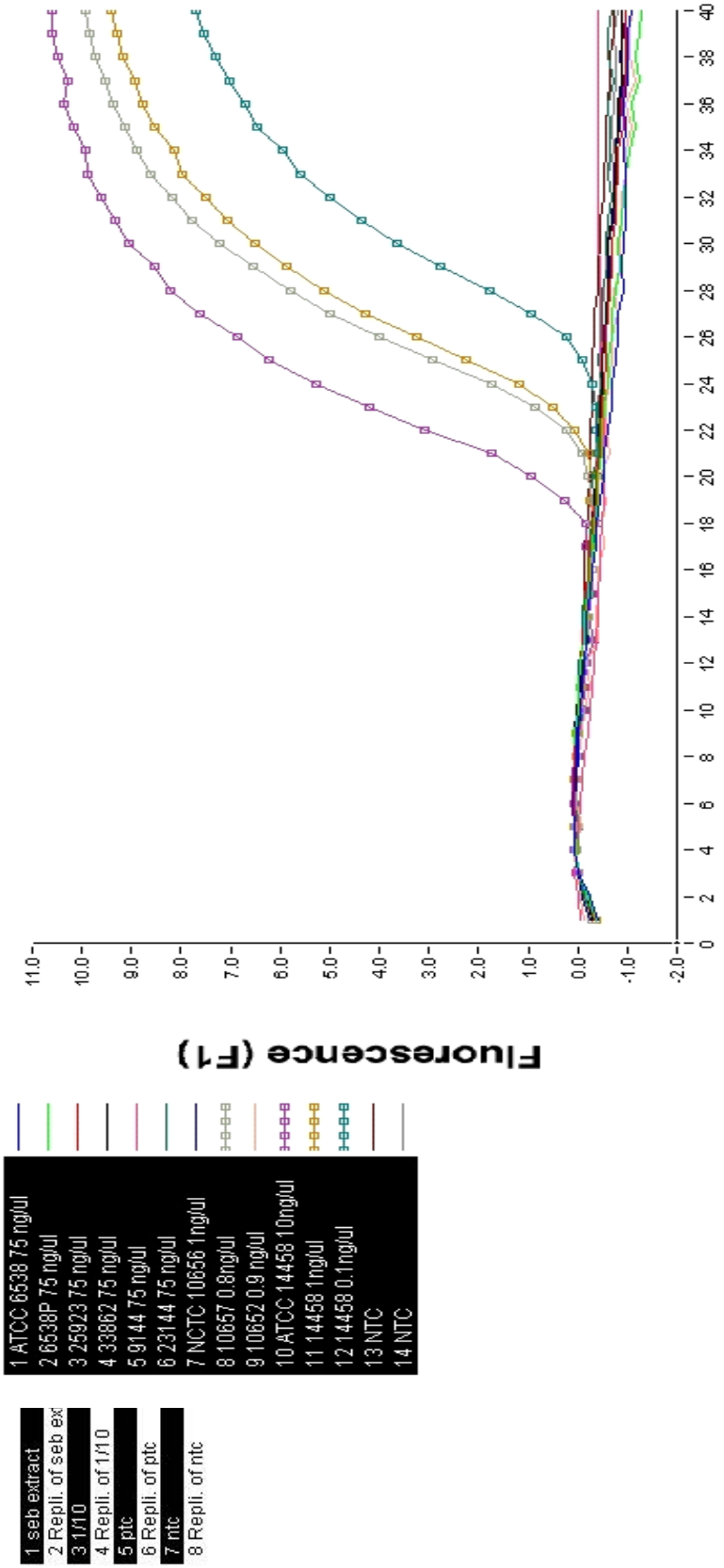


Figure 3.23 Real-time PCR analysis of SEB toxin obtained from Sigma using the *seb* Taqman® primer and probe set. DNA was extracted from an SEB sample at a concentration of 3.5mg/ml using a QIAamp kit 2 µl of DNA was then tested by PCR. An increase in fluorescence was seen with the positive template control sample containing *S. aureus* NCTC 14458 DNA.

3.7.3 Detection of Sigma *Ricinus communis* aggutinin (RCA_{60})

Ricin toxin, bought commercially in 2001 from Sigma, was available in two formats, either with or without the agglutinin subunit (RCA_{120} and RCA_{60} respectively). The exact process used to purify the toxin at Sigma was not known although it was stated to be 'affinity purified, electrophoretically pure RCA_{60}'. It was therefore assumed that affinity chromatography was used in the same way as at Dstl, exploiting the lectin property of the toxin to bind it to sepharose media. Only a small amount of toxin was sampled, in accordance with Dstl safety regulations. A 7.8 μg sample was reconstituted in 200 μl dH_2O and 2 μl analysed by PCR. No DNA was detectable in the untreated sample, as was the case with the toxin purified at Dstl. The rest of the sample was then subjected to a Qiagen DNA extraction procedure and eluted in the smallest volume permissible by the manufacturer, 50 μl. However, no DNA was detected in the treated sample.

3.7.4 Detection of Sigma phospholipase C (alpha toxin)

Four types of phospholipase C from *C. perfringens* were obtained from Sigma, UK. The crudest preparation, type I, was supplied without additional information but was presumably extracted and lyophilised. The toxin was reconstituted at a concentration of 3.5 mg/ml and analysed by *plc* PCR. DNA was not detectable from the neat sample due to inhibition of the reaction, but the 1/10 dilution was estimated to contain 3.24 pg/μl, a relatively high DNA concentration compared to other toxins sampled previously. Thus, it was not necessary to extract DNA from the toxin sample in order to obtain a positive PCR signal.

The second toxin sample studied was an aseptically filtered derivative of type I, type I-S. This was processed and sampled in the same way as type I and found to contain significant amounts of DNA, 3 ng/μl.

Type IX was partially purified before lyophilisation and as a result DNA was not detectable, either in the initial preparation or the DNA extracted sample. Several PCR reactions were carried out with this sample in an attempt to determine whether it contained any DNA. However, abnormal fluorescence readings were obtained on the Lightcycler, masking the real-time PCR results (not shown). Agarose gel electrophoresis confirmed that weak amplification had occurred, therefore a small amount of DNA was present in the toxin. However, Q-PCR analysis was not possible as a result of the spurious fluorescence readings. It is thought that the high fluorescence readings were due to the presence of a fluorescent amino acid such as tryptophan (348 nm), tyrosine (303 nm) or phenylalanine (282 nm), however the cause is unknown and has not been observed with other samples.

Phospholipase C type XIV was chromatographically purified using an undefined method and DNA was not detectable by PCR either in its native or DNA extracted state.

In summary, the commercially available toxins purified chromatographically or or electrophoretically were not detectable by PCR. Toxins that were less pure were detectable.

In order for PCR to be used as a toxin screening method a large number of assays must be run. One way of reducing this logistic burden would be to combine assays

and perform multiplex analysis. With this in mind, multiplex PCR was carried out using *C. botulinum* A and B DNA.

3.8 MULTIPLEX PCR

Most of the singleplex assays developed in this study can be run using the same thermal cycling parameters. A sample can therefore be tested rapidly for the presence of SEB, ricin and possibly botulinum neurotoxins A and F at once by using all four singleplex assays on one machine. However, some of the Clostridiae assays have a lower annealing temperature and longer amplicon than most assays due to their low GC content. Therefore, botulinum neurotoxins B and F require another protocol utilising a lower annealing temperature. *C. perfringens* α toxin requires a third protocol which makes use of a low annealing temperature combined with a low extension temperature. Although inconvenient, this should not present a problem providing the real-time PCR machine used is able to provide the necessary flexibility. However, differences in thermal cycling parameters between assays may prevent multiplexing of certain assays. Nevertheless, multiplex assays would reduce the assay costs and setup time, making PCR more suitable for routine use.

The *C. botulinum* A and B assays were chosen for use in a multiplex PCR experiment. It was postulated that achieving sensitive detection of both toxin genes would be challenging, since their thermal cycling profiles differ. Experiments were carried out on the Cepheid Smartcycler® and Corbett Rotorgene machines as it was not possible to perform multiplex PCR on the Roche Lightcycler using TaqMan® chemistry. The *C. botulinum* B TaqMan ® probe was labelled with 5' FAM and 3'

BHQ. The *C. botulinum* A assay was also quenched with BHQ but labelled with ROX at the 5' end. Internal controls for types A and B were labelled with CY5 and CY3 respectively.

Initially the sensitivity and efficiency of the *C. botulinum* A assay (Figure 3.24) prevented amplification of low copy numbers of the *C. botulinum* B gene (Figure 3.25), with the detection limit being increased from 1 fg/µl to 0.1 ng/µl. However, when optimal ratios of primers and PCR reaction components were used, the detection limit for both assays was 1fg/µl (Figures 3.26 and 3.27). *Taq* polymerase (5U) was added to the multiplex reaction, compared to 0.8U per singleplex assay. The concentrations of dNTPs and magnesium chloride were also increased, from 200 to 600 nM and 3 to 8 mM, respectively. The concentration of each *bont/A* primer was reduced from 500 nM to 50 nM without affecting the sensitivity of the assay. The concentration of *bont/B* primers remained unchanged, at 500 nM.

Quadruplex PCR was then used to amplify both *bont* genes with their respective internal controls, a composite DNA comprising a portion of the human β actin flanked by the *bont* primers. Using the dye regimes described previously, there was no cross-talk between channels and all four targets were amplified simultaneously (data not shown).

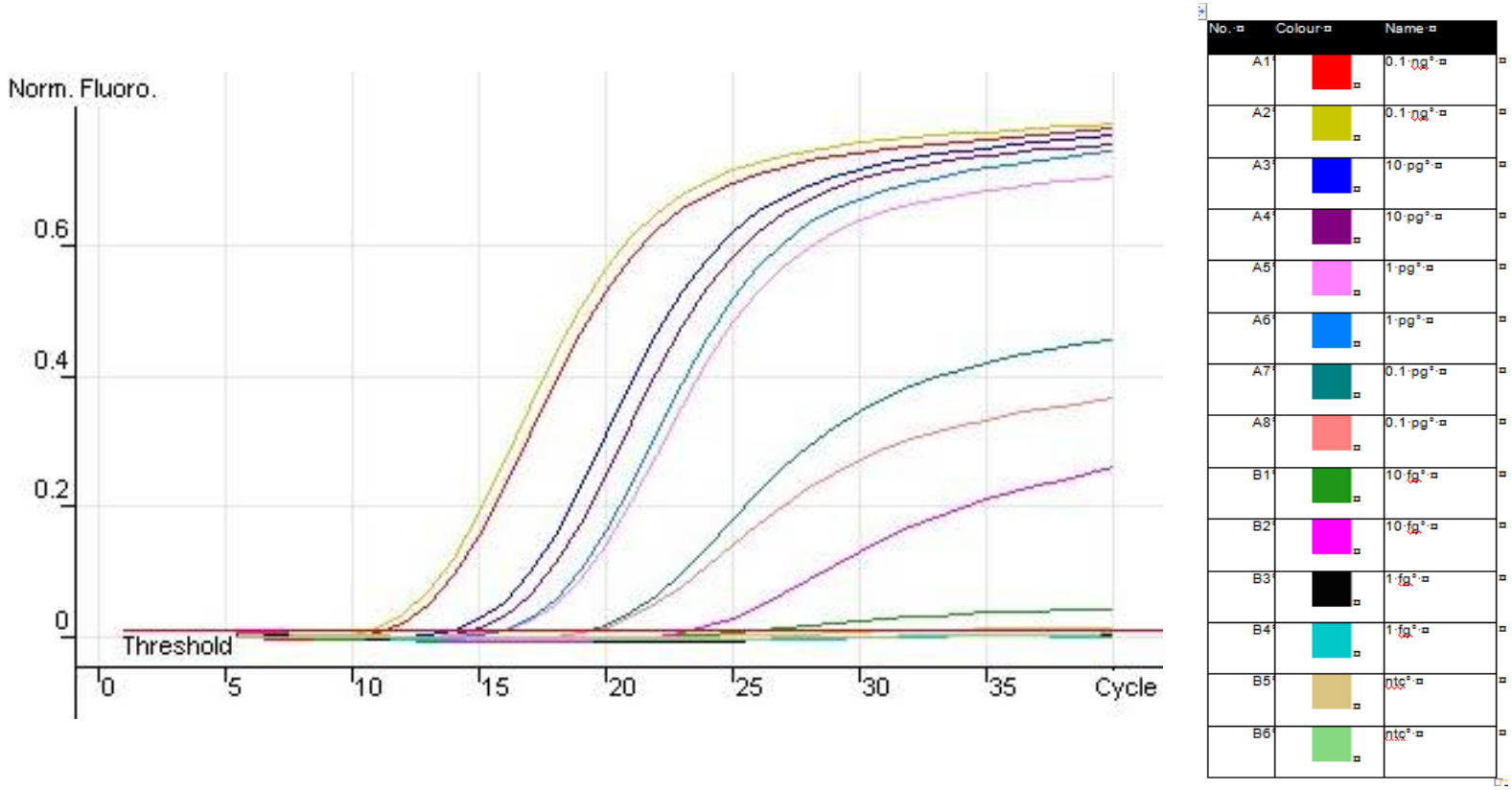


Figure 3.24 Detection limit of un-optimised *C. botulinum* A DNA in a multiplexed A/B PCR assay. DNA standards were prepared from *C. botulinum* A NCTC 2916 and amplified with the *bont* A/B multiplex using 500nm of each type A primer. An increase in fluorescence was observed with DNA concentrations between 0.1 ng/µl and 10fg/µl

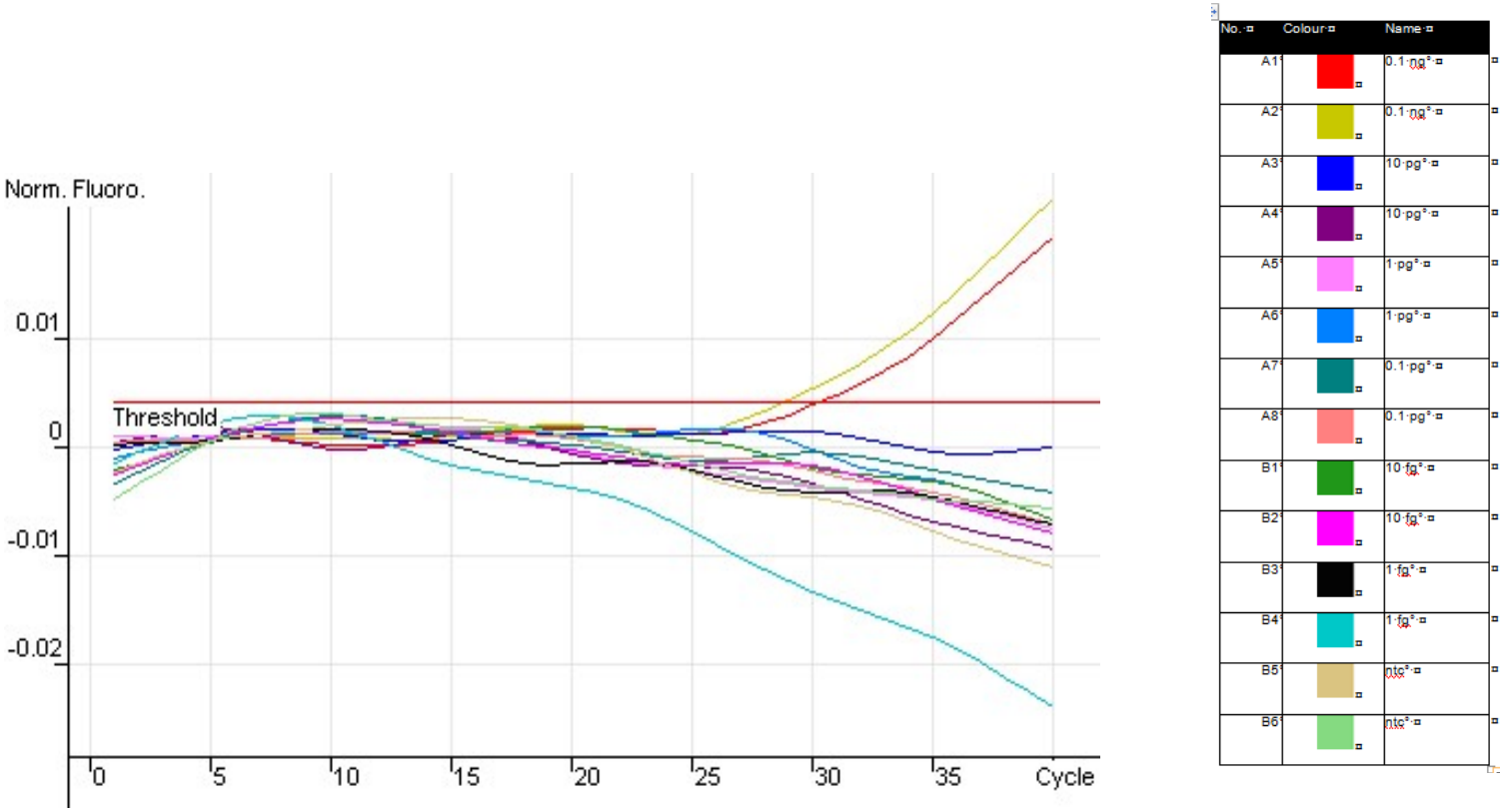


Figure 3.25 Detection limit of un-optimised *C. botulinum* B DNA in a multiplexed A/B PCR assay. DNA standards were prepared from *C. botulinum* A NCTC 2916 and amplified with the *bont* A/B multiplex using 500nm of each type B primer. An increase in fluorescence was observed with DNA concentrations between 0.1 ng/µl and 10fg/µl

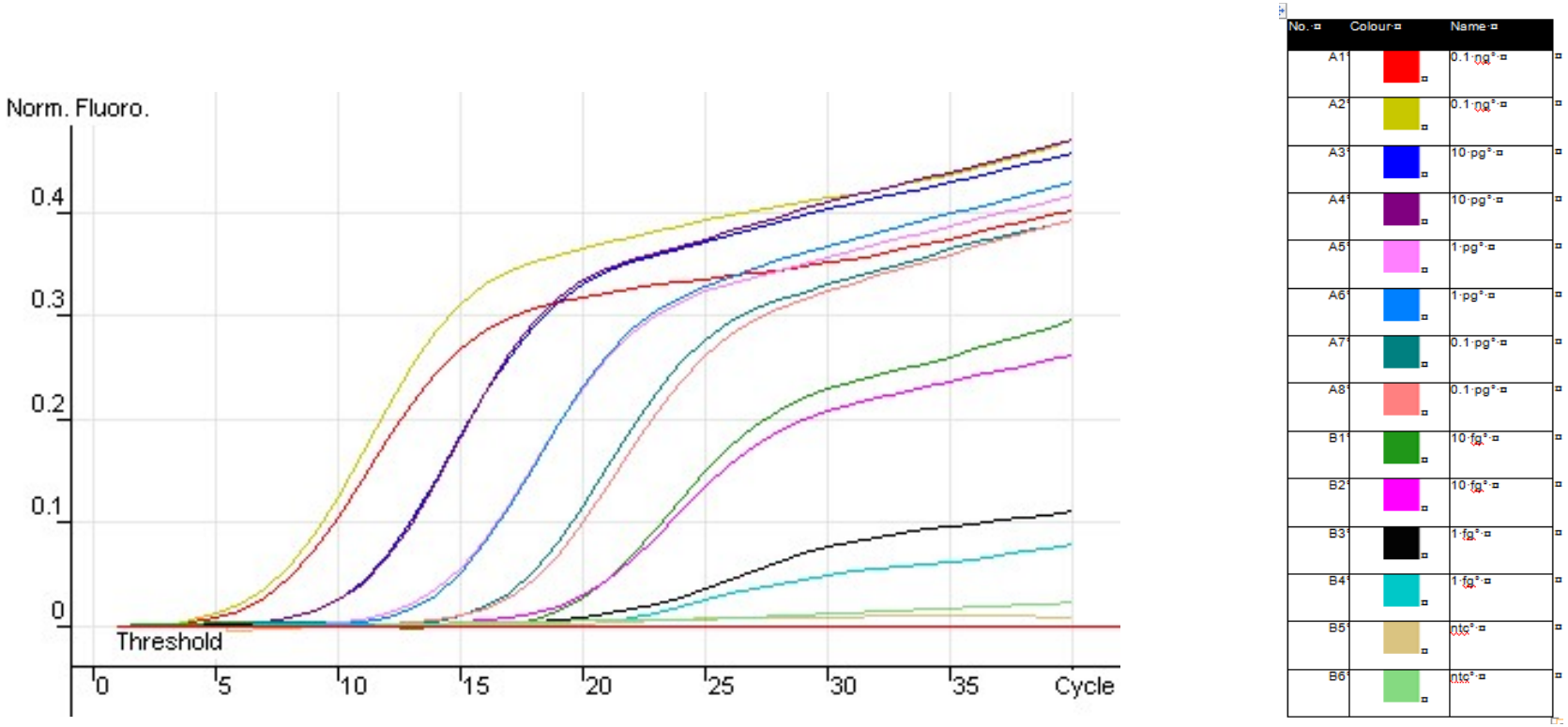


Figure 3.26 Detection limit of *C. botulinum* A DNA in an optimised multiplexed A/B PCR assay.
DNA standards were prepared from *C. botulinum* A NCTC 2916 and amplified with the bont A/B multiplex using 50nm of each type A primer, with increased amounts of *Taq* polymerase, dNTPs and $MgCl_2$. An increase in fluorescence was observed with DNA concentrations between 0.1 ng/µl and 10fg/µl.

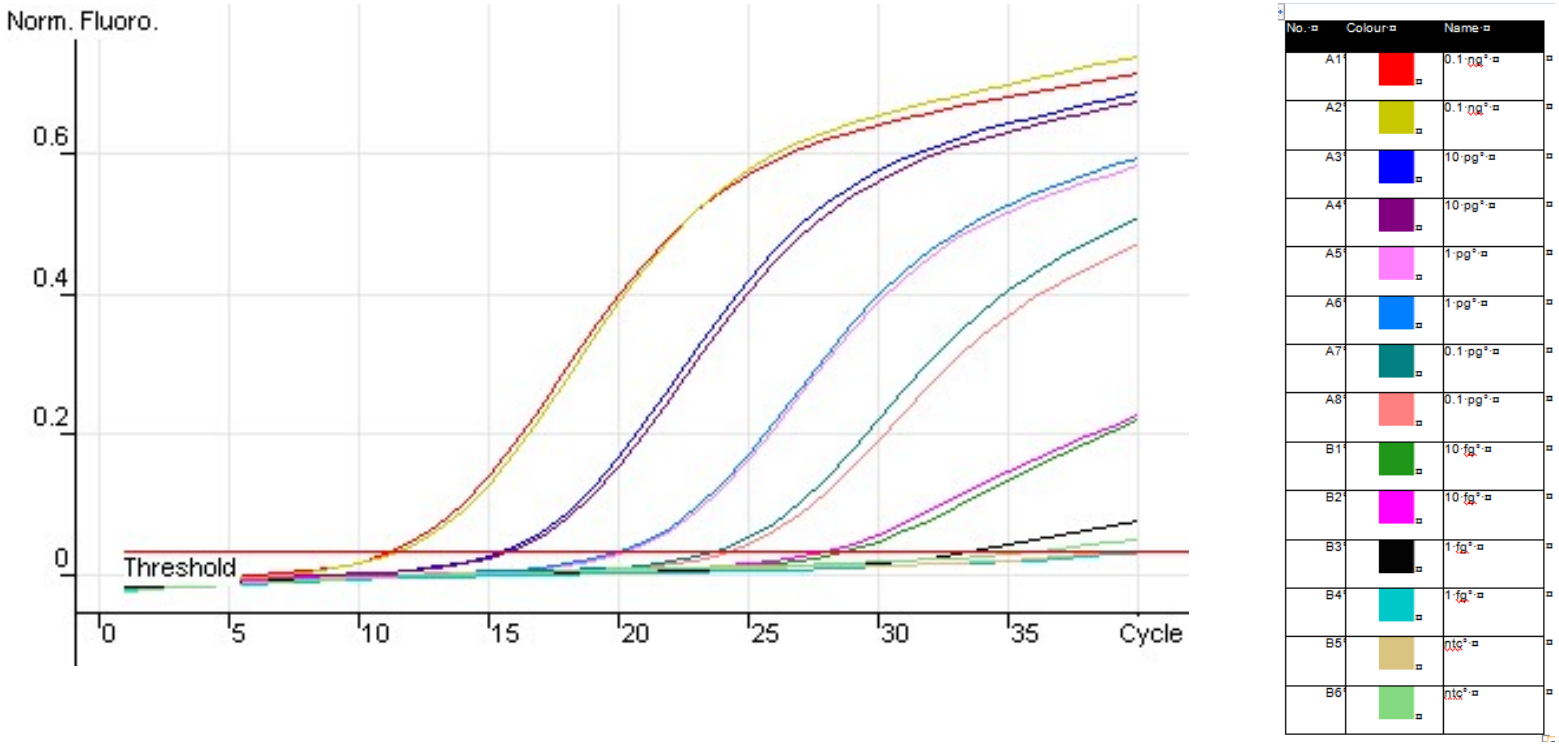


Figure 3.27. Detection lit of *C. botulinum* B DNA in an optimised multiplexed A/B PCR assay. DNA standards were prepared from *C. botulinum* B ATCC 17841 and amplified with the *bont* A/B multiplex using 500nm of each type B primer with 50 nM of type A primer. Extra *Taq* polymerase, dNTPs and $MgCl_2$ were also added to the multiplex assay. An increase in fluorescence was observed with DNA concentrations between 0.1 ng/µl and 1fg/µl.

3.9 CHAPTER SUMMARY

The PCR primer and probe sequences and assay run conditions used in this chapter are detailed in Table 3.12. A summary of PCR toxin detection results are given in Table 3.13. In general terms toxins prepared crudely using basic precipitation protocols were detectable by PCR. Only four toxins were detectable following DNA extraction treatment of the purified toxin, eight samples did not contain sufficient DNA to allow PCR detection.

Table 3.12. Summary of the polymerase chain reaction assays used in this study.

