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Development of novel methods for the detection of *Vibrio parahaemolyticus* in seafood

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ABSTRACT

Vibrio parahaemolyticus is marine bacterium found worldwide. It is most commonly associated with seafood related gastroenteritis, but can also be found naturally occurring in the estuarine environment. Infection is most common in the summer months, when the water temperature is suitable for the growth of bacteria. The infection is usually self limiting but morbidity can result in some cases. Currently very few methods are in use for rapid detection of this organism. In addition, these methods are not always specific and can give false positive reactions.

This study describes the development of a novel method, based on phage antibody display, for the detection of *V. parahaemolyticus* in seafood. The initial part of the study focuses on the use of the Polymerase Chain Reaction (PCR) as a rapid method for the detection of *V. parahaemolyticus*. Four sets of primers were used for PCR, of which three were targeted to the toxin producing genes. The sensitivity and specificity of these primers in PCR was tested using pure cultures of *V. parahaemolyticus* and non-*parahaemolyticus* spp. Homogenised oyster tissue was then seeded with these cultures and they were tested again by PCR.

A semi-synthetic scFv bacteriophage antibody library was used to isolate bacteriophage antibodies against *V. parahaemolyticus*. After five rounds of panning, the clones were further tested by ELISA and flow cytometry. At least four of the clones showed a strong binding profile with strains of *V. parahaemolyticus*. These clones also did not cross-react with other *Vibrio* spp. Similar to the PCR reaction, the clones were tested by seeding oyster homogenate with bacterial cultures. It was found that the ELISA reaction was inhibited by the presence of oyster homogenate. Therefore, a 3 h pre-enrichment step was necessary before testing for the presence of bacterial cells. The phage antibody clones which showed strong binding in pure culture still retained their binding capacity, when tested in the presence of seafood (oyster homogenate). The

sensitivity of the assay was further increased by using chemiluminescence detection of phage binding. The data also suggests that this method has the potential sensitivity to detect *V. parahaemolyticus* at or below the current action level.

Publications and Presentations associated with this Thesis

Publications:

(1) **Pai**, N., Topping, K. P., Greenman, J. and Paget, T. A. (2000) Detection of pathogens by phage antibody display. Disease Markers 16: 99-100.

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(2) **N. Pai**, G. Temple and T. Paget. (2001) A rapid assay for the detection of *Vibrio parahaemolyticus* in seafood. 9th International Symposium on Microbial Ecology (ISME-9), Amsterdam.

(3) **N. Pai**, G. Temple and T. Paget . (2001) Detection of *Vibrio parahaemolyticus* in seafood. Microbial Genome-Environment Interactions. Society for General Microbiology(SGM) Conference, Belfast, UK.

(4) **N. Pai**, J.Greenman, P.J.Large and T. Paget. (2002). A novel method for the detection of *V. parahaemolyticus* in seafood. American Society for Microbiology, 102nd General Meeting, Salt Lake City, Utah, USA.

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Chapter 1

Introduction

The family *Vibrionaceae* consists of Gram-negative, non-sporing non-acid fast rods, which may be straight or curved (Elliot *et al.*, 1998). There are currently four genera accepted in the family. The genus *Vibrio* is the type genus, the other genera being *Aeromonas, Plesiomonas* and *Photobacterium* (Colwell *et al.*, 1986). Members of the family *Vibrionaceae* are natural inhabitants of seawater but can be found in fresh water The GC content of the DNA for this family is 38-63 mol % (Barrow and Feltham, 1993).

The Genus Vibrio: There are currently many species recognized within this genus with *Vibrio cholerae* as the type species. The other reconized species include *V. alginolyticus, V. damsela, V. fluvialis, V. furnissii, V. hollisae, V. metschnikovii, V. mimicus, V. parahemolyticus* and *V. vulnificus* (Barrow and Feltham 1993). *Vibrio* species are oxidase-positive, Gram-negative facultative anaerobes (Bauman and Schubert, 1984). Many *Vibrio* spp. are pathogenic to humans and have been implicated in food borne disease (Madigan *et al.*, 2000). Other than *V. cholerae* and *V. mimicus*, all other *Vibrio* spp. do not grow in media that lack added sodium chloride and are referred to as halophiles (Elliot *et al.*,1998).

Bergeys Manual of Systematic Bacteriology (Bauman and Schubert, 1984) classifies the genus *Vibrio* as follows:

Name: VIBRIO

Authors: Pacini 1954

Status: Approved Lists

Type species: *V. cholerae*

Literature: Int. J. Syst. Bacteriol. 30:417

Name: *Vibrio parahaemolyticus*

Authors: Fujino *et al.* 1951, Sakazaki *et al.* 1963

Status: Approved Lists

Literature: Int. J. Syst. Bacteriol. 30:417 (AL)

Risk group: 2 (German classification)

Type strain: ATCC 17802, DSM 10027

Other names: *Beneckea parahaemolytica* (objective synonym)

V. parahaemolyticus is a halophilic bacterium found naturally in estuarine waters and animals. It has worldwide distribution in estuarine and coastal environments and has been isolated from many species of fish, shellfish, and crustaceans (Hlady, 1997).

1.1 <u>Biochemistry</u>:

V. parahaemolyticus is a facultatively anaerobic chemoautotroph, with an optimum growth temperature of 37°C. It is halophilic, that is, sodium chloride is an absolute requirement for growth (Elliot *et al.*, 1998). A selective media such as MacConkey agar containing sodium chloride can be used for presumptive analysis of *V. parahaemolyticus*, which grows as non-lactose fermenting colonies. Thiosulfate-citrate-bile salts-sucrose (TCBS) agar is a highly selective media, containing sucrose and bile salts, which is specifically designed for the isolation of *Vibrio* spp. from stool specimens. *Vibrio* organisms appear as green or yellow colonies, depending on the isolate's ability to ferment sucrose while most other enteric bacteria are inhibited. As *V. parahaemolyticus* does not ferment sucrose, it will grow as green colonies on the TCBS agar. Some of the biochemical properties of *V. parahaemolyticus* are outlined in Table 1.1.

Table 1.1: Biochemical characteristics of some common *Vibrio* spp. (Elliot *et al.*, 1998)

Biochemical tests	***V. alginolyticus***	***V. cholerae***	***V. harveyi***	***V. hollisae***	***V. mimicus***	***V. parahaemolyticus***	***V. vulnificus***
TCBS agar	Y	Y	Y/G	NG	G	G	G
0% NaCl	-	+	-	-	+	-	-
3% NaCl	+	+	+	+	+	+	+
6% NaCl	+	-	+	+	-	+	+
8% NaCl	+	-	V	-	-	+	-
10% NaCl	+	-	V	-	-	-	-
Growth at 42°C	+	+	V	nd	+	+	+
Lactose	-	-	V	-	-	-	+
Oxidase	+	+	+	+	+	+	+
ONPG	-	+	V	-	+	-	+
Voges-Proskauer	+	V	-	-	-	-	-
Arginine	-	-	-	-	-	-	-
Lysine	+	+	+	-	+	+	+
Ornithine	+	+	+	-	+	+	+
10 µg 0/129	R	S	R	nd	S	R	S
150 µg 0/129	S	S	S	nd	S	S	S
Urease	-	+	-	V	-	-	V

Key: Y= yellow colonies, G = green colonies, NG = no growth

R = resistant, S = sensitive to vibrio-static agent 0129 (2,4-diamino-6,7-di-isopropyl-pteridine phosphate)

+ = 80% or more of strains positive, - = 80% or more of strains negative

nd = not determined, V= variable

ONPG = o-nitro-β-D-galactopyranoside hydrolysis by β-galactosidase

1.2 Serotyping:

V. parahaemolyticus can also be classified on the basis of its antigenic structure. It has three major antigens - the thermostable cell wall-associated O antigen, the thermolabile capsular K antigen, and the flagellar H antigen. Serotyping is based on the 12 heat stable O and 70 heat labile K antigens (Table 1.2).

Table 1.2: Antigenic scheme of *V. parahaemolyticus* (Twedt, 1989)

O Group	K Antigen
1	1, 25, 26, 32, 38, 41, 56, 58, 64, 69
2	3, 28
3	4, 5, 6, 7, 29, 30, 31, 33, 37,
	43, 45, 48, 54, 57, 58, 59, 65
4	4, 8, 9, 10, 11, 12, 13, 34, 42,
	49, 53, 55, 63, 67
5	15, 17, 30, 47, 60, 61, 68
6	18, 46
7	19
8	20, 21, 22, 39, 70
9	23, 44
10	19, 24, 52, 66, 71
11	36, 40, 50, 51, 61
12	52

Until 1994, the most prevalant serotype of *V. parahaemolyticus* associated with food poisoning outbreaks in Japan was O4:K8 (IASR report, 1996). The upsurge in the

worldwide incidence of *V. parahaemolyticus* infection in the last 5 years has been attributed to the recent appearance of three serotypes with pandemic potential: O3:K6, O4:K68, and O1:K untypable (KUT) (K antigens from environmental isolates are often untypable). Since 1996 a new clones of serovar O3:K6 was reported to cause pandemics in Southeast Asia, Japan and North America (Okuda *et al.*, 1997; Matsumoto *et al.*, 2000; Wong *et al.*, 2000). While most serotypes are primarily associated with sporadic cases, the O3:K6 strain has been classified by the Centre for Disease Control (CDC) as an outbreak strain and the O4: K8, seen predominantly in Japan, may also merit special concern (FDA report, 1999).

1.3 Epidemiology:

Infections caused by *Vibrio* species are largely classified into 2 distinct groups: *Vibrio cholera* infection and noncholera *Vibrio* infections. Historically, the noncholera *Vibrio* species were further classified as halophilic *Vibrio* species and nonhalophilic *Vibrio* species, depending on their requirement of sodium chloride for growth.

The earliest reported outbreaks of *V. parahaemolyticus* were in Japan, in 1950, from the post-mortem specimens of patients who died during an outbreak of food poisoning associated with the consumption of semi-dried sardines (Fujino *et al.*, 1951). Reports also indicate outbreaks in Britain in the early 1970's (Ayers and Barrow, 1978; Barrow and Miller,1972). It was first found in the United States by Baross and Liston (1976) in the estuarine waters of Puget Sound. Most major epidemic outbreaks occur in Japan and the United States (Table 1.3), though minor outbreaks have been reported worldwide. These include Taiwan (Wong *et al.*, 1999), India (Bag *et al.*, 1999), Brazil (Matte *et al.*, 1994) and Canada (Fyfe *et al.*, 1997). The great majority of these infections are associated with the consumption of contaminated food; therefore, *V. parahaemolyticus* infections predominantly are a food-borne disease (Miwatani and

Takeda, 1976). This organism exhibits seasonality, that is, infections are more common in the summer months when the water temperature is optimal for the organisms to multiply (CDC report, 1999; Doyle, 1998). The combination of increased water temperature and increased salinity in places where shellfish have been harvested has been suggested to contribute to the increased contamination rate of shellfish with noncholera *Vibrio* species. In the United States, the CDC estimates that 8000 *Vibrio* infections and approximately 60 deaths related to *Vibrio* infections may occur annually. A study by Daniels *et al.* (2000) shows that between 1973 and 1998, 40 outbreaks of *V. parahaemolyticus* infections were reported in the United States and these outbreaks included more than 1000 illnesses. Similarly, between 1988 and 1997, 345 sporadic *V. parahaemolyticus* infections were reported. Table 1.3 shows the worldwide outbreaks of *V. parahaemolyticus* in the past decade.

Table 1. 3: Worldwide outbreaks of *V. parahaemolyticus* during the past decade

Year	Country	Summary	Reference
1994-1995	Japan	224 and 245 outbreaks were reported in 1994 and 1995 respectively. Both years showed summer prevalent tendency. O4:K8 was the most important serotype.	IASR report, 1996
1996-1998	Japan	292 oubreaks were reported in 1996, with 568 oubreaks in 1997 and 850 oubreaks in 1998 respectively. Again,summer months showed the highest cases.The predominant serotype changed from O4:K8 to O3:K6	WHO report, 1999
1997	Canada (British Columbia)	43 cases were reported from a period of July to August. Testing of oyster harvesting sites showed that the oysters contained less than 100-200 colony forming units(CFU) per gm oyster meat,which is far lower than the level (10,000 cfu/g) used as the threshold limit for safe consumption	Canadian Communicable Disease Report, 1997
1998	United States (Galveston Bay,Texas)	139 case were identified. Majority of the cases were reported from May to July. The O3:K6 serotype was predominant and the major source of infection was raw oysters	Disease prevention news, 1997
1998	United States (New York and New Jersey)	23 cases were reported. 11% of the patients showed blood stream infection along with gastroenteritis. The cases were reported from July-September. The most dominant serotype was O3:K6	MMWR Morbidity and Mortality Weekly Report, 1999

V. parahaemolyticus adsorbs to chitin, which is a major component of the exoskeleton of shellfish and plankton. It is therefore not hard to see why it is so often associated with the consumption of raw or contaminated shellfish or oysters - either by affinity to the chitinous shell of the main food item or to plankton upon which oysters and clams filter out and feed upon. It is also chitinoclastic, that is, it can degrade chitin and therefore can persist over the winter on the estuarine floor on chitinous material from plankton (Venkateswaran, 1990).

1.4 <u>Transmission</u> :

Vibrio species are natural inhabitants of seawater, therefore, states or countries bordered by large bodies of seawater frequently report high incidence of *Vibrio* infections.

Foods that have been implicated in outbreaks include oyster (Aronson, 1998), shrimp (Hernandez-Lopez *et al.*, 1997), toothcarp (Alcaide *et al.*, 1999), mussels (CDC report, 1998), lobster (CDC report,1999), crab (Cassels *et al.*,1994) and crayfish (Bean *et al.*, 1998). In one survey of shellfish from the coastal water of the U.S in the summer, 100% of the oysters tested contained detectable *V. parahaemolyticus* (Doyle, 1998). In addition, exposure of wounds to contaminated seawater and injury caused by contaminated seashells are potential sources of infections (Table 1.4).

Table 1.4: Clinical presentation rates of pathogenic *Vibrio* infections (Ho *et al.*, 2002)

Vibrio Species	Gastroente-ritis (%)	Wound Infection (%)	Septicemia (%)	Miscellaneous (%)
V. parahaemolyticus	59	34	5	2
V. vulnificus	5	45	43	7
Non-01 *V. cholerae*	67	9	15	...
V. alginolyticus	5-12	71	1	10-15
V. mimicus	85	3	3	...
V. fluvialis	73	10	6	...
V. damsela	Rare	>95	Rare	...
V. furnissii	>90	Rare	Rare	...
V. metschnikovii	Common	Rare	Rare	...
V. hollisae	85	7	5	...
V. cincinnatiensis	Rare	Rare	Rare	Meningitis

Key : ... = not determined

The current action level (maximum level of bacteria allowed in food for consumption) for this organism is less than 10,000 CFU (colony forming units) per gram of fish or shellfish (Sanyal and Sen, 1974). However reports of outbreaks in Oyster Bay (Texas Department of Health report, 1998) and in the Pacific Northwest Coast of the United States (CDC report, 1998) showed the levels of *V. parahaemolyticus* in oysters taken from these sites was less than, or equal to,

200 CFU per gram oyster meat. This may indicate that human illness can occur at levels much lower than current action levels.