***perf1* demonstrated cross reactivity with *C. histolyticum, C. novyi* and *C. bifermentans*. *perf2* cross reacted with *C. histolyticum*.**

Primer set and target gene	Primer sequences (F and R)	TaqMan® probe sequence and concentration		Mg 2+	PCR Conditions
Bot A *bont/A*	F:TTTGAGGAGTC ACTTGAAG R:TTACTGCTGGA TCTGTAGC	CCTCTTTTAGGTGC AGGCAAA 0.3 µM		3	95 °C 0 sec 55 °C 30 sec, 72 °C 30 sec
Bot B *bont/B*	F: CGTTTCTACGAA TCTGG R: TTCAGTCCACCCT TCAT	TTGGGATGTAATT GGCAGTTT 0.3 µM		3	95 °C 0 sec 50 °C 30 sec 72 °C 30 sec
Bot E2 *bont/E*	F: TTAACTTTTGGA GGTACTGATT R: TATAAGGATTAA GTAGTGGATTAG ATAC	ACTAGTGCTCAGT CCAATGATATCTA TACTAATCTTCTAG 0.3 µM		4	95 °C 0 sec 50°C 30 sec 72°C 30 sec
Bot F *bont/F*	F:TGGATAATTCC TGAGAGAA R: GCACTGCTTCCG TTC	TTTTGAT CCACCG GCTTC	0.4 µM	3	95 °C 0 sec 55 °C 30 sec 72 °C 30 sec

Perf1 *plc**	F: AGTGCAAGTGTT AATCGTTATCA R: ACCAAGTAAAAA CCTGTCTA	CATCAACTAAAGT CTACGCTTGGG 0.3 µM	4	95 °C 0 sec 55 °C 30 secs, 65 °C 60s
Perf2* *plc*	F: AGTGCAAGTGTT AATCGTTATCA R: TGGACAGATCAT TTTCTAAGAT	AATGAAAAGAAAG ATTTGTAAGGCGC T 0.3 µM	4	95 °C 0 sec 55 °C 30 secs, 65 °C 60s
Ord *ord*	F: CAGTCGGTTCGG CAGTTTTC R: GGTCTTCTCACAT ATCGGGAAGA	CACATCTTCTCGCC AGGAGAGTGGAGT G 0.3 µM	4	95 °C 0 sec 60°C 30 sec
Ricin *rta*	F: GACTGTAGCAGT GAAAAGGCTGAA R: CTCGGTTTGCTG AGGACGT	AACAGTGGGCTCT TATGCAGATGGTT CAA 0.3 µM	3	95 °C 0 sec 60°C 30 sec
SEB *seb*	F: TGAATTTAACAA CTCGCCTTATGA A	AAATGAGAATAGC TTTTGGTATGACAT GATGCCTG 0.2 µM	5	95 °C 0 sec 60°C 30 sec

	R: AATCAACCATTTTATTGTCATTGTACATC			
Ntnh *ntnh*	F: CTCCTGGACCAAAAATAAC	AATTCAATTTTTTATTAGATTTTGGTGCT	N/A	
	R:ATCTTTGATTTCTACAGCTATTC			
rV *lcrV*	F: CGCCGAATACACAATGGGA	TGCGGGCGTTCATGGCAGTAATG3	95 °C 0 sec 60°C 30 sec	
	R: TACGATCGGCGGTTAAAGAGA			
Bot E1(cross reacted with F)	F: AATATTGTTTCTGTAAAAGGCATAAGGAA	Not used ND	ND	
	R: AAGTTACTGTATCGTCAATTTCTTTAGGAG			

Table 3.13 General Summary of Q-PCR Toxin Detection Results

Protein	Protein Conc	Purification method	PCR result Protein sample	PCR result: DNA extracted from protein sample
Clostridium botulinum neurotoxin A	Not determined	Acid Precipitation	N/A	Positive
Clostridium botulinum neurotoxin B	Not determined	Acid Precipitation	N/A	Positive
Clostridium botulinum neurotoxin E	Not determined	Acid Precipitation	N/A	Positive
Recombinant V antigen	Not determined	11 stage affinity chromatography method	Negative	Positive (15.2ng/µl)
Alpha toxin in house fraction 1	2.8 mg/ml	18 step ion exchange/gel filtration protocol	Negative	Negative
Alpha toxin fraction 2	1.1 mg/ml		Negative	23 pg/µl
Ricin	Not determined	Affinity chromatography	Negative	Negative
Sigma Alpha toxin Type I – crude prep	3.5 mg/ml	Not known	Positive (32.4 pg/µl)	Negative
Sigma Alpha toxin Type I-S – purified from type I	3.5 mg/ml	Not known	Positive (3ng/µl)	Negative
Sigma Alpha toxin Type IX – partially purified	3.5 mg/ml	Not known	Negative	Very low, not quantifiable using Q-PCR
Sigma Alpha toxin Type XIV highly purified		Chromatography	Negative	Negative
Sigma Ricin	7.8 µg	Affinity chromatography.	Negative	Negative
Sigma SEB	3.5 mg/ml	Unknown	Negative	Negative

3.10 DISCUSSION

PCR was used successfully to detect toxin genes from residual DNA in toxin preparations, with high sensitivity and specificity, with the exception of the *Clostridium botulinum* DNA samples. A number of the *C. botulinum* B DNA extractions may have failed or were of poor quality, although this was unlikely since favourable spectrophotometry results at $A_{260/280}$ were obtained. *C. botulinum* characteristically produces high levels of DNases, which could cause the DNA to degrade quickly, although this is not generally thought to cause a problem with PCR amplification of short products (Keto-Timonen *et al.,* 2005). The neurotoxin gene is carried chromosomally on an integrated prophage which may be susceptible to instability. However, the most likely theory was that the bacteria had mutated and lost their virulence through years of subculturing. To corroborate this theory, further analysis of DNA obtained from the original type B stock cultures was obtained. PCR was successful with all but one strain, strongly suggesting that a lack of selective pressure caused the toxin genes to be lost or mutated within the bacterial population. It should also be noted that the *bont/b* PCR results were inconsistent and it was not possible to reproduce many of the results (hence the need for individual results in Figures). Although it is assumed that toxins can be produced with similar ease from group I and II strains, it would have been desirable to test toxins from both types of B strain, however it was not possible using the culture stocks held in house. A negative mouse lethality or ELISA test performed with fresh culture supernatant would confirm the lack of toxin. However, it was not possible to test this theory during this study as funding for antibody or animal work was unavailable. Furthermore, as the utility of toxin detection by PCR was in question following the

results obtained in this study, the *Clostridium botulinum* PCR assays were not investigated further.

In order to complement the majority of gene probe assays used in this study, a generic *bont* assay would have been highly desirable. It was not possible to design such an assay using the parameters used in this study, however it should be noted that several research groups have more recently designed generic *bont* real time PCR assays, including De Medici *et al.,* (2009), Satterfield *et al.,* (2010), Hill *et al.,* (2010), and Kirchner *et al.,* (2010). These researchers have encountered problems similar to those identified in this study, such as poor homology between strains and low GC content. However, they have used elegant solutions to the problem. Kirchner *et al.,* (2010) used MGB probes which are similar to TaqMan® probes but use a unique minor groove binding (MGB) chemistry supplied by Applied Biosystems that allows short probes (that would have a low melting temperature if used with standard TaqMan® chemistry) to be used in place of the usual 20 bp probe. Hill *et al.,* (2010) used degenerate primers targeting a highly conserved region of the *ntnh* gene to facilitate detection of toxin in types A to G. Both approaches were successful, proving that generic detection is possible.

In the current study BoNTs were prepared using a simple acid precipitation method. PCR analysis showed that DNA was present in the purified toxin samples, indicating that genomic DNA was co-precipitated. Further protein purification such as affinity chromatography or gel filtration was not carried out, limiting the amount of DNA that was removed. The fate of DNA during protein purification processes is not known. It is conceivable that DNA was also precipitated using ammonium sulphate but it seems likely that a number of factors allowed the presence of DNA in the

BoNT samples. Ammonium sulphate may have sufficient ionic strength to precipitate DNA to a degree, however DNA is more commonly precipitated with a combination of acid and alcohol, such as sodium acetate and ethanol. Therefore, it seems most likely that DNA was present in the toxin sample because it was bound electrostatically to the protein. It may also have been free in solution as it would have a high molecular weight if the extraction process had not caused degradation or shearing. Therefore some may have been retained and recovered by the dialysis process. Acid precipitation of *C. botulinum* neurotoxins is thought to have been used as a method of preparing biological weapons for use in the Iraq war (Zilinskas 1997). Therefore, the fact that the toxin was detectable by PCR is of significant importance.

Affinity chromatography was used to purify recombinant V antigen and ricin. Although DNA was removed during each stage of the LcrV purification process, the final protein contained a high concentration of DNA in spite of its intended use as a vaccine candidate. Nanograms of DNA were detected in the pure *lcrV* protein sample. In contrast no DNA was amplified in the ricin sample. It was interesting to note that one of the first steps in clarifying the LcrV protein preparation was to use enzymatic degradation of nucleic acids, although the aim of this step was to reduce viscosity rather than completely remove DNA. Consequently, complete removal of DNA was not achieved and the nuclease-treated sonicated samples contained 90 to 150 pg/µl of DNA. Ricin samples were not nuclease-treated and yet contained less DNA, with no trace being detectable after the initial petroleum ether extraction. In other words, although ricin was affinity purified, it was not the sepharose purification that eliminated DNA from the toxin, but the solvent and salt extraction used initially. This was surprising given the effect of acid precipitation on the DNA concentration of BoNT. The use of petroleum ether was governed by the fact that a major

component of *R. communis* seeds is castor oil which must be removed from a preparation of ricin if it is to be used *in vitro*. Castor oil is insoluble in petroleum ether, hence the formation of a lipid and aqueous phase. The DNA analysis results indicate that all *R. communis* DNA was removed to the lipid layer. If an alternative solvent was used this may not be the case. Affinity purified ricin from Sigma was also not detectable by PCR. Therefore, the results presented for this study show the ease with which it is possible to prepare ricin toxin that is undetectable by PCR, although expertise is required to produce pure toxin.

Alpha toxin was purified using a capture, intermediate, purification and polishing (CIPP) ion exchange chromatography protocol. DNA was detected in a number of samples taken during the process. The first elution from the HiTrap Q column contained a high DNA and low toxin concentration whilst subsequent elutions contained less DNA and more toxin. This is due to the nature of the ion exchange medium used. HiTrap Q is a quarternary ammonium anion exchanger often used at the capture stage of the purification process. As DNA carries a negative charge it is likely to bind to an anion exchange column and be eluted at a different ionic strength and higher pH than the toxin. Another anion exchange column was used for intermediate purification with similar effects on the protein and DNA concentrations. Gel purification was used to 'polish' the toxin. Interestingly, no DNA was detectable by PCR in the major protein eluate from the gel filtration column; only the second eluate had DNA associated with it. This may be because molecules are eluted from a gel filtration column according to size, with the largest molecules being eluted first. Alpha toxin is a 42.5 kDa protein whereas contaminating DNA is expected to be smaller due to severe degradation by DNase I and sonication. Consequently, DNA would be eluted from a gel filtration column after the major protein eluate had been

obtained. Although a significant amount of DNA was lost during each purification step and some of the samples from the final stage of the process did not amplify, contaminating DNA within the pure protein was detectable after sample processing. This was in contrast to the highly pure samples of SEB and alpha toxin obtained from Sigma. Unless the commercially produced toxin were cloned it seems likely that they were purified by a type of ion exchange chromatograpy as the properties of the proteins do not immediately suggest an alternative method (Johansson *et al.,* 2007). In spite of this, the Sigma alpha toxin was not detectable in its purest form, again suggesting that the suitability of PCR as a toxin detection method is entirely dependent on the method used to process the toxin.

A sensitive multiplex PCR assay for the detection of *Clostridium botulinum* A and B was developed from the corresponding singleplex assays. The sensitivity of the multiplex assay was unexpected as there was a 5 degree difference in annealing temperature of the *C. botulinum* A and B primers and probes. Furthermore, this success has not been observed with multiplex assays developed subsequently at Dstl (Mitchell *et al*. 2010). It was a useful technique as it doubled the number of genetic tests that were performed per reaction. This is particularly useful in situations where generic assays are unavailable for a target, as is the case with botulinum neurotoxin. Although real-time multiplex PCR is currently limited by the reporting chemistry and instrumentation, it is expected that this field of science will advance in future. This may permit the detection of all seven *bont* genes in one reaction

In conclusion, many research groups have successfully used PCR to detect toxin genes in food. It is widely accepted as indicative of the presence of toxin. However, previously it was unknown whether PCR would be a useful method for the detection

of toxins on the CDC threat list prepared as biological warfare agents. The results obtained in this study show that expertise is required to remove DNA from samples containing toxin. A number of protein preparations contained enough contaminating DNA to allow detection by PCR unless highly purified by a specialised laboratory. However, the majority of commercially produced toxins sampled were not detected by PCR. The DNA extraction protocol could be further optimised for low copy number detection by the addition of carrier DNA. Additionally, it woud be advantageous to take larger toxin samples for analysis. The use of PCR for detection of toxins needs to be considered carefully, depending on the scenario. The user needs to make an educated guess as to whether a toxin sample is likely to contain DNA. For example, aggressors may not have the laboratory equipment necessary to purify toxins sufficiently to evade PCR detection. Theoretically however, a well-equipped aggressor may have the capability to produce toxins to such a high purity that PCR detection is prevented. Although it is unlikely that an aggressor will remove DNA from a toxin preparation, the prospect exists. For this reason, PCR is not considered a useful approach to toxin detection unless used with an orthogonal technology.

4 IMMUNO-PCR

4.1 INTRODUCTION

The results presented in Chapter Three showed that PCR was not a reliable method of toxin detection if contaminating DNA was present in low quantities. Therefore an alternative nucleic acid approach was required. Immuno-PCR (I-PCR) was investigated as the alternative method as it offers advantages over conventional PCR and ELISA technologies. It was first described by Sano *et al.,* (1992) who used PCR to detect DNA labelled antibody binding in an immunoassay format. The way in which the antibody is labelled with DNA can vary, as described in more detail in Section 4.4. Although the technique is less widely used than PCR or ELISA it offers distinct advantages over both. Unlike PCR, I-PCR is able to detect antigen directly, so results obtained using this method lead to conclusions rather than inferences. In contrast with ELISA, I-PCR offers exponential signal amplification, potentially increasing the sensitivity of the assay (Sano *et al.,* 1992).

4.2 AIMS

The aim of this part of the study was to develop and evaluate I-PCR systems as an alternative to PCR detection of toxins. Firstly, an existing ELISA for the detection of the toxin simulant ovalbumin was converted to an I-PCR format using antibodies labelled with DNA. Secondly, a more novel approach using an anti-*Bacillus cereus* single chain antibody (scFv) in place of a conventional antibody, termed immuno-phage-PCR, was also investigated, since scFv carry intrinsic genetic material that avoids the need for a DNA labelling step. Finally, DNA aptamers were developed for detection of ovalbumin using various methods, as summarised in Table 4.1.

Table 4.1 Summary of immuno-PCR assays developed for this study.

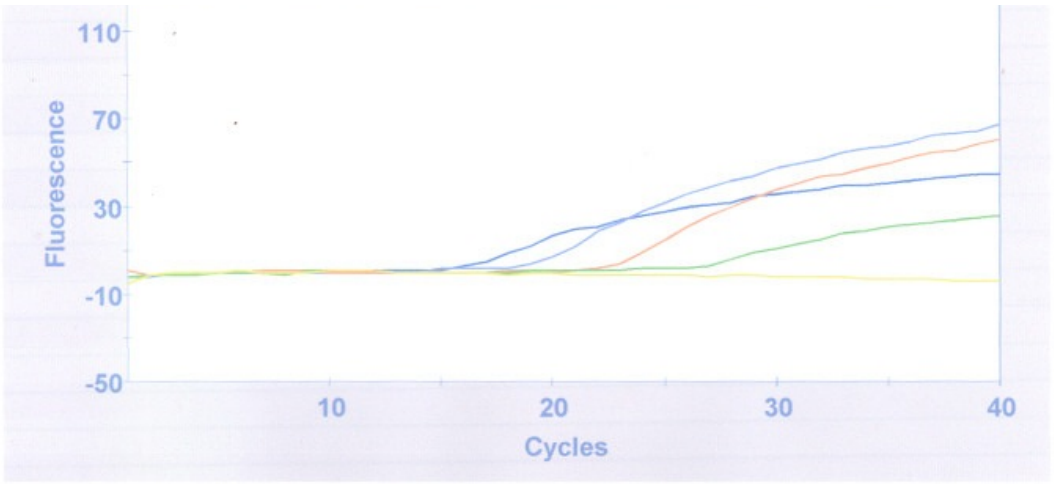
Antigen/Assay	Primary ligand	Secondary ligand	DNA element
Ovalbumin I-PCR	Anti-ovalbumin rabbit polyclonal antibody	Anti-ovalbumin rabbit polyclonal antibody	Biotin labelled amplicon
Ovalbumin I-PCR	Anti-ovalbumin rabbit polyclonal antibody	Anti-ovalbumin rabbit polyclonal antibody	Neutravidin labelled amplicon
Ovalbumin I-PCR	Anti-ovalbumin rabbit polyclonal antibody	Anti-ovalbumin rabbit polyclonal antibody	Covalently labelled secondary antibody
B. cereus spores I-ϕ-PCR	Anti *B. cereus* ScFv antibody	N/A	scFv/M13 phage DNA
Ovalbumin	Aptamer	Anti-ovalbumin rabbit polyclonal antibody	Colourimetric detection of ovalbumin
Ovalbumin	Anti-ovalbumin rabbit polyclonal antibody	Aptamer (Biotinylated)	Colourimetric detection of biotin labelled aptamer
Ovalbumin	Anti-ovalbumin rabbit polyclonal antibody	Aptamer	Aptamer PCR

4.3 IMMUNO-PCR ASSAYS

A number of PCR assays were used with the immuno-PCR work described in this chapter. Each was designed using Primer Express v1.0 as described previously, with the exception of the *pgi* assay which had been designed at Dstl prior to the onset of this study. An internal control was constructed for each assay using composite primers, as detailed in Table 4.2, targeting a region of the human β actin gene. The internal control was designed to be larger than the target amplicon, at 120 basepairs, so as not to be preferentially amplified over the target sequence. The internal control was amplified using the composite primers and then cloned into a pT7 plasmid vector containing a TA overhang in order to facilitate large scale production of DNA. The optimal concentration of internal control DNA for use with the immuno-phage PCR assays used in this study, determined empirically, was 10 fg (Figure 4.1). The thermal cycling conditions and sequences are summarised in Table 4.3. Quadruplex PCR was developed for simultaneous detection of the *bce* and *scFv8* gene and internal control targets. To ensure sensitive detection of all four amplicons, the 60 °C annealing and extension phase was extended from 30 seconds to 1 minute.

Table 4.2 Composite primer sequences used to construct internal PCR controls (where $X_{(n)}$ represents the toxin simulant specific region of the primer).

Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')
$X_{(n)}$AGTACTCCGTGTGGATCGGA	$X_{(n)}$GCTGATCCACATCTGCTGGA



Site Legend

Site ID	Sample ID	Cy5 Ct	Protocol
A1	-3	10.41	b cereus
A2	-4	11.02	b cereus
A3	-5	20.25	b cereus
A4	-6	10.83	b cereus
A5	ntc	10.73	b cereus

Data Type	Line Type
Primary Curve	———

Channel	Symbol
Cy5	None

Figure 4.1 Serial dilution of internal control DNA for the immuno-phage-PCR assay. A synthetic DNA construct was made by PCR using a region of the b actin gene, flanked by scFv8 specific primers. Internal control DNA was added to the PCR reaction at various concentrations, from 1 pg to 10 fg.

Table 4.3 Summary of the thermal cycling conditions used with the immuno-PCR assays designed for this study.

Target organism/gene	Gene name	[Probe]	[Mg]	Thermal cycling conditions	Forward primer	Reverse primer	Taqman ® probe
B. cereus entero-toxin	*bce*	0.3 µM	4	95 °C 1 sec 60°C 30 sec	AAGAGTTAGTTTCAACAGCGCGT	CACTCGGTTTTTGCGTAGCTAAT	TCGGTCGTTCACTCGGGATCCATT
P.agglomerans phosphoglucse isomerase[1]	*pgi*	0.3 µM	3	95 °C 1 sec 60°C 30 sec	GTTTTGCCGCCTATTTCC	CACGATATGGGCTACAGACTC	
Aspergillus flavus oxidoreductase gene	*ord*	0.3 µM	3	95 °C 1 sec 60°C 30 sec	CAGTCGGTTCGGCAGTTTTC	GGTCTTCTCACATATCGGGAAGA	CACATCTTCTCGCCAGGAGAGTGGAGTG
B. cereus specific single chain antibody CMAN 8.	ScFv 8	0.3 µM	3	95 °C 1 sec 60°C 30 sec	CCTATTGCCTACGGCAGCC	TCTTTGTAGTCCGCCATGGC	
Aptamer F and R primers	AP	N/A	3	95 °C 1 sec 60°C 30 sec	CCGGATCCGTTGATATATAAAATTC	GGGAGCCAACACCACATTCAA	

4.4 DETECTION OF OVALBUMIN USING IMMUNO-PCR

4.4.1 Anti-ovalbumin ELISA

An existing, well characterised ELISA for detection of ovalbumin was converted to an immuno-PCR (I-PCR) format as described in detail in Section 2.5.2.1. Briefly, an ELISA plate was coated in a rabbit polyclonal capture antibody and non specific binding to the plate was prevented by filling each well with 2 % (w/v) milk powder in PBS. Ovalbumin was then diluted three-fold across the plate. A biotin-labelled detection antibody specific for ovalbumin was subsequently bound, followed by the addition of a streptavidin-horse radish peroxidase (HRP) conjugate. Positive detection of ovalbumin was determined following the addition of a colorimetric HRP substrate and an absorbance reading of three times above background level at A414 nm. The approach used is depicted in Figure 4.2a. The detection limit of the ELISA for ovalbumin used in this study was routinely 1.3ng/ml (Figure 4.2b). This assay had been well studied previously at Dstl and was deemed suitable for conversion to an immuno-PCR format as it performed reliably and had low non-specific background signal.

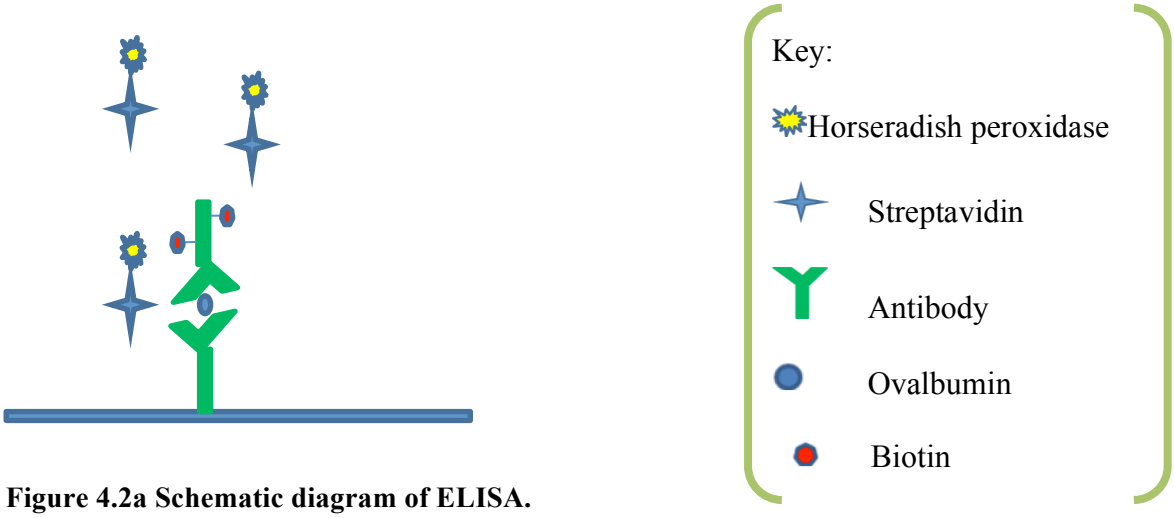


Figure 4.2a Schematic diagram of ELISA.

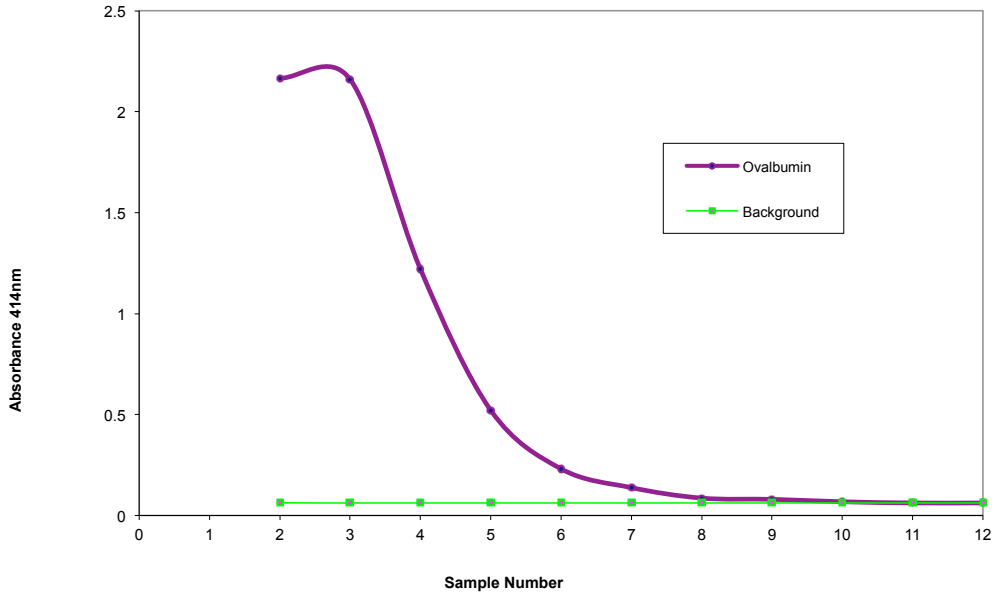


Figure 4.2b. Example ELISA A414 nm results for ovalbumin concentrations diluted 3-fold, starting at 1000 ng/ml. The primary antibody was bound to the surface of the ELISA plate. Ovalbumin was subsequently allowed to bind to the antibody. A secondary biotin-labelled antibody was bound to the immobilised ovalbumin. Streptavidin labelled with horseradish peroxidase (HRP) was bound to the sandwich. This was followed by the addition of the HRP substrate hydrogen peroxide plus ABTS resulting in a colorimetric change which can be measured at A414 nm and plotted on a graph. The background fluorescence was 0.062.

4.4.2 Immuno-PCR using biotin labelled DNA

Immuno-PCR was initially carried out using the same format as the ELISA, except a biotinylated PCR amplicon was added to the ELISA after the Streptavidin-HRP conjugate had been added to the sandwich (Figure 4.3). Although the background using colorimetric detection on a control row was low (0.067 OD), the background crossing point of the I-PCR was cycle 26, as observed with sample seven and onwards (Figure 4.5), limiting the sensitivity of the assay to 1.3 ng/ml. Therefore, in this format, immuno-PCR did not have any advantage over ELISA. It was thought that the lack of sensitivity may have been caused by the biotinylated antibodies sequestering any available streptavidin in the penultimate stage of the immunoassay, thus preventing binding between streptavidin and biotin labelled DNA in the final stage of the process. This method was therefore disregarded.

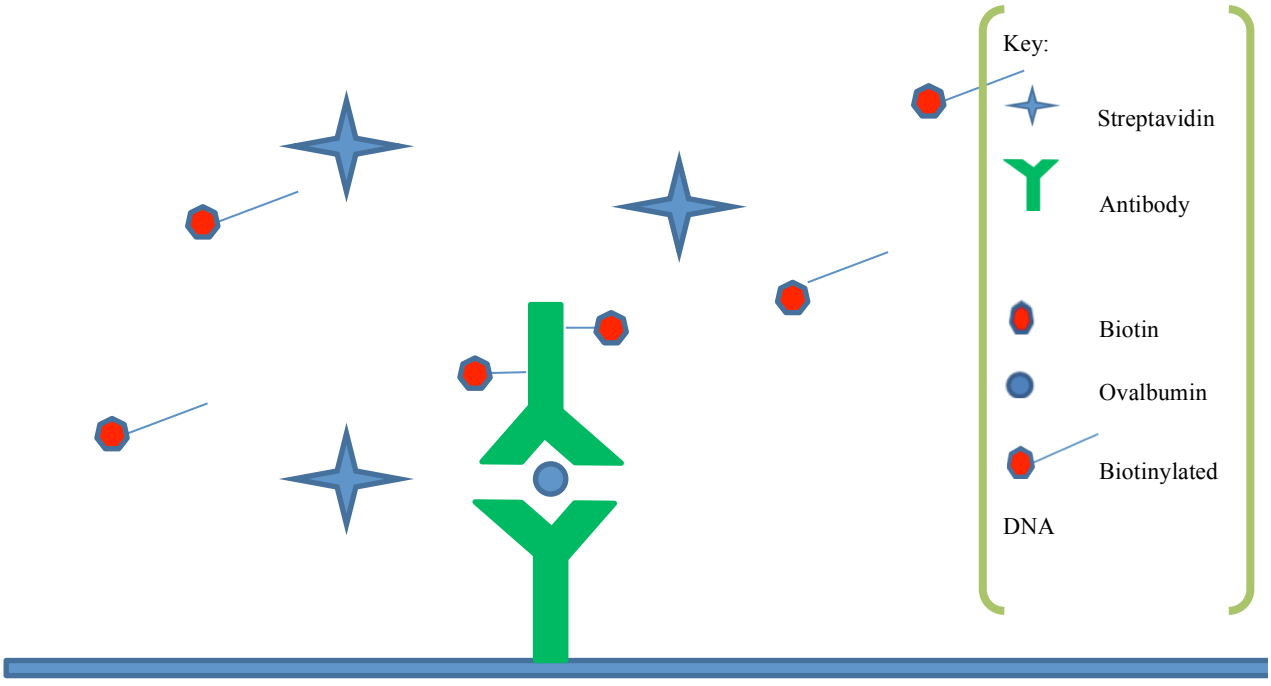


Figure 4.3 Schematic diagram of immuno-PCR using biotin labelled DNA. The primary antibody was bound to the plate. Ovalbumin was captured by the primary antibody and then detected using a biotin labelled antibody. Streptavidin was then

added to the plate, followed by biotin labelled DNA. PCR was used as the end-point detection method.

4.4.3 Immuno-PCR using pre-conjugated biotinylated DNA-streptavidin.

To overcome this problem, biotinylated DNA was directly bound to Streptavidin-HRP by incubating the two together for one hour at 37 °C. The pre-formed conjugate was then added to the ELISA plate following the removal of unbound biotinylated secondary antibody (Figure 4.4). The amount of DNA used in the method was optimised at 70 pg/well by performing a series of negative control reactions and determining the maximum concentration at which the DNA could be used without giving non specific signal (Table 4.4). DNA concentrations between 0.7 and 70 pg per well gave the same crossing point value as the no amplicon control, however the end point fluorescence value was low with 0.7 and 7 pg. Therefore 70 pg of biotin-streptavidin conjugated DNA per well were used in further I-PCR experiments. The number of final plate washes in dH_2O was also determined and the most reliable results were obtained when 70 pg DNA were used per well with 2 final dH_2O washes. It was necessary to stop the PCR at cycle 33 rather than allowing it to proceed for 40 cycles owing to the signal obtained from non-specific binding of the proteins to the ELISA plate. Using only 30 PCR signals was not sufficient to increase the sensitivity with 1.3ng/µl being the detection limit. The results obtained using the optimised method are summarised in Figure 4.4. In general terms the detection limit obtained using immuno-PCR was 50 pg/µl, a 9-fold increase in sensitivity compared to ELISA. When a ten fold dilution series of ovalbumin was used in immuno-PCR the detection limit of the assay was 10 pg/µl. Therefore the crude and simple method used here was successful. It should be noted, however, that the repeated PCR amplification of amplicon-labelled antibodies caused the background of the immuno-PCR became indistinguishable from the positive

signal with each successive experiment (data not shown). Steps to prevent this re-occuring would be necessary if the method were to be used in future.

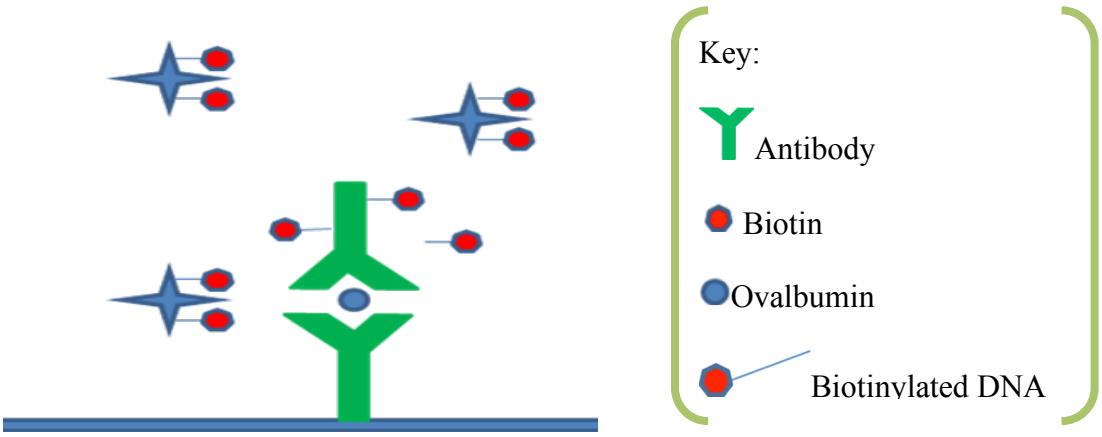


Figure 4.4 Schematic diagram illustrating immuno-PCR for the detection of ovalbumin using pre-formed conjugates of biotin labelled DNA and streptavidin. Ovalbumin was captured by the primary antibody and then detected using a biotin labelled antibody. Streptavidin and biotin labelled DNA we pre-conjugated prior to addition to the plate. PCR was used as the end-point detection method.

DNA concentration (ng per well)	Mean crossing point value (n=12)	Standard deviation
7000	26.36944	0.298651
700	28.40833	0.742135
70	33.41667	0.759203
0.7	34.65	0.617117
0.07	35.03333	0.174801
0.007	34.96667	0.334996
0.0007	35.11667	0.276385
0	35.1	0.742135

Table 4.4. Optimisation of the DNA concentration to be used in Immuno-PCR experiments. Biotin laballed *pgi* amplicon conjugated to streptavidin was added to a 96-well plate at concentrations ranging from 7000 ng to 0.7 pg, in replicates of 12. The DNA was bound to the plate at 37 ^{o}C for 1 hour. Following 5 washes the plate was analysed by PCR on the Roche Lightcycler. The average crossing point values were obtained, with standard deviation. Background signal was high and the no amplicon control amplified with a crossing point value of 35 due to non-specific binding of streptavidin to the plate.

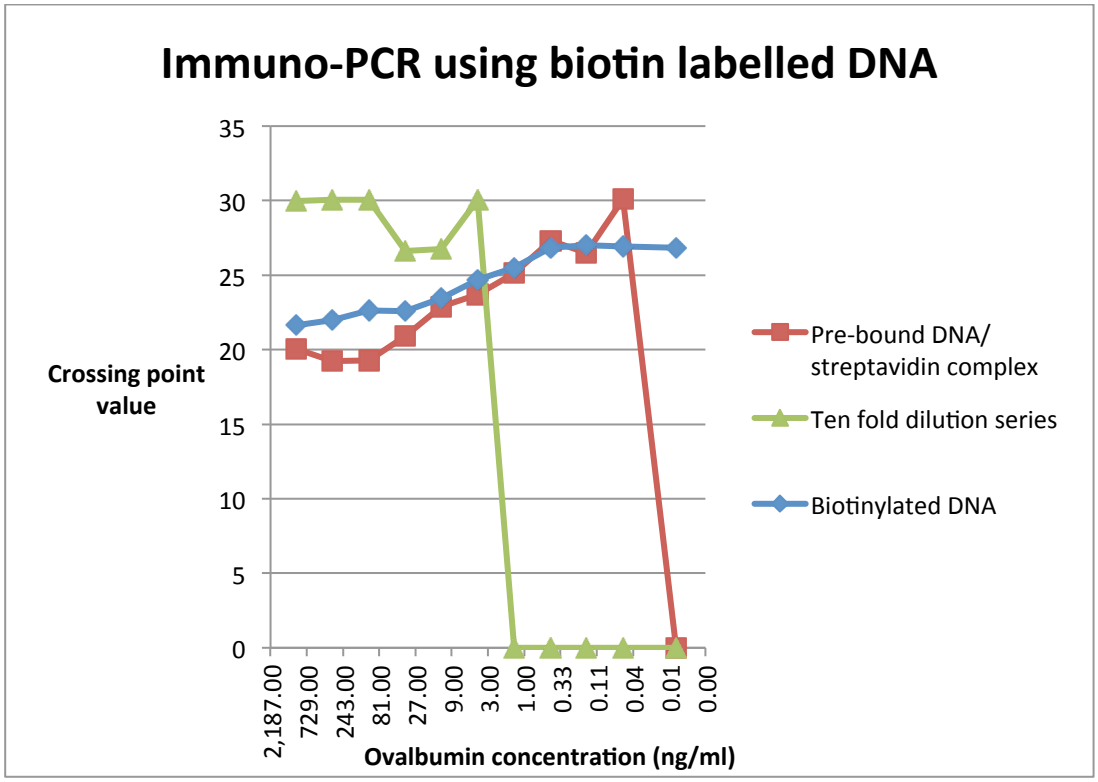


Figure 4.5 Immuno-PCR using biotin labelled DNA and a biotinylated DNA-streptavidin complex. Plates were coated in 5 µg/ml of anti-ovalbumin polyclonal capture antibody and blocked in 2 % milk powder solution. Ovalbumin was added to columns 1 to 12 at the concentrations stated in the graph. 2.5 µg/ml of biotinylated anti-ovalbumin antibody was then was added to each well. Streptavidin was added to each well at a dilution of 1:2000. Biotin labelled was added to the plate. Following the final incubation period, the plate was washed 5 times in PBST and 3 times in dH_2O. DNA was eluted from the plate by heating it to 100 ^{o}C for 30 secs; this was immediately amplified by real-time PCR on the Roche Lightcycler.

4.4.4 Immuno-PCR using neutravidin labelled DNA

The addition of the biotin-labelled amplicon to the immunoassay described in Section 4.4.2 required an extra hour-long incubation step compared to ELISA. The approach in Section 4.4.3, using preconjugated bioton labelled DNA and streptavidin was quicker but was inelegant. An alternative approach was investigated for conversion of the ovalbumin ELISA to an I-PCR format. Neutravidin has the same affinity for biotin as streptavidin and is commonly available as an oligonuceotide modification. The use of neutravidin labelled DNA would reduce the assay time in comparison to the approach used in Section 4.4.2, since it would bind directly to the biotin labelled secondary antibody. Consequently, although the approach involved the use of PCR amplicon, it was deemed worthy of investigation. The immuno-PCR scheme is summarised in Figure 4.6.

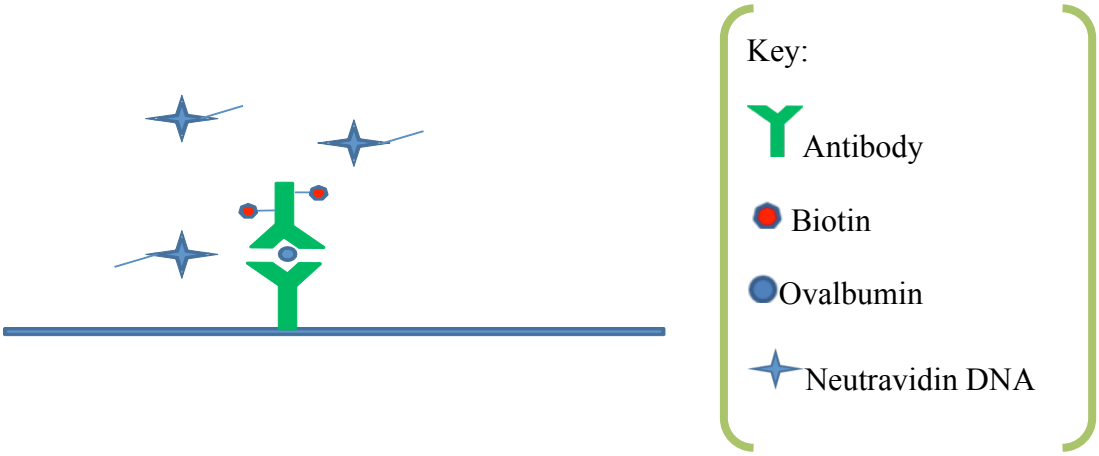


Figure 4.6. Summary of the I-PCR scheme using neutravidin labelled DNA.

Neutravidin labelled *pgi* PCR primers were synthesised by Thermo Hybaid (UK), with 1.5 mg being delivered to the thiol modified oligonucleotides, followed by HPLC purification. The neutravidin labelled *pgi* oligonucleotides, at a concentration of 100 μM, were used to produce a labelled amplicon by PCR. Amplification using these primers was inefficient compared to that obtained using unlabelled primers, however amplicon was collected, purified by microconcentration and used in further work. Initial pilot experiments showed that the neutravidin labelled amplicon was able to bind to the biotinylated secondary antibody sufficiently to give a good PCR signal (data not shown).

The neutravidin amplicon was tested in I-PCR by using a final concentration estimated from Q-PCR data at 10^8 and 10^7 copies per μl. The initial results using this approach showed no discrimination between positive and negative samples (Figure 4.7), although in general terms the crossing point values increased in relation to the ovalbumin concentration. The crossing point values in Table 4.4 are difficult to interpret, demonstrating the lack of reproducibility of the method. However, the negative control samples had high background signals (12.54 or 11.43). Therefore, only samples with a value above these thresholds could be considered positive. Accordingly, the limit of detection of the assay was between 4.1 and 1.3 ng/ml, which was the same as the original colorimetric ovalbumin ELISA. It was therefore necessary to carry out further optimisation experiments in an attempt to exploit the sensitivity of PCR whilst reducing the level of background signal.

The primary capture antibody concentration was varied from 5μg/ml to 0.039μg/ml in a two-fold dilution series. The concentration of secondary detection antibody remained constant at 2.5 μg/ml. Ovalbumin was diluted across the plate as with the

standard protocol. The results showed that reducing the concentration of capture antibody had no effect on the amount of non-specific binding occurring with the neutravidin labelled oligonucleotides (Figure 4.8). Furthermore, reducing the amount of capture antibody used on the plate had an adverse effect on the immuno-PCR and such results were less consistent than those obtained originally.

The detection antibody concentration was then varied between 2.5 μg/ml and 0.0195 μg/ml, with all other reagent concentrations unchanged. The ovalbumin concentration was diluted threefold across the plate as in other experiments. The results in Figure 4.9 demonstrate again the inconsistency of the approach, however an overall trend can be seen, with an increase in crossing point value being observed in relation to a decrease to ovalbumin. However background was excessively high, therefore samples containing less than 0.45 μg/ml of ovalbumin were not analysed by PCR.

A third immunoassay was carried out using lowered concentrations of both capture and detection antibody, with the ovalbumin concentration remaining the same as used previously. The background signal obtained with this plate was lower than observed previously, but was still excessive, preventing the full sensitivity of the technique being exploited (Figure 4.10).

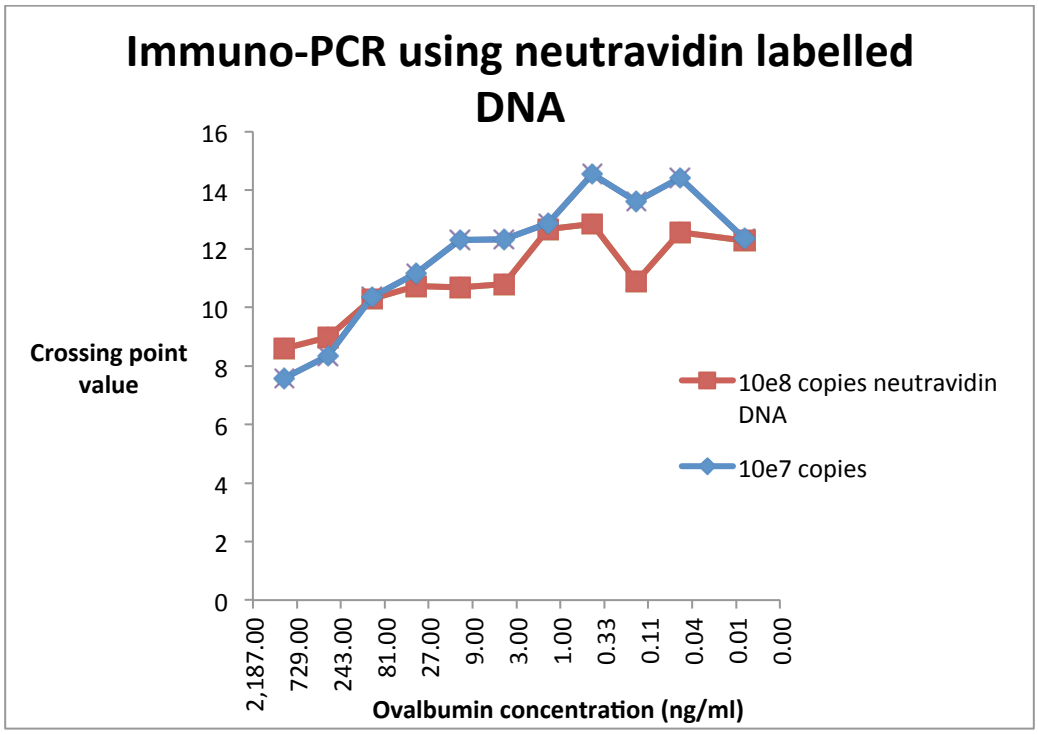


Figure 4.7 Crossing point values obtained using I-PCR with neutravidin labelled DNA. Plates were coated in 5 µg/ml of anti-ovalbumin polyclonal capture antibody and blocked in 2 % milk powder solution. Ovalbumin was added to columns 1 to 12 at the concentrations stated in the graph. 2.5 µg/ml of biotinylated anti-ovalbumin antibody was then was added to each well. Neutravidin labelled DNA was added to each well at a concentration of 40 ng/µl or 4ng/µl. Following the final incubation period, the plate was washed 5 times in PBST and 3 times in dH_2O. DNA was eluted from the plate by heating it to 100 ^{o}C for 30 secs; this was immediately amplified by real-time PCR on the Roche Lightcycler.

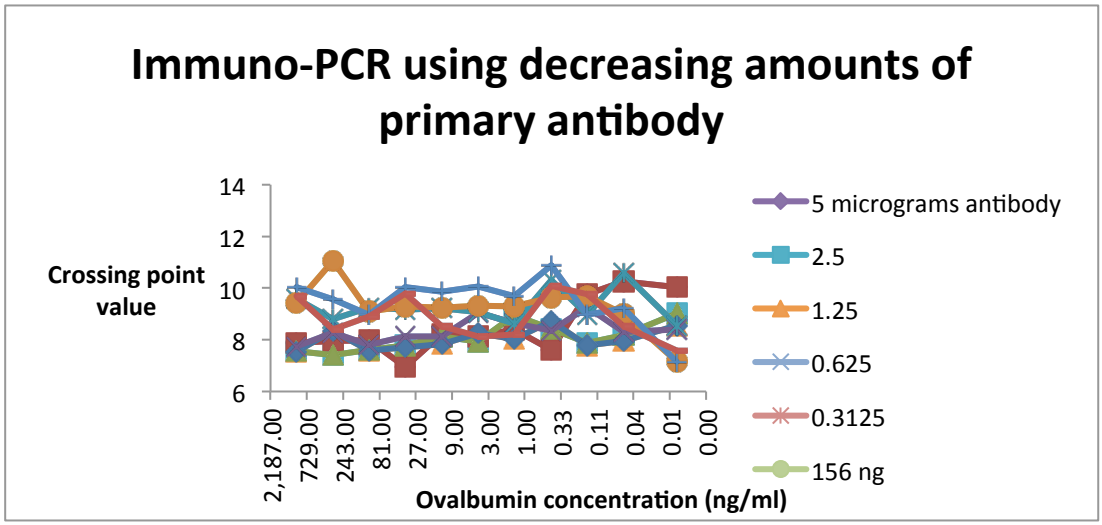


Figure 4.8. Crossing point values obtained using 10^7 copies/µl neutravidin labelled *pgi* amplicon in I-PCR. Plates were coated in anti-ovalbumin polyclonal capture antibody at the concentrations described in the graph and blocked in 2 % milk powder solution. Ovalbumin was diluted across the plate. 2.5 µg/ml of biotinylated anti-ovalbumin antibody was then was added to each well. Neutravidin labelled DNA was added to each well at a final concentration of 10^7 copies/ul. Following the final incubation period, the plate was washed 5 times in PBST and 3 times in dH_2O. DNA was eluted from the plate by heating it to 100°C for 30 secs; this was immediately amplified by real-time PCR on the Roche Lightcycler.

Further experiments used a dilution series of neutravidin down the plate with standard concentrations of primary and secondary antibodies. The crossing point values are summarised in Figure 4.11. The 10 and 1 copies/µl dilutions of neutravidin labelled amplicon did not amplify as the concentration of DNA template was too low. Therefore, the detection limit of the PCR assay was 100 copies of neutravidin labelled *pgi*. Despite using the neutravidin label at the minimum concentration detectable by PCR the immuno-PCR was still subject to inconsistent Ct values. In spite of this, all samples containing ovalbumin amplified with a Ct value above the background level using 100 or 1000 copies of amplicon. Although the Ct values obtained using high concentrations of ovalbumin and amplicon were distinguishable from the background, the detection limit of the I-PCR assays were no lower than using the colourimetric immunoassay.

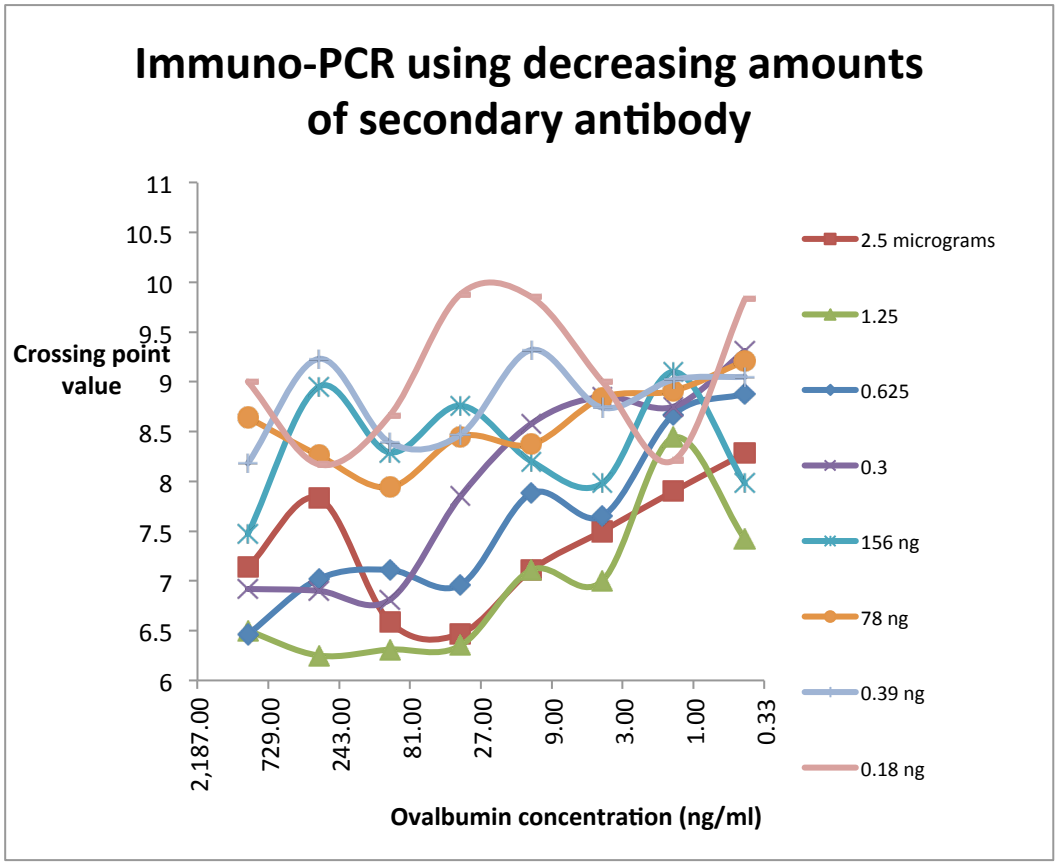


Figure 4.9 Crossing point values obtained using 10^7 copies/µl of neutravidin labelled *pgi* amplicon in I-PCR. Plates were coated in 5 µg/ml of anti-ovalbumin polyclonal capture antibody and blocked in 2 % milk powder solution. Ovalbumin was added to columns 1 to 12 at the concentrations stated in the table. Biotinylated anti-ovalbumin antibody was then was added to each well at the concentrations described in the graph. Neutravidin labelled DNA was added to each well at a concentration of 10^7 copies/µl. Following the final incubation period, the plate was washed 5 times in PBST and 3 times in dH_2O. DNA was eluted from the plate by heating it to 100°C for 30 secs; this was immediately amplified by real-time PCR on the Roche Lightcycler or Corbett Rotorgene®.

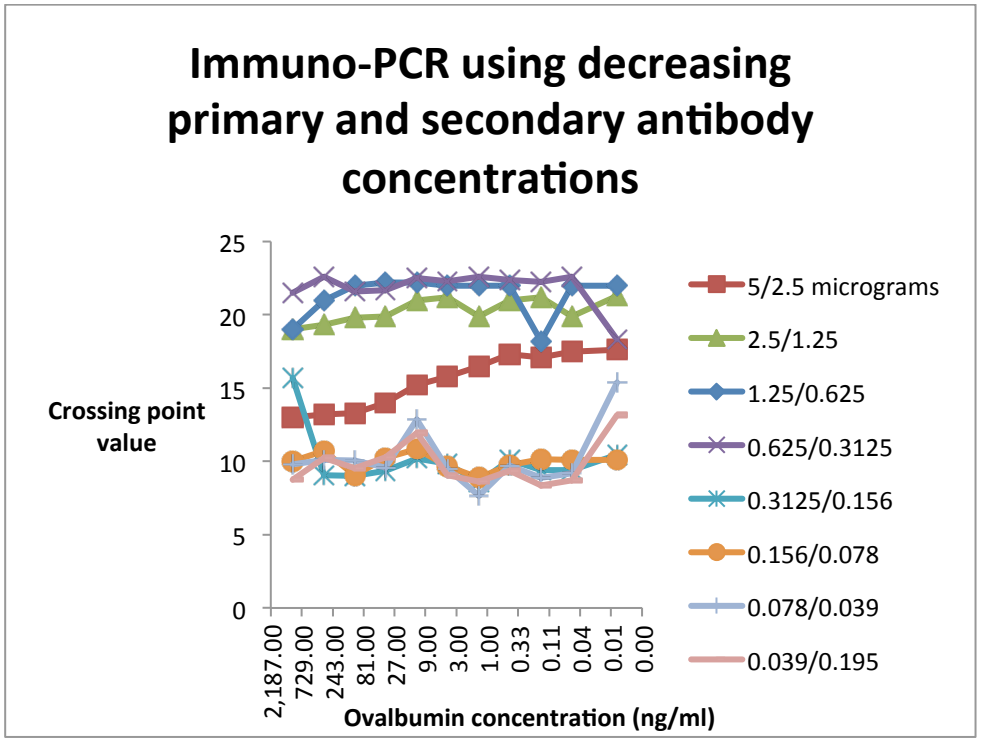


Figure 4.10. Crossing point values obtained using 10^7 copies/µl of neutravidin labelled *pgi* amplicon in I-PCR and a ten fold dilution series of capture and detection antibody. Plates were coated in 5 µg/ml of anti-ovalbumin polyclonal capture antibody and blocked in 2 % milk powder solution. Ovalbumin was added to columns 1 to 12 at the concentrations stated in the graph. Biotinylated anti-ovalbumin antibody was then was added to each well at the concentrations described in the table. Neutravidin labelled DNA was added to each well at a concentration of 10^7 copies/µl. Following the final incubation period, the plate was washed 5 times in PBST and 3 times in dH_2O. DNA was eluted from the plate by heating it to 100°C for 30 secs; this was immediately amplified by real-time PCR on the Roche Lightcycler.