1.5 <u>Toxins</u>:

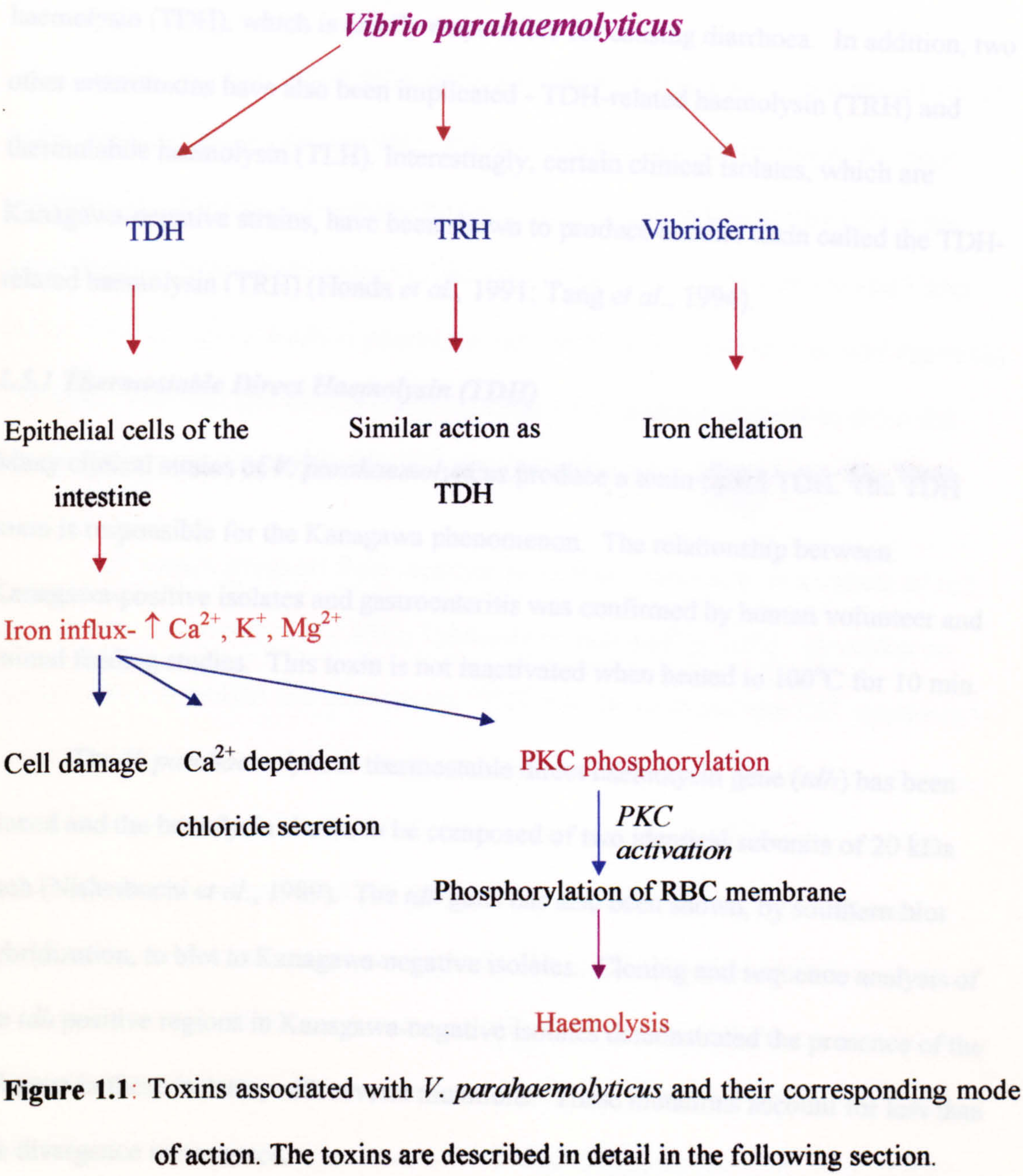


Figure 1.1: Toxins associated with *V. parahaemolyticus* and their corresponding mode of action. The toxins are described in detail in the following section.

Infection is initiated when the organism attaches itself to the small intestine and excretes toxins (Akeda *et al.*, 1997). Clinical strains associated with gastroenteritis usually cause β-haemolysis *in vitro* on Wagatsuma blood agar. This is called the

Kanagawa phenomenon (Joseph *et al.*, 1982; Honda *et al.*, 1976; Wagastsuma, 1968). It has been shown that most (>96%) clinical isolates from *V. parahaemolyticus* are Kanagawa-positive, whereas only 1% of environmental isolates are Kanagawa-positive. Kanagawa-positive strains produce a heat stable cytotoxin called the thermostable direct haemolysin (TDH), which is chiefly responsible for causing diarrhoea. In addition, two other enterotoxins have also been implicated - TDH-related haemolysin (TRH) and thermolabile haemolysin (TLH). Interestingly, certain clinical isolates, which are Kanagawa-negative strains, have been shown to produce another toxin called the TDH-related haemolysin (TRH) (Honda *et al.*, 1991; Tang *et al.*, 1994).

1.5.1 Thermostable Direct Haemolysin (TDH)

Many clinical strains of *V. parahaemolyticus* produce a toxin called TDH. The TDH toxin is responsible for the Kanagawa phenomenon. The relationship between Kanagawa-positive isolates and gastroenteritis was confirmed by human volunteer and animal feeding studies. This toxin is not inactivated when heated to 100°C for 10 min.

The *V. parahaemolyticus* thermostable direct haemolysin gene (*tdh*) has been cloned and the hemolysin shown to be composed of two identical subunits of 20 kDa each (Nishcibuchi *et al.*, 1989). The *tdh* gene has also been shown, by southern blot hybridization, to blot to Kanagawa-negative isolates. Cloning and sequence analysis of the *tdh* positive regions in Kanagawa-negative isolates demonstrated the presence of the *tdh* gene in these isolates, with several mutations. These mutations account for less than 3% divergence in sequence.

Yoh *et al.* (1991) have cloned the *tdh* genes from two Kanagawa-postive isolates and Kanagawa-negative isolates, showing that all the sequences were not identical but also did not differ by more than 3%. The four TDH proteins were not inactivated by

heating at 100°C for 10 min and could bring about β-haemolysis in erythrocytes *in vitro*. In addition, further studies showed that all the four TDH stimulated vascular permeability in rabbit skin and were lethal to mice. The four proteins were also antigenically indistinguishable.

It has been shown that many Kanagawa-positive strains carry two non-identical copies of the *tdh* gene, *tdh*1 and *tdh*2. Protein isolation and sequencing of the major secreted TDH protein and comparison with both *tdh* genes indicated that *tdh*2 is the gene which is preferentially expressed for production of TDH (Nishibuchi and Kaper, 1995). The Kanagawa-positive phenotype and >90% of the TDH protein was expressed from *tdh*2. This was also confirmed by the creation of isogenic mutants in either the *tdh*1 or *tdh*2 genes (Nishibuchi and Kaper, 1995)

TDH causes intestinal fluid secretion as well as cytotoxicity in a variety of cell types *in vitro* [Raimondi *et al.*, 2000; Takahashi *et al.*(a and b), 2000]. In studies on human colonic epithelial cell monolayers, TDH was shown to activate Ca^{2+} (primarily), in addition to K^+, Na^+ and Mg^{2+} transporters in a dose-dependent manner. By elevating cation permeability, it can cause cell damage *in vivo* (Douet, 1996; Huntley and Hall, 1996). The exact mechanism of cell damage has not been elucidated, though severe histopathological changes have been observed. The mode of action of TDH has been shown in Figure 1.2. TDH has been shown to bind directly to erythrocytes (Yoh *et al.*, 1995). Once TDH has bound to erythrocyte membranes, PKC cause phosphorylation of proteins on the surface of the erythrocytes, followed by haemolysis (Yoh *et al.*, 1995). The significance of this effect may relate to the fact that *V. parahaemolyticus* can use both heme and haemoglobin as iron sources (O'Malley *et al.*, 1999). The biological activities of TDH are various and include haemolysis of erthrocytes, cytotoxicity and

increased vascular permeability in rabbit skin (Ljungh and Wadstorm, 1982; Honda and Iida, 1993; Takeda *et al.*, 1983).

Studies have been done to identify the possible receptors for TDH. The exact method of action of the toxins on the cell membrane is unknown. Takeda *et al.* (1978) and Yoh *et al.* (1995) have suggested that GT1 gangliosides may play a role in acting at toxin receptors. Tang *et al.* (1997) carried out a study to determine possible receptors for TDH. They used a mutant cell line of *V. parahaemolyticus*, which was over 200 times more resistant to TDH than the original parent cell line. They then tested both cell lines using native TDH and a toxin called R7, which has retained the ability to bind to erythrocytes but cannot form pores in the cell membrane. The experiments showed that R7 did not bind to the mutant cell line, thus indicating that receptor binding is necessary for cytotoxic action of the toxin. They further tested TRH against the same cell lines, and found similar results. Thus they hypothised that TDH and TRH may have similar binding receptors; and the difference in their mode of action is due to different post-binding activity.

1.5.2 TDH-related haemolysin (TRH)

Shirai *et al.* (1990) have also shown the presence of a TDH-Related Hemolysin (TRH) in Kanagawa-positive and Kanagawa-negative clinical isolates of *V. parahaemolyticus*. TRH acts in a similar way to TDH in that it triggers a calcium-dependent chloride secretion followed by elevation of calcium ions associated with protein kinase C (PKC) phosphorylation (Raimondi *et al.*, 2000). TRH is biologically and immunologically similar, but not identical to TDH. The two cysteine residues necessary for interchain bonds in the toxins are conserved in both haemolysins. In addition, TRH is produced under a limited range of conditions and not in Wagatsuma agar or other media used for the production of TDH.

Comparative analysis has shown that the *tdh* and *trh* gene sequences share 69% homology. Use of both *tdh* and *trh* as gene probes showed that 76% of all the Kanagawa-negative clinical isolates had either the *tdh* or *trh* gene, or both. Considering that 96% of clinical isolates are Kanagawa-positive, the results of this study would show that the *trh/tdh* genes would also be present in 76% of the remaining Kanagawa-negative isolates, bringing the co-relation of TDH/TRH with *V. parahaemolyticus* intestinal diarrhoea up to 99% (Shirai *et al.*, 1990).

Plasmid borne-*tdh* genes are present in *V. parahaemolyticus,* but in a few strains (Baba *et al.*, 1991; Nishcibuchi and Kaper, 1995). The toxin-producing genes- *tdh* and *trh* are located on the chromosomal DNA and not plasmid DNA (Vadivelu *et al.*, 1996). Yoh *et al.* (1992) have compared the *V .parahaemolyticus* TRH from both environmental and clinical isolates and showed that TRH from environmental strains was indistinguishable from toxins of clinical origin.

1.5.3 Thermolabile haemolysin(TLH)

Thermolabile haemolysin (TLH) has not been directly implicated in pathogenicity but is universally present in all *V. parahaemolyticus* strains (Sakurai *et al.*, 1974). The toxin shows haemolytic activity against human erythrocytes at 37°C but is inactivated by heating at 60°C for 10 min (Taniguchi *et al.*, 1985). McCarthy *et al.* (1999) used the *tlh* gene to develop a nucleotide probe for the detection of *V. parahaemolyticus.*

1.5.4 Other virulence factors

In addition, some Vibrios, including *V. cholerae* and *V. parahaemolyticus* possess a mechanism whereby they can acquire iron from haemoglobin. This is based on the production of two heme-chelating siderophores, vibrioferrin and vibriobactin. The heme utilization system of *V.cholerae* has been studied extensively but the one for *V.*

parahaemolyticus has not been well characterized. A study by O'Malley *et al.* (1999) showed that the gene array for heme utilization in this organism is very similar to the genes found in *V. cholerae*.

In a recent publication Lee *et al.* (2002) reported the detection of a serine protease as a putative virulence factor. The strain of *V. parahaemolyticus* carried neither the *tdh* nor the *trh* genes. The protease had a molecular size of 50 kDa (Ishihara *et al.*, 2002). Studies indicated that this protease is a heat-labile protein, and shows cytotoxic effect *in vitro*.

1.6 Phage display of antibodies:

The exquisite specificity of monoclonal antibodies has long provided the potential for creating new reagents for the *in vivo* delivery of therapeutic drugs or toxins to defined cellular targets or improved methods of diagnosis (Breitling, 1991). However, there are a number of limitations to the current hybridoma based approach to monoclonal antibody production, which has largely restricted their application. Conventional antibody production methods require working with animals (immunisation) followed by screening millions of antibody producing bacteria to select for an antibody binding to a particular antigen. Also, one can only raise antibodies towards antigens that are immunogenic (non-self) and not towards antigens that the animal is already tolerant to. Naïve antibodies, i.e. antibodies that have not had any previous contact with the antigen, cannot be obtained. In addition, monoclonal antibody production is restricted to the mouse and the rat, in other species low production rates are obtained and the antibodies are inherently unstable (Griffiths *et al.*, 1994).

With the recent developments in applied molecular techniques, it has been possible to engineer genes that encode antibodies rather than the manipulation of intact antibodies. This has broadened the scope of using antibodies in different formats.

Phage display is a novel technology that can be used to display antibody libraries on the surface of filamentous bacteriophages. DNA sequences of interest are inserted into the genome of the bacteriophage, such that it is expressed or "displayed" on the surface, fused to one of the coat proteins (Azzazy *et al.*, 2002). Tailor-made antibodies can be synthesized and selected to acquire the desired affinity of binding and specificity, both for *in vitro* and *in vivo* diagnostics.

The main advantages of phage display are that one can screen a large number of clones in a short time period, which saves time and labour, since it does not involve working with animals. Antibodies against virtually any antigen can be directly isolated from a single pot naïve library, thus bypassing immunization. Using human antibody libraries, entirely human antibody fragments can be isolated directly from these repertoires, making humanisation strategies unnecessary (Kontermann *et al.*, 1997). The antigen targeted need not be immunogenic. The small size of the antibody (~25kDa) makes it easier to work with; in addition they can be stored for long periods at –80°C.

One can also enhance the affinity of isolated antibodies by *in vitro* affinity maturation, which allows us to design tailor-made antibodies from scratch, with an option to choose its building blocks, its affinity (up to picomolar concentrations), its format (size and valency), its effector function [natural (IgG) or novel (enzymes),etc.] (Hoogenboom *et al.*, 1998). It gives us an unrestricted availability of large and stable phage libraries towards new drug targets, cell receptors and ligands for *in vitro* and *in vivo* diagnosis and therapy. Besides offering a source of antibodies for therapy, phage display technology is very well suited for high-throughput generation of antibodies for research purposes such as massive target identification and validation (Kretzschmar and Ruden,2002)

1.6.1 The structure of the filamentous phage

Bacteriophages are single or double stranded DNA or RNA viruses that infect bacteria. The most common route of entry into the bacterium is using a pilus as a receptor. The filamentous phages that infect *E. coli*, using the F pilus, are M13, fd and f1 phage, all of which have a single strand of DNA enclosed by a protein coat.

The M13 phage particle consists of a long loop of ssDNA, which codes for around 10 different proteins. This is coated with multiple copies (~2700) of the major coat protein pVIII (Figure 1.2). In addition, there are four minor coat proteins on the surface of the phage present at about five copies each: pVI and pIII located at the proximal absorption end of the phage and pVII and pIX at the distal end (George, 1995).