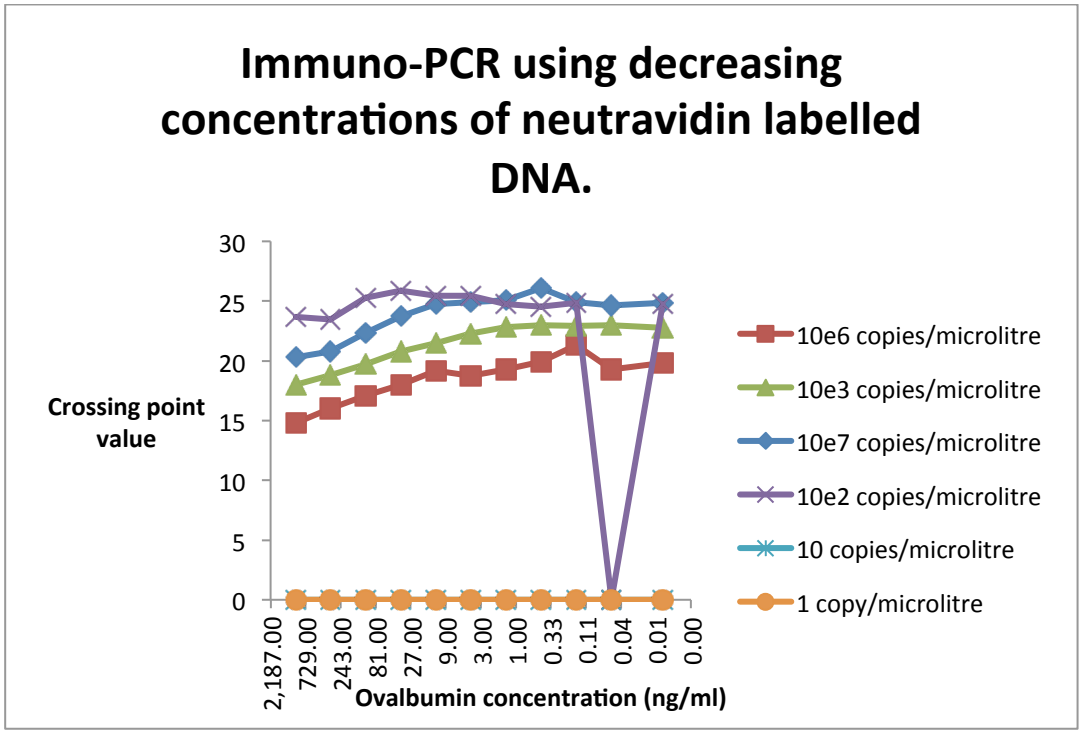


Figure 4.11 Crossing point values obtained using a dilution series of neutravidin labelled DNA in I-PCR. Plates were coated in anti-ovalbumin polyclonal capture antibody at the concentrations described in the table and blocked in 2 % (w/v) milk powder solution. Ovalbumin was added to columns 1 to 12 at the concentrations stated in the graph (ng/µl). 2.5 µg/ml of biotinylated anti-ovalbumin antibody was then was added to each well. Neutravidin labelled DNA was added to each well at the concentrations stated. Following the final incubation period, the plate was washed 5 times in PBST and 3 times in dH_2O. DNA was eluted from the plate by heating it to 100 °C for 30 secs; this was immediately amplified by real-time PCR on the Roche Lightcycler.

4.4.5 **Detection of ovalbumin using directly labelled antibodies**

It was thought that the crude methods used to label antibodies with DNA in previous experiments may have prevented the I-PCR methods used from fully exploiting the sensitivity of PCR detection. Therefore a more controlled labelling method was investigated. The anti-ovalbumin antibodies used in previous experiments were covalently labelled with a 78 bp oligonucleotide amino-modified tag by Alchemy (UK). An oligonucleotide label was used in comparison for these experiments as it could be synthesised directly rather than amplified by PCR, as used by Hendrickson *et al.,* (1995). The simplicity of the assay is summarised in Figure 4.12.

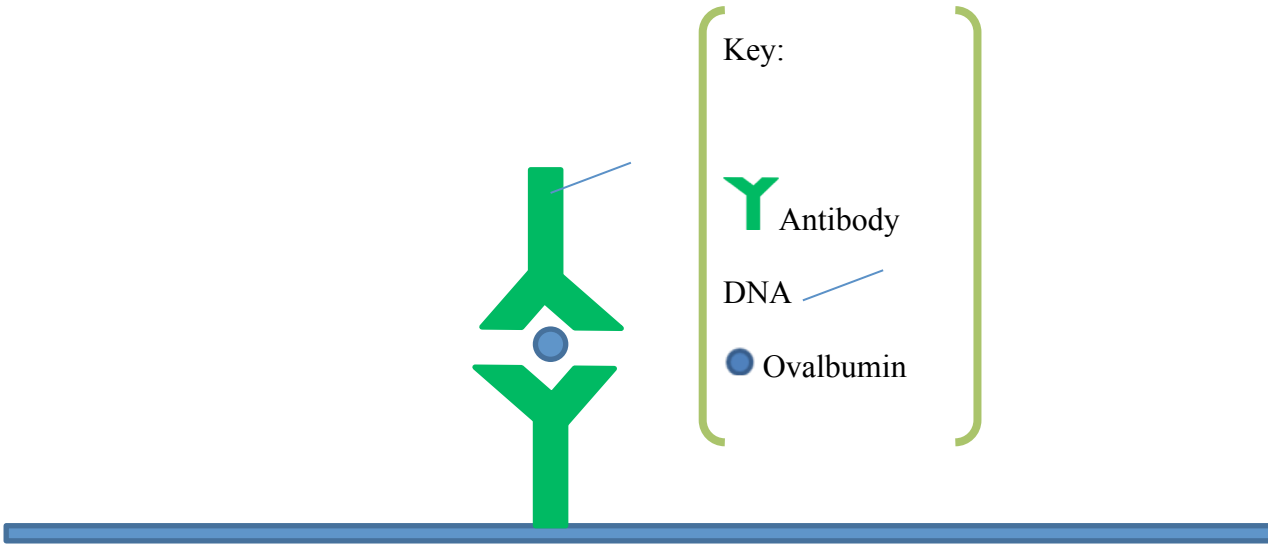


Figure 4.12. Immuno-PCR scheme using DNA labelled antibodies. Plates were coated in anti-ovalbumin polyclonal capture antibody, followed by the addition of ovalbumin. Covalently DNA labelled anti-ovalbumin antibody was then was added to each well. DNA was eluted from the plate by heating it to 100 °C for 30 secs; this was immediately amplified by real-time PCR on the Roche Lightcycler.

4.4.5.1 **Detection using high antibody concentration**

Immuno-PCR using 0.85 mg/ml amino-modified DNA labelled antibodies, with ovalbumin concentrations ranging between 1000ng/ml and 0.01 ng/ml, was carried out. The anti-ovalbumin capture antibody was bound to the plate, washed and followed by ovalbumin addition. The plate was re-washed and the DNA labelled secondary antibody added. Following further washes the plate was heated briefly to disrupt antibody binding and samples were analysed by PCR. Initial results showed

that the DNA coupling reaction had worked well and that the concentration of DNA bound to the antibody was suitable for use in immuno-PCR. However, the background signal was excessive, once again leading to false positive results. The experiment was repeated with an additional three washes of the microtitre plate in dH_2O. This resulted in a reduction in background signal, however the Ct value of sample 7 was lower than that of the negative control, leading to this sample being incorrectly classified as negative (Figure 4.13).

Experiments to lower the background signal were then carried out, using six PBS-T washes, followed by numerous washes in dH_2O. An ovalbumin concentration range between 1 µg and 0.1 pg/ml was tested in the presence and absence of capture antibody, with the aim of quantifying the amount of non-specific antibody binding occurring on the plate. The signal obtained from the row containing capture antibody was higher than that of the negative control row, however a high background signal was observed. This indicated that the amount of DNA-labelled antibody used in the I-PCR assay was excessive. Two washes in dH_2O were then carried out, which significantly reduced the amount of background signal, as did three washes. However, it was not possible to reduce the background signal further using this approach (Figure 4.14). Therefore, further experiments were carried out using less DNA labelled antibody.

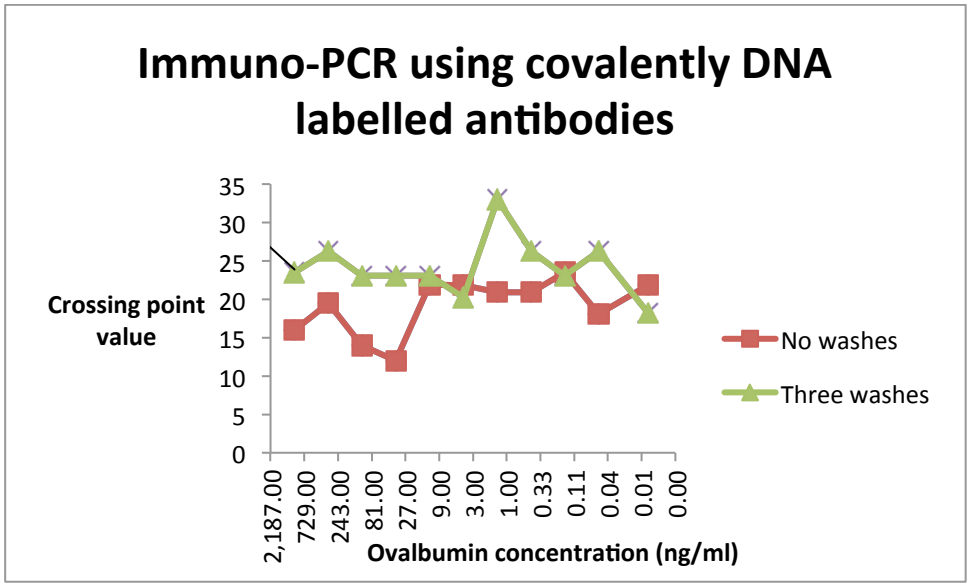


Figure 4.13 Immuno-PCR results obtained using 0.85 mg/ml DNA labelled antibody as the detection ligand in an ovalbumin immunoassay. The immunoassay was carried out using a standard ovalbumin ELISA, however colorimetric detection was replaced by PCR. Additionally, three extra washes of the microtitre plate were carried out with distilled water in order to remove unbound antibody.

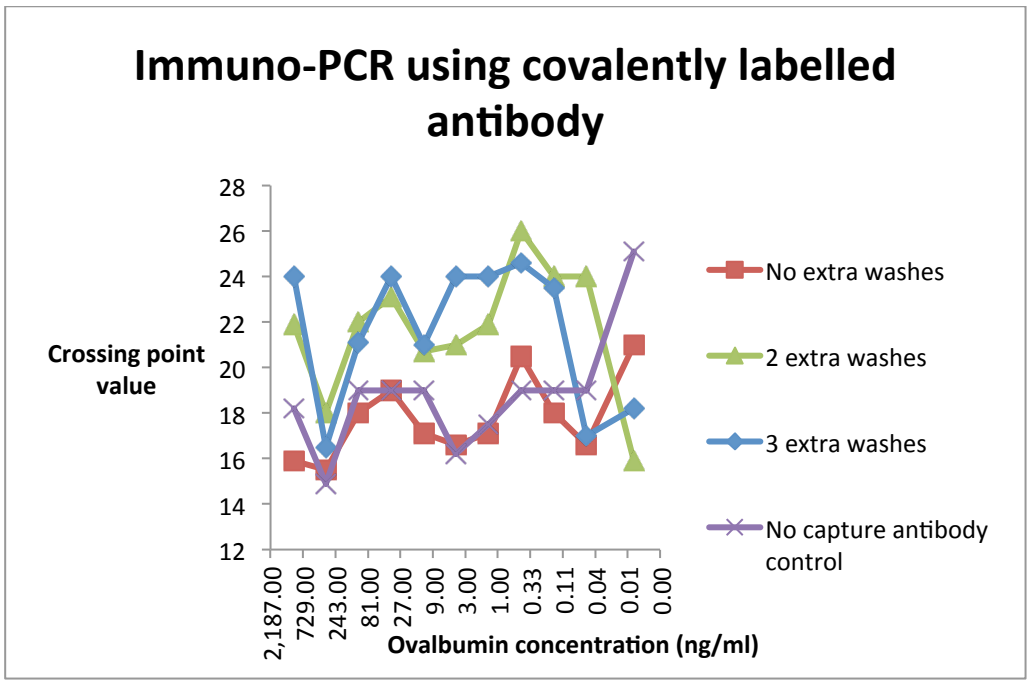


Figure 4.14. Immuno-PCR results obtained using 0.85 mg/ml DNA labelled antibody as the detection ligand in an ovalbumin immunoassay. The immunoassay was carried out using a standard ovalbumin ELISA, however colorimetric detection was replaced by PCR using *ord* specific primers and probe. Additional washes of the microtitre plate were carried out, as detailed above, with distilled water in order to remove unbound antibody.

4.4.5.2 **Detection of ovalbumin using low covalently labelled antibody concentrations.**

Theoretically, it should be possible to use a lower concentration of detection antibody in I-PCR compared to conventional ELISA. Accordingly, as with conventional I-PCR, the concentration of detection antibody used was reduced. The average Ct value obtained using 1µg of ovalbumin with 2.5 µg detection antibody and three dH_2O washes was 25. This was increased to 26 using 1.25 µg of antibody, however the background signal was excessive. Results using 0.25 µg of detection antibody were promising, with many of these samples being negative following three stringency washes in dH_2O. However, these results were not reproducible once the plate had been heated to release the antibody-DNA molecules. Further experiments were carried out using 0.125 and 0.025 µg of detection antibody. However, false positive and negative results were obtained using 0.125 µg of antibody. Furthermore, the no template controls amplified at cycle 30, an unfortunate consequence of performing the same reaction an excessive number of times in one laboratory, as previously experienced in this thesis (Section 4.4.3).

A final experiment was performed using 0.025 µg of detection antibody. Results were inconsistent, with no predictable pattern being observed across the dilution series of ovalbumin. Only samples 1, 7 and 6 gave a higher signal than the background, with false negative results being observed with all other concentrations. Therefore, the concentration of detection antibody used in this experiment was too low to allow reliable detection. These results are summarised in Figure 4.15. Further experiments utilising a low concentration of capture antibody were not carried out as the approach was unsuccessful with neutravidin labelled antibodies. Instead, a different approach was used which also eliminated the DNA labelling step.

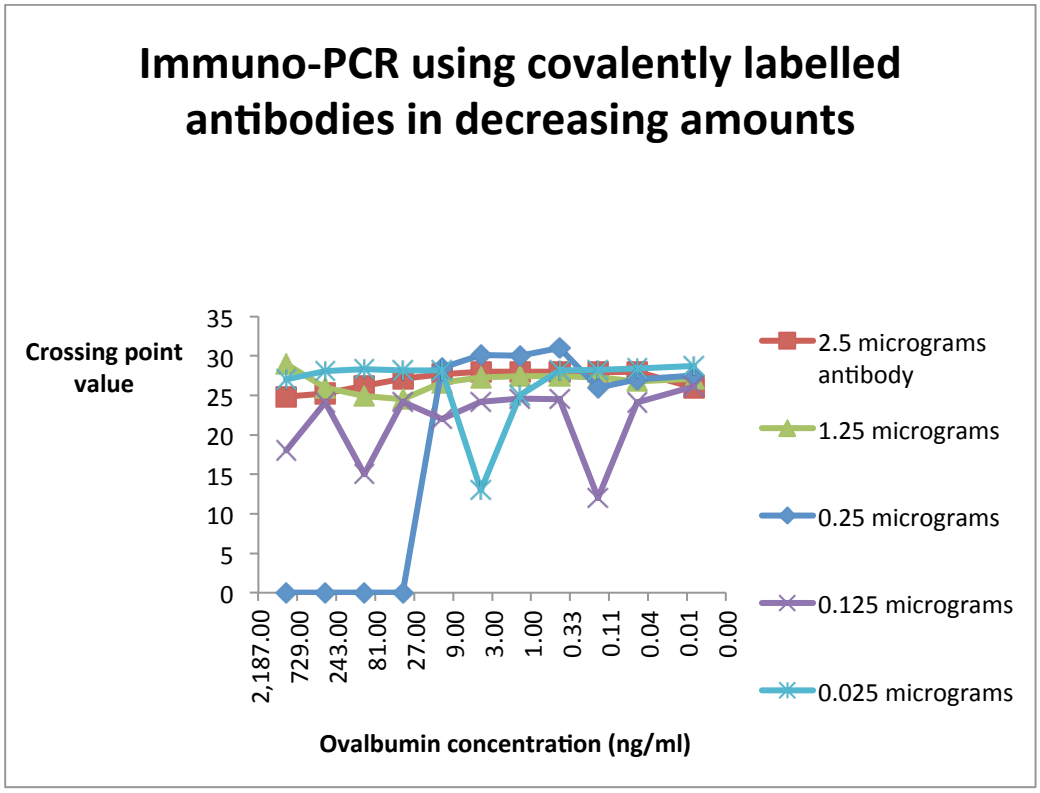


Figure 4.15. Immuno-PCR results obtained using varying concentrations of covalently labelled antibodies for the detection of ovalbumin. The immunoassay was carried out using a standard ovalbumin ELISA, however colorimetric detection was replaced by PCR. Additionally, three extra washes of the microtitre plate were carried out with distilled water in order to remove unbound antibody. The amount of detection antibody used was varied as described above.

4.5 DETECTION OF *BACILLUS CEREUS* BY I-ϕ-PCR

4.5.1 Introduction to Immuno-phage-PCR

Immuno-PCR was demonstrated to offer increased sensitivity over ELISA (Section 4.4.2) when performance was optimal and background signal was sufficiently low. However, the results also showed that the way in which DNA was bound to the detection antibody affected the sensitivity and reproducibility of the method. Therefore, an alternative approach was investigated, using single chain antibodies (scFv) in place of poly- or mono-clonal antibodies. Single chain antibodies are produced by cloning and expressing the mRNA of a mouse immunised with an antigen of interest. They are expressed in *E. coli* cells as phage displayed particles, enabling the antibody to be presented to an antigen (Krebber *et al.,* 1997). The antibodies are usually purified using recombinant protein purification methods and would therefore be expected to carry a small quantity of contaminating DNA. However, the scFv used for this study were not purified extensively, simply being precipitated from the culture supernatant instead. The aim of using scFv in immuno-PCR was to eliminate the need for a separate DNA labelling reaction. The approach is depicted in Figure 4.16.

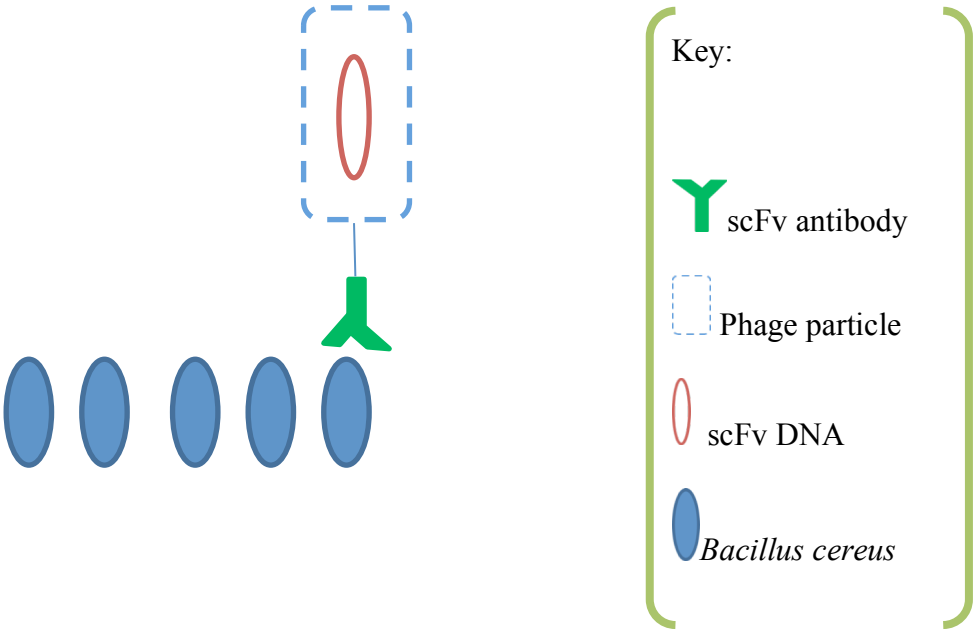


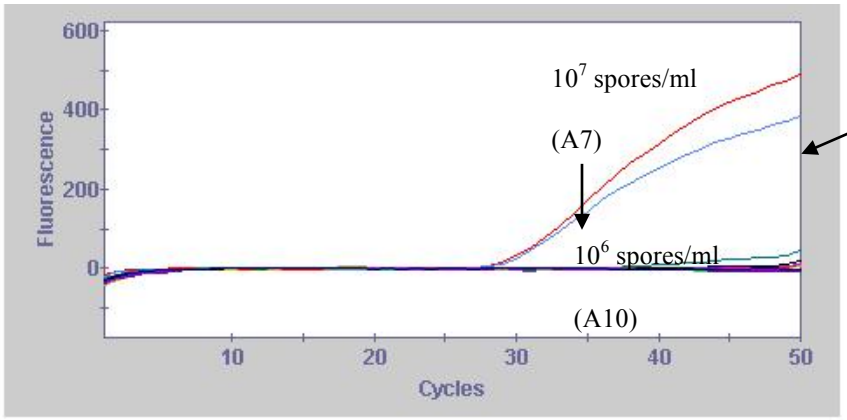
Figure 4.16. Summary of immuno-phage-PCR. The *Bacillus cereus* spores were bound directly to the ELISA plate at 4 °C overnight. The phage particles displaying *B. cereus* specific scFv were bound to the spores. DNA was released from the phage particles by heating it to 100 °C for 30 secs; this was immediately amplified by real-time PCR on the Cepheid™ Smartcycler.

As scFv to ovalbumin or bacterial toxins were unavailable, a scFv to *Bacillus cereus* spores, designated CMAN8, was used instead. CMAN8 was obtained as a cross reactant of antibodies developed for the detection of *Bacillus anthracis* spores. CMAN8 had previously been shown to work well in ELISA by colleagues at Dstl and was thought therefore suitable for conversion to an I-ϕ-PCR format. Detection of a positive immunoassay result was obtained using a CMAN8 PCR assay specific for the DNA encoding the single chain antibody used in this experiment, as detailed in Table 4.2.

4.5.2 **PCR detection of spores**

A real time TaqMan® PCR assay was designed to target the enterotoxin (*bceT)* gene using Primer Express to identify suitable oligonucleotide sequences. The PCR worked using standard PCR conditions (Table 4.2) once optimised with DNA from *B. cereus* NCTC 2566. The *bceT* gene is carried by only a few *B. cereus* strains, therefore, the specificity of the assay was determined by testing the *B. cereus* strains held at Dstl. DNA from strains NCTC 11124, FRI 41, FRI 42, FRI 43 and FRI 13 were all negative for the *bce* gene as expected, as was the standard Dstl test panel. The PCR assay was therefore demonstrated to be specific for enterotoxigenic *B. cereus.*

Bacillus cereus spore samples were treated with 5 % sodium hypochlorite solution and then washed three times in distilled water in order to remove vegetative cell debris and extracellular DNA. The detection limit of the PCR assay for the detection of the *B. cereus* enterotoxin gene was 10^6 purified spores / ml (Figure 4.17).



Site Legend

Site ID	Sample ID	FAM Ct	Protocol
A1	10e6/ml	0.00	PCR 1 min
A2	10e5	0.00	PCR 1 min
A3	10e4	0.00	PCR 1 min
A4	10e3	0.00	PCR 1 min
A5	10e2	0.00	PCR 1 min
A6	0	0.00	PCR 1 min
A7	bce 10e7	29.94	PCR 1 min
A8	ntc	0.00	PCR 1 min
A9	10e7 p-	30.20	PCR 1 min
A10	10e6	47.77	PCR 1 min
A11	10e5	0.00	PCR 1 min
A12	10e4	0.00	PCR 1 min
A13	10e3	0.00	PCR 1 min
A14	10e2	0.00	PCR 1 min
A15	10e1	0.00	PCR 1 min

Data Type	Line Type
Primary Curve	———

Channel	Symbol
FAM	None

Figure 4.17 - Detection limit of real-time *bce* PCR assay with *Bacillus cereus* spores. PCR was carried out on a dilution series of *B. cereus* spores ranging from 10^7 (positive control) to 10^2 spores per ml using primers specific for the *bce* enterotoxin gene. 50 cycles of denaturation at 94 ^oC and annealing / elongation at 60 ^oC were carried out on the Cepheid SmartCycler®.

4.5.3 **Singleplex I-ϕ-PCR detection of spores**

Single chain antibodies were available in limited supplies as they were donated from a colleague in kindness, for proof-of-principle experiments. They were an unwanted bi-product of an antibody generation programme, discovered to cross react with *Bacillus cereus*. It was not possible to express large volumes of this antibody due to financial constraints, so pilot experiments were carried out with a view to carrying out further experiments if the method showed potential. A preparation of phage particles was expressed in recombinant *E. coli* cells and precipitated in sodium chloride and PEG 8000. It was hypothesised that a crude preparation would contain sufficient levels of contaminating DNA to facilitate immuno-PCR without the need for a DNA labelling step. Spores were bound to an ELISA plate during an overnight incubation. The plate was then washed, non-specific binding to the plate was prevented using 2 % milk powder solution in PBS and the plate was re-washed. The crude single chain antibody preparation was subsequently added to the plate. Following incubation for one hour the plate was washed five times and then boiled briefly to release phage particles and their associated DNA.

Initial results showed that the Immuno-Phage assay offered increased sensitivity over the *bce* PCR assay, with 10^4 washed spores per ml being detected reliably using the *lac* gene as the immuno-PCR target (Figure 4.18). However, the positive results (Ct 19-23) were difficult to distinguish from negative results (Ct 24) as the *lac* gene is commonly found in M13 based cloning vectors, used routinely in the laboratory in which the immuno-PCR work was carried out. In this case, laboratory contamination, together with the use of *lac* and *bce* internal control plasmids, comprising an M13 backbone, contributed to a high background signal.

The results shown in Figure 4.18 also indicate that the phage preparation worked best as a detection ligand when used undiluted (10^7 transforming units per microlitre), as samples using diluted phage were indistinguishable from the background signal. Owing to the high background signal obtained using the *lac* PCR assay, the 10^7 to 10^0 spore/ml samples detected using 10^7 TU/ml of phage were also used in a singleplex PCR assay using scFv specific primers. The melt curve analysis of PCR products showed that 10^5 spores/ml were strongly detected, with 10^3 spores/ml being the limit of detection (Figure 4.19). Therefore I-ϕ-PCR was able to lower the limit of detection of *B. cereus* spores from 10^6 washed spores/ml by PCR to 10^3 spores/ml.

The results obtained using the scFv assay were far superior to those obtained using the *lac* assay as it was not subject to a background signal. I-ϕ-PCR was repeated one last time, using TaqMan® PCR. The background was high on this occasion with a Ct value of 25.2, but was clearly different to the positive samples, demonstrating the potential for sensitive detection of spores using this technique. When Ct values were plotted against spore concentration, the regression co-efficient was 0.9884, demonstrating a trend between the two variables (Figure 4.20). Although high, the background signal was significantly different to those obtained using low spore concentrations, but was high. The use of a sandwich immunoassay format and additional washes in water may reduce the background signal in further work, however it was not possible to test this theory within the confines of this study.

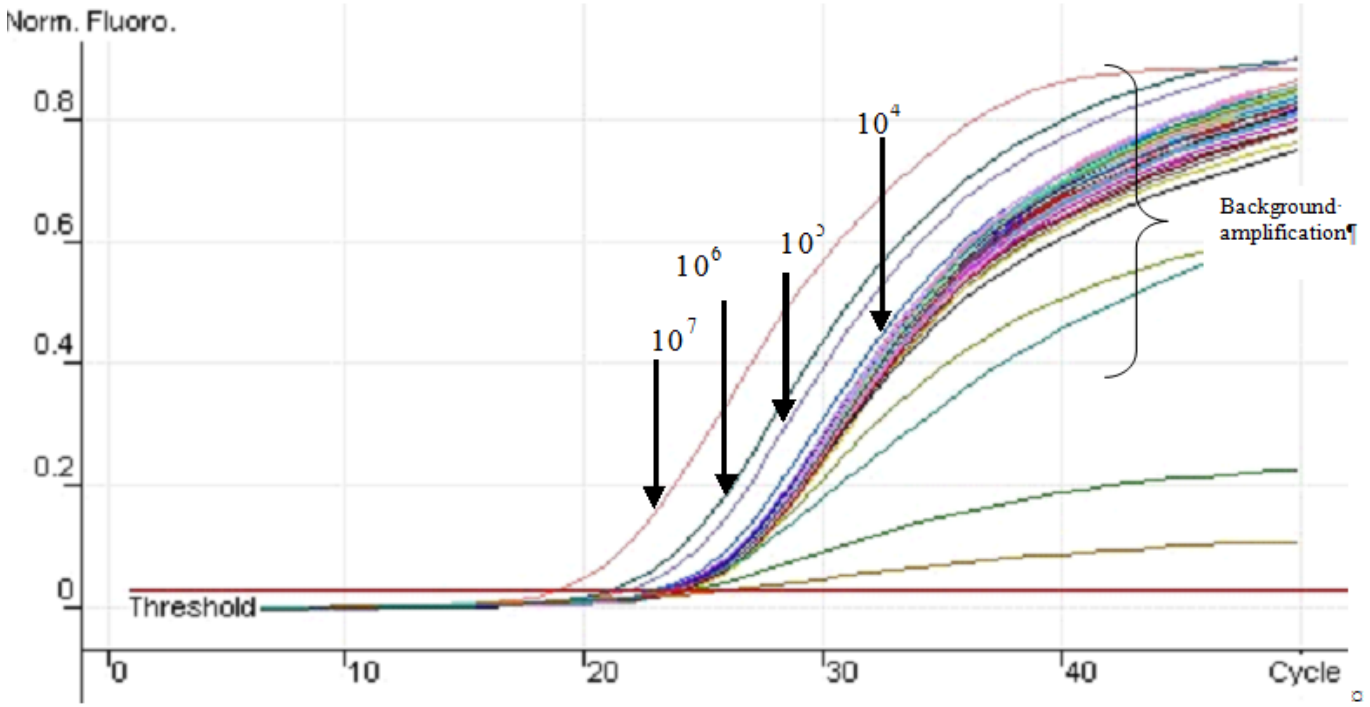


Figure 4.18 - Detection limit of *B. cereus* immuno-phage PCR. Immuno-phage PCR was carried out using a range of *B. cereus* spore concentrations from 10^7 to 10^0 / ml. Phage concentration ranged from 10^7 transforming units (TU)/ml to 10^5 TU/ml. Positive amplification was observed using 10^7 tu/ml only; using diluted phage resulted in background amplification.

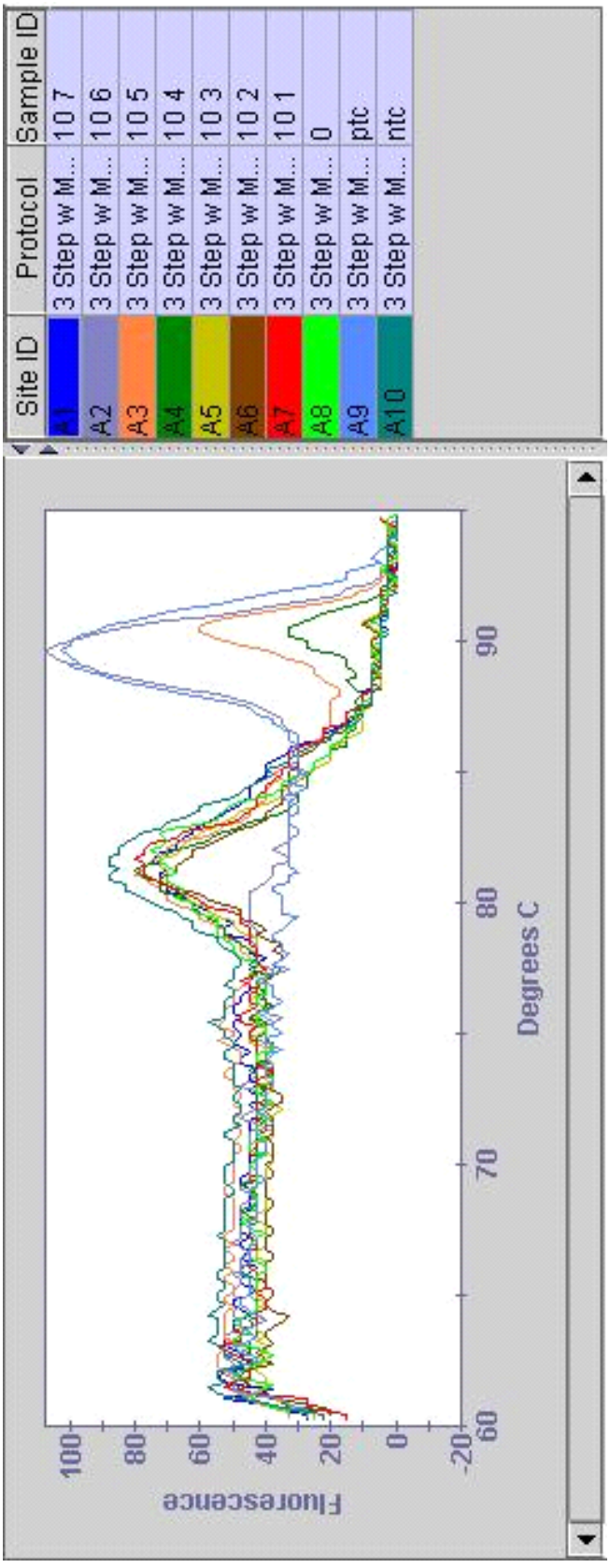


Figure 4.19 - Melt curve analysis of scFv PCR products PCR of I-ϕ-PCR samples containing 10^7 to 10^3 spores/ml produced a specific product with a Tm of 90°C. Non specific products were also observed, with a Tm of 81 °C.

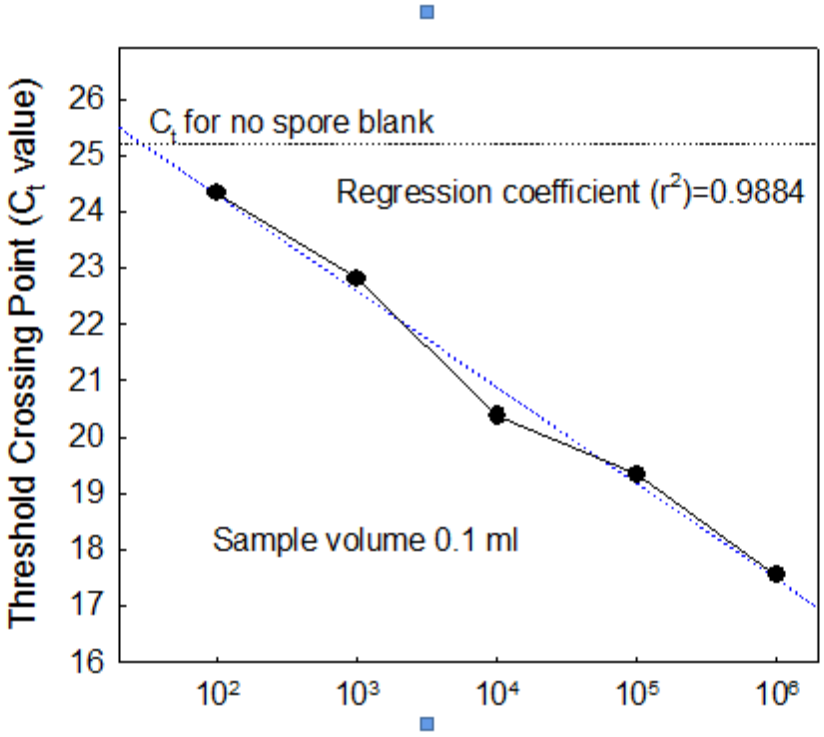


Figure 4.20. I-ϕ-PCR detection of *B cereus* spores. The I-ϕ-PCR signal, measured as Crossing point value, is proportional to the spore concentration. The background signal of the assay is significantly different from the positive samples.

4.5.4 **Quadruplex PCR of *Bacillus cereus* spores**

Quadruplex PCR was carried out for the detection of spores using four channels on the Cepheid® Smartcycler, as summarised in Table 4.5.

Channel	Target Gene
1 Fam	*Bce* (*Bacillus cereus*)
2 Cy3	*bce* internal control
3 Texas Red	CMAN8 (scFv)
4 Cy5	CMAN8 internal control

Table 4.5. Summary of the quadruplex PCR strategy used for detection of *Bacillus cereus* spores. Channels 1 and 2 were used to detect the toxin gene directly and channel 3 and 4 were used simultaneously for immuno-phage-PCR.

As it was not possible to repeat the I-ϕ-PCR due to the limited supply of phage the same samples used to produce the results shown in Figure 4.19 were used in quadruplex PCR. The results in Figure 4.21 would ideally be a replicate of those obtained previously; however, the background was very high (Ct value 20.21). The reason for this is thought to be a result of contamination between wells of the 96 well plate. All other Ct values were slightly lower than in the duplex assay as a result of competition for reaction components, but the sensitivity of detection was not reduced. This method demonstrates potential for sensitive detection of samples where genetic material is limited, such as washed spores or toxins. In summary, Immuno-phage-PCR is considered to be a useful approach to toxin detection.

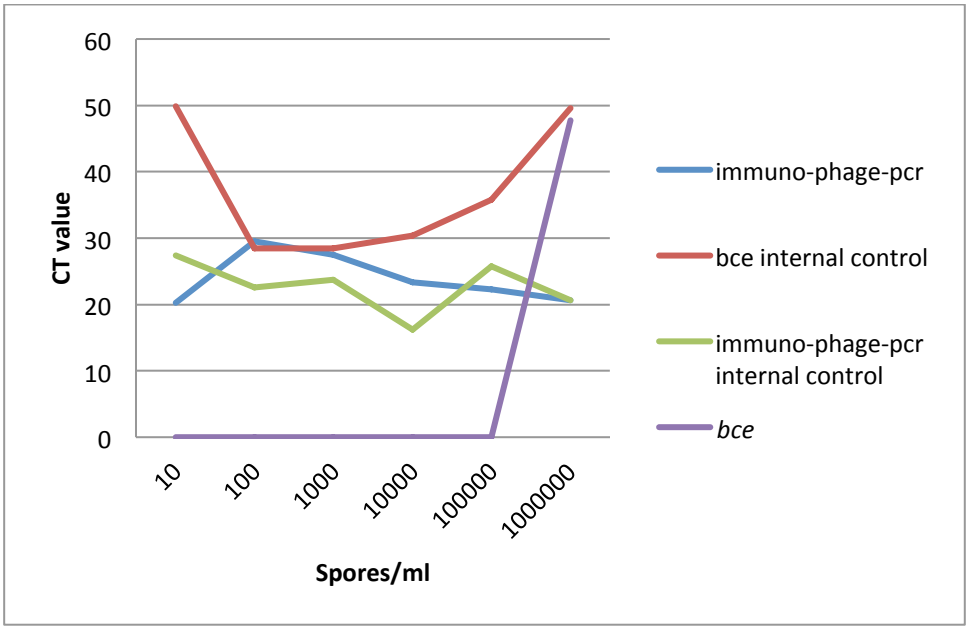


Figure 4.21. Summary of cycle threshold values obtained from immuno-phage-PCR detection of *Bacillus cereus* spores. Detection of *bce* and *scFv* genes, plus their respective internal controls, was carried out using real time PCR.

4.6 IMMUNO-PCR USING ANTI-OVALBUMIN APTAMERS

4.6.1 Introduction to SELEX

Aptamers are short random DNA sequences that have a high affinity for specific antigens, developed using a process known as Selective Evolution of Ligands by Exponential Enrichment (SELEX). The method is not widely used in comparison to methods using traditional ligands such as antibodies, however, aptamers have the advantage of being cheap to produce *in vitro*. For that reason, they were chosen for investigation as part of this study.

Aptamers can be RNA, double stranded or single stranded DNA. RNA aptamers have the advantage of a higher degree of secondary structure compared to DNA, however RNA is inherently unstable and therefore more difficult to work with. Therefore, DNA aptamers were developed to ovalbumin using the method described by Bruno and Kiel (1999).

4.6.2 SELEX results

Ovalbumin was bound to Tosyl activated Dynabeads® in order to provide a suitable platform for washing and removal of unbound aptamer species. A negative pre-selection round of SELEX was carried out to eliminate aptamers with an affinity to the paramagnetic beads. The unbounds aptamers were amplified using PCR with aptamer specific primers (AP F and R; Section 2.9.1). The amplified aptamers were then used in the first SELEX experiment using ovalbumin immobilised on Tosyl Dynabeads®. The aptamer pool was incubated with the ovalbumin beads for an hour, unbound species were removed and aptamers with an affinity for ovalbumin were immobilised by magnetic separation. The beads were washed to remove

residual species with low affinity for ovalbumin. Anti-ovalbumin aptamers were eluted from the beads by boiling for thirty seconds and then amplified using aptamer PCR. Sybr Green I was added to the aptamer real time PCR reactions in order to facilitate monitoring of the progress of the SELEX experiment overall. The amplification cycle threshold value of the ovalbumin-specific round 1 aptamers was lower than that of the unbound species. This indicated that, as expected, the majority of the aptamer pool initially had poor affinity for ovalbumin. However, it was assumed that the concentration of ovalbumin specific aptamers would increase with each round of selection, until finally the majority of the pool contained a majority of ovalbumin specific aptamers. PCR analysis of samples taken during various stages of round 1 showed the presence of several species (Figure 4.22). Bound and unbound species had the same melting temperature (87.5 ^{o}C). Similar amplification products were obtained from each round.

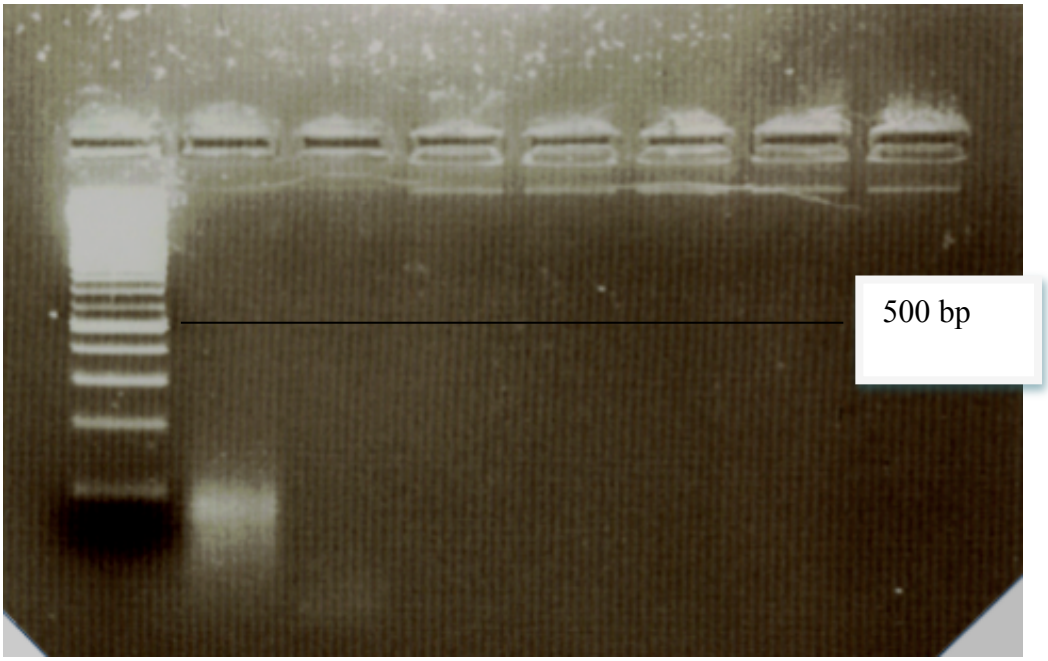


Figure 4.2.2 Agarose gel electrophoresis of SELEX products. Lane 1: Roche marker XIV (100 bp ladder), Lane 2: round 17 aptamers, Lane 3: negative control. A 2 % (w/v) agarose gel was prepared with 0.25ng/ml ethidium bromide in 1 x TAE buffer. The gel was electrophoresed for 30 minutes at 90 volts.

In total, 17 rounds of selection were performed, however, after 17 rounds the majority of the DNA in the pool was not bound to ovalbumin at the point of sampling. At this point, the theory that bound species should be in excess to unbound species was re-examined. It may have been a result of poor affinity to ovalbumin as previously thought, but more likely, it was an effect of the binding reaction kinetics. Therefore, the round 17 pool was purified by microconcentration and then cloned into a pT7 blue TA cloning vector. The recombinant plasmid was then transformed into competent *E.coli* JM109 cells, with 115 clones resulting from the experiment. Real time PCR was used to screen clones for inserts using aptamer (AP F and R) primers. In total, 90 positive clones were obtained (78 %) and screened. The amplicons were concentrated and purified by microconcentration and then sequenced in both orientations at the Dstl sequencing.

The first 32 sequences were aligned in SeqMan, in order to determine whether any of the sequences were duplicated. Seven of the 32 sequences aligned to one contig, including the forward and reverse complement of clone 5. All other contigs contained single sequences, affirming the dominance of clone 5 within the aptamer pool. Clone 5 was therefore used in subsequent experiments. The secondary structure was modelled using Gene Quest software. Although the software models RNA rather than DNA, the degree of secondary structure is demonstrated in Figure 4.23.

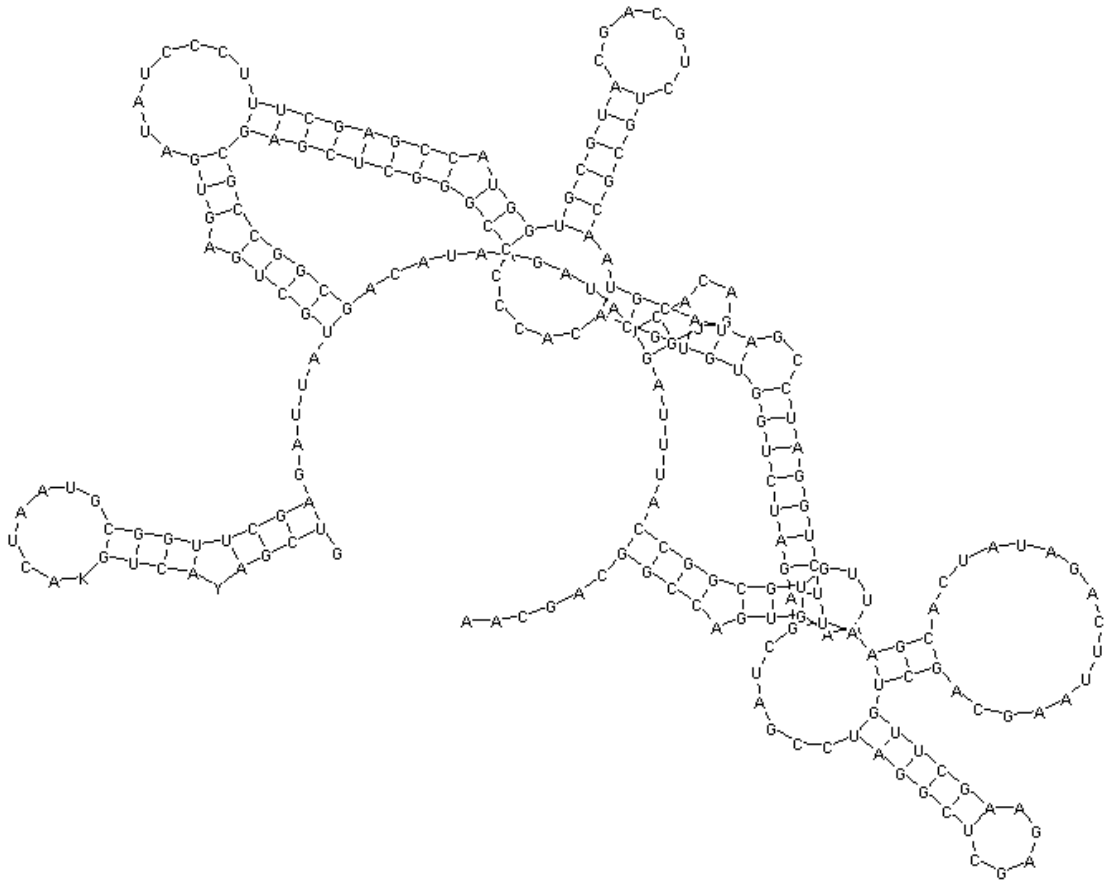


Figure 4.23 Predicted secondary structure of the dominant anti-ovalbumin aptamer (clone 5), as determined by Gene Quest software (Lasergene, DNAstar). The clone 5 DNA sequence was imported into Gene Quest as RNA and folded using Vienna-RNA folding parameters of 37 ^{o}C, with the 'specially stable tetra loops' and 'use dangling energies' options selected.

4.6.3 **ELONA**

The purified clone 5 aptamer was used in an ELISA-like assay termed Enzyme Linked OligoNucleotide Assay ELONA, (Drolet, 1996). The double stranded clone 5 DNA was used as a capture ligand with 100 μg of ovabumin and 2.5 μg detection antibody. Streptavidin-HRP was used to report aptamer binding, however, no signal was observed (A_{414}=0.05, n = 84) following the addition of the ABTS substrate apart from in the control row which used ovalbumin antibodies as the capture ligand (detection limit 0.1 ng/ml; A_{414}= 0.128). Immuno-PCR was also carried out with these samples. Results were inconsistent, with cycle threshold values between 9 and 15 being obtained arbitrarily.

The clone 5 aptamer dsDNA pool did not function effectively as a capture ligand in the previous experiment, presumably as it had low affinity for ovalbumin in spite of 17 rounds of evolution being carried out. As affinity was expected to be higher an alternative use of the aptamer was evaluated. In order for it to function as a detection ligand it was biotin labelled to facilitate enzymatic detection. Labelled aptamers were produced by PCR using biotinylated AP F and AP R primers, followed by purification by microconcentration. An Immulon 2 96 well plate was coated with ovalbumin ranging in concentration from 1 μg/ml to 0.1pg/ml. The plate was then blocked in 2 % (w/v) skimmed milk powder, followed by the addition of clone 5 aptamers, labelled either at the 5', 3' or both ends. Signal was generated by the addition of a streptavidin horseradish peroxidase enzyme conjugate, followed by the addition of ABTS substrate. The control wells, utilising biotinylated antibodies rather than aptamers, gave an A414 signal of between 1.9 and 2.4 with an ovalbumin concentration of 1 μg/ml. One well, the 5' and 3' labelled aptamer with 1 μg/ml of

ovalbumin, gave an A414 reading of 0.443. However, this result was not reproduced when the experiment was repeated, indicating that the colour change was spurious. All other aptamer containing wells gave a reading similar to that of the background (0.05-0.07). The false negative results obtained by using aptamers in place of antibodies also indicated the aptamers were not able to bind ovalbumin sufficiently.

The same protocol was then followed using an initial ovalbumin concentration of 100 μg/ml, but again no signal was generated by aptamer binding.

The 96 well plates described in Section 4.6.3 were also subjected to PCR analysis, using the aptamer primers to amplify clone 5 DNA bound to the ovalbumin on the plate. The results were non-specific, confirming the ELONA results. This approach was also tried with unlabelled aptamers, in case steric hinderance was caused by the late introduction of the biotin molecule to the experiment. Ovalbumin was added to the plate at a concentration ranging between 1 mg/ml and 0.1 fg/ml. The aptamer was added at a concentration of 28 ng per well. The results using unlabelled dsDNA aptamers were also non-specific and subject to a high background, with amplification occurring indiscriminately at a cycle threshold value of between 12 and 15 on the Corbett Rotorgene.

4.6.4 **Anti-ovalbumin aptamers as capture ligands**

A final investigation of the affinity of clone 5 aptamers for ovalbumin was undertaken, using them as capture ligands in an ELISA-type assay. A 96 well Immulon 2 plate was coated with 200 ng of aptamer dsDNA in Reacti-Bind™ DNA Coating Solution (Pierce, UK). The plate was then blocked with 2 % (w/v) skimmed milk powder and ovalbumin was added at a concentration of 100 μg per well.

Colorimetric detection of antigen binding was carried out with the biotin labelled polyclonal antibodies used in I-PCR experiments. Colour was observed throughout the whole plate, with an absorbance reading of 2, indicating that either blocking was insufficient or that the aptamer binds non-specifically to a component of the reaction. The aptamer concentration was reduced to 20 ng with the aim of reducing the background signal. However, colour was observed throughout the plate again, with a reading of 0.2. Diluting the aptamer concentration further simply reduced the absorbance reading proportionally, with 2 and 0.2 ng of aptamer giving readings of 0.073, the same as the background signal. These experiments therefore indicated that the aptamer had poor specificity for ovalbumin and the colour change was assumed to be a cross reaction with one of the immunoassay components. To determine which reaction component the aptamers had affinity for, the assay was repeated with several extra controls. The assay was carried out without the biotin labelled detection antibody, which should have given a negative result. However, an aborbance reading of 1.9 was observed. As streptavidin-HRP is very commonly used in immunoassays it was thought that the possibility of non-specific binding to ovalbumin or the plastic ELISA plate was unlikely. However, the plate had been treated with Reacti-Bind™ which may have affected the protein binding properties of the plastic. A second control experiment was carried out, eliminating the use of ovalbumin. The absorbance reading was also 2, again suggesting that the streptavidin-HRP was binding to the aptamer or plate. To discount the possibility of the aptamer having affinity for the plate a final set of experiments was carried out with varying concentrations of aptamer. Eliminating the capture aptamer resulted in an absorbance reading of 0.3 which, at three times the normal background signal of 0.07, would be falsely interpreted as positive. Similarly, the use of a low aptamer concentration with varying ovalbumin concentrations resulted in a reading of 0.3.

Therefore excess background signal was caused by insufficient blocking. As the absorbance reading was only high when the aptamer concentration was high, therefore it can be assumed that streptavidin-HRP was able to bind to the aptamer. The aptamer was therefore not considered suitable for ovalbumin detection in an assay using biotin and streptavidin and was not investigated further. These results are summarised in Table 4.6.

Aptamer Concentration (ng)	Ovalbumin Concentration (μg)	A414 (mean)
200 ng	100	2.12 (strong positive)
20	100	0.236 (weak positive)
2	100	0.073 (background)
0.2	100	0.076 (background)
Neat aptamers (200ng), no biotinylated antibody control	100	1.91 (strong positive, demonstrating poor specificity of the aptamer)
Neat aptamers (200ng) , no antigen control	100	2.07 (strong positive, demonstrating poor specificity)
No aptamer (H_2O control)	100	0.30 (weak positive/high background
20	100	0.2-0.5 (weak positive/high background)
20	10	0.2-0.5 (weak positive/high background)
20	1	0.2-0.5 (weak positive/high background)
20	0.1	0.2-0.5 (weak positive/high background)
20	0	0.2-0.5 (weak positive/high background)

Table 4.6 Summary of data obtained using an anti-ovalbumin aptamer as a capture ligand.

4.6.5 **Immuno-PCR using ssDNA aptamers**

The experiments described previously were performed using double stranded DNA. It was unknown whether this would present a problem, since other researchers have found that single and double stranded DNA from the SELEX pool bind equally well to their targets (Bruno and Kiel 2002). However, as SELEX experiments were performed with ssDNA, an experiment was performed to isolate either the sense or antisense strands for further use. Biotin labelled aptamer-specific forward or reverse oligonucleotides were bound to Dynabeads Streptavidin as described in Section 2.7. The oligo coated beads were then used to capture their complementary strands from the heat denatured SELEX pool. The removal of dsDNA from the pool was then verified using Sybr Green I agarose gel electrophoresis. The single stranded DNA pools were further supplemented using asymmetric PCR.

Immuno-PCR using ss-DNA aptamers was carried out as described in Section 4.6.3. Following incubation of the aptamers, the plate was washed three times in PBST, then three times in dH_2O, soaking the wash for 1 minute each time to reconstitute as much free DNA as possible. However, PCR samples taken at this stage were positive, indicating the presence of a large amount of unbound DNA. A further three washes were performed, however PCR results were still indiscriminately positive, regardless of whether the sense or antisense aptamer was used.