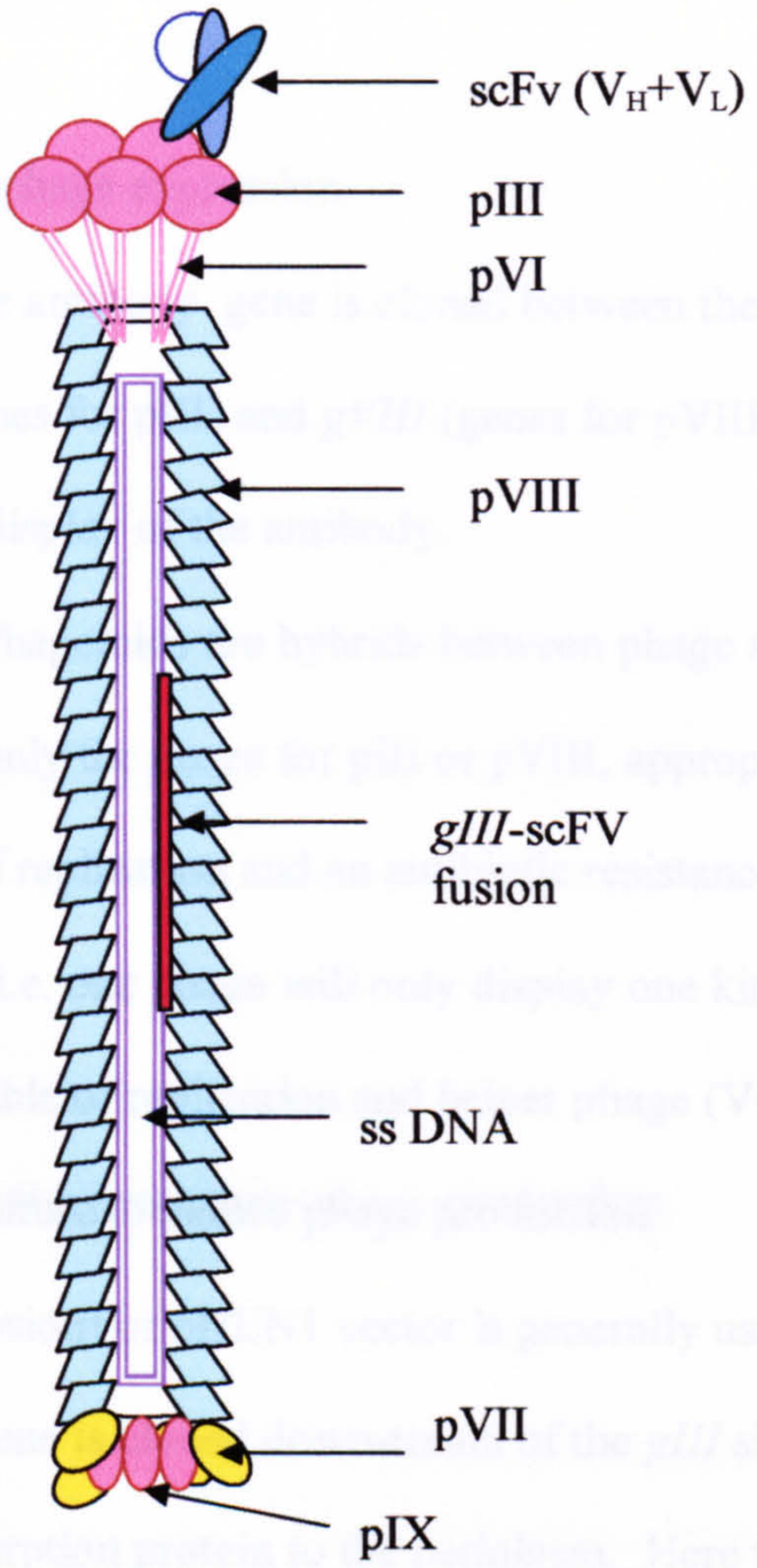


Figure 1.2: The structure of the filamentous phage.The phage consists of a single strand of DNA encoded by coat proteins. The major coat protein is pVIII; in addition there are four minor coat proteins- pIII, pVI, pVII and pIX. Phage antibody can be expressed in conjunction with of the major or one of the minor coat proteins.

Insertion of the antibody fragment is either with the major coat protein pVIII, or at the tip, fused with the minor coat protein. The minor coat protein pIII has three domains. The N-terminal domain is responsible for penetration, the middle domain is resposnisble for the recognition of the F-pilus and the C-terminal domain is needed for morphogenesis and membrane anchorage and is hidden in the phage evelope. Assembly of the virus takes place in the periplasmic space. Around 100-300 phage are produced in a bacterial life cycle, therefore 1ml of culture typically contains 10^{12}phage.

Phage expression system:

Two types of vector systems are used for phage expression

(A) The phage system - In this system,the antibody gene is cloned between the L (leader) and the coding region of *gIII* (genes for pIII) and *gVIII* (genes for pVIII) of the phage vector. This results in multivalent display of the antibody.

(B) The phagemid system (Figure 1.3) - Phagemids are hybrids between phage and plasmid vector. A phagemid consists of only the genes for pIII or pVIII, appropriate cloning sites, bacterial and viral origins of replication and an antibiotic resistance gene. This results in monovalent phage display i.e. one phage will only display one kind of antibody. However phagemids are incapable of replication and helper phage (VCS-M13, M13-KO7) has to be added to the culture to induce phage production.

The pSEX (plasmid surface expression) or pHEN1 vector is generally used for display of the scFv molecule. The scFv gene is cloned downstream of the *gIII* signal sequence, which normally directs the adsorption protein to the periplasm. Here the V_H and V_L domains will fold correctly. ScFv is fused at the N-terminal end of the mature pIII protein (infective phage) or at the N-terminal of a truncated pIII lacking the first 2 N-terminal domains (non-infective phage). The phage are grown up in cultures of *E. coli* strains having the F pilus. Infection is initiated by binding of the minor coat protein pIII to the tip of the F-pilus followed by internalization of the phage particle. The phage genome is under the control of the *lacZ* promoter, in the presence of glucose as a catabolite repressor, phage lysis genes are not expressed. Expression of pIII prevents infection of *E. coli* with the helper phage, therefore glucose is removed from the medium only after helper phage infection (Breitling, 1991, Neri *et al.*,1998). This leads to the expression of sufficient fusion product to generate monovalent phage particles.

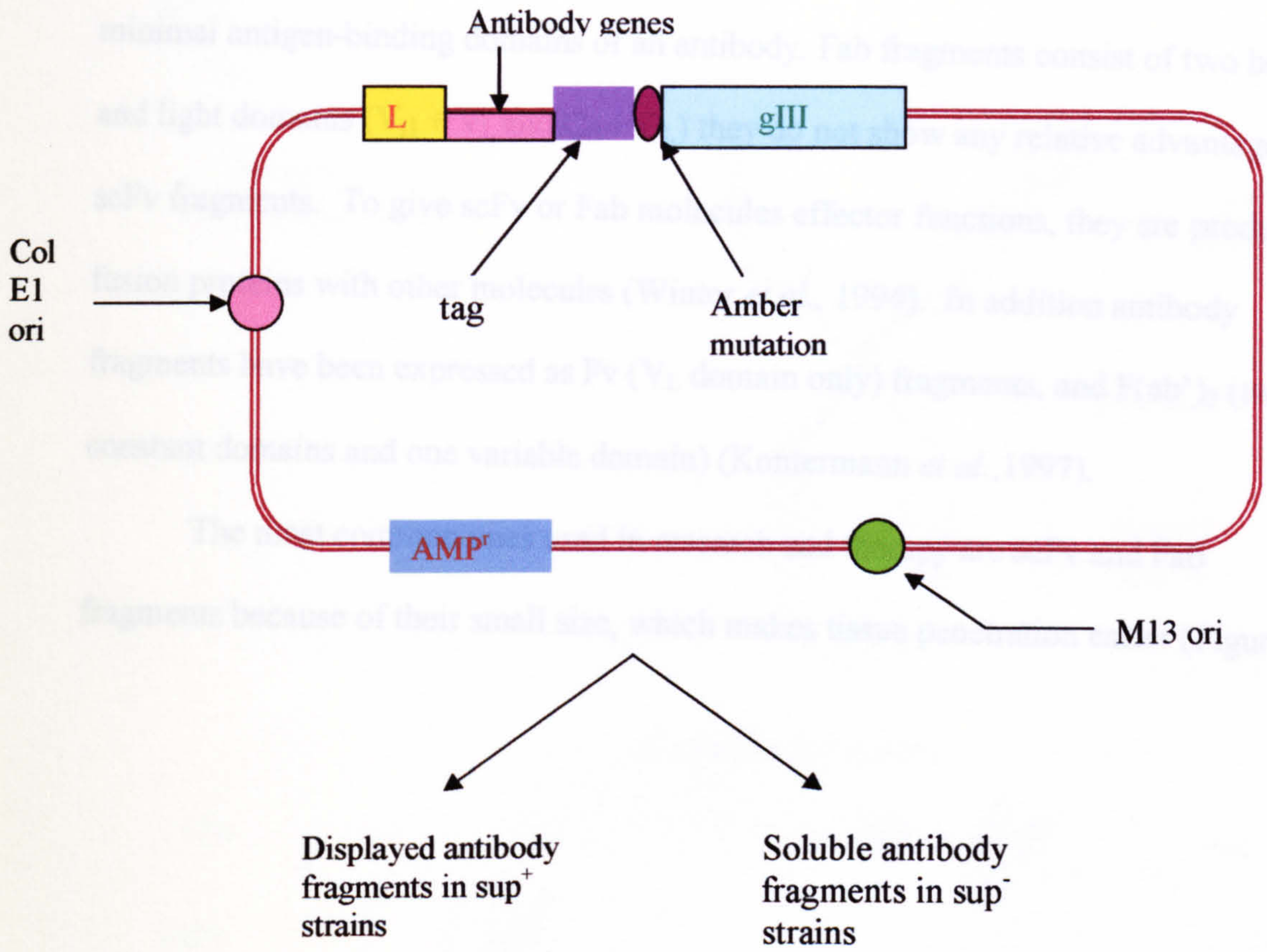


Figure 1.3: The phagemid system, pHEN1. This is used to express displayed (surface-bound) or soluble phage antibodies *in vitro*. It consists of an M13 origin of replication and *E. coli* origin of replication, an ampicillin resistant marker, genes for either pIII or pVIII and an amber mutation to produce soluble antibody fragments.

The antibody fragment is expressed either fused with pIII or pVIII protein and displayed on the surface of the phage. The antibody gene is inserted into the N-terminal regions of the protein. Insertion of antibody fragments has little or no deleterious effect on phage structure and function. In addition to antibody fragments, other proteins, such as hormones (Venkatesh *et al.*, 2002) or enzymes (Gram, 1999; Sun *et al.*, 2003) and enzyme inhibitors (Sblattera *et al.* 2002) can be fused and displayed on the surface of the phage.

Various recombinant antigen-binding fragments of immunoglobulins have been successfully expressed using phage display. A scFv molecule contains the V_H and V_L

domains of an immunoglobulin linked by a peptide spacer; therefore it contains the minimal antigen-binding domains of an antibody. Fab fragments consist of two heavy and light domains ($V_H + V_L$ and $C_H + C_L$) they do not show any relative advantage over scFv fragments. To give scFv or Fab molecules effector functions, they are produced as fusion proteins with other molecules (Winter *et al.*, 1994). In addition antibody fragments have been expressed as Fv (V_L domain only) fragments, and $F(ab')_2$ (two constant domains and one variable domain) (Kontermann *et al.*,1997).

The most common ones used in research and therapy are scFv and Fab fragments because of their small size, which makes tissue penetration easier (Figure 1.4).

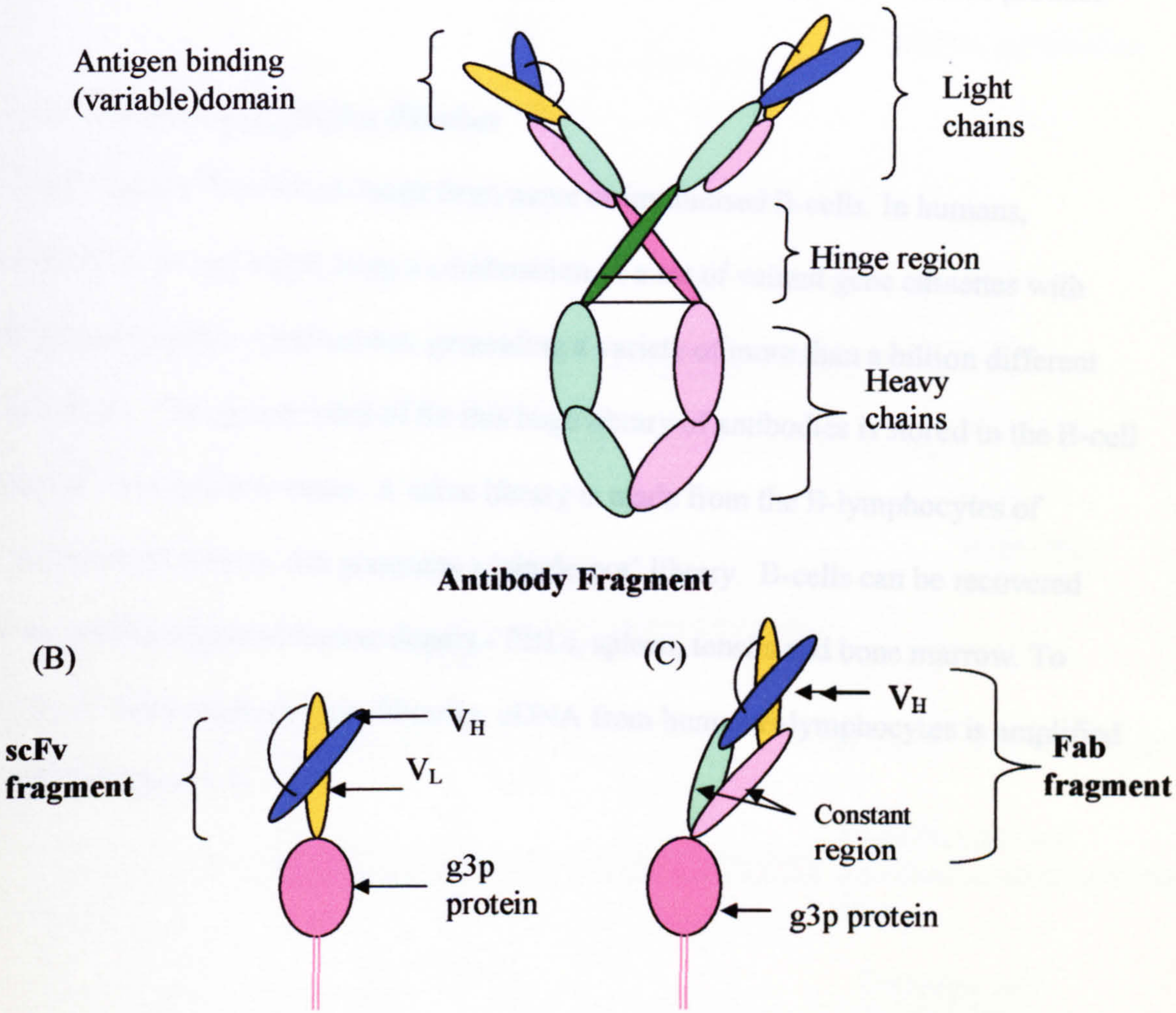


Figure 1.4: Schematic representation of an antibody molecule showing (A) the antigen binding domain in an antibody fragment,(B) the scFv and (C) the Fab fragment

These fragments are expressed in *E. coli* with a bacterial leader peptide sequence at the N-terminus responsible for export of the protein into the periplasmic space. Here the V_H and V_L domains fold into the active protein including formation of disulphide bonds present in the two domains.

Phagemid DNA encoding the antibody-pIII fusion will be preferentially packaged into the phage particle using a helper phage like M13KO7 or VCS-M13, which supplies all structural proteins. Since the helper phage genome encodes wild

type pIII, typically over 90% of the rescued phage display no antibody at all, and the vast majority of rescued phage will display only a single copy of the fusion product.

1.6.2 Making phage display libraries

Phage display libraries are made from naïve or immunised B-cells. In humans, antibodies are generated using a combination of a set of vairant gene cassettes with additonal mutation mechanisms, generating a variety of more than a billion different sequences. The genetic pool of for this huge library of antibodies is stored in the B-cell pool of our lymphatic tissue. A naïve library is made from the B-lymphocytes of unimmunised donors; this generates a 'single-pot' library. B-cells can be recovered from various organs of human donors - PBLs, spleen, tonsils and bone marrow. To construct huge antibody gene libraries, cDNA from human B-lymphocytes is amplified by PCR (Figure 1.5)

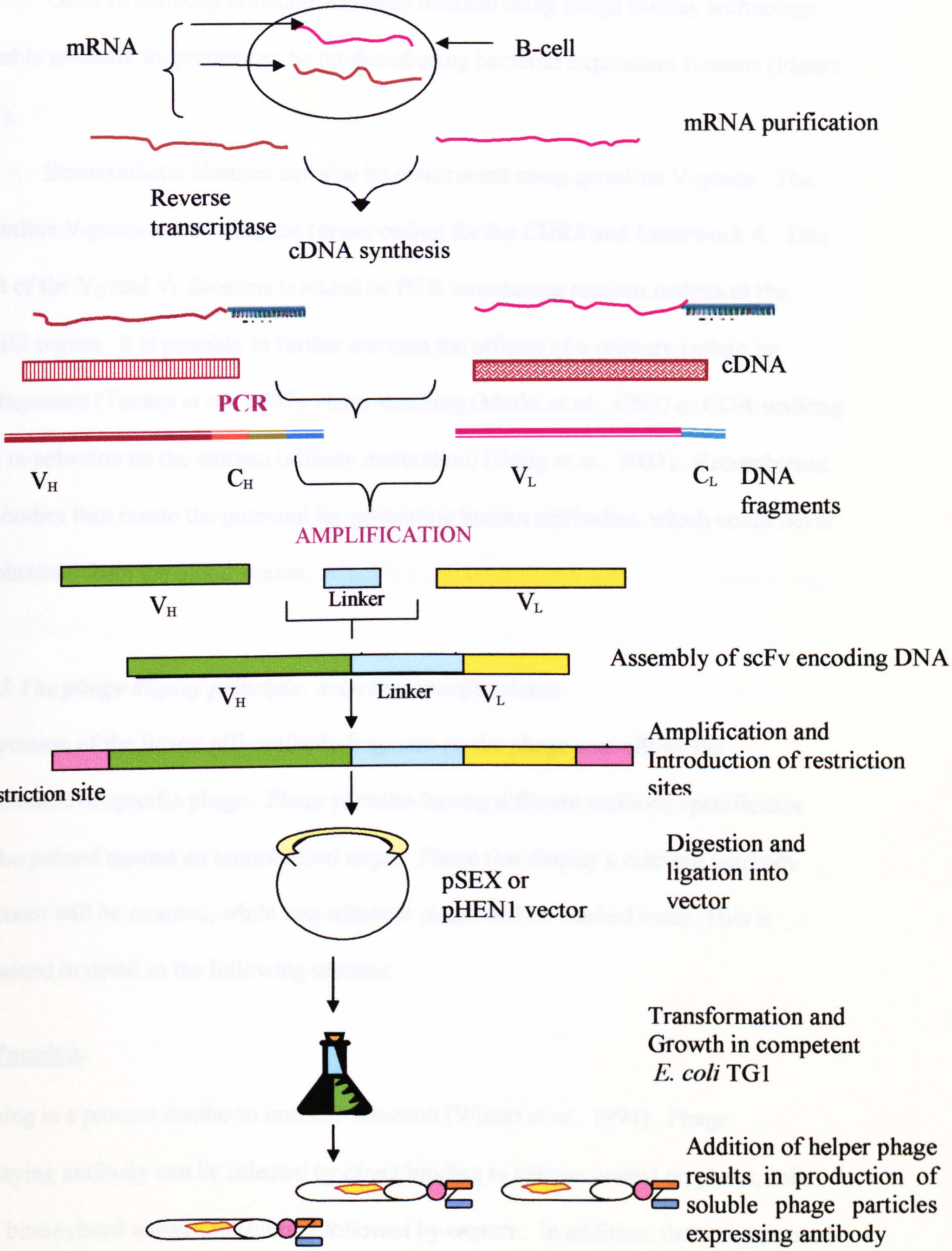


Figure 1.5: Making phage display libraries using naïve or immunised B-cells. mRNA is purified from the B-cells and used to synthesize cDNA. The DNA fragements are amplified by PCR and cloned into appropriate phagemid vector. The vector is introducted into competent *E. coli* TG1. The *E.coli* are infected with helper phage, which results in production of soluble phage particles expressing antibody.

Once an antibody molecule has been selected using phage display technology, soluble antibody fragments can be produced using bacterial expression systems (Figure 1.3).

Semisynthetic libraries can also be constructed using germline V-genes. The germline V-genes are missing the region coding for the CDR3 and framework 4. This part of the V_H and V_L domains is added by PCR introducing random codons at the CDR3 region. It is possible to further increase the affinity of a primary isolate by mutagenesis (Tuckey *et al.*, 2002), chain shuffling (Marks *et al.*, 1992) or CDR walking and re-selection on the antigen (affinity maturation) (Dong *et al.*, 2003). Recombinant antibodies thus create the potential for generating human antibodies, which could never be obtained from the blood stream.

1.6.3 The phage display principle: Selecting specific clones

Expression of the fusion pIII-antibody fragment on the phage coat allows the enrichment of specific phage. Phage particles having different antibody specificities can be panned against an immobilized target. Phage that display a relevant antibody fragment will be retained, while non-adherent phage will be washed away. This is explained in detail in the following sections.

1.7 Panning:

Panning is a process similar to immune selection (Winter *et al.*, 1994). Phage displaying antibody can be selected by direct binding to antigen coated on plates, cells or as biotinylated antigen in solution followed by capture. In addition, the antigens used for selecting phage antibodies can be either in the pure or impure form. Several studies have used purified antigens for the isolation of epitope-specific antibodies (Lee *et al.*, 2002). Impure antigens are used when the target epitope is unspecified or difficult to

purify. The use of purified antigens ensures that high-affinity antibodies can be generated for a specific known epitope, whereas with impure antigens (like on whole cells) it is difficult to regulate the amount of antigen presented for selection, and its association with other cell proteins. However the main advantage is that it selects for antigen in its native *in vivo* form, which is particularly useful for diagnostic antibodies. If the antibody is able to target antigen on cells without the need to purify the antigen, it would tremendously enhance the utility of diagnostic antibodies. A study was carried out by Hoogenboom *et al.* (1999) using two subsurface glycoprotein antigens present in the cell membrane. They used a naïve phage library to pan against the cell-surface expressed 7-TM and CD36 antigens. No dominant antibodies were generated against 7-TM but the CD36 molecule showed a single immunodominant epitope, to which a large collection of antibodies were readily selected. This study shows that the selection of epitope-specific phage antibody is largely dependent on the properties of the antigen itself.

When the phage has been mixed with the antigen, unbound phage is removed by washing, then the bound phage is eluted by changing the pH e.g.: - using a mild acid. This allows the enrichment of the phage antibody 20-1000 fold in a single round of selection (Figure 1.6).

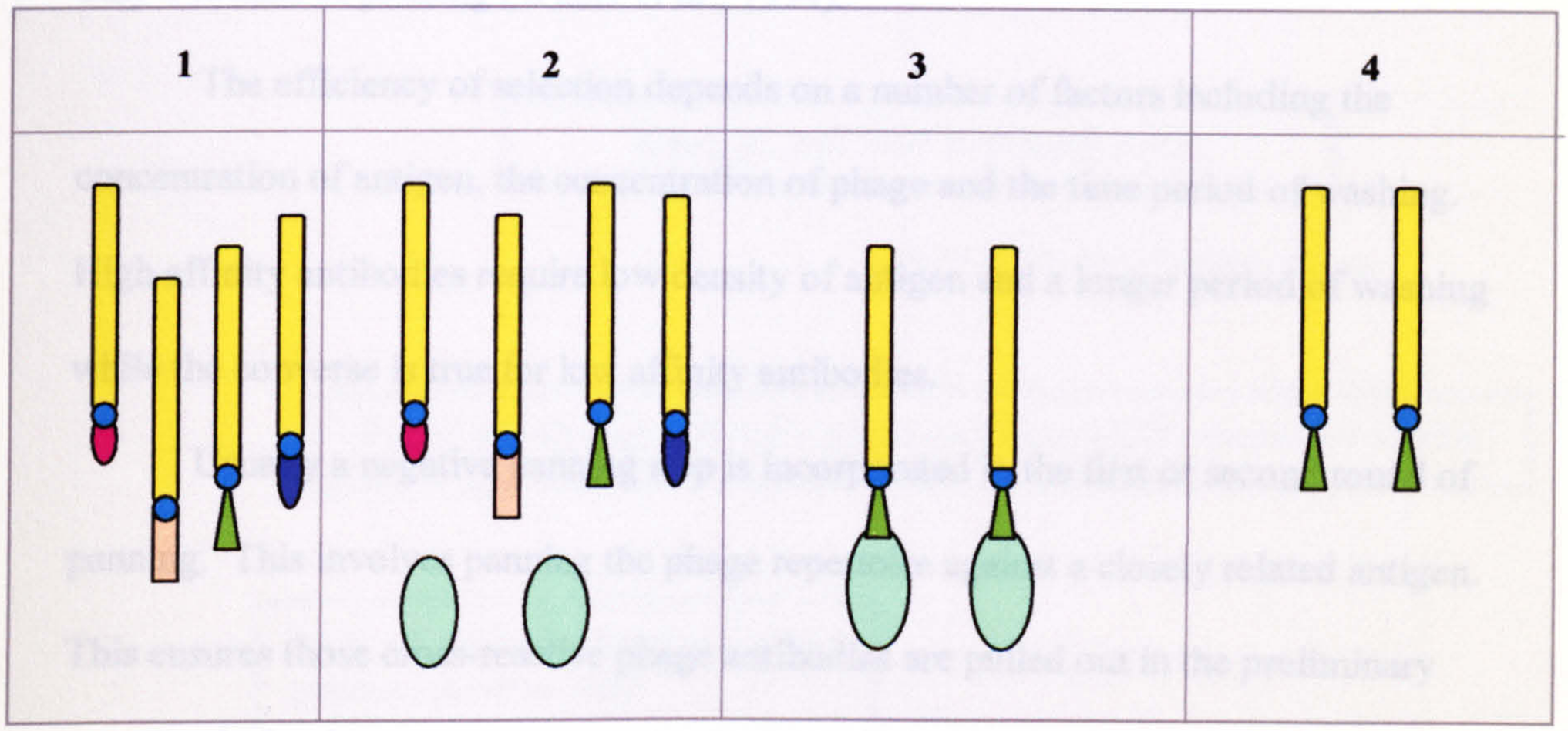


Figure 1.6: Selection of phage antibodies by the process of panning

Step 1 = Filamentous phage with scFv fused to pIII

Step 2 = Incubation of phage antibodies with antigen;

Step 3 = Elimination of unbound phage by washing;

Step 4 = Elution of bound phage using a mild acid.

= antigen (bound/unbound)

= unique scFv fragments fused to filamentous phage

The enriched phage can be grown in bacterial culture and subjected to further rounds of selection. Thus at least a 10^7 fold enrichment of phage can be obtained in only 4 rounds of panning (Winter *et al.*, 1994).

The efficiency of selection depends on a number of factors including the concentration of antigen, the concentration of phage and the time period of washing. High affinity antibodies require low density of antigen and a longer period of washing while the converse is true for low affinity antibodies.

Usually a negative panning step is incorporated in the first or second round of panning. This involves panning the phage repertoire against a closely related antigen. This ensures those cross-reactive phage antibodies are pulled out in the preliminary rounds of selection.

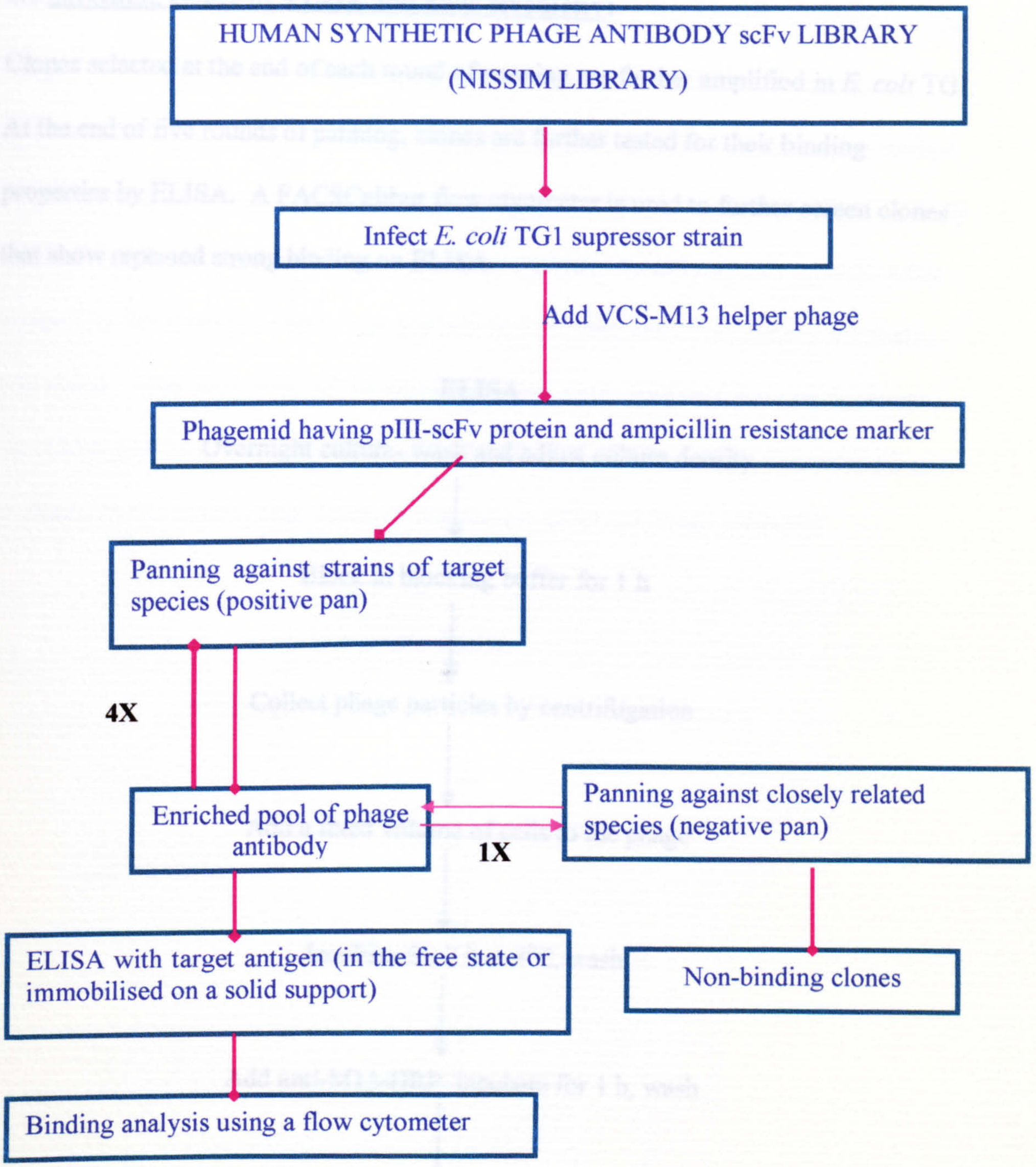


Figure 1.7: Outline of the panning process. The phage is incubated with antigen and unbound antibody is washed off. The bound phage are eluted using a mild acid. This process is repeated at least three times, to enrich for antigen-specific antibodies. A negative panning step may be incorporated within these rounds to remove cross-reacting antibody. The phage clones are then tested using ELISA, flow cytometry and other techniques.

1.8 Screening clones by ELISA and Flow ctyometry:

Clones selected at the end of each round of panning are further amplified in *E. coli* TG1. At the end of five rounds of panning, clones are further tested for their binding properties by ELISA. A FACSCalibur flow ctyometer is used to further screen clones that show repeated strong binding on ELISA.

ELISA

Overnight culture- wash and adjust culture density

↓

Block in blocking buffer for 1 h

↓

Collect phage particles by centrifugation

↓

Add a fixed volume of cells to the phage

↓

Incubate for 2 h at 4°C, wash

↓

Add anti-M13-HRP, incubate for 1 h, wash

↓

Add TMB substrate; incubate in dark for 30 min

↓

Record readings using an ELISA reader

ELISA is based on the interaction between antigen and specific antibodies. The antigen may be the whole cell or part of a purified component. Either the antigen (indirect ELISA) or the antibody (sandwich ELISA) can be immobilised on the surface. For estimating phage antibodies we used the direct ELISA method. The phage antibody was incubated with whole cells of *V. parahaemolyticus* for a fixed time period. After incubation, the excess antibody was washed, and enzyme-linked secondary antibody was added. After separation of the non-bound secondary antibody, enzyme substrate was added and the colour reaction measured using any ELISA reader. The extinction produced by the dye is proportional to the amount of bound phage antibody.