The SELEX experiments presented here were unsuccessful. Agarose gel electrophoresis was performed on DNA samples taken from experiment rounds 1, 4, 5, 6, 11 and 17 which showed the presence of smears in each lane. This indicated that the diversity of the pool was high, indicating that the aptamer pool was not the

cause of the problem. The failure of the aptamer to capture ovalbumin when used with Reacti-Bind® solution may have been caused by steric hinderance. The possible cross reaction with streptavidin is likely to have contributed to the poor results observed when the aptamer was used as a detection ligand. Similarly, the addition of biotin to the aptamer may have changed its structure, preventing it from binding effectively to ovalbumin. The use of real time PCR in SELEX was intended to provide more information about the experiment, using changes in melting temperature and crossing point to indicate changes occurring within the pool. However, the use of Sybr Green I may have affected the binding of the aptamer to ovalbumin when ds-DNA was used. With this in mind, a second SELEX experiment was carried out, this time using biotin labelled AP primers.

4.6.6 **SELEX to develop biotin labelled aptamers**

Tosyl activated beads were used as described in Bruno and Kiel (2002) to produce ovalbumin coated magnetic particles. Briefly, the SELEX library (23 μg) was amplified by PCR as described in Section 2.9.1 using biotin labelled AP F and AP R primers. A negative pre-selection round of SELEX was carried out to eliminate biotinylated aptamers that bound to Tosyl activated magnetic beads. The unbound species were denatured and added to ovalbumin coated beads for 1 hour at room temperature on a rolling platform, and then washed three times in 1 ml of binding buffer. The aptamer bound beads were then added directly to a 100 μl PCR reaction. The supernatant containing amplified biotinylated aptamers was recovered by magnetic bead separation in a Dynal MPC. The aptamers were then used in 5 rounds of SELEX.

The biotinylated aptamers produced following five rounds of SELEX were briefly tested in several immunoassays for the detection of ovalbumin. Non-specific binding was observed when using the round 5 aptamer pool as a capture or detection ligand (data not shown). It is thought that immobilisation of ovalbumin on paramagnetic beads may have influenced the selection process adversely, by reducing the number of binding sites available to the aptamers. This is in contrast to the results reported by Bruno and Kiel (2002), who successfully used magnetic bead bound toxin to evolve aptamers for the detection of toxins. Furthermore, they were able to amino-modify the resulting aptamers without their binding efficacy being adversely affected. However, in their work reported in since then (Fan *et al*., 2008), they note that the speed at which an experiment is carried out may have an effect on the specificity of the aptamer.

4.7 DISCUSSION

A number of immuno-PCR methods were developed for detecting toxin simulants. The simplest method involved converting an existing ELISA to I-PCR using biotin labelled DNA. It was discovered that the method by which the biotin labelled DNA was bound to streptavidin affected the sensitivity and background signal of the assay significantly. Binding streptavidin to an antibody-antigen sandwich, followed by addition of biotinylated DNA lead to high levels of non-specific binding. It was thought that adding streptavidin and biotinylated DNA in succession favoured the formation of biotin labelled antibody-streptavidin complexes, leaving limited binding capacity for biotin labelled DNA molecules. When biotinylated DNA and streptavidin molecules were pre-incubated together prior to their addition to the immunoassay, the results obtained were in line with those discussed in the open literature (including but not limited to Sano *et al.,* 1992; McKie *et al.,* 2002; Lind

and Kubista 2005; Babu *et al.,* 2011). Although the I-PCR format seemed inelegant compared to others used in this study, the labelled DNA was simple to synthesise and could be used in conjunction with any biotin labelled antibody. This would facilitate the conversion of any ELISA to immuno-PCR with relative ease, meaning that the technique could be accessed by any laboratory when the extra sensitivity of I-PCR is required. Therefore, I-PCR using biotin labelled DNA would be a useful toxin detection method for future experiments.

Another simple I-PCR conversion was tested using neutravidin labelled amplicon. This was added directly to the biotin labelled secondary antibody and therefore did not increase the assay time compared to ELISA, in contrast to I-PCR using biotinylated DNA. Although results obtained using this method were inconsistent compared to those obtained with the biotin labelled I-PCR system, the detection limit was comparable once the concentration of amplicon used was optimised at 100 copies/μl. It is thought that the optimal concentrations of biotin and neutravidin labelled DNA were different as a result of the method used to produce the labelled oligonucleotide primers. Biotin-TEG oligonucleotides are simpler to produce in comparison to thiol modified molecules which must then be further treated to bind to neutravidin. Both oligonucleotides were purified by HPLC, however the average concentration of neutravidin may have been low compared to the number of biotin molecules per oligonucleotide. Therefore it is conceivable that, although the method used to bind the biotin labelled DNA to the antibody was crude, the DNA labelling method may have been more efficient. Further work to reduce the non-specific binding could involve increasing the stringency of the wash steps, for example by using a higher concentration of Tween-20. Alternatively, other non-ionic detergents such as Triton-X100 or Pluronic® could be used in future work.

It was thought that covalently linking DNA to antibodies may increase the sensitivity of I-PCR further, since the number of DNA molecules bound per antibody would be less variable than those exploiting the affinity of biotin for avidin. The covalent conjugation of the amine modified DNA with the NHS ester allowed formation of amide bonds. Furthermore, the number of components that could bind non-specifically to the Immulon 2 plate was lower than with the other I-PCR methods tested. However, the method used to produce the DNA conjugated antibodies was complex. Furthermore, it was not possible to optimise the antibody labelling reaction since only one attempt could be made at Alchemy. The assumption was made that one DNA molecule was bound to each antibody, however this may not have been the case. The results obtained using this method were poor compared to the other I-PCR methods tested, with the detection limit on average remaining the same as with the ELISA or higher. This was unexpected and contradicted observations made in the open literature at the time of this study (Hendrickson 1995, Lind and Kubista 2005, Niemeyer 2007). It is thought that the high background was a consequence of being unable to optimise the reaction to the same degree as with the non-covalent methods of labelling. Furthermore, some researchers are still opting to use the 'crude' biotin labelled DNA I-PCR method (He *et al.,* 2011). Therefore, the assumption that directly labelling the antibody would be the quickest and most sensitive I-PCR method was incorrect. If future I-PCR work were to be carried out as an extension of this study, the simplicity of the 'crude' method would be exploited.

I-ϕ-PCR was demonstrated to be successful for the detection of *B. cereus,* with the signal obtained from the negative control samples comparable to that obtained with

optimised I-PCR methods using biotin and neutravidin labelled antibodies described previously. However, this method was advantageous since it eliminated the need for antibody labelling as single chain antibodies could be detected using a PCR reaction specific for the phage they were displayed on. Since the DNA was packaged within phage the assay may have been less prone to non-specific binding of DNA.

The sensitivity of the I-ϕ-PCR assay was not compared to the conventional immunoassay, since it was not a fully characterised antibody, being designed to target *Bacillus anthracis* rather than *Bacillus cereus*. Instead the sensitivity of the I-ϕ-PCR assay was compared to the spore PCR. I-ϕ-PCR was demonstrated to be more sensitive than PCR with samples containing low concentrations of DNA. This would make the method particularly suitable for detecting purified toxins. It is possible that the method may be further improved by the use of single chain antibodies displayed on the surface of hyperphage, since the number of antibodies per hyperphage would be greater. However it was not possible to test this within this study. Phage display mediated I-PCR was also used successfully by Guo *et al.,* (2006). However, the method is not widely used as it is thought that single chain antibodies do not have the affinity for their target molecules that their parent monoclonal antibodies have. Therefore, although the use of single chain antibodies is becoming more widespread, their use in I-PCR is unlikely to increase.

The SELEX process used in this study was unable to produce aptamers suitable for use in ovalbumin detection assays, although the SELEX process itself had been, and continues to be used successfully by numerous research groups. Most SELEX experiments use 5 to 10 cycles of selection. 17 rounds of selection were carried out in this study due to assumptions made incorrectly by monitoring the reaction by real

time PCR. However, this is not thought to be the cause of the failed experiment. Sequence analysis of the round 17 pool showed that one species, clone 5, was represented more commonly, indicating that the SELEX experiment was successful.

Steric hinderance caused by the use of Reacti-Bind™ solution may have been a problem when using clone 5 as a capture ligand, as the method used to bind the DNA to the plate was proprietory. It may have changed the conformation of the aptamer or bound the ovalbumin-binding site to the plate making it non-functional as a capture ligand. Similarly, the ovalbumin immobilised on paramagnetic beads may have been presented to the aptamers in a different orientation on a 96 well plate. However, this effect has not been observed by other researchers including Bruno and Keil (2002), who successfully switched between magnetic bead and polystyrene supports. Magnetic beads are commonly used to immobilise antigens as they facilitate rapid removal of unbound aptamers and are used by numerous research groups. The automated method developed by Famulok *et al.,* (1996), using magnetic bead immobilised antigen and a robotic platform has been used commonly since then (Hunsicker *et al.,* 2009).

A lack of secondary structure of the DNA has also been ruled out. Although RNA aptamers are commonly used, the decision to use DNA aptamers was taken due to the expense of stabilising the molecule to avoid nuclease degradation. dsDNA was used in the SELEX experiments in this study, which is not common, however Fan *et al.,* (2008) proposed that, once bound to their target, dsDNA aptamers are denatured, leaving ssDNA bound to the target. Therefore, a lack of secondary structure is not thought to be the cause of the lack of affinity of the clone 5 aptamers for ovalbumin. It should be noted that some of the aptamers developed by Fan *et al.,* (2008)

exhibited cross reactivity with other toxins. When their experiment was repeated the same cross reaction was observed. The reason for the failure of some of their experiments is unknown, but may be attributed to the speed at which the whole process was completed (10 hours). The method in which SELEX is carried out may therefore be crucial to the success of the experiment. The use of Sybr Green I, a DNA intercalater, may have prevented efficient binding to ovalbumin, therefore end-point PCR should be used in future experiment, with samples being taken for real time –PCR analysis if required.

The cross-reaction of clone 5 with streptavidin-HRP is interesting. The control experiments summarised in Table 4.3 showed that the aptamer did not bind indiscriminately to the ELISA plate or reaction components, with the exception of streptavidin-HRP. Ovalbumin and avidin are both present in hen egg white, so it seems possible that the ovalbumin purchased from Sigma (type III; 90 % purity) also contained avidin. It is possible that failure of the SELEX experiment could partly be attributed to the evolution of aptamers to egg white rather than ovalbumin specifically. However, immuno-PCR using an anti-ovalbumin capture antibody with clone 5 as the detection ligand also resulted in false positive results, despite the lack of streptavidin. This indicates that the aptamers generally had poor specificity for ovalbumin and they were not suitable for further use.

Other research groups have recently published papers describing immuno-PCR strategies using aptamers which indicates that the method has potential for sensitive detection of proteins despite the results presented here (Yoshida *et al.,* 2009; Pinto *et al.,* 2009). Ding *et al.,* (2009) showed that the length of the aptamer is important to

the success of the experiment and had the most success with a 93 bp aptamer. The aptamers used in this study were of a similar length, at 96 bp.

Further SELEX experiments should be performed with a different antigen; however this was not possible within the funding constraints of this study. Furthermore, the new library could be biotin labelled, in order to ensure steric hinderance is not caused by the addition of a label once the aptamer has been evolved. The use of a competitor protein must also be considered to improve the specificity of aptamers introduced to the experiment. The most vital lesson to be learnt from this study however, is that the purity of the antigen is crucial to the success of the experiment.

The data presented here demonstrated that a fully optimised immuno-PCR assay can offer a significant increase in sensitivity of detection compared to ELISA, although it should be noted that the method used to DNA label antibodies is crucial to the success of the assay. This may not be necessary for routine use in most laboratories, particularly as there are increased reagent costs and assay time. However, immuno-PCR offers an advantage over ELISA for detection of samples containing low concentrations of protein, such as toxin. Additionally, I-PCR was also able to detect samples where PCR failed due to the low concentration of DNA in the sample. This is particularly important in samples that may have been extensively treated, such as purified toxins, which evade PCR detection. In conclusion, I-PCR is considered to be a useful approach for detecting biological toxins.

5 GENETIC DIFFERENTIATION OF *R. COMMUNIS*

5.1 INTRODUCTION

The results presented in Chapter Three showed that PCR was not a reliable method of toxin detection if contaminating DNA was present in low quantities, therefore it is not recommended that it be used routinely. Conversely, if DNA was present in sufficient quantity such as in culture, plant material or crude toxin preparation samples, PCR could be used to successfully infer the presence of toxin. Therefore PCR is considered a useful approach to toxin detection in specific situations. A positive result obtained by PCR could be a starting point for a researcher to perform further analyses with the aim of determining the origin of the causative agent. The use of PCR as a strain differentiation method is well described for bacterial and plant species and has been used routinely for many years. More relevant to this study, it has also been used for typing toxin DNA, for example Macdonald *et al.,* (2008) used variable number tandem repeat (VNTR) analysis to differentiate between species of *Clostridium botulinum* A using contaminating DNA in crude toxin preparations. However, at the time of starting the research for this thesis, similar methods for the differentiation of *Ricinus communis* strains had been unsuccessful as it is assumed to have low genetic diversity, making differentiation problematic (Allan *et al.,* 2008). Numerous techniques exist for genetic typing of plants, for example those based on hypervariability of satellite DNA within the genome. Those chosen to complement the research carried out by other groups are summarised in Table 5.1. However, the main emphasis of this Chapter was to evaluate the potential of rapid pyrosequencing, specifically 454™ as a single nucleotide polymorphism (SNP) discovery tool (Barbazuk 2007; Sugarbaker 2008; Vera *et al.,* 2008).

Table 5.1. Nucleic acid methods chosen for differentiation of *R. communis* cultivars.

Name	Acronym	Description
Roche 454™ Sequencing	454	High-throughput, rapid pyrosequencing with long reads (>400 bp).
Random amplified polymorphic DNA	RAPD	Amplification of genomic DNA using a random sequence 10 bp primer.
Restriction fragment length polymorphism	RFLP	Restriction enzyme digestion of genomic DNA which is agarose gel electrophoresed to produce a DNA fingerprint.
Amplified fragment length polymorphism	AFLP	As RFLP, however digested fragments are ligated to specific primers and then amplified by PCR.
Simple sequence repeat	SSR	Short regions of repeated sequence consisting of 2 or more nucleotides. Also known as microsatellites, they are easily detected and quantified by PCR.

5.1.1 **454 sequencing™**

The genome of *R. communis* Hale strain was Sanger sequenced by the J. Craig Venter Institute (JCVI) in 2008. Using a whole genome shotgun strategy, Chan *et al.,* (2008) achieved a four-fold coverage of the *R. communis* genome. This was assembled and annotated using multiple gene prediction programs and alignments derived from 50,000 expressed sequence tags along with cDNA and protein sequences. The genome assembly is publicly available (http://castorbean.jcvi.org) as are the Expressed Sequence Tags (ESTs) (http://plantta.jcvi.org). These data could be useful to help devise PCR primers specific to *R. communis* for the genetic differentiation between cultivars: regions of repeat sequence, for example, can be found *de novo* using repeat search programmes. However, previous attempts to differentiate between cultivars have been unsuccessful using such methods (Allen *et al.,* 2008), presumably due to the lack of genetic diversity amongst crops. SNP discovery by high-throughput sequencing has been reported for species other than *R. communis* (Barbuzuk 2007). The method is ideal for rapid genome annotation and analysis and is capable of producing mega-, if not giga-bases of sequence per experiment. High-throughput sequence analysis of the *R. communis* genome was previously unstudied; therefore the method was investigated as part of this study.

5.1.2 **Aims**

The aim of this part of the study was to evaluate the potential of 454 Sequencing™ for identification of regions of genetic variability within the *R. communis* genome. For simplicity, the software supplied with the 454 Sequencer™ was investigated as a SNP discovery method, as the Roche *De novo* Assembler and Reference Mapper software provided are reportedly suitable for this application. The secondary aim

was to investigate more traditional genetic approaches for differentiating between cultivars for comparison.

5.2 SNP DISCOVERY BY *454 SEQUENCING™*

5.2.1 *R. communis* Library Preparation

In order for the *R. communis* genome to be sequenced it was necessary to extract high quality DNA from plant material. The MoBio Powerplant™ kit was used successfully to extract 5 μg of DNA. DNA was quantified using the Nanodrop® spectrophotometer and the Qubit fluorimeter as the concentration used for library preparation had been finely optimised by Roche. The library was then prepared using the method described in detail in Section 2.11. Briefly, the genomic DNA was sheared by nebulisation and then purified on a silica column. The eluted DNA was size separated, ligated to sequencing adapters and then analysed on a high resolution gel matrix to confirm that it had been sheared to the optimal fragment size for sequencing. The library was quantified once more to ensure that a titration could be carried out to determine the number of copies of DNA per sequencing bead that would give optimal enrichment. A small volume emulsion PCR was then carried out using 2, 4, 8 and 16 copies per bead. The PCR reactions were recovered by aspiration and washed in isopropanol and ethanol. Beads carrying the DNA library were annealed to an enrichment primer-bound magnetic particle and then quantified. The enrichment results should show a correlation to the number of copies of DNA per bead. This was not the case. Therefore a second small volume emulsion PCR was repeated. The results of the second experiment were also not as expected, with the percentage enrichment for 1 copy per bead, at 14 % (Table 5.2), being excessive according to the guidance issued by Roche. It should be noted, however, that since the conclusion of this study, Roche have issued new guidance stating that enrichment

values of up to 20 % are likely to give high quality sequence output. It is very important to titrate the number of copies of DNA per bead as too few would lead to empty picotitre plate wells and too many would cause mixed sequence reads, however, in the absence of useful emPCR titration results, 4 copies per bead were chosen for conducting a large volume PCR in consultation with Roche technical support. The results are shown in Table 5.2.

Table 5.2. Library preparation results for *R. communis* strains Jianxiang and Zibo 1 using 454™ Sequencing emulsion PCR. Jianxiang emPCR results for regions 1 and 2 are shown in grey and white respectively.

Copies per bead	Percentage Enrichment (small or large volume emulsion PCR)									
	Jianxiang SV		Jianxiang LV						Zibo 1 LV	
1	5.46	14.76								
2	4.16	7.5								
4	3.5	10.78	4	2.2	3.2	3.8	1.8	1.3		
8	6.29	6.1								
16									2	1.7
32									7.1	3.3

5.2.2 *De novo* assembly and SNP analysis

In total 179 megabases of sequence were obtained from one Jianxiang LV emPCR. The second and third emPCR reactions did not produce sufficient DNA to run on the sequencer (data not shown). It is thought that the DNA extracted from plants grown overseas degraded during shipping as a result of nuclease release, as it was also unsuitable for production of PCR amplicons over 500 basepairs. Therefore, a second shotgun library was prepared using existing Zibo 1 genomic DNA using updated Phase C Chemistry released by Roche. Both the small and large emPCR kits were improved to include 4, 8, 16 or 32 copies per bead. The small volume PCR enrichment results did not follow a trend; therefore the data was not used to define the DNA concentration required in the large volume experiments. For that reason, the large volume PCR was carried out using different DNA concentrations, resulting in 2 % and 7.1 % enrichment for 16 and 32 copies per bead respectively (Table 5.2). The DNA from this reaction was loaded onto the sequencer, yielding 146 Mb of Zibo 1 sequence which was subsequently analysed using the Roche GS *De Novo* Assembler. Although this level of coverage would not be sufficient to produce reliable genome sequence for complete annotation, the amount of data produced was suitable for discovery of a number of high confidence differences with 20 times coverage using the GS Reference Mapper software. Accordingly, SNPs with a minimum of 20 times coverage of Jianxiang sequence were investigated further. However, the contigs containing putative SNPs did not align with 100 % homology, due to ambiguous base calling (N). Therefore, these SNPs were not investigated further.

In the same way 37 high confidence differences were identified by the GS Reference Mapper software using Zibo 1 DNA. The six most useful SNPs, in terms of depth of coverage and homology amongst Zibo 1 contigs, are summarised in Table 5.3. BLAST analysis was carried out using the sequence from each contig (Table 5.3). Four out of six SNPs had homology to conserved hypothetical proteins encoded by mitochondrial DNA. One SNP was identified within 26S rRNA sequence, another SNP-rich target commonly used in genotyping studies. A further SNP was identified in a retrotransposon carried within the *R. communis* genome.

In order to validate and determine whether the SNPs were present in other *R. communis* cultivars it was necessary to perform further sequence analysis. The sequences flanking the SNPs detailed in Table 5.3 were exported to the Biotage Pyrosequencing™ Assay Design Software (Qiagen, UK). This software facilitates the design of PCR assays that flank the SNP, plus a sequencing primer located upstream of the SNP site. The output of the assay design software is summarised in Table 5.4.

Table 5.3 Location of SNPs identified by the Roche GS reference mapper software in Zibo cultivars.

Hale strain accession number	Hale SNP	Depth	Zibo 1 Contig	SNP	Zibo or Jianxiang SNP	BLAST nucleotide results
53166	1857	84	154654	GTTAAA deletion	1332	*Ricinus communis* conserved hypothetical protein, mRNA; accession numbers gi|255591121|XM_002535395.1 and gi|255581851|XM_002531680.1 (100% identity). Other matches to mitochondrial genes.
38467	1487	70	19819	G>T	1115	*Ricinus communis* conserved hypothetical protein, mRNA; gi|255560297|XM_002521120.1 84% identity. Mitochondrial gene.
30056	948913	84	03509	C>T	588	*Ricinus communis* retrotransposon RC-Tekay, complete sequence; 75% identity GQ294573.1; *Ricinus communis* conserved hypothetical protein, mRNA XM_002533692.1 85% identity
27949	34984	66	60110	T>G	551	*Euphorbia polychroma* and other species 26S ribosomal RNA gene, complete sequence is the best hit, no *R. communis* sequences were matched.
29016	32575	60	122881	A>C	2040	*Ricinus communis* conserved hypothetical protein, mRNA; gi|255582548|XM_002532012.1; (12 similar loci identified in total, all JCVI annotations)
51309	3404	54	63875	A>C	3061	*R. communis* conserved hypothetical protein (JCVI annotation), gi|255589077|XM_002534779.1

Table 5.4 Small amplicon sequencing assays designed using Biotage Pyrosequencing™ Assay Design Software for six SNPs identified by 454™ sequencing.

Jianxiang or Zibo contig number	Sequence (5'-3')	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3'; 5' biotin labelled)	Sequencing primer (5'-3'); biotin labelled
154654	TAAGGAAGGAGGGACGAAGAGGAACGACCAAAACAAAAGAGGGATCAGACCCCCT	GGCAAGAACGCCTTATTACAACT	TGCCTAAAGGGGGTCTGATCC	CTTTCCCTAATACATTAAAT
19819	GGGGATTTGGATCAGGCTGCTGTTGTTGAACTAGG	GTCCGTCAATTCCGAAAATG	TGGGAGAGGTAGCCGAATTAG	CGTCATGCTGGAGCG
03509	GACATTAAGAATTGGCGTAAAGAATCATGGGAATATAATTAACAAGCTTGACCGG	TGCACCATTAGGTTCTACATCATT	TAAGTCACGCCCTAATGCTACC	GCATGAGGACTCTTGAT
60110	CTCGTGTGGAACAAAAGGGTAAAAGCTCGTTTGATTCTGATTTCCAGTACGAATA	ACATTGTCAGGTGGGGAGTT	CACGCTTTCACGGTTCGTAT	CTCAACGAGAACAGAAAT
122881	AGCCTACCATAAGACTGACAATAAAGTCCCAGGCGCACCTGAAGAGTTCTAACTA	GCCCTGTTTTCCATACGGAGATC	CCCTTGCTTTTCGCCACAT	TCATGAAAGATTGTTGGT
63875	CTGGTCCATTCTGGATTTTGGATGACCGGTTCTTTGTCGGAGTTCCCGGTAGATCTG	TCGACAGCCAGGAGACCTC	CCTACTGAGCTTGGGCTGGTAA	GCCTCAGGTGCTGAA

5.2.3 SNP validation by targeted pyrosequencing (PyroMark)

The six putative SNPs were investigated further using targeted pyrosequencing. Briefly, PCR amplicons were produced and purified using a Promega Wizard® SV PCR clean-up kit. Biotinylated PCR amplicons were then immobilised onto streptavidin-coated magnetic beads and denatured to produce single-stranded DNA by using the PSQ 96 sample prep tool supplied with the sequencer. The sequencing primers were hybridised to the single stranded products and then sequencing was performed according to the PyroMark™ instructions. The dispensation order of nucleotides was calculated by the instrumentation when the sequence to be analysed was entered, thereby allowing identification of the SNP. Four strains were analysed using the method. Initially all of the sequences were aligned in Megalign (Figure 5.1), with the aim of verifying that the sequences from each row of the plate aligned with each other as expected. Separate phylogenetic trees were subsequently produced for each Hale contig number studied. The results for Hale references 53166 (Figure 5.2) and 29016 (Figure 5.4) were as expected, with sequence being read immediately downstream from the sequencing primer.

The sequence around the SNP site for contig 53166 showed that the predicted deletion was present only in Zibo 1 and downstream sequence varied between all four strains, hence the decision to present the complete alignment rather than highlight the putative SNP data. This SNP locus and surrounding sequence may prove to be useful for generating data to facilitate differentiation in future. However, other cultivars should be examined in further work.

The SNP identified in contig 29016 was carried by two of the cultivars examined, with the other two cultivars having the same sequence as the reference Hale strain.

Accordingly, the locus may serve to aid differentiation of cultivars in conjunction with other loci.

One sequencing reaction failed to yield a product (30056). The sequences shown in Figure 5.3, Figure 5.5 and Figure 5.6 should have been from homologues of contigs 38467, 27949 and 51309 respectively. However, extensive analysis of the PyroMark data using BLASTN at Pubmed and the castor bean genome assembly at JCVI, plus alignment of the parent contigs in Seqman and Megalign could not match the data to any known sequence. It is noteworthy however that the original 454 data (Table 5.3) showed only 85 % identity to the nearest match in the BLASTN database for 38467 and 30056. The Zibo 1 sequence with homology to Hale contig 27949 showed no identity to known *R. communis* sequence, with the nearest match being *Euphorbia* 26S rRNA. Contig 30056 showed homology to a retrotransposon found within the *R. communis* genome. It is a possibility that this sequence is highly unstable and may not be found within the strains used in this study, explaining the failure of the primer set specific to contig 30056 to produce a sequencing amplicon. The alignment in Figure 5.5 shows that numerous unexpected homopolymer stretches were yielded. These may be genuine as the stretches are short, however, is a known error associated with pyrosequencing (Oliver *et al.,* 2011). The relationship between the fluorescence and number of bases when called by pyrosequencing is generally only linear up to 10 identical nucleotides (Gong *et al.,* 2010). Although it was not possible to validate four of the SNP assays during this study, they should not be excluded from further study. In general, the pyrosequencing reactions generated useful data which serve as an interesting starting point from which to base further differentiation assays, however extensive further work would be required to validate

the data generated by 454 Sequencing™, possibly using a germplasm database. Accordingly, more traditional genotyping methods were examined.