FACS

Overnight culture - wash and adjust culture density

↓

Block in blocking buffer for 1 h

↓

Add 10^{13} phage per 1ml of culture

↓

Incubate for 2 h at 4°C

↓

Add anti-M13 to each tube

↓

Incubate for 1 h, wash

↓

Add FITC (fluorescent dye),incubate for 1 h, wash

↓

Run the samples through a flow cytometer

Flow cytometry is the analysis of cells by detection of their light-absorbing or fluorescing properties after passing in a narrow liquid stream through a beam of laser light (Figure 1.8). In flow ctyometry, the light, after leaving the laser source is focussed through a lens into a beam of about 50μm cross-sectional diameter as it approaches the liquid stream, which flows perpendicular to the light beam. This ensures that the centre of the beam is uniformly illuminated. The point of intersection of the beam and the liquid stream is called the analysis point, which is surrounded by lenses, which collect light as it emerges from the analysis point. This light is then transmitted to a photodetectoer which converts the light energy to an electric signal. The greater the amount of light, the stronger the signal. In front of each photodetecter, there is a filter, which ensures that only light of a specific wavelength passes through (Givan, 1992).

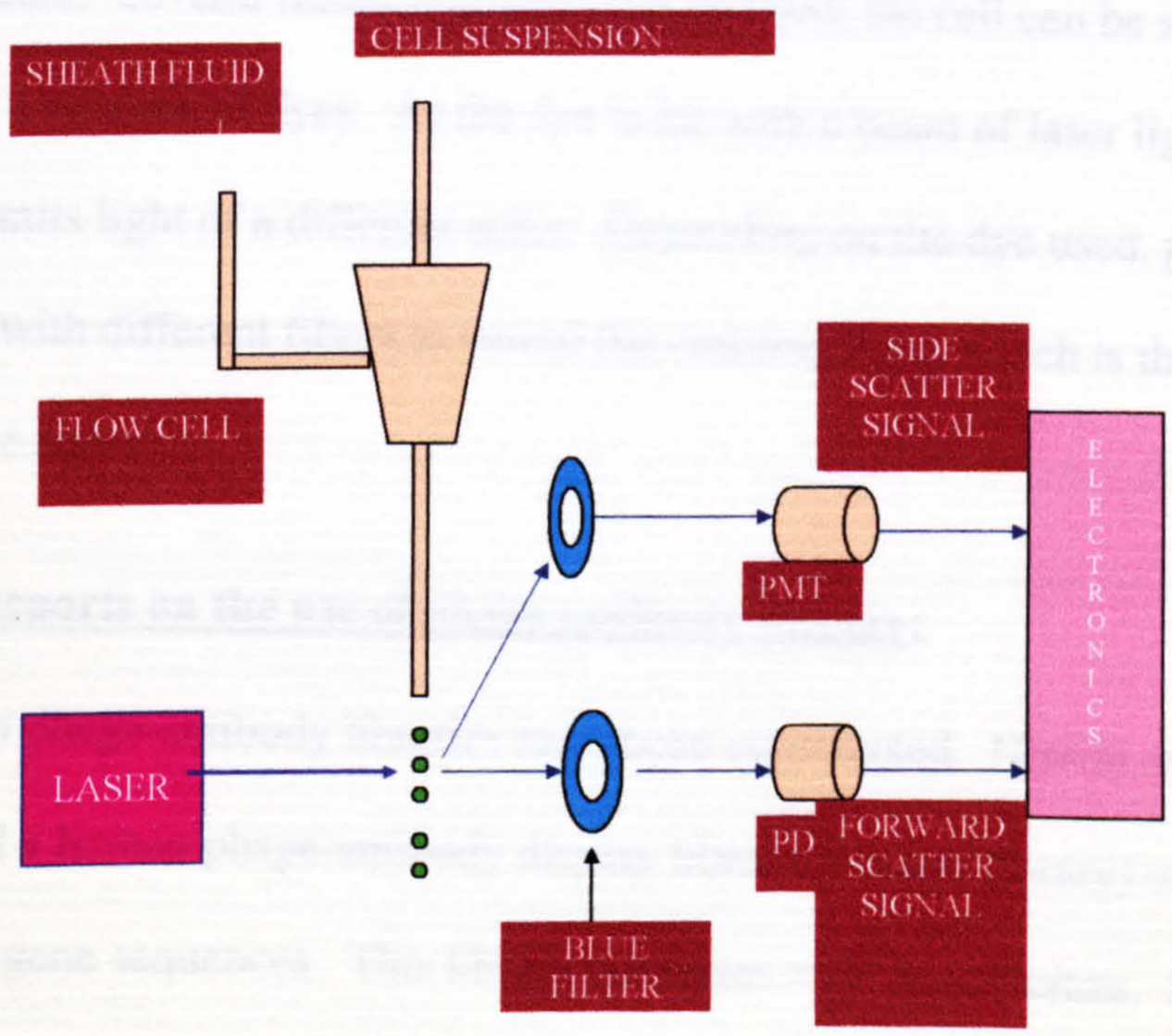


Figure 1.8: Basic principles of flow cytometry. The sample is passed through a beam of laser light as tiny droplet particles. The light scatter is then measured using a photo detector and converted to an electric output.

One of the detectors is located directly opposite the source of light; it captures light as it emerges from the liquid stream. This detector has a narrow metal strip in front of it, so that only light that has been bent as it passes through a particle will be able to impinge on the detector and generate an electric signal. Thus if two particles have the same size, the one with a higher refractive index will bend more light and generate a stronger singal. This signal is called the Forward Scatter Signal (FSC).

Other photodectors are located at right angles to the source of illumination. Thus only light which has been deflected or generated by the particle itself, will hit the detectors. Usually a same-color filter is fitted in front of one of the photodetectors. This detector will recognise any light that has been deflected to the side by particles in the stream. This indicates the granularity of the particles. Higher the granularity, greater is the light deflected, and stronger is the signal. This is called Side Scatter Signal (SSC). In addition, it is possible to detect fluorescence generated by particles as they move in the liquid stream. Several fluorescent dyes can be used; the cell can be stained with a single dye or a mixture of dyes. As the dye is hit with a beam of laser light, it gets excited and emits light of a different color. Depending on the dye used, photodetectors are equipped with different filters to record the emitting light, which is then converted into an electric signal.

1.9 Current reports on the use of phage antibody display:

Several human phage-antibody libraries have been constructed. Nissim *et al.* (1994) first developed a human phage-antibody display library (Nissim library) using naïve rearranged V_H gene sequences. This library possesses $>10^8$ specificities. In 1994, Griffiths *et al.* developed another phage antibody library which had diversity in both the heavy (V_H) and light (V_L) chains. Marks *et al.* (1993) have used a human phage antibody library for differentiating between blood group antigens of the ABO and I

blood group systems, of the Rh system and of the Kell system. Recently, Tomlinson (Griffiths *et al*, 1994) developed a phage library containing $>10^9$ antibody specificities. The phagemid vector used contains a HIS myc tag, which allows direct purification of soluble antibodies on a column.

Phage antibody display has been used extensively to differentiate between surface markers on phenoypically different eucaryotic cells (Neri *et al.*, 1998; Topping *et al.*, 2000). They have also been used to construct libraries towards specific ligands, since virtually any oligopeptide can be exposed on the surface of the phage. Barban *et al.* (2000) used phage display to identify an epitope in hepatitis C virus (HCV) core protein using cloned antibody repertoire from a non-symptomatic, anti-HCV-positive carrier. This could help identify antibodies with potential clinical value.

Grothaus *et al.* (2000) have used phage antibody display to select for an immunogenic peptide mimic of the capsular polysaccharide of *Neisseria meningitides* serogroup A. The isolation of such peptide mimics would facilitate the development of a vaccine towards *N. meningitides*.

Kasman *et al.* (1998) used phage display to express the *Bacillus thuringiensis* toxin. The toxin was produced as a fusion protein with the minor coat protein of filamentous phage. The toxin was shown to be antigenically equivalent to native toxin by ELISA and Western blotting analysis. This would allow the construction of large libraries of toxin variants for screening or biopanning.

Sowa *et al.* (2001) have used phage display to target antigens for the malarial parasite *Plasmodium falciparum.* This organism presents different antigens on its surface associated with its different life-cycle stages. Antibodies towards the major antigens will help in developing potential anti-malarial vaccines. They constructed a phage library from pooled peripheral blood lymphocytes of patients with clinical malaria and development of an anti-malarial vaccine. Antibodies were selected for

specific adhesion to *P. falaciparum* MAD20/B12 type schizont-infected red blood cells, which have shown to have protective effect in patients. These antibodies will be useful for characterisation of the parasite's antigenic properties and nature of the human immune responses to malaria infection.

Very few reports exist on the use of phage antibody display to differentiate between two bacterial cultures. Boel *et al.* (1998) used competitive phage antibody display to differentiate between serum complement-resistant and complement-sensitive molecular weight outer membrane protein, which was found only in complement-resistant but not in complement sensitive strains of *Moraxella catarrhalis.* This paper showed that phage antibody display could be used to differentiate surface molecules on prokaryotic cells.

Recently, De Greeff *et al.* (2000) reported the selection of recombinant antibodies specific for pathogenic *Streptococcus suis* by subtractive phage display. They isolated antibodies against whole cells of pathogenic *S. suis*. Using subtractive selection methods they also isolated a distinct phage antibody which was directed against the extracellular factor (EF) protein. This showed that EF is differentially expressed by pathogenic and non-pathogenic strains.

One of the most interesting developments in phage display technology has been the generation of fluobodies - green fluorescent scFv fusion proteins (Griep *et al.*, 1999). This system allows single step characterization of selected antibodies by flow cytometry of fluorescent stained-cells. Also, unlike FITC-conjugated antibodies they do not fade upon illumination. These fluobodies have been used to identify lipopolysaccharide of the bacterium *Ralstonia solanacearum.*

Chapter 2

Detection of *Vibrio parahaemolyticus* by Polymerase Chain Reaction (PCR)

2.1 Media and Cultures:

Media was obtained from Oxoid laboratories,UK and chemicals were obtained from Sigma-Aldrich, UK, unless otherwise stated. The media composition is outlined in Appendix I.

Cultures were obtained from the National Collection of Type Cultures (NCTC), London, UK and the American Type Culture Collection (ATCC), Virginia, USA.

The following strains were used-

STRAIN	SOURCE	OPTIMUM GROWTH TEMPERATURE
Vibrio parahaemolyticus		
↓ HRY VP1	An isolate from Hull Royal hospital,Hull,UK	37°C
↓ NCTC 10903	Food poisoning outbreak in Shirasu, Japan	37°C
↓ NCTC 10884	Food poisoning outbreak in Tokyo, Japan	37°C
↓ NCTC 10441	Food poisoning outbreak in Cornwall, England	37°C
↓ ATCC 27969	Blue crab haemolymph	25°C
↓ ATCC 17802	Food poisoning outbreak in Shirasu, Japan	37°C
V. harveyi NCTC 11346	Cooked prawns, Malaysia	30°C
V. mimicus NCTC 11435	Food poisoning outbreak in Atlanta, USA	37°C
V. alginolyticus NCTC 12160	Raw oysters ,Japan	25°C
V. vulnificus ATCC 27562	Human blood, Florida	30°C
V. logei ATCC 29985	Gut of mussel, Artic region	14°C

2.2 Culture Maintenance:

Cultures obtained from the ATCC and NCTC were stored as lyophlised ampoules at -20°C, or as glycerol stocks at -70°C. To determine the purity of the cultures, they were isolated on nutrient agar and thiosulphate bile citrate sucrose agar (TCBS) plates and incubated at 37°C overnight. They were then examined by Gram-staining and colony characteristics. All isolates showed green colonies on TCBS agar with a colony diameter of 1-2mm except *V. alginolyticus*, which is a sucrose fermenter and shows yellow colonies with a diameter of 2-3mm.

To propagate cultures, the contents of the lyophlilized ampoules were transferred into 50ml APW. The cultures were grown for 24 h on an orbital shaker at 37°C. Culture purity was checked routinely by streaking them on TCBS agar. Working stocks were maintained in APW.

2.3 Detection of *V. parahaemolyticus* using Polymerase Chain Reaction (PCR):

The PCR reaction consists of two steps - DNA extraction and the PCR reaction. These have been outlined below. The reagents and media used for DNA extraction were sterilised by autoclaving at 121°C, 15 psi for 20 min.

2.3.1 *DNA extraction using lysozyme and proteinase K*

This method was modified from Nowak and Kur (1995); Barski *et al.* (1996) and Sambrook *et al.* (1989). The cultures used for extraction were grown overnight in APW at 37°C on an orbital shaker. Next day the cells were collected by centrifugation at 12000xg for 10 min. The cells were washed 2X with PBS and the cell density was adjusted to 1 at A_{600} using PBS. This corresponds to a cell density of 8×10^8 cfu/ml. Cells were pelleted by centrifugation and resuspended in 200 µl of 2% (v/v) Triton-X100 in 2M sodium chloride solution. This suspension was dispensed into 1.5ml

microcentrifuge tubes and 2.5 µl lysozyme (stock solution 50 mg/ml) was added to the suspension. Cultures were incubated in a water bath at 37°C for 30 min and 1 µl of proteinase K (stock solution 20 mg/ml) was then added to the suspension.

Finally, cells were incubated at 65°C for 15 min in a dry heating block with occasional mixing by inversion. Chloroform: isoamyl alcohol (24:1) (400 µl) was added to the tubes and they were mixed by inverting them several times. After centrifugation at 6000xg for 15 min at 4°C the top phase was removed to a fresh tube. For each 100µl of extract 200 µl of isopropanol was added. The suspension was mixed well and incubated at 4°C for 15 min. After centrifugation at 11,200xg for 20 min at 4°C the supernatant was discarded. Any traces of isopropanol were removed by blotting the tubes on tissue paper at room temperature for 15 min. To further purify the DNA, it was washed 1X with 70% (v/v) ethanol. After allowing the ethanol to evaporate, 50-75 µl of pre-warmed Tris-EDTA (TE) buffer was added per tube and tubes were incubated in a 37°C water bath for 10 min and then overnight at 4°C.

The concentration of DNA was measured at A_{260} using a spectrophotometer. The DNA samples were diluted in TE buffer and stored at –20°C for further use.

2.3.2 *DNA extraction by Chelex method*

This method was based on the protocol of Khan *et al.*, 2000. Chelex (iminodiacetic acid) is an ion-exchange resin used for purification of DNA. The chelex beads were suspended uniformly by stirring on a magnetic stirrer. An overnight culture was prepared by growing the cells in APW at 37°C on an orbital shaker. The culture (1ml) was centrifuged at 1200xg for 10 min at 4°C and then washed 2X in PBS. The optical density was adjusted to 1 at A_{600}. The culture (200 µl) was then transferred to 0.5µl microcentrifuge tubes and 50 µl Chelex was added per tube. The tubes were transferred

to a PCR block and heated to 56°C for 20 min, followed by 99°C for 10 min. The tubes were centrifuged at 10,000xg for 5 min. The supernatant, containing DNA, was collected and used for PCR.

2.3.3 *DNA extraction by boiling method*:

This method was modified from Venkateswaran *et al.*, 1998. The cultures were grown in APW at 37°C overnight on an orbital shaker. The culture (1 ml) was transferred to a 1.5 ml microcentrifuge tubes and placed in a heating block at 100°C for 10 min. The tubes were then centrifuged at 13,000xg on a bench top centrifuge for 5 min. The supernatant; containing the DNA, was collected and diluted 10-fold in TE buffer. DNA concentration was estimated spectrophotometrically and 3 µl used for the PCR reaction.

2.3.4 *Optimising PCR detection of V. parahaemolyticus*

(a) *Estimation of DNA concentration and purity*

DNA concentration can be determined by using a spectrophotometer. At 600 nm an O.D. of 1 corresponds to 50 µg/ml of double stranded DNA (Sambrook *et al.*, 1989). Therefore, by measuring absorption values for the sample at 600 nm, its DNA concentration can be determined.

To determine the purity of DNA, the $A_{260/280}$ ratio is used and a value greater than 1.8 indicates that the sample is relatively pure and free of proteins.

(b) *Optimising the primer concentration and magnesium concentration*

The primer concentration can be optimised by carrying out the PCR using a range of concentrations from 1 to 10 mM at 1mM intervals. Similarly, the magnesium concentration can be optimised using a range of concentrations from 1-5 mM at 0.5mM intervals.

(c) *Optimising annealing temperature - Temperature gradient PCR*

This experiment was carried out to determine the optimum annealing temperature for PCR. Temperature Gradient PCR was performed for the *V.parahaemolyticus* and non-*parahaemolyticus* strains listed in **section 2.1**. The melting temperature(Tm) values of each set of primers was determined and an annealing optimum was set 5°C below the Tm. The PCR gradient was set at ±5°C of the annealing temperature.

From this experiment, the primer pairs were determined to have the following optimum annealing temperature:

Gene probe	**Optimum annealing temperature°C**
tdh	55
trh	55
toxR	63
gyrB	60

(d) *Optmising DNA concentration*

To optimise concentration of DNA, the PCR was carried using DNA concentrations ranging from 100-10 µg/ml at 10 µg intervals and 100-10 ng/ml at 10 ng intervals.

In the first stage of the study, PCR conditions were optimised for each set of primers using *V. parahaemolyticus* NCTC 10903 and NCTC10884. PCR products were run on a 1.2% agarose gel containing 3 µl of ethidium bromide (stock = 10 mg/ml). The bands were visualized on an UV transillumintor (Syngene, UK).

2.3.5 *Detection of the tdh gene*

The oligonucleotide primers used in this PCR were derived from the *tdh* gene sequence for *V. parahaemolyticus* (Nishibuchi and Kaper, 1995; Lee and Pan, 1993). The primer sequence is as follows (5'-3') TGG TTG AGA TCC TAC ATG AT GTG (VP21/forward) and GGG GAT CCC TCA GTA CAA AGC CTT (VP22/reverse).

PCR conditions were as follows: 37.9 µl of ddH_2O, 2.5 µl of 10X PCR buffer, 2 µl of 10mM dNTP, 2.5 µl each of the primers, 3.8 µl of $MgCl_2$, 0.12 µl of Taq polymerase and 5 µl of DNA template (containing at least 10 ng DNA). The PCR program was as follows: initial denaturation step at 94°C, followed by 34 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 10 min. The PCR product had a size of 400 bp.

2.3.6 *Detection of trh gene*

Primers for this PCR were based on the sequence of the *trh* gene for *V. parahaemolyticus* (Tada *et al.*, 1992). Primers were obtained from Maxim Biotech,UK. The primer sequences used were (5'-3') CAT TTC CGC TCT CAT ATG C (VP 1003/forward) and GGC TCA AAA TGG TTA AGC G (VP 1004/reverse).

PCR conditions were as follows: 27.8 µl of ddH_2O, 5 µl of 10X PCR buffer (containing $MgCl_2$), 5 µl of each of the primers, 0.2 µl of Taq DNA Polymerase, 2 µl of 2.5mM dNTP and 5 µl of template DNA (containing at least 10 ng DNA). The PCR program was as follows: an initial denaturation temperature of 94°C, followed by 34 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, with a final extension step of 72°C for 10 min. The size of the amplicon was 250 bp.

The *toxR* primers were designed using the gene sequence by Lin *et al.* (1993) and Matsumoto *et al.* (2000).

Sequence of primers used for PCR was as follows (5'-3') - GTC TTC TGA CGC AAT CGT TG (VPT1/forward) and ATA CGA GTG GTT GCT GCT ATG (VPT2/reverse).

PCR conditions were as follows: 33.75 µl of ddH_2O, 3 µl of 10X PCR buffer , 2µl of 20mM $MgCl_2$, 4 µl of 2.5mM dNTP mix, 2 µl each of primer, 0.25 µl Taq polymerase and 3 µl of template DNA (containing at least 10 ng DNA). The PCR cycle was carried out at 94°C for 1 min, 63°C for 1.5 min and 72°C for 1.5 min for 20 cycles. There was no initial denaturation step or a final extension step. The amplicon generated by PCR had a size of 386 bp.

2.3.8 *Detection of the gyrB gene*

The primers were derived from the gene sequence of the *gyrB* gene (Venkateswaran *et al.*, 1998). Primers sequence was as follows (5'-3') CGC CGT GGG TGT TTC GGT AGT (VPG1/forward) and TCC GCT TCG CGC TCA TCA ATA (VPG2/reverse). The PCR conditions were as follows: 28.5 µl ddH_2O, 3 µl of 10X PCR buffer, 2 µl of 50mM $MgCl_2$, 1 µl of 10mM dNTP, 5 µl of 10mM primer each, 0.5 µl Taq polymerase and 5µl of template DNA. The PCR reaction had to be modified to include a hot-start step, because the primer VPG1 tends to form primer dimers at lower temperatures, preventing the binding of primer to DNA. The basis behind hot-start PCR is to keep the Taq polymerase enzyme inactive until the PCR reaction temperature is above normal primer annealing temperature. In hot-start PCR, all the components of the reaction, except Taq polymerase are added to the tube and the tubes are heated up in the PCR block to the desired denaturation temperature. The Taq polymerase is then added at this step. This ensures that the PCR reaction will start after the primers have bound to

denatured DNA. The PCR was modified as follows: initial denaturation of primers and DNA at 100°C for 2 min followed by a holding period of 94°C for 2 min (when Taq polymerase is added). Then the PCR reaction is run for 20 cycles at 94°C for 1 min, 60°C for 1.5 min and 72°C for 2.5 min followed by a final extension step at 72°C for 7 min. The amplicon size was 285 bp.

2.4 Seeding Oysters with *V. parahaemolyticus* and detection by PCR:

Pacific oysters (*Crassostrea gigas*) were seeded with cultures of *V. parahaemolyticus* and the recovery and detection of the cells in oyster meat was determined using PCR. Oysters were obtained from the local fish monger and stored at -20°C. To prepare oyster samples, the oysters were first brought to room temperature and rinsed with distilled water. The oysters were scrubbed, surface sterilized with 100% ethanol and then shucked. The oyster meat was collected in a sterile container and was homogenized in 1 % (w/v) NaCl solution in a ratio of 1:10 (oyster meat: to 1% (w/v) NaCl).

V. parahemolyticus cultures (NCTC 10884, 10441 and 10903; ATCC 27969 and 17802) were grown overnight in APW at 37°C in an orbital shaker. The cultures were collected by centrifugation at 6000xg for 10 min at 4°C. The cultures were washed 2X with PBS and resuspended to give a cell density of 1 at A_{600}.

Serial dilutions of *V. parahaemolyticus* of known cell densities were prepared in PBS. The oyster homogenate was resuspened in PBS and aliquoted in tubes. To this the diluted cultures of *V. parahaemolyticus* were added. The culture was mixed well with the homogenate on a plate shaker. Depending on the experiment, the bacteria were either recovered immediately and used, or incubated with the oyster homogenate for varying periods of time.

As a negative control, cultures of *V. mimicus* NCTC 11435, *V. alginolyticus* NCTC 12160 and *V. harveyi* NCTC 11346 were used. Cultures were prepared in the same way as the *V. .parahaemolyticus* cultures.

2.4.1 *Immediate recovery of cells*

The suspension of oyster homogenate and bacteria was mixed on the plate shaker for 5 min to ensure even distribution of the culture. The larger particles were collected by centrifugation at 1500xg for 10 min. The supernatant was separated and bacterial cells were further separated from the supernatant by centrifugation at 11,000xg for 5 min. The cells were then used for DNA extraction or the whole cells were used directly for PCR.

The DNA was purified from these cells using the boiling and the chelex methods, both of which have been described previously in **section 2.3.2** and **2.3.3.** In addition, DNeasy columns (Qiagen) were also used for purification of DNA. The protocol for DNA extraction using DNeasy columns is outlined below -

The cells were harvested in a microcentrifuge tube by centrifugation at 5000xg for 10 min and resuspended in 180 µl of Buffer ATL. Proteinase K (20 µl) was added to the tubes, and they were mixed by vortexing briefly and incubated at 55°C, for 1 hour. The sample was mixed occasionally during the incubation period. At the end of the incubation period the sample was vortexed for 15 s and 200 µl buffer AL was added to the sample. It was then mixed again by vortexing and incubated at 70°C for 10 min. 200µl of 100% ethanol was added to the sample and it was mixed thoroughly by vortexing. The mixture was pipetted into a DNeasy mini column sitting in a 2 ml collection tube and centrifuged at 6000xg for 1 min. The column was transferred to a fresh collection tube and 500 µl of AW2 was added to it. It was centrifuged again for 3 min at full speed (13000xg) to dry the DNeasy membrane. The column was then

transferred into a sterile 1.5 µl microcentrifuge tube and 150 µl buffer AE was pipetted onto the membrane. It was incubated at room temperature for 1 min and centrifuged at 6000xg for 1 min to elute DNA. The DNA was stored at 4°C for short-term storage and -20°C for long term storage.

2.4.2 *Incubation with oyster tissue*

The oyster homogenate and bacterial cultures were mixed on a plate shaker at room temperature for 5 min. The larger debris were pelleted by centrifugation at 1500xg for 10 min. The supernatant (1 ml) (containing the bacterial cells) was aliquoted into individual tubes of 10 ml APW which were labelled 0 h, 3 h, 6 h and 24 h respectively. The tubes were then incubated at 37°C on an orbital shaker for the required time period. At the end of this time interval, the bacterial cells were collected by centrifugation at 11,000xg for 5 min, washed 2X with PBS and optical density recorded at 600 nm. The DNA was purified from these cells using the DNeasy columns. PCR was performed using approximately 40 ng of the DNA and using the *toxR* primers. The products were run on a 1.2 % agarose gel containing ethidium bromide and visualized using a UV transilluminator.

Chapter 3

Detection of *Vibrio parahaemolyticus* using phage display

Phage display was used to identify and differentiate between different strains of *V. parahaemolyticus.* The phage were panned against whole cells of *V. parahaemolytiucs* and binding phage antibodies were selected and enriched. The binding of the phage antibodies was further analysed by ELISA, FACS and Western Blot analysis.

The media used for the experiment were sterilized by autoclaving at 121°C at 15psi for 20 min. The media and reagents used for this study are listed in Appendix 1.

3.1 Phage display library:

3.1.1 *The NISSIM library*

The human synthetic ScFv library #1(Nissim Library) used in this study was the kind gift of Dr. Greg Winter ,MRC centre, Cambridge.(Nissim *et al.*, 1994). This library consists of a single Vλ3 light chain paired with a bank of *in vitro* rearranged V_H gene fragments containing a random V_H-CDR3 of 4-12 amino acids in length. It possesses $>10^8$ specificities. The phagemid used for cloning was pHEN-Vλ3. The bacterial strain used for the propagation of phage particles was *E. coli* TG1 supressor strain (K12,D(lac-pro), supE, thi, hsdD5/F'traD36, $proA^+B^+$.$lacI^q$,lacZDM15). The helper phage used for the rescue of the phagemid libraries was VCS-M13 (Stratagene), it carries a kan^r (kanamycin resistance) gene.

3.1.2 *Bacterial strains*

The following cultures were used for selection of phage antibodies:

V. parahaemolyticus NCTC 10903, NCTC 10884 and NCTC 10441,

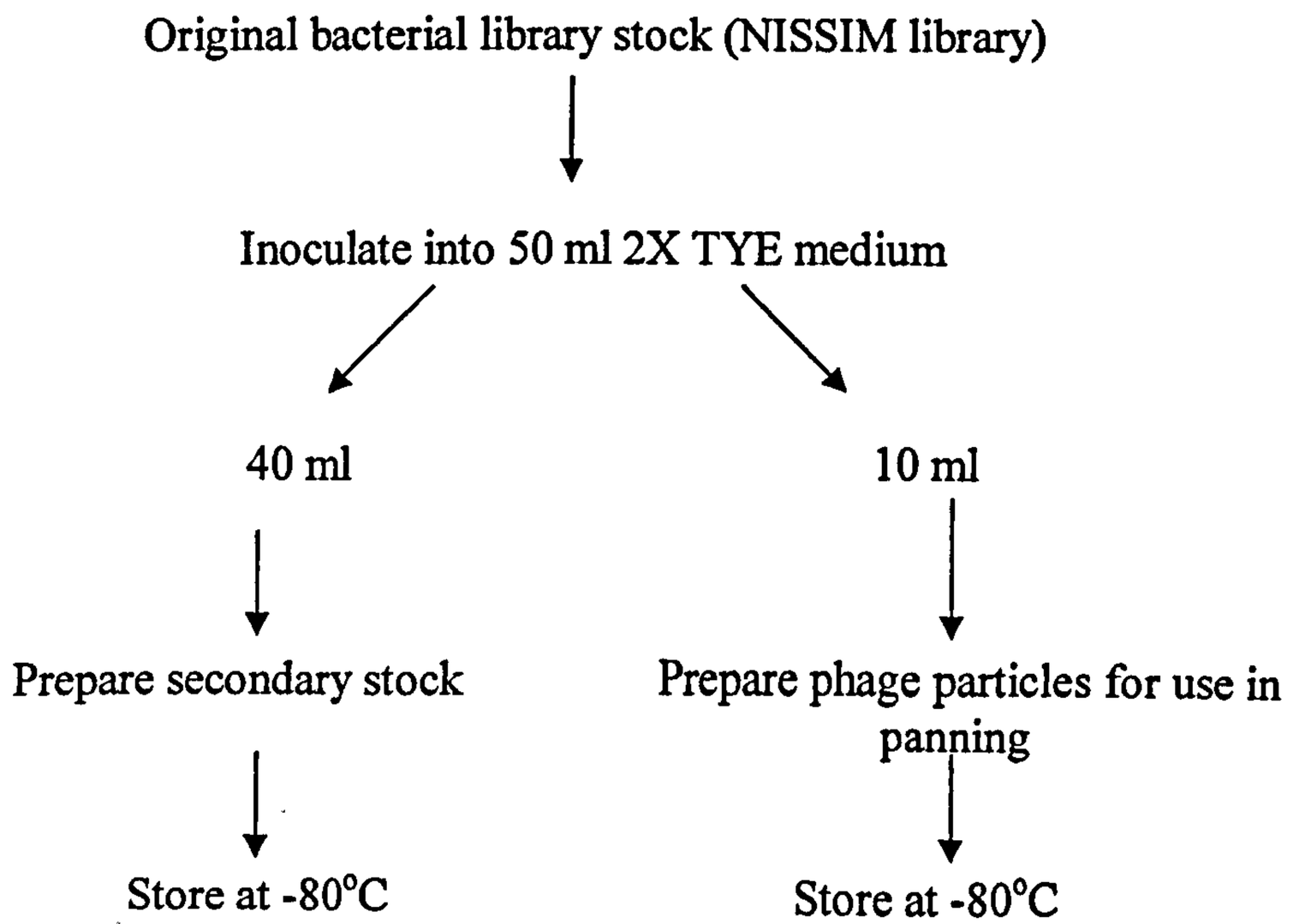
V. mimicus NCTC 11435, *V. harveyi* NCTC 11346, *V. alginolyticus* NCTC 12160.

3.1.3 *Titrating phage particles*

A single colony of *E. coli* TG1 was grown overnight in 5 ml 2X TYE medium at 37°C. Next day the culture was sub-cultured by diluting it 1:1000 into fresh 2X TYE medium and placed in an orbital shaker untill it reached log phase. The phage preparation was diluted in 10-fold serial dilutions in round-well microtitre plates, using PBS as the diluent. Serial dilutions (10^9, 10^{10} and 10^{11}) were used for estimating phage after a phage preparation. The phage titres are lower after a panning round than a phage preparation, therefore lower dilutions (10^3,10^4 and 10^5) were used for estimating phage titres. Log phase *E. coli* (200 µl) was mixed with 10 µl of phage and the cultures incubated at 37°C for 30 min in a water bath. The infected cultures (0.1 ml) were then spread on 2X TYE agar plates and left at 37°C overnight. Titrations were performed in duplicate. The phage titre was calculated as follows:

Average number of colonies per plate x dilution factor x 100 = number of phage /ml of phage preparation.

3.1.4 *Phage preparation for panning and secondary stock from the NISSIM library*



This method has been derived from Harrison *et al.*, 1996. Phage-carrying *E. coli* TG1 bacterial library stock (50 µl), containing approximately 5 x 10^8 specificities, was inoculated into 50 ml 2X TYE containing 100 µg/ml ampicillin and 1% (w/v) glucose. The culture was grown at 37°C in an orbital shaker till it reached log phase i.e.-the optical density at A_{600} was between 0.4-0.6.