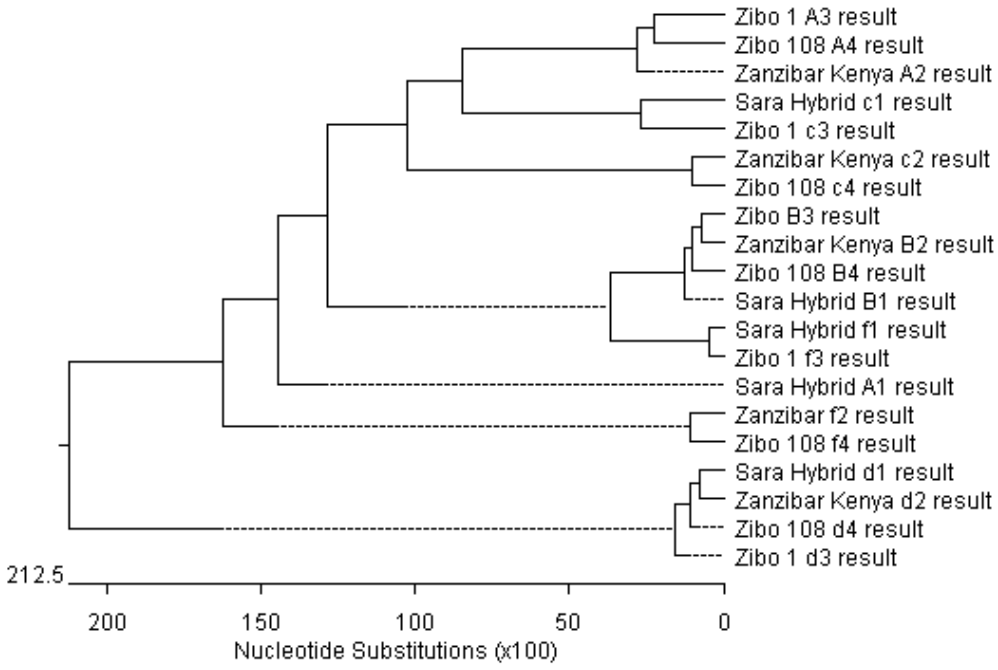


Figure 5.1. Phylogenetic tree illustrating the relationship between each SNP assay. Sequences obtained from 6 targeted pyrosequencing assays were aligned in Megalign using Clustal W. Dotted lines represent a distant relationship whereas solid lines denote a close relationship between two species.

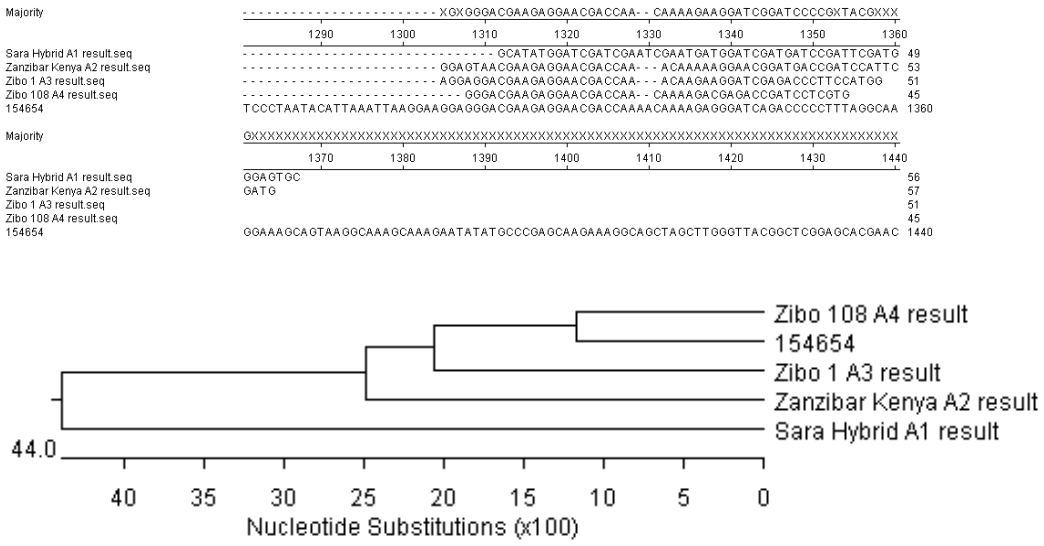


Figure 5.2. Biotage generated sequence alignment and dendrogram aligned to Hale reference contig 53166 for four cultivars of *R. communis*, generated using Clustal W (DNAstar, US). A putative deletion is underlined in red and shown above at position 1331 to 1332 (GAACGACCAAAAC<u>AA</u>AAGAG).

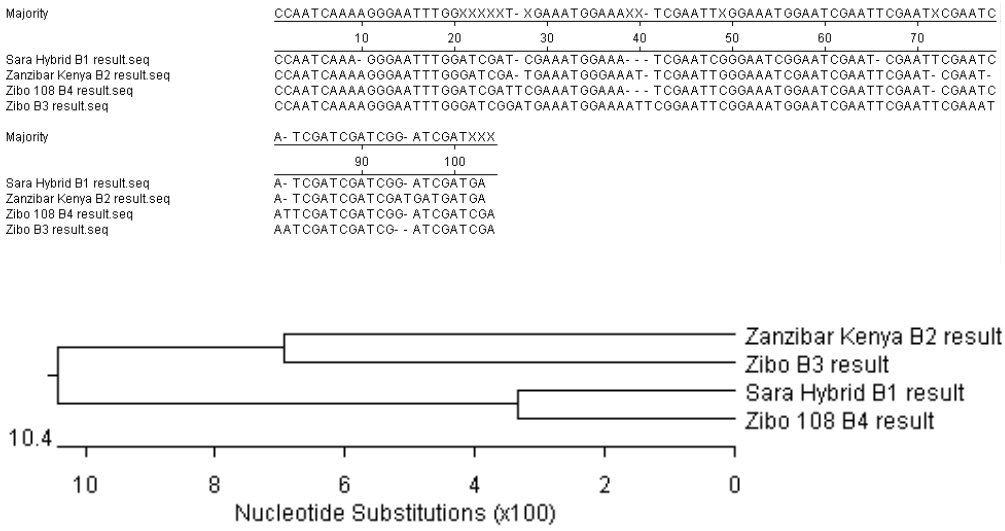


Figure 5.3. Biotage generated sequence data alignment and dendrogram for four cultivars of *R. communis*.

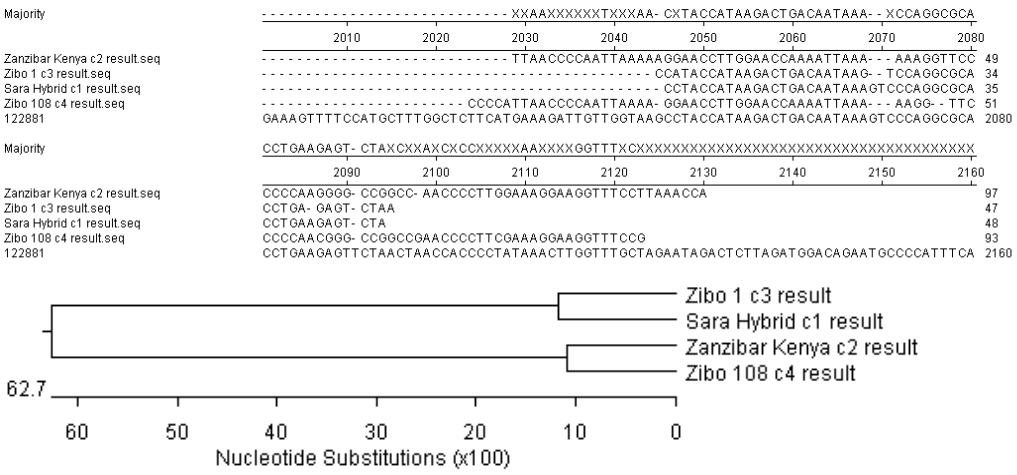


Figure 5.4 Biotage generated sequence alignment and dendrogram around the SNP (2063 A>C) site in Hale reference contig 29016 (Zibo 1 122885) for four cultivars of *R. communis*.

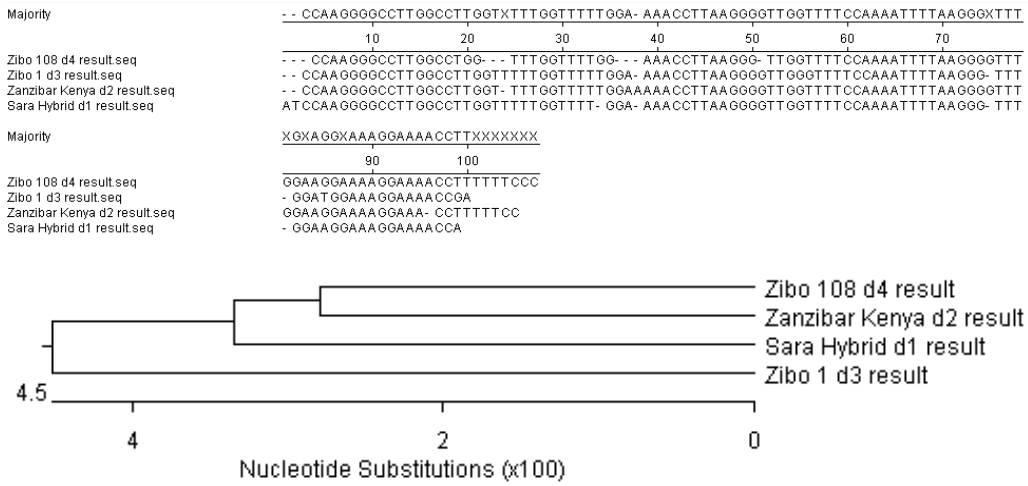


Figure 5.5. Biotage generated sequence alignment and dendrogram for four cultivars of *R. communis*.

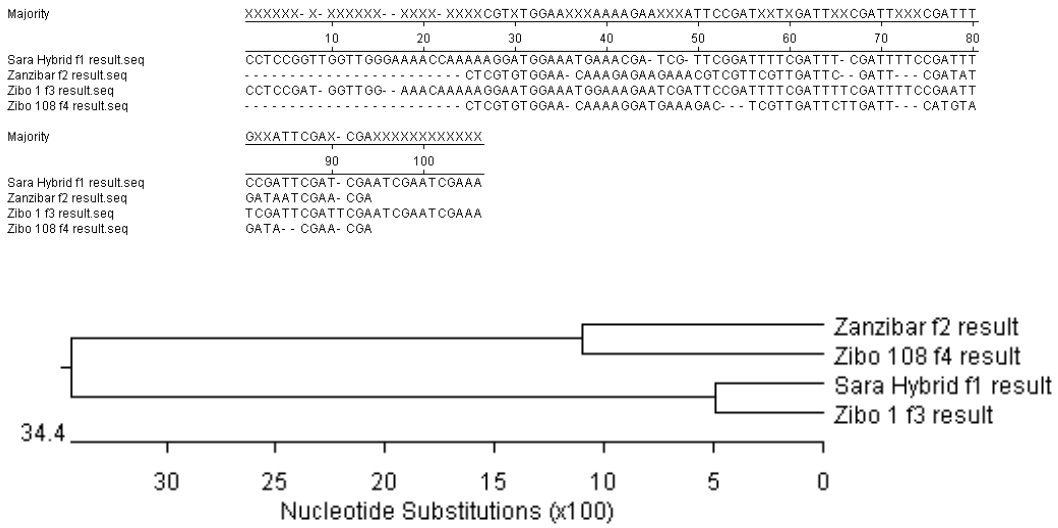


Figure 5.6. Biotage generated sequence alignment and dendrogram for Hale reference contig 27949 for four cultivars of *R. communis*.

5.3 ALTERNATIVE APPROACHES

The sequence analysis showed that 0.85 X coverage interrogation of a genome was not suitable for the discovery of unique SNPs in *Ricinus communis*, although its utility has been demonstrated with other genomes (Vera *et al.,* 2008; Oliver *et al.,* 2011) with greater coverage. It would have been desirable to continue preparing samples for sequencing using emulsion PCR. However, this was not possible as the sequencing chemistry was only updated and used successfully very close to the end of this study (Roche alert number AA0110PAN GS Titanium 12/3/10). At the time of this study 454 Sequencing™ was an emerging technology and a new capability to Dstl, therefore it was designated as high risk. Accordingly, a more traditional approach was developed for comparison to the 454 SNP data.

A review of the literature confirmed that there was no single genetic locus that would enable genotyping of *R. communis* plants. However, several research groups had initiated genotyping studies. Allen *et al.,* (2008) assessed the genetic diversity of 41 castor bean germplasm accessions using AFLP and simple sequence repeats (SSRs). Three AFLP primer sets were used which identified 43 polymorphisms overall. However, no banding patterns revealed cultivar specific markers. Similarly, SSR was able to identify several polymorphic loci but unable to differentiate between cultivars within their germplasm database. O'Connell *et al.,* (2006) sequenced the preproricin genes from 63 cultivars and found 14.4% variability across 1262 bases. Hinckley (2006) used 25 primer sets to investigate a number of loci commonly used for plant genotyping, including *trn*, *rpo*, and *psb*, however none were able to distinguish between cultivars. With this in mind, several alternative approaches were evaluated.

5.3.1 **Random Amplified Polymorphic DNA (RAPD)**

Six commercially available RAPD primer sequences were obtained from Eurofins MWG Operon (Germany) (OPAA1, OPAB5, OPT05, OPAB18, OPAA10 and OPAA20; Section 2.10.1). The primers were demonstrated to work effectively, however none of the six primers was able to produce distinctively different fingerprints with the *R. communis* DNA samples tested (Figure 5.7). Although RAPD is well regarded as a tool for producing DNA fingerprints, the limited number of RAPD primers evaluated in this study were not suitable for this purpose with *R. communis* DNA, again suggesting its genetic diversity is low.

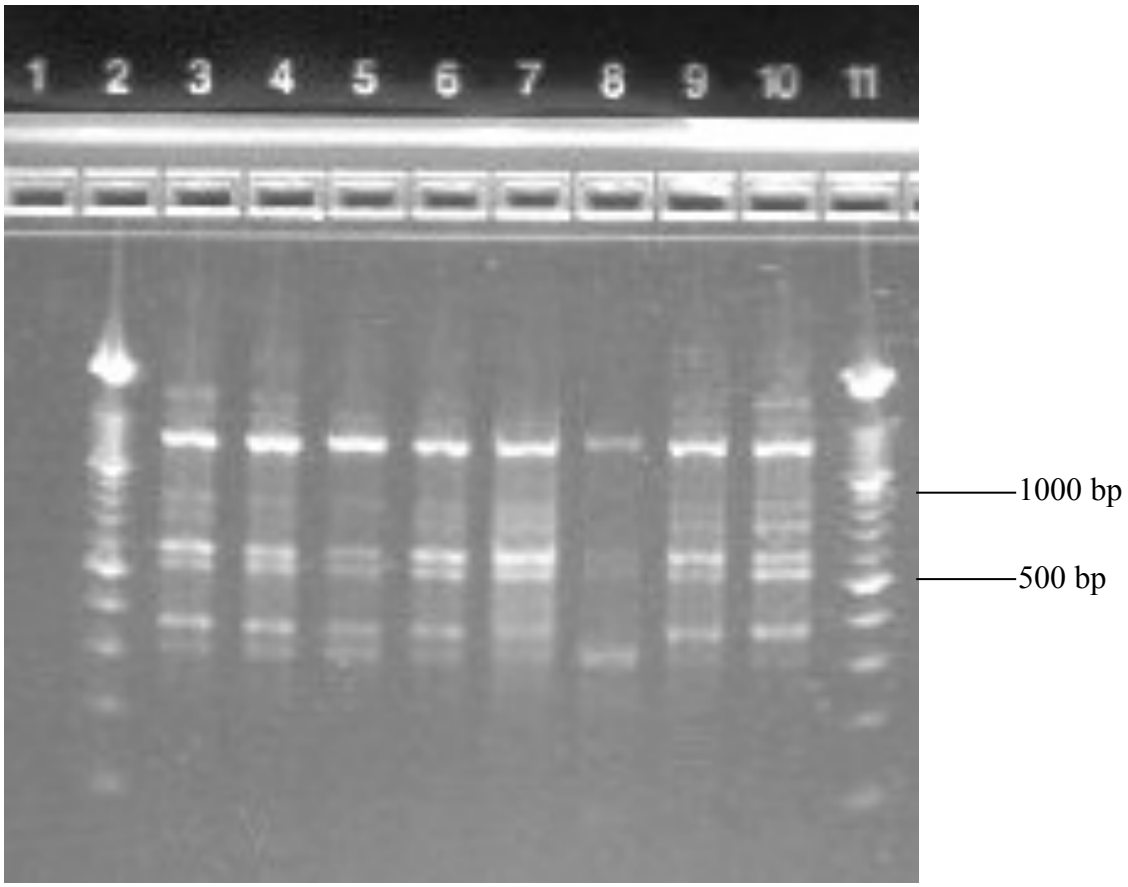


Figure 5.7 RAPD PCR of *R. communis* cultivars using primer OPC-10 resolved on a 2 % agarose E-gel (invitrogen) run for 30 minutes on an E-gel iBase™ (Invitrogen). Lanes 2 and 11: 100 bp molecular weight marker; lane 3, Shang Dong 108; Lane 4, Jin Castor 2; Lane 5, Black Diamond; Lane 6, Sanguinis; Lane 7, Kunming 202; Lane 8, Zanzibar; Lane 9, Zibimas; Lane 10, Zibo 1.

5.3.2 **Restriction Fragment Length Polymorphism (RFLP) analysis**

A new *rca* assay was designed in order to obtain the larger amplicon required for RFLP, in comparison to the *rca* assay used for toxin detection in Chapter 3. Amplification was achieved using primers R1 F and R2 R which produced the expected 1.6 kb fragment (Section 2.10.2). It is of note that some of the cultivars gave rise to a pair of bands around 1.6 kb (data not shown). This was previously reported by O'Connell *et al.,* (2006) who attributed the doublet to truncated versions of the preproricin gene occurring in the genome of some cultivars along with the full length gene. The genome of *R. communis* is either haploid, diploid or tetraploid (Timko *et al.,* 1980), therefore multiple bands may be expected. Although the plants were assumed to be diploid, the ploidy of the samples in question is unknown.

Single digests of *R. communis* DNA were performed with *Bst*NI, *Hae*III and *Taq*I. Double digests were carried out with *Hae*III and *Taq*I and also *Eco*RI and *Hind*III. For all enzymes there were fewer restriction sites in the EST fragment than there were in the *rca* gene fragment. As Figure 5.8 demonstrates, even with just six ricin cultivars and using a double digest with two frequent cutter enzymes, only two finger-print patterns were produced. This illustrates the lack of differentiation achieved by this technique. Similarly, a double digest with *Eco*RI and *Hind*III (Figure 5.9) did not adequately differentiate between cultivars.

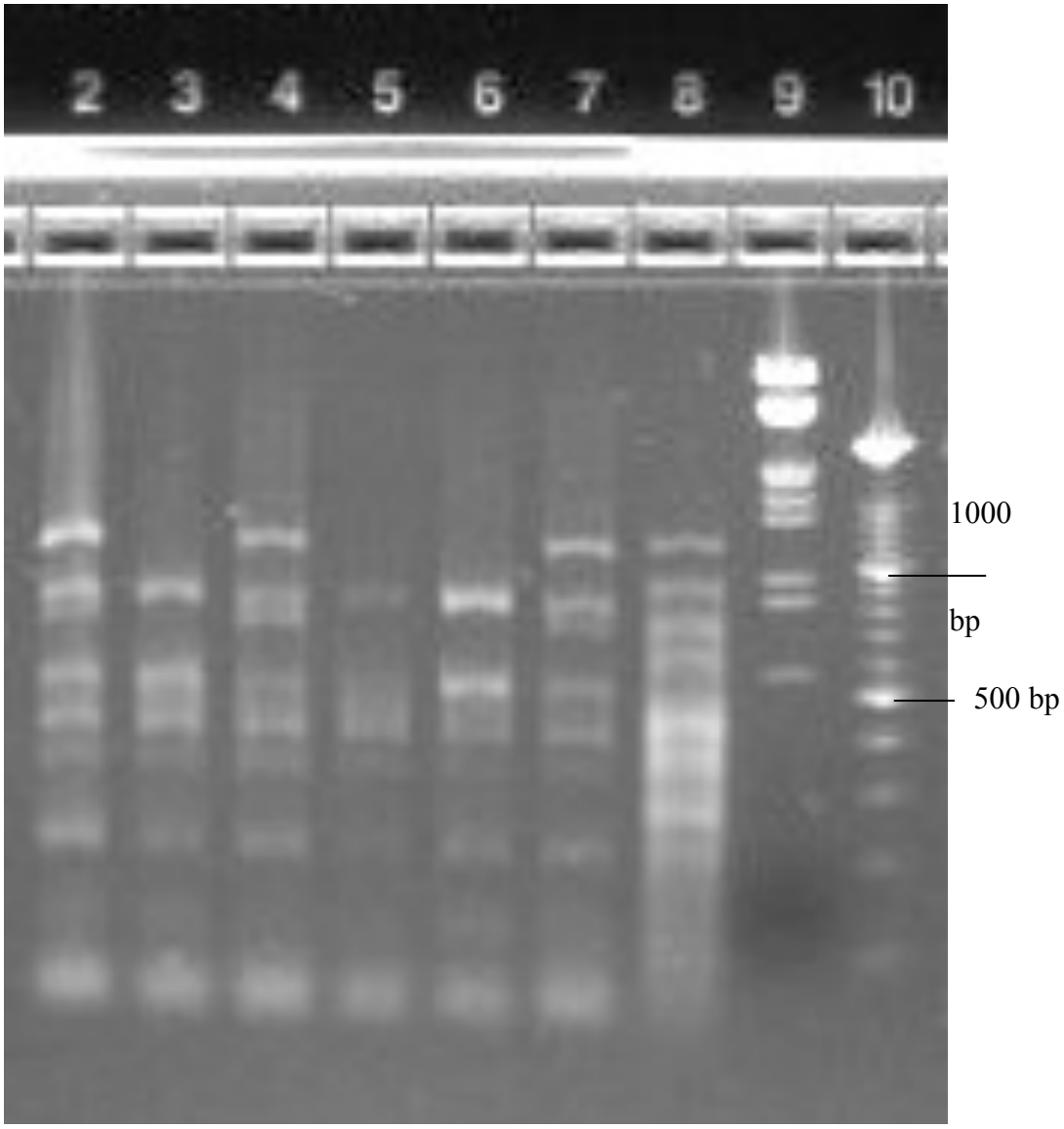


Figure 5.8. RFLP analysis of a 1.6 kb fragment of the *rca* gene of *R. communis* cultivars. The DNA was cut with *Hae*III and *Taq*I and resolved on a 2% agarose E-gel (Invitrogen), run for 30 minutes on an E-gel iBase™ (Invitrogen). Lane 2, Shang Dong 108; Lane 3, Jin Castor 2; Lane 4, Sanguinis; Lane 5, Zanzibar; Lane 6, Zibimas; Lane 7, Zibo 1; Lane 8, *Hae*III and *Taq*I digest of molecular weight marker XIV (Roche; enzyme control); Lane 9, uncut molecular weight marker XIV (Roche); Lane 10, 100 bp marker.

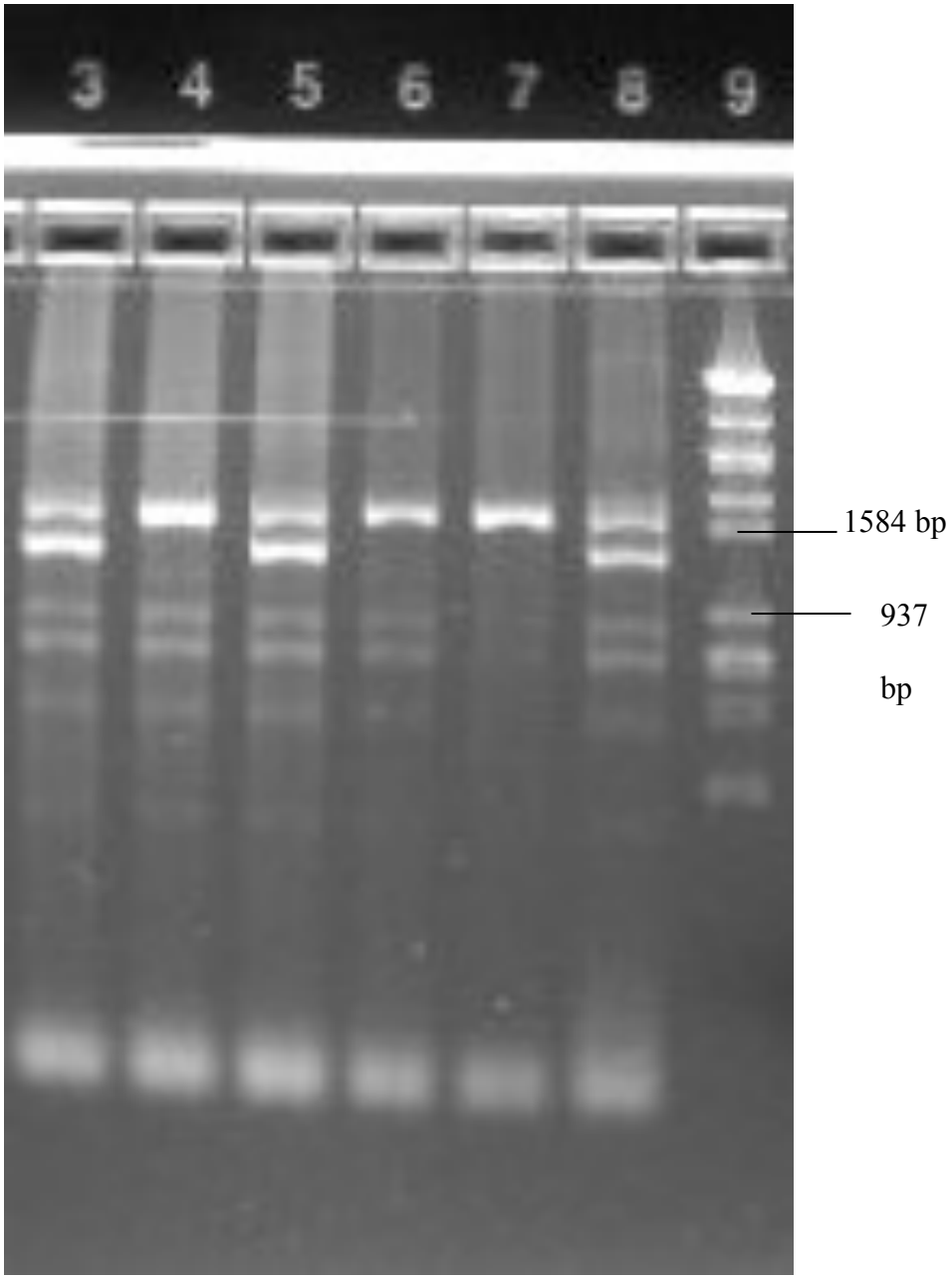


Figure 5.9. RFLP analysis of a 1.6 kb fragment of the preproricin gene of *R. communis* cultivars. The DNA was cut with *Eco*RI and *Hind*III and resolved on a 2 % agarose E-gel for 30 minutes. Lane 3, Shang Dong 108; Lane 4, Jin Castor 2; Lane 5, Sanguinis; Lane 6, Zanzibar; Lane 7, Zibimas; Lane 8, Zibo 1; Lane 9, Molecular weight marker III (Roche).

5.3.3 **Simple Sequence Repeat (SSR) analysis**

Two previous publications have used SSR primers successfully for genomic analysis of ricin (Allan, *et al.,* 2008; Gedil *et al.,* 2009). Allan *et al.,* (2008) used 9 microsatellite primer sets, however neither published the amplification conditions nor the sequences of the primers used. The six SSR primer pairs used in this work (Table 5.4) were obtained by searching the castor bean genome using the publicly available FASTA Simple Sequence Repeat finder software http://espressosoftware.com/sputnik/index.html (Jewell *et al.,* 2006).

The SSRs primers were combined into two sets of three primers in order to make the finger-printing process more rapid and cost-effective: Primer set A (SSR26, SSR40 and SSR47) and Primer set B (SSR52, SSR61 and SSR189). The thermal cycling profiles for the triplex reactions are shown in Table 5.5.