The culture (10 ml) was infected with VCS-M13 helper phage in the ratio of 1:20, where the phage concentration was 20 times greater than that of bacterial cells. The phage were allowed to infect the cells in a water bath at 37°C for 30 min and the mixture was centrifuged 3300xg for 10 min, then resuspended in 300 ml of 2X TYE tryptone-yeast extract medium (TYE) containing 100 µg/ml of ampicillin and 25 µg/ml kanamycin. The phage were incubated at 30°C in an orbital shaker. Next day the cells were collected by spinning them at 3300xg for 30 min. A mixture of polyethylene glycol and sodium chloride (PEG/ NaCl) (1/5th volume) was added to the supernatant to precipitate phage. The mixture was left on ice for 1 h. The supernatant was centrifuged at 10,800xg for 30 min to collect the phage particles. The supernatant was discarded and the phage particles were resuspended in 40 ml water and 8 ml of PEG/NaCl. The mixture was again left on ice for 20 min. The phage were pelleted by centrifugation at 3300xg for 30 min and the supernatant aspirated. PEG/NaCl was drained completely by blotting. The pellet was resuspended in PBS/ glycerol (2 ml) and centrifuged at 3000xg for 10 min in a microfuge to remove any bacterial debris. The phage preparation was then filtered through a 0.45 µm filter and titred using a log phage culture of *E. coli* TG1. The phage stock solution was diluted to about 10^{13} ptu/ml (see Appendix 1) and stored at –80°C.

To prepare a secondary stock, 40 ml of the remaining phage-infected culture was used. The cells were collected by centrifugation at 3300xg for 10 min. They were then resuspended in a 0.5 ml 2X TYE and spread on a square bioassay dish containing 2X

TYE agar with 100 μg/ml ampicillin and 1 % (w/v) glucose. The plate was incubated overnight at 30°C. Next day, cells were scraped with a sterile spreader and resuspended in 2 ml 2X TYE. The stock was aliquoted and stored at –80°C.

3.2 Panning:

Vibrio spp were grown overnight in APW at 37°C in an orbital shaker. Next day, the cultures were washed 2X in PBS and resuspended to give an A_{600} of 1, which corresponds to concentration of 8×10^8 cells/ml . Individual cultures were pooled together for panning. Cultures were blocked with MPBS blocking buffer. Cells were collected by centrifugation and resuspended in 2 ml blocking buffer. Phage library suspension was added to the cells at a final concentration of 10^{11} ptu/ml. The suspension was mixed for h at room temperature. To remove the unbound phage the cells were pelleted and washed once in 0.5% (w/v) PBS/0.1% (w/v) Tween and 5X in PBS alone. Bound phage particles were eluted by incubation the cells with 500 μl of 76mM citric acid for 20 min at room temperature. The acid was neutralized with 500 μl of 1 M Tris-HCl, pH 7.4 (Marks, 1993). The phage were amplified in *E. coli* TG 1 cells (Harrison *et al.*, 1996) and used for further rounds of panning.

A total of five panning rounds were carried out, four positive (using *V. parahaemolyticus* cultures) and one negative panning (using non-*parahaemolyticus* cultures (Figure 3.1 and 3.2).

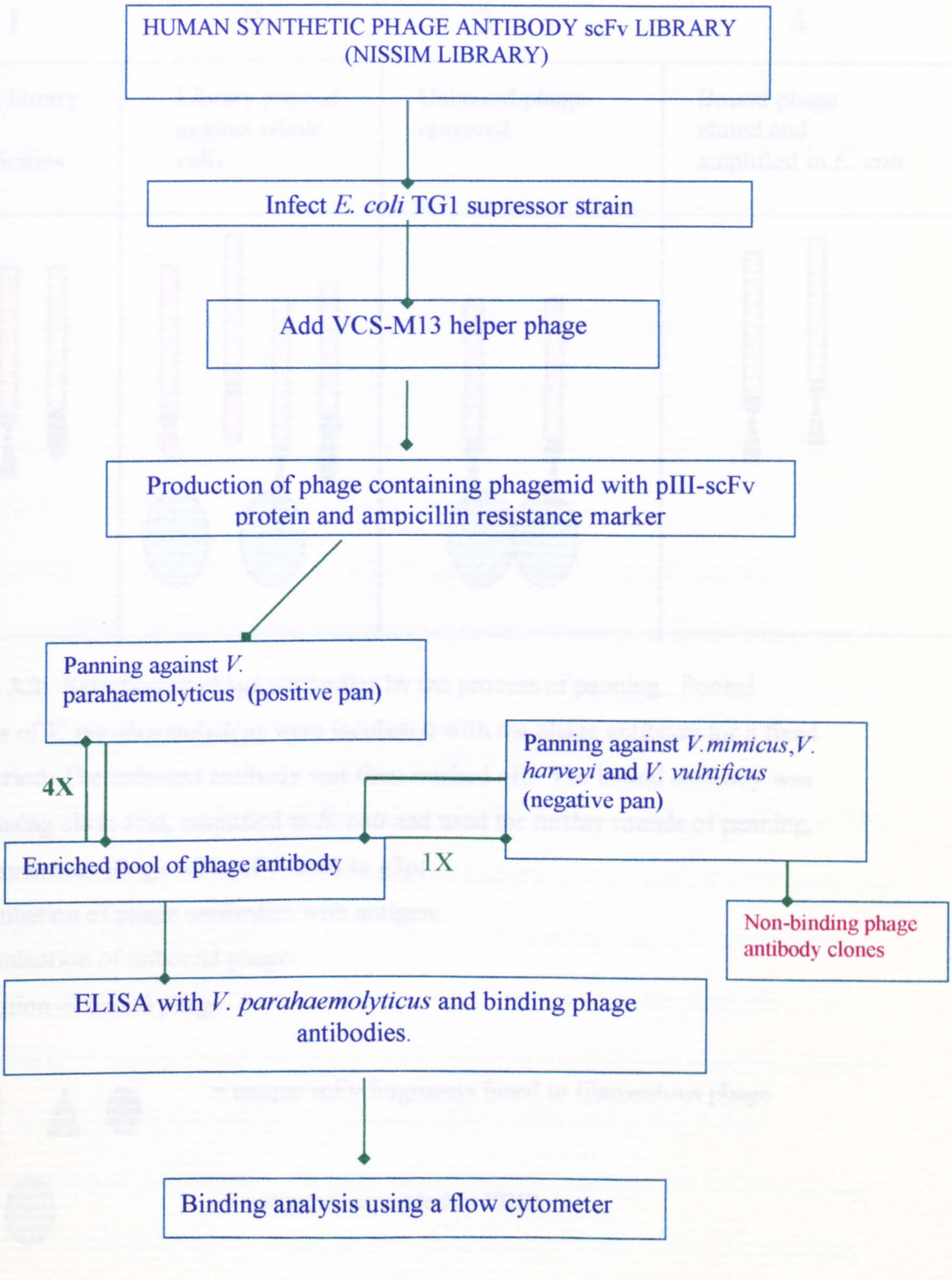


Figure 3.1: Outline of the panning process. The panning was carried out using the NISSIM library. Four rounds of positive panning were carried out using pooled cultures of *V. parahaemolyticus*. A negative panning round, using non-parahaemolyticus cultures was incorporated into second step. At the end of 5 rounds, clones were picked for ELISA and then further analyzed using flow cytometry and other techniques.

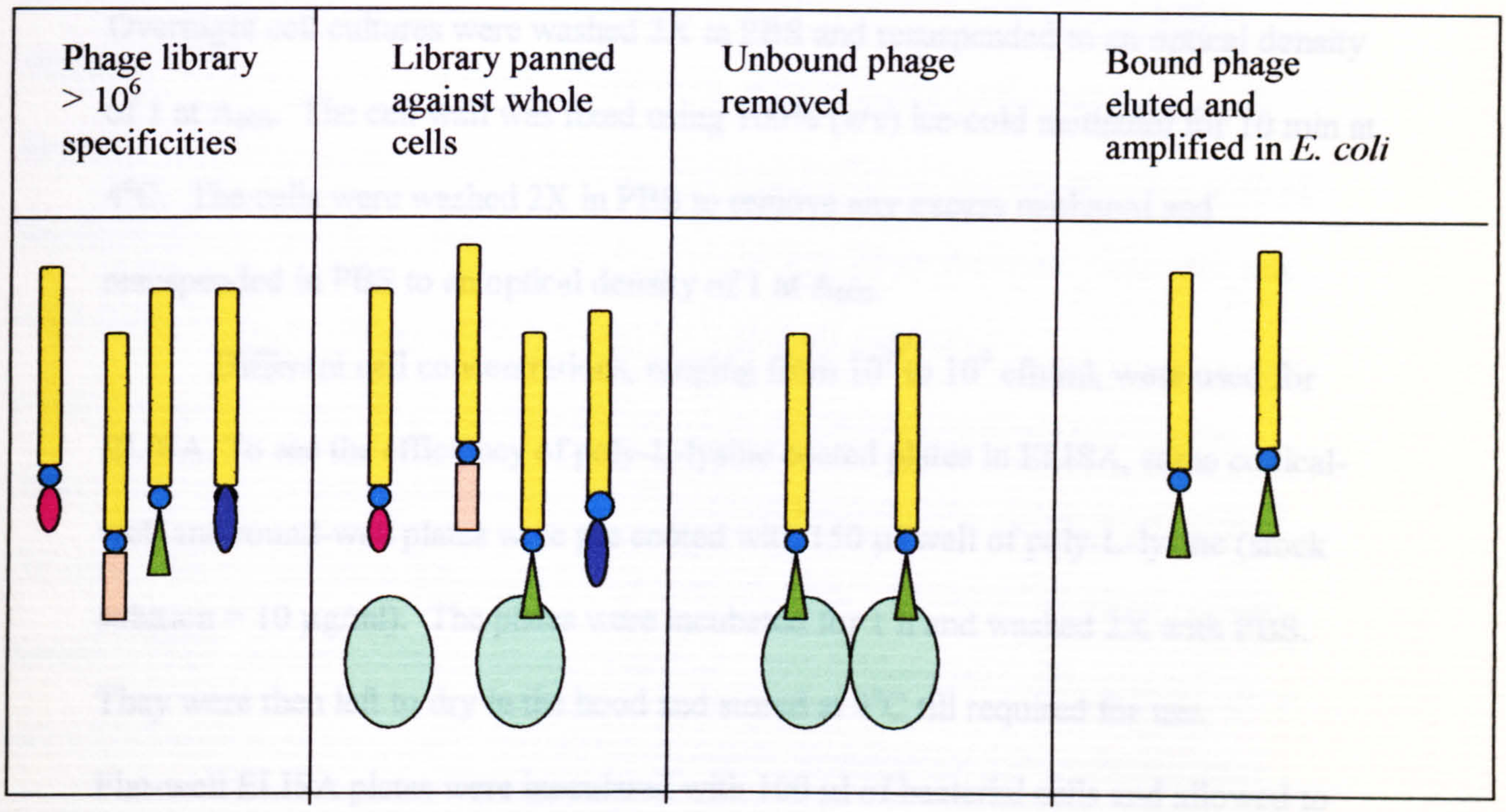


Figure 3.2: Selection of phage antibodies by the process of panning. Pooled cultures of *V. parahaemolyticus* were incubated with the phage antibody for a fixed time period. The unbound antibody was then washed off. The bound antibody was eluted using citric acid, amplified in *E. coli* and used for further rounds of panning.

1 = Filamentous phage with scFv fused to g3p;

2 = Incubation of phage antibodies with antigen;

3 = Elimination of unbound phage;

4 = Elution of bound phage

= unique scFv fragments fused to filamentous phage

= antigen (suspended in PBS)

3.3 ELISA:

Overnight cell cultures were washed 2X in PBS and resuspended to an optical density of 1 at A_{600}. The cell wall was fixed using 100% (v/v) ice-cold methanol for 10 min at 4°C. The cells were washed 2X in PBS to remove any excess methanol and resuspended in PBS to an optical density of 1 at A_{600}.

Different cell concentrations, ranging from 10^7 to 10^9 cfu/ml, were used for ELISA. To see the efficiency of poly-L-lysine coated plates in ELISA, some conical-well and round-well plates were pre coated with 150 µl/well of poly-L-lysine (stock solution = 10 µg/ml). The plates were incubated for 1 h and washed 2X with PBS. They were then left to dry in the hood and stored at 4°C till required for use. Flat-well ELISA plates were inoculated with 100 µl of bacterial cells and allowed to adsorb overnight at 4°C. Excess culture was drained off and the wells were blocked in 250 µl MPBS blocking buffer for 45 min at room temperature.

Phage suspensions for ELISA were prepared as follows - At the end of the fifth round of panning individual colonies were inoculated into 150 µl of 2X TYE medium in a 96-round-well microtitre plate. Clones were grown overnight at 37°C on an orbital shaker. This was the stock plate. Next day, 3 µl of culture from the stock plate was transferred to a second round-well microtitre plate and incubated on an orbital shaker at 37°C for 1 hour. Helper phage (10 µl) was added to each well giving a final concentration of 10^9 ptu/well. The plate was allowed to stand at 37°C for 30 min and then transferred to an orbital shaker at 37°C for a further 90 min. The plate was then centrifuged at 400xg for 10 min and the supernatant aspirated. The pellet was resuspended in 150 µl of 2X TYE containing 100 µg/ml ampicillin and 50 µg/ml kanamycin and grown overnight at 30°C on an ortbital shaker. The *E. coli* TG1 cells were separated from the phage particles by spinning down at 400xg for 10 min and supernatant used as the source of phage.

Next day, the antigen (whole cells of *V.parahaemolyticus*) coated microtitre plates were washed 2X with PBS. The wells were then blocked with 250 µl MPBS blocking buffer for 45 min at room temperature. The blocking buffer was tipped off from the microtitre plates. Double strength blocking buffer (75µl) was added to each of the wells along with 75 µl of the supernatant containing phage particles. One well was used as the blank, by using anti-NIP phage (10^{13} pfu/ml) instead of *V. parahaemolyticus*-specific phage preparation. The plates were incubated on a plate shaker (100 rpm) at room temperature for 2 h with gentle rotation. Plates were washed 1X with 0.5% (w/v)PBS/0.05% (v/v) Tween and 2X with 0.5%(w/v)PBS alone. 1:500 anti-M13-HRP antibody (Pharmacia) was diluted 1:500 in blocking buffer and 100 µl was added to each of the wells. The plates were then incubated on a plate shaker for 1 h. Plates were then washed 1X with 0.5%(w/v)PBS/0.05% (v/v) Tween and 2X with 0.5%(w/v)PBS alone. Freshly prepared ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) or TMB (3,3',5,5'-tetramethylbenzidine); (Vector Laboratories) (150 µl) was added to each well and the plates were incubated at room temperature for 30 min in the dark. The readings were measured at A_{630}-A_{405} (for ABTS) or at A_{450} (for TMB) using an ELISA reader (Dynex), blanking on the well with the anti-NIP phage.

3.3.1 *PCR for the CDR3 region, to check for phage clone diversity*

PCR was used to check the size of the CDR3 inserts in the phage clones. The PCR protocol was based on Nissim *et al.* (1994). Primers used for PCR were as follows (5'→3') CAG GGT ACC TTG GCC CCA (CDR3 FOR/forward) and GTC TAT TAC TGT GCA AGA (CDR3 BACK/reverse). The PCR reaction consisted of 5 µl of 10X PCR buffer, 2.5 µl of 50mM magnesium chloride, 1 µl of 10mM dNTPs and 0.5 µl of each of the primers at a concentration of 1 µg/µl. The final volume was made up to 50 µl with PCR-grade water. Whole cells were added as a source of PCR template, by

gently touching the colonies with a sterile toothpick and inoculating this into the PCR tubes. The PCR program ran as follows: an initial denaturation step at 94°C for 2 min, followed by 34 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 30 s. PCR products were separated on a 5% (w/v) metaphor agarose gel (Flowgen), containing ethidium bormide,at 100 V for 1 h. The products were visualised using an UV transilluminator.