Each reaction was demonstrated to work well with a variety of DNA samples, demonstrated by the presence of numerous amplicon bands on an agarose gel (not shown). In order to discriminate between the number of repeats it was necessary to finely separate amplicons by size using a PAGE gel. Amplicon volumes were reduced to 10 μl by microconcentration. The gels were electrophoresed in 1x Tris Borate EDTA (TBE) buffer, stained in ethidium bromide and de-stained in dH_2O (Figure 5.10).

Cluster analysis was carried out using the Uviband Map software included with the gel documentation apparatus used for this study (UVTec, UK.) with each of the band sets normalised. The Jaccard coefficient method was used to identify the presence or

absence of bands, creating a binary profile. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm was used to create a phenetic dendrogram (Figure 5.11). There was sufficient discriminatory power using just one set of primers to differentiate between all of the cultivars tested.

All of the Zibo cultivars clustered together using the UPGMA analysis. Furthermore, Shang Dong 108 is the closest relative to the Zibo cluster. It would seem to be more than a coincidence that Zibo is a city in Shang Dong province, in the People's Republic of China. Presumably the clustering of these cultivars from one Chinese region reflects some genetic commonality, reflected in this analysis. It must be recognised, however, that the UPGMA analysis presented here is based on phenotypic differences, which cannot be used to determine genetic divergence. Nevertheless, Figure 5.7 serves as an example of the data handling that is possible from the SSR analysis.

Locus	Repeat Type	Primer sequence (5'-3')
SSR26	GA	Forward: TGCTAATTGCAGGAAATAGGAT Reverse: GCAGCTTTTTAGCATAACAATCAA
SSR40	GA	Forward: TGCATCATGGTCCACTCACT Reverse: CATTCTTTTCGGCATTCCAT
SSR47	CA	Forward: GGAGCACCTTTTGCTGAGTT Reverse: TTGGAACAAAGCAGCATCAC
SSR52	GT	Forward: GCCAGCAAGGTTTGCTACAT Reverse: AACTGTCAAACCATTCTACTTGC
SSR61	CA	Forward: GGCTGCTTTACCTTCTACTCAGA Reverse: CAAGAACGCCAATATGCTGA
SSR189	TTTTA$_{(2)}$TTA$_{(3)}$TTTTA	Forward: GATGACTGGTCTCAAGTGGTGA Reverse: AAGAAGAGCTCCTCCTGCAA

Table 5.4. Genetic location, repeated nucleotides and primer sequences used to amplify the SSRs used in this study.

Temperature	Duration	Number of cycles
94 ^{o}C	2 min	
94 ^{o}C	30 seconds	35 cycles
51 ^{o}C (Primer set A) 53 ^{o}C (Primer set B)	30 seconds	
72 ^{o}C	30 seconds	
72 ^{o}C	5 min	

Table 5.5 Thermal cycling conditions for the two triplex reactions used to amplify the selected SSR loci.

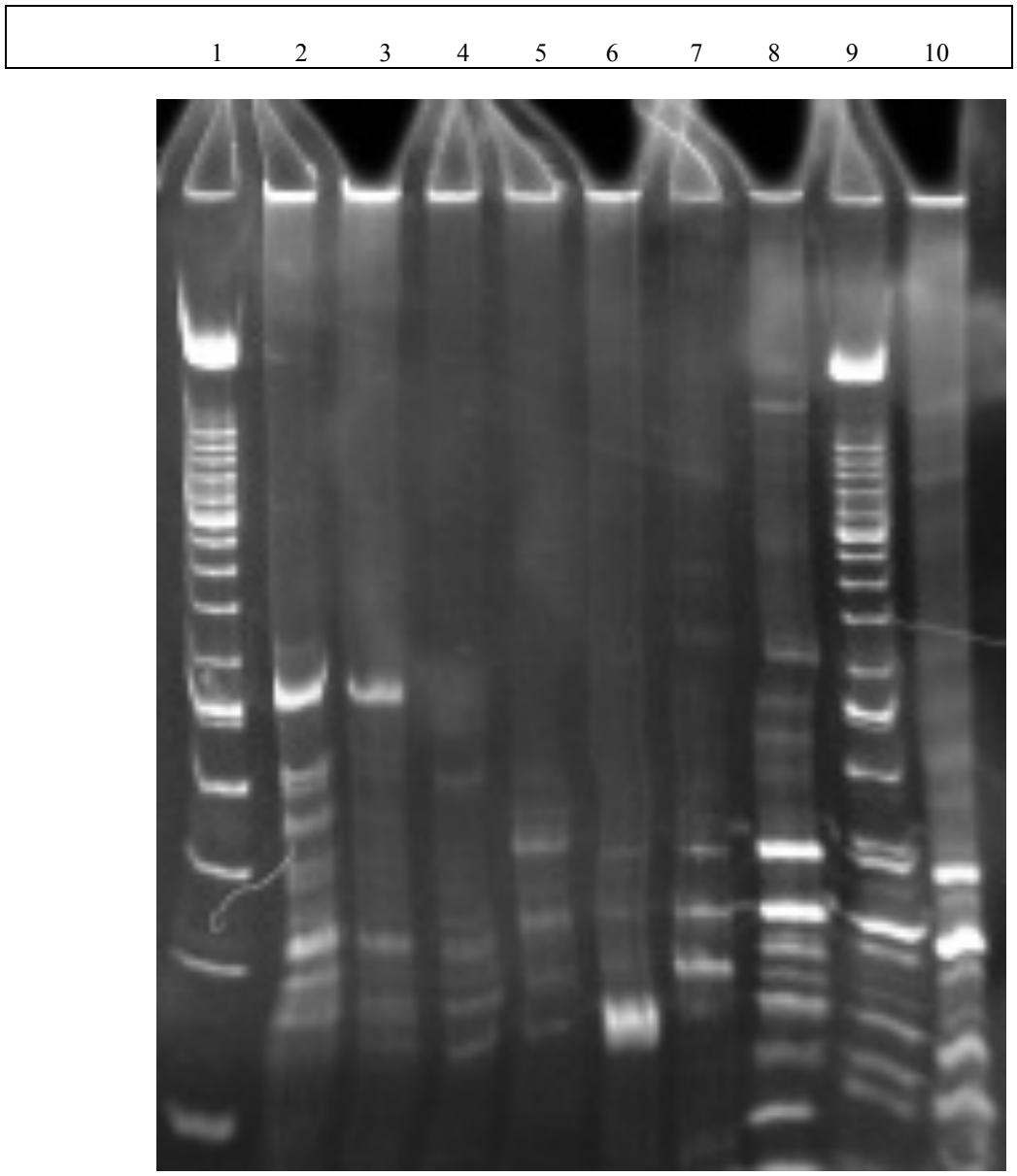


Figure 5.10. SSR amplification of *R. communis* cultivar DNA using primer Set A resolved on a 6% polyacrylamide gel. Lane 1, 100 bp marker; Lane 2, Zanzibar Kenya; Lane 3, Zibo 305; Lane 4, Zibo 1; Lane 5, Zibo 101; Lane 6, Zibo 108; Lane 7, Zibo 2; Lane 8, Shang Dong 108; Lane 9, 50bp marker; Lane 10, Kungming 202.

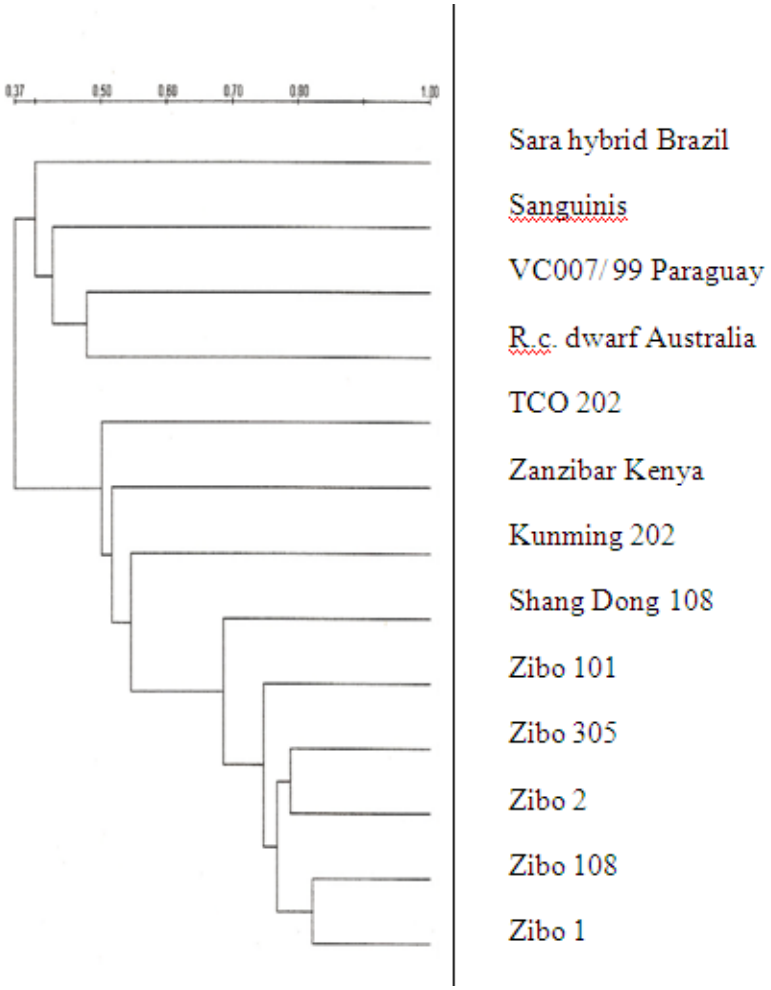


Figure 5.1 Error! No text of specified style in document..**6 Cluster analysis of SSR data by UPGMA, generated using UVIBand Map software (Uvitec, UK) to analyse PAGE gel banding patterns produced with SSR primers.**

5.4 DISCUSSION

The data resulting from 454™ sequencing reactions were suitable for identification of SNPs when compared to the *R. communis* Hale reference genome assembly. Six SNPs were chosen for further study using targeted sequencing, of which only two yielded sequence with homology to the Hale reference. Un-attributable sequence was obtained from three SNP assays, as analysed by BLASTN searches of the Pubmed nucleotide and castor bean whole genome assembly. The additional polymorphisms observed when comparison was made to Hale reference contigs 51366 and 122885 may be genuine and could be confirmed by additional targeted re-sequencing of the same and alternative cultivar DNA samples. However, they may be a result of sequencing or PCR aberrations. The SNP results derived from reference contigs 51309, 38467 and 27948 were attributed to sequencing error, as they had no identity to the reference Hale strain. Each of the six SNPs investigated by targeted pyrosequencing was obtained using a 454 read depth of 20 times, which should be ample for identification of genuine SNPs. This is far greater coverage than used by Oliver *et al.,* (2011) and Foster *et al.,* (2010) however their interrogation approach was entirely different to that used in this study. Foster *et al.,* (2010) used Nucmer analysis of the castor bean assembly, with a cut-off of 3 times coverage or greater, to generate SNP typing assays. Putative SNPS were subsequently validated using a Sequenom iPlex mass array typer to sequence 48 SNPs. Oliver *et al.,* (2011) used 454 Sequencing™ of the oat genome with a read depth of greater than four. They eliminated 55 % of the predicted SNPs based on conservation between reads of a single germplasm, eventually choosing SNPs that varied between 4 oat cultivars. Although the SNP calling criteria used in this study differed from the approaches used by other researchers, 20 X coverage with 100 % identity between contigs

should have been stringent enough to eliminate pseudo-SNPs. The high level of polymorphism surrounding the putative SNP sites and the lack of identity to the reference strain may indicate that sequencing errors caused spurious results. SNP discovery if commonly performed using 454 Sequencing™ but there are other platforms, such as the Illumina Solexa or MiSeq which may be better suited for the task as, although the read lengths are shorter, they offer increased depth of coverage. The data obtained by Foster *et al.,* (2010) using Sanger sequencing were more robust than the data generated for this study. In spite of this, it was unsuitable for differentiation of *R. communis*, which confirms the theory that the *R. communis* populations have low genetic diversity (Allan *et al.,* 2008; Foster *et al.,* 2010; Chan *et al.,* 2010). Therefore targeted sequencing experiments could be repeated to enable conclusions to be formed. However, this work was beyond the scope of this study since it was apparent that using six SNPs would not be sufficient to differentiate between cultivars of *R. communis*.

More traditional approaches of population differentiation were investigated as an alternative to 454 Sequencing™, namely AFLP, RAPD, RFLP and SSR. Low genetic diversity was observed with the cultivars tested with each method, with the exception of SSR analysis. These results are in concordance with those reported by Allan *et al.,* (2008). The SSR assays designed for this study were able to differentiate between 13 cultivars, with a unique profile being generated for each one. The dendrogram generated using the electrophoresis data was interesting as the Zibo cultivars clustered together, indicating that the technique is suitable for geographical differentiation of cultivars to a degree, however the sample size used in this study is much smaller than that used by other researchers. In spite of this, the genomes assumed to originate in South America, Africa and China clustered closely

to each other. This is in contrast to the data reported by Allen *et al*. (2008) who used UPGMA analysis to represent the SSR data that they produced from 41 cultivar accessions from around the world. They found a lack of geographical structure in their phylogenetic tree: accessions even from the same continent did not cluster together. It is to be expected that different SSR assays would give different results and, accordingly, the SSR data obtained for this study was suitable for differentiation between each cultivar studied. However the number of samples examined was extremely small compared to the populations used by other researchers. It is not anticipated that the SSR assays developed here would be more suitable for use than those developed by Allan *et al.,* (2008), rather they may serve as a useful addition to their array. It is also of note that a germplasm collection was not used, rather samples obtained from a commercial seed grower. The utility of the six SSR assays developed for this study for differentiating between samples obtained from a germplasm database remains untested and was not appropriate for inclusion in this study. However it would serve as an interesting starting point for further investigation of the technique.

5.5 CONCLUSIONS

Evidently, the subject of differentiating between strains of an organism with low genetic diversity, such as *R. communis,* is complex and is not going to be easily solved using one technique. Microsatellite data were the most useful in terms of discriminatory power, however further investigation is required to determine whether the assays have the potential to differentiate between large numbers of strains. Additionally, it is more difficult to automate microsatellite analysis than SNP analysis which can be carried out on a larger scale using array technology or high resolution melt curve analysis. Therefore, it is felt that a combination of classical

genetics and next generation sequencing will provide the information required for differentiation of *R. communis* strains in the near future.

6 GENERAL DISCUSSION

6.1 OVERVIEW

Almost all biological toxins are present on the Centers For Disease Control and Prevention (CDC) category A, B and C threat lists. In the event of a biological attack it would be necessary to identify the causative agent. An investigation of an unusual outbreak of disease may be undertaken using clinical or environmental samples using a variety of methods. Microbiological culture is the gold standard for detection of human pathogens; however it is not suitable for detecting non-viable organisms or extracted toxins. Culture may not therefore be suitable for detecting biological weapons as the organisms may not survive for prolonged periods in samples such as dust, soil or water. Additionally, not all biological toxins are of bacterial or fungal origin and, as such, the use of a culture-based method would preclude the identification of plant or animal toxins. Antibody methods are routinely used for toxin detection as they do not rely on the survival of the causative agent for positive identification. Antibody assays such as ELISA are sensitive, specific and simple to use once developed. However it can be difficult to obtain good quality antibodies to toxins, since they kill their host before eliciting an effective immune response, requiring toxoids to be used in immunisation schedules instead. Ultrasensitive detection would be desirable in a BTWC scenario, in order to avoid false negative results. Additionally, it would be important to use a variety of techniques for analysis of biological weapons, since both identification and confirmatory analysis using an orthologous method may be required. For these reasons three nucleic acids based approaches were investigated, namely PCR, immuno-PCR and genotyping using a variety of techniques.

6.1.1 **PCR detection of toxins**

One of the initial areas of investigation was based on the premise that residual DNA could be detected in purified toxin and that this could be detected by PCR. The way in which a toxin was purified was demonstrated to affect the quantity of DNA present in the sample, directly determining whether or not PCR was suitable as a detection method. Acid and ammonium precipitation, the simplest form of toxin purification investigated and reportedly deployed during the Gulf war for the preparation of *C. botulinum* neurotoxin (Zilinskas, 1998), did not remove DNA, allowing PCR detection of the toxins. This is a significant finding as PCR is known to be capable of amplifying trace amounts of DNA. It is conceivable that trace analysis of toxin gene DNA would be extremely useful following a biological attack as it would facilitate detection in environmental samples such as soil or water (Romanowski *et al.,* 1992; Paget *et al.,* 1998), in turn informing forensics, attribution and disease surveillance. However, not all of the toxins investigated contained sufficient DNA to allowed detection of the gene using PCR. Ricin extracted in PET ether and purified using sepharose affinity chromatography did not contain any detectable DNA. Similarly, SEB and alpha toxins produced commercially were not detectable using PCR. With this in mind the decision to use PCR as a toxin detection method would have to be very carefully considered. The origin of the sample could be used determine whether a suspect sample could have been purified by an expert capable of using technology that would be regarded as highly complex to non scientists, such as HPLC. This in itself makes PCR analysis of toxins less suitable for routine use than the initial results obtained with *C. botulinum* neurotoxins would suggest. In spite of this, the utility of PCR for analysis of toxin gene DNA should not be underestimated. For example, it may be possible to take swab samples from

laboratory equipment or surroundings and obtain a positive PCR signal from residual toxin gene DNA. Therefore PCR is considered to be a useful toxin detection method in specific scenarios. With the aim of providing unequivocal and ultrasensitive detection of the toxin however, immuno-PCR was investigated.

6.2 IMMUNO-PCR

Six variants of immuno-PCR were developed and tested as part of this study, each using a different method to label a detection antibody with DNA. This demonstrated that the specific method used to label the antibody with DNA was crucial to the success of the method. The simplest method of I-PCR evaluated was originally reported by Sano *et al.,* (1992). Other more elegant I-PCR approaches were evaluated with less success. The covalent linkage of a DNA tag to the antibody offered less sensitivity than the initial approach. This phenomenon has also been observed by other researchers (He *et al.,* 2007). The addition of a biotin labelled DNA tag to a streptavidin containing immunoassay 'sandwich' already containing biotin was crude and may be difficult to reproduce, however it offered a nine-fold increase in sensitivity. The use of scFv antibodies in a more novel approach termed immuno-phage-PCR was also shown to offer increases of sensitivity of three orders of magnitude over the conventional ELISA. This could be significant when studying samples that contain toxins at low concentrations, as may be found in suspect BW agent samples. However, the generation of single chain antibodies for the specific purpose of developing an immuno-phage-PCR assay would be labour intensive and require the use of animal work that would be difficult to justify if existing monoclonal or polyclonal antibodies were available. Therefore, although the techique showed promise it would only be suitable for converting existing

immunoassay utilising scFvs. It may be that researchers would favour emerging technologies, such as those using gold nanoparticles or SERS (Graham and Faulds, 2010) for example, over immuno-phage-PCR unless they have a particular requirement to generate single chain antibodies. Nevertheless, immuno-phage-PCR was demonstrated to offer a significant increase in sensitivity and, as such, should be considered for future BW sample analysis.

In contrast to the practical and ethical difficulties faced by researchers when generating antibodies, there would be few objections to a research team generating aptamers to a target molecule. Aptamers are theoretically simple and rapid to generate without the use of animals or expensive cell line maintenance. Therefore they have many advantages over antibodies. As such, they were investigated as part of this thesis with the aim of testing aptamers for detection of ovalbumin in ELISA and immuno-PCR formats. One clone dominated the aptamer pool after 17 rounds of selection, indicating that the process had been successful. However, all experiments carried out utilising the clone 5 aptamer resulted in a high non-specific background, including immuno-aptamer-PCR. Generally, aptamers are reported to be generated with ease although the success of an experiment can vary depending on aptamer length (Fan *et al.* 2008) and the number of cycles carried out (Bruno and Keil 2002). The failure of the experiment could have been attributed to a number of things including steric hinderance caused by a change in solid support or the addition of biotin. It is thought that the aptamer may have had an affinity for streptavidin, an avidin derivative also from egg white. It may also be possible that the Sybr Green I used to report aptamer amplification may have remained bound to the double stranded aptamers, limiting secondary structure formation. Single stranded, Sybr Green I-free aptamers were also demonstrated to have poor affinity for ovalbumin,

however they were derived from the double stranded clone 5 aptamers. As such, they would not represent single stranded aptamers developed using a ss-DNA SELEX experiment. A second experiment was carried out using end-point PCR to amplify a biotin labelled aptamer pool, however it was not possible to evaluate the resulting aptamers fully during this study.

The failure of the experiment means that the question of whether aptamers have a useful role in immuno-PCR experiments remains unanswered. Although immuno-aptamer-PCR had not been reported at the start of this study, it has subsequently been used very successfully by Yoshida *et al.,* (2009) and Pinto *et al.,* (2009). Yoshida *et al*. (2009) used a trace amount of unmodified RNA aptamers from round 18 to detect 16 attomoles of vascular endothelial growth factor, thereby proving the utility of the technique. Likewise, Pinto *et al.,* (2009) used 50 nM of aptamer to detect 450 femtomoles of thrombin. Although it was not possible to evaluate immuno-aptamer-PCR for this thesis, it would be interesting to generate aptamers to a different toxin with the aim of re-evaluating the method, this time including near neighbour and competitor proteins in the screening process. Immuno-aptamer PCR may not gain wide acceptance as a detection method for toxins where antibodies already exist. However, they have numerous advantages over antibodies which should make them attractive to researchers. They can be generated within a week if efforts are focused, in contrast to the lengthy immunisation schedule required for antibody production. A Home Office license is not required for SELEX experiments. Aptamers can be generated to highly toxic materials, therefore they may be useful for trace analysis of toxins, chemicals or haptens that are not detectable by other means. SELEX can be carried out in the presence of competitor and near neighbour molecules, potentially increasing the specificity of the aptamers generated. Similarly, aptamers could be

designed to function in non-physiological conditions by simply changing the pH or solvent used as the binding buffer. Therefore, although the experiments presented here were unable to demonstrate the utility of aptamers, their use in immuno-aptamer-PCR for BW agent analysis scenarios is recommended.

6.3 GENETIC DIFFERENTIATION OF *R. COMMUNIS*

As discussed previously PCR may not always be suitable for toxin detection. However, on the occasions it enables the positive identification of a toxin, further genetic analysis would be useful. Genetic differentiation of bacterial species is well established and numerous methods exist for genotyping bacteria including the BW agents *Bacillus anthracis* (Rasko *et al.,* 2011), *Yersinia pestis* (Vogler *et al*. 2011) and *C*. *botulinum* (Macdonald *et al.,* 2008*)*. However there have been difficulties in developing similar methods for *Ricinus communis* as it has low genetic diversity (Allan *et al.,* 2008; Foster *et al*. 2010; Chan *et al.,* 2010). The use of 454™ Sequencing as a SNP discovery tool has been reported for plant species other than *R. communis* (Foster *et al.,* 2010; Oliver *et al.,* 2011). On this occasion, the 454™ Sequencing generated 179 megabases of data from *R. communis* Hale strain. Although this is a vast amount of sequence that would have taken years to generate a decade ago, it represents coverage of only 85 % of the genome. It would have been preferable to work with between two and five times coverage, to ensure that the data were high quality.

Six SNPs identified using the Roche reference mapper software were investigated further, each occurring in twenty contigs or more with 100 percent homology. However, further investigation of the SNPs using targeted DNA sequencing eliminated four of the SNP sites, with two yielding sequence with homology to the

Hale strain. The sequence data generated using the two genuine SNP sites was suitable for phylogenetic analysis, however two SNPs are not sufficient to differentiate between cultivars of a species with such low diversity. Similar results were obtained using the more traditional genotyping methods AFLP, RAPD and RFLP. SSR (or microsatellite) analysis was the only method suitable of discriminating between the limited number of strains. Therefore, it would be interesting to carry out the same SSR analysis on a larger *R. communis* sample set in future, such as a germplasm database. It would also be ideal to repeat the 454™ Sequencing analysis of *R. communis* Zibo 1 to obtained more coverage of the genome, if only to conclude that the complete genome does not contain sufficient SNPS to enable strain differentiation. It would also be worthwhile repeating the 454™ Sequencing with a different strain for comparison. In summary, the *R. communis* genotyping experiments presented in this study were insufficient to allow discrimination on their own. Nevertheless, ricin remains high on the CDC threat list and it is expected that efforts to continue this work will be made by a number of research groups.

6.4 CONCLUDING REMARKS

PCR is widely acknowledged as suitable for inferring the presence of a toxin from the amplification of its coding gene in clinical or food samples. However, this thesis has demonstrated that it is not suitable for detecting biological weapon samples that have been purified using molecular biology techniques such as HPLC or affinity chromatography. As biological weapons may be used in which ever form an aggressor chooses, PCR can not be used as a primary method of toxin detection as there is the chance that the technique would produce false negative results. Therefore, PCR is not considered to be a useful primary method for toxin detection.

However, the sensitivity of PCR and the stability of DNA in the environment suggest that PCR would be appropriate for forensic analysis, either with or without the trigger of a positive result using a method such as ELISA. Furthermore, a positive PCR result may serve as the starting point of a full genotyping investigation; therefore PCR is thought to be a useful approach to toxin detection. Genetic differentiation of biological weapon samples may be useful for attribution of an attack to individuals or nations so it was also studied as part of this thesis. Although the volume of data generated here was insufficient for complete discrimination of *R. communis* cultivars, it may prove to be crucial as this area of science continues to develop. Immuno-PCR is considered to be a useful method for toxin detection in samples of low concentration. Although time consuming, the additional sensitivity of detection obtained using I-PCR may be useful in certain scenarios. For example, I-PCR may not be suited to routine use in laboratories in which adequate detection limits are achieved using a well optimised sample extraction method. However, it may be the only method available to detect a sample of low concentration. Therefore it is particularly applicable to BW agent detection where a false negative would have far reaching implications. Additionally, I-PCR lends itself well to automation and it is conceivable that many antigens would be detectable simultaneously once the instrumentation necessary for multiplex PCR is developed further. In summary, both the PCR and immuno-PCR assays developed here may prove useful in future biological weapons investigations. The 454™ sequence and SSR data may be useful for differentiating between *R. communis* cultivars when used with other techniques. Therefore, nucleic acids are considered to be useful for toxin detection.

7 APPENDICES

Table 7.1 General Bacterial DNA test panel used in this study.

Strain	Origin (if known)
Bacillus anthracis	Ames
Coxiella burnettii	9 mile
Yersinia pseudotuberculosis	NCTC 10275
Yersinia enterocolitica	NCTC 10461
Pseudomonas aeruginosa	NCTC 606
Pseudomonas maltophila	NCTC 10257
Pseudomonas mendocina	NCIMB 10541
Pseudomonas pickettii	NCTC 11149
Pseudomonas testoni	NCTC 10698
Burkholderia mallei	NCTC 10229
Burkholderia pseudomallei	NCTC 4845
Burkholderia cepacia	NCTC 10743
Staphylococcus aureus	FRI 361
Staphylococcus typhimurium	SL1344
Shigella sonnei	9744
Shigella boydii	9327
Shigella dysemberiae	9952
Shigella flexneri	8517
Vibrio cholera 01	E105239
Vibrio cholera 01EL	E87019
Escherichia. coli 0109	E8017

Erwinia herbicola	490291A
Francisella novicida	
Bacillus globigii	
Bacillus subtilis	10073
Bacillus subtilis	3610
Bacillus cereus	10320
Bacillus cereus	11143
Bacillus cereus	11145
Bacillus cereus	
Bacillus cereus	9945
Bacillus cereus	9939
Bacillus cereus	7464
Bacillus cereus	2599
Bacillus thurengiensis	
Clostridium botulinum B	
Clostridium botulinum E	
Clostridium botulinum F	
Clostridium bifermentans	
Clostridium sordeii	

Clostridium histolyticum

Clostridium chauvoi

Clostridium perfringens

Clostridium perfringens

Clostridium perfringens

Clostridium perfringens

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