Clones were also analysed by *BstN1* restriction digestion of the PCR fragment covering the whole scFv region. The protocol for PCR has been described in Marks *et al.* (1991). Primers used for the PCR were (5'→3) CAG CAA ACA GCT ATG AC (LMB3/forward) and GAA TTT TCT GTA TGA GG (fd-SEQ1/reverse). The PCR reaction was set up in the same way as above. The PCR program consisted of 35 cycles of 94°C for 30 sec; 55°C for 30 sec and 72°C for 30 sec. PCR products were separated by electrophoresis on a 5% (w/v) Metaphor agarose gel (Flowgen) containing ethidium bromide. PCR products were visualized using a UV transilluminator. The DNA from the PCR products was further purified using phenol:chloroform extraction method (Sambrook *et al.*, 1989) followed by precipitation with 100% (v/v) ethanol and washing with 70% (v/v) ethanol. The final pellet was then rehydrated in 20 µl of TE buffer, pH 8.0. This DNA was used as the template for restriction digestion. The reaction consisted of 1.5 µl each of *Bst*N1 restriction enzyme, 10X NE buffer and 100X purifiedBSA. DNA template was added to the reaction to give a final volume of 15 µl. It was then incubated at 60°C for 1 h and the restriction products were separated on a 2.5% (w/v) metaphor agarose gel containing 3 µl ethidium bromide (10mg/ml) and visualised using UV light.

3.4 Flow cytometry:

Phage clones were prepared as described in **section 3.1.4**

V. parahaemolyticus were grown overnight and washed 2X in PBS, and the optical density adjusted to1 at A_{600} which corresponds to 8×10^8 cfu/ml. Culture (1 ml) was dispensed into individual FACS tubes and blocked with MPBS for 1 h at room temperature. Cells were washed once in PBS and resuspended in 1 ml PBS. Phage preparation containing at least 10^{10} ptu/tube was added to the tubes. Anti-NIP phage was used as the negative control. Cells were incubated at 4°C for 2 h and washed once with PBS/BSA/Azide buffer (Appendix I). A 1:100 dilution of anti-M13 antibody (50 µl) (Amersham Pharmacia) in PBS/BSA/Azide was added to each of the tubes. Tubes were incubated at 4°C for an 1 h and washed as above. A1:100 dilution of anti-mouse FITC (50 µl) (Fluorescein isothiocyanate;Vector laboratories) was added to each tube and they were incubated at 4°C for 1 h and washed as above. Cells were resuspended in 400 µl of PBS/BSA/Azide buffer and run through a FACSCalibur Flow Cytometer, running Cell Quest Software..

The cells (10 µl) were also observed using a UV microscope with a DAPI (4',6-diamidine-2-phenylindole,dihyrochloride) /FITC filter. Cells stained with DAPI were used as the positive control.

3.5 Western blotting:

Western blotting was used to investigate the phage-binding repertoire. The cells were lysed by two methods:

(1) Freeze-thawing in liquid nitrogen

(2) Sonication

Cultures of *V. parahaemolyticus* were grown overnight at 37°C in APW. The cells were harvested by centrifugation at 13000xg for 5 min. The cells were washed 2X with PBS and resuspended in PBS to get an optical density of 1 at A_{600}. The cells were dispensed into 1.5 ml microcentrifuge tubes and kept on ice for sonication. Sonication was carried out at 4 µl peak to peak amplication twice for 20 s intervals. The cell debris was collected by centrifugation at 1000xg for 5 min. The supernatant was used for Western blotting.

For freeze thawing the microcentrifuge tubes containing the samples were immersed alternatively in liquid nitrogen and in a 37°C water bath five times for 10 s intervals. The tubes were then centrifuged at 1000xg for 5 min to remove cell debris. The supernatant was used for Western blotting.

To prepare the cells for SDS-PAGE electrophoresis, 30 µl of cell lysate was mixed with 30 µl of reducing (with β-mercaptoethanol) or non-reducing sample buffer.

The cells were heated to 100°C in a PCR block for 5 min. The lysates were cooled prior to use, to allow for renaturation of the proteins. The samples were then loaded onto the polyacrylamide gel, as described in **section 3.5.2**. Any unused sample was stored at –20°C.

3.5.1 *Preparation of scFv for Western blotting.*

Clones which showed a strong binding on ELISA and FACS analysis were checked for the presence of the scFv insert by PCR. These clones were then further used for Western blot analysis. *E. coli* TG1 scFv-containing clones (10 µl) were inoculated in 5 ml of 2X TYE containing 100 µg/ml ampicillin and 1 % (w/v) glucose. The culture was incubated overnight on an orbital shaker at 37°C. The overnight culture was diluted 1:100 into fresh 2X TYE -ampicillin-glucose medium and incubated on the shaker at 37°C until the OD was between 0.4 to 0.6 (log phase culture). Helper phage was added

in a 1:20 ratio (bacteria: phage) from a stock solution containing at least 10^{13} ptu/ml. The tubes were incubated at 37°C in a stationary water bath for 30 min. The cells were then collected by centrifugation at 1000xg for 10 min and resupended in 5 ml 2X TYE containing 100 µg/ml ampicillin and 50 µg/ml kanamycin. This culture was grown overnight at 30°C in an orbital shaker. The culture was aliquoted into 1.5 ml microcentrifuge tubes and the cells collected by centrifugation at 13000xg at 4°C for 5 min.

The supernatant was transferred to a fresh tube, and 200 µl of PEG/NaCl was added to each tube. The tubes were mixed well by inverting and placed on ice for 15 min. The precipitated phage were separated by centriguation at 13000xg for 5 min at 4°C. The supernatant was used as the source of scFv.

3.5.2 *Polyacrylamide gel electrophoresis*

PAGE mini-gel apparatus (BioRad) was used for preparing gels. A 10% (w/v) separating gel was prepared for SDS-PAGE and Native PAGE. The components of the gel are listed in Appendix. 1. Stacking gel 3 % (w/v) was used as the top layer to load the samples. Once the gels had been cast, 30 µl of sample was loaded in each well, in addition 10 µl of Benchmark rainbow protein ladder (Invitrogen) was also loaded in one well. The protein standard contains proteins of molecular mass between 172.6 kDa and 9.3 kDa. The gel was allowed to run at 200V till the dye front had run off from the gel (approximately 1 h). The gel was removed from the cast and either (1) transferred to Coomassie blue or to SimplyBlue™ SafeStain (Invitrogen), for visualization of proteins or (2) allowed to equilibriate in transfer buffer for Western blotting.

3.5.3 *Staining protein gels*

Protein gels were run in duplicate and stained with Coomassie blue and Safe stain (Invitrogen) for visualization of bands.

(a) Coomassie blue staining: The gel was placed in a shallow tray on a rocking platform and covered with Coomassie stain. After 1 h the excess dye was poured off and recycled. The gel was then transferred to a destaining solution (acetic acid + methanol), and left on a rocking platform till the bands were clear on the gel. The destaining solution was changed once after 3 h and then left to destain overnight. Next day, the excess destaining solution was poured off and the gel was then photographed using the white light in a transilluminator.

(b) Safe stain: The gel was placed in a shallow tray on a rocking platform and covered with Safe stain. After 1 h the excess stain was poured off and the gel was washed with distilled water 3X times. The gel was then left to destain in distilled water overnight on a rocking platform. Next day, the excess distilled water was poured off and the gel was then photographed using the white light in a transilluminator.

3.5.4. *Western blotting*

Proteins were transferred from the PAGE gel to a nitrocellulose membrane (Amersham-Pharmacia) by blotting with a Trans-blot apparatus (BioRad). Blotting was carried out at 100 V for 1h at 4°C. The transfer solution was mixed using a magnetic stirrer during the blotting process. The nitrocellulose membrane was carefully separated from the gel and transferred to a 10% MPBS blocking solution for 1 hour at room temperature on a rocking platform or 4°C overnight. The membrane was washed 3X in 0.5-1% (v/v)Tween-PBS, the final wash was for 5 min on a platform shaker. Blocking buffer (15 ml) and 500 µl of phage preparation containing at least 10^{13} ptu/ml was added to the membrane. It was incubated at room temperature for 1 h on the platform shaker. The

membrane was washed as before and anti-M13-HRPconjugated antibody (10 μg/ml) diluted1:5000 in 15 ml of blocking buffer, was added. The membrane was again incubated as before and washed. HRP was detected using a chemiluminescent kit (ECL Western blotting detection system, Amersham Pharmacia Biotech). Briefly, reagent 1 and reagent 2 were mixed in equal volumes and added to the membrane. The membrane was incubated for 1 minute with the detection reagent. Luminescent signal was detected using an X-ray film (Hyperfilm ECL, Amersham Pharmacia Biotech), using different exposure times as appropriate. The film was then developed using developing and fixing solutions from Sigma-Aldrich, according to the manufacturer's instructions..

3.6 Sequencing :

An ALF Express sequencer (Amersham-Pharmacia) was used for sequencing.

3.6.1 *Preparing the gel*

The glass plates were cleaned thoroughly and assembled according to the manufacturer's instructions. Sequagel XR and sequagel complete buffer were mixed along with APS to prepare the gel. The gel was cast using a 50 ml plastic syringe, ensuring that no air bubbles were formed. The gel was wrapped in cling film and allowed to set for 2 h.

3.6.2 *Sequencing reaction*

PCR products of scFv amplification of the clones were used for sequencing.

For the sequencing reaction, Thermo Sequenase Primer Cycle Sequencing Kit (Amersham-Pharmacia) was used. To prepare the master mix, 1 μl of the cy5-primer (5 μM stock) was mixed with 5-10μl of template. The final volume was adjusted to 13 μl

with distilled water. For each template to be sequenced four tubes were prepared containing di-deoxy-NTPs- ddA,ddT,ddG,ddC. The master mix (3μl) was aliquoted into each tube. Thus each reaction had four tubes, one of each of the base pairs. The PCR cycle was run for 20 cycles as follows: 95°C for 30 s, 55°C for 30 s, 72°C for 1 min. At the end of the cycling sequence the tubes were spun briefly and kept of ice. Stop solution (6 μl) (formamide loading dye) was added to each tube.

3.6.2 *Sequencing run*

Prior to use, the glass plates and the outer surfaces of the pre-poured gel were wiped gently with tissue containing isopropanol. The gel cassette was attached to the instrument according to the manufacturers instructions. TBE buffer (0.6 X) was added to the upper and lower chambers. The instrument was switched on and the laser readings were allowed time to stabilise. The comb was removed from the gel and the wells were flushed to remove excess urea. The sample (5 μl) was loaded in each well. The electrodes were connected and the computer program started. Run time was adjusted depending on the length of the sample and lasted approximately 3-4 h.

In addition, DNA was extracted from the clones using the Wizard plus minipreps DNA purification system (Promega) and sent to Invitrogen for sequencing, using the LMB3 and fd-SEQ1 primers.

3.7 Chemiluminescent assay :

The ELISA test was modified using a chemiluminescent dye. As for the ELISA, *V. parahaemolyticus* cultures were mixed with phage antibody and anti-M13-HRP was added as the secondary antibody. The detection reagent was the ECL Western Blotting Kit (Amersham Pharmacia). The plate was then covered with an X-ray film (Hyperfilm

ECL, Amersham Pharmacia) and exposed for different time periods ranging from 5 s to 30 min. The films were developed as described in **section 3.5.4**.

Once the optimum exposure time was recorded, the test was repeated using varying concentrations of *V. parahaemolyticus* and phage.

The same test was also carried out using a Gel Cam system (Polaroid) to record the chemiluminescence using a black and white film.

3.8 Detection of *V. parahaemolyticus* in seeded oyster samples using phage display:

Phage antibody technology was applied for the detection of *V. parahaemolyticus* from seeded oyster tissue samples. The clones which were used for ELISA in **section 3.3** were used for this experiment.

Vibrio cultures (*V. parahaemolyticus* and non-parahaemolyticus strains) were grown and washed as described in **section 3.3.** The oyster tissue was homogenised using 1%(w/v) NaCl and 1 ml of the oyster tissue was added to 10 ml of the culture which was then mixed on a plate shaker for 5 min. The larger debris were pelleted by centrifugation at 1500xg for 10 min. The supernatant was aliquoted into 10 ml APW which were labelled 0 h, 3 h, 6 h and 24 h respectively. The oyster supernatant (1 ml) was added to each tube which was then incubated for the required time period. At the end of this time interval, the bacterial cells were collected by centrifugation at 3000xg for 10 min, washed 2X with sterile PBS and optical density recorded.

Phage were prepared for ELISA as described in **section 3.3.** The bacterial cells were incubated with the phage, and the assay carried out as described in **section 3.3.**

Chapter 4

Results: Detection of *V. parahaemolyticus* by PCR

4.1 DNA extraction:

DNA was purified from cultures of *V. parahaemolyticus* and other strains using the proteinase K/lysozyme method (**section 2.3.1**), the Chelex method (**section 2.3.2**) and boiling method (**section 2.3.3**). In addition, DNeasy columns were used to purify DNA from pure cultures and from seeded oyster tissue (**section 2.4.1**). The table below compares the yield and purity of DNA after extraction by boiling method, proteinase K/lysozyme method and DNeasy method for four strains in pure culture of *V. parahaemolyticus*.

Table 4.1: Yield and purity of DNA from four *Vibrio* spp.. Three different methods of DNA extraction were used. The yield and purity was compared by spectophotometric analysis using absorption values at 260 nm and 280 nm (**section 2.3.4**).

Strain number	Yield of DNA			Purity of DNA		
	DNeasy method (µg/ml)	Boiling method (µg/ml)	Proteinase K method (µg/ml)	DNeasy method (%)	Boiling method (%)	Proteinase K method (%)
V. parahaemolyticus NCTC 10903	330	66.4	230	99	73	96
V. parahaemolyticus NCTC 10884	280	51.6	178	99	72	95
V. alginolyticus NCTC 12160	300	67.8	150	99	75	81
V. mimicus NCTC 11435	250	68.3	194	99	80	87

Highest yields and the most pure DNA was obtained by DNeasy method. DNA extracted by the boiling method showed low $A_{260/280}$ ratios, as compared to the other methods, indicating higher protein contamination. In addition, the boiling and Chelex methods also failed to remove any inhibitors of PCR reaction, when the PCR was carried out on oyster tissue seeded with *Vibrio* spp..

4.2 Detection of the *tdh* gene :

The *tdh* primers were tested against the strains listed in **section 2.1**. The primer concentration was standardized to 2.5 μM and magnesium concentration to 1.5 mM. Expected amplicon size was 400 bp. The PCR was positive for *V. parahaemolyticus* strains NCTC 10903, 10441 and ATCC 27969 but negative for strain NCTC 10884. In the absence of the *tdh* gene, the strains show a Kanagawa-negative phenomenon and cannot be recognized by culture on blood agar plates. However, these *tdh*-negative strains are still potentially pathogenic as they may possess other toxin genes (Honda *et al.*, 1991; Tang *et al.*, 1994). Two-fold dilutions of DNA from *V. parahaemolyticus* NCTC 10903 and NCTC 10884 were used to check sensitivity of the PCR assay. The PCR worked at a concentration of 40 ng DNA (data not shown). This agrees with what has been reported in literature (Lee and Pan, 1993).

4.3 Detection of *trh* gene :

Optimum conditions for the PCR were standardized using *V. parahaemolyticus* NCTC 10903. An amplicon of 250 bp was observed after gel electrophoresis. The temperature optimum for annealing was also determined to be 50°C. The results of a temperature gradient PCR are shown in the Figure 4.1.

After optimisation of the PCR, *V. parahaemolyticus* and non-*parahaemolyticus* strains were tested. All strains of *V. parahaemolyticus* were positive for the *trh* gene.

However, *V. mimicus* NCTC 11435 showed a non-specific amplicon at 250 bp, which was indistinguishable from the amplicons of *V. parahaemolyticus* (Figure 4.2)

The sensitivity of the assay was determined using two fold dilutions of *V. parahaemolyticus* DNA. The lowest detection limit for the PCR assay was found to be between 57 ng DNA and 28 ng DNA. The results are shown in Figure 4.3.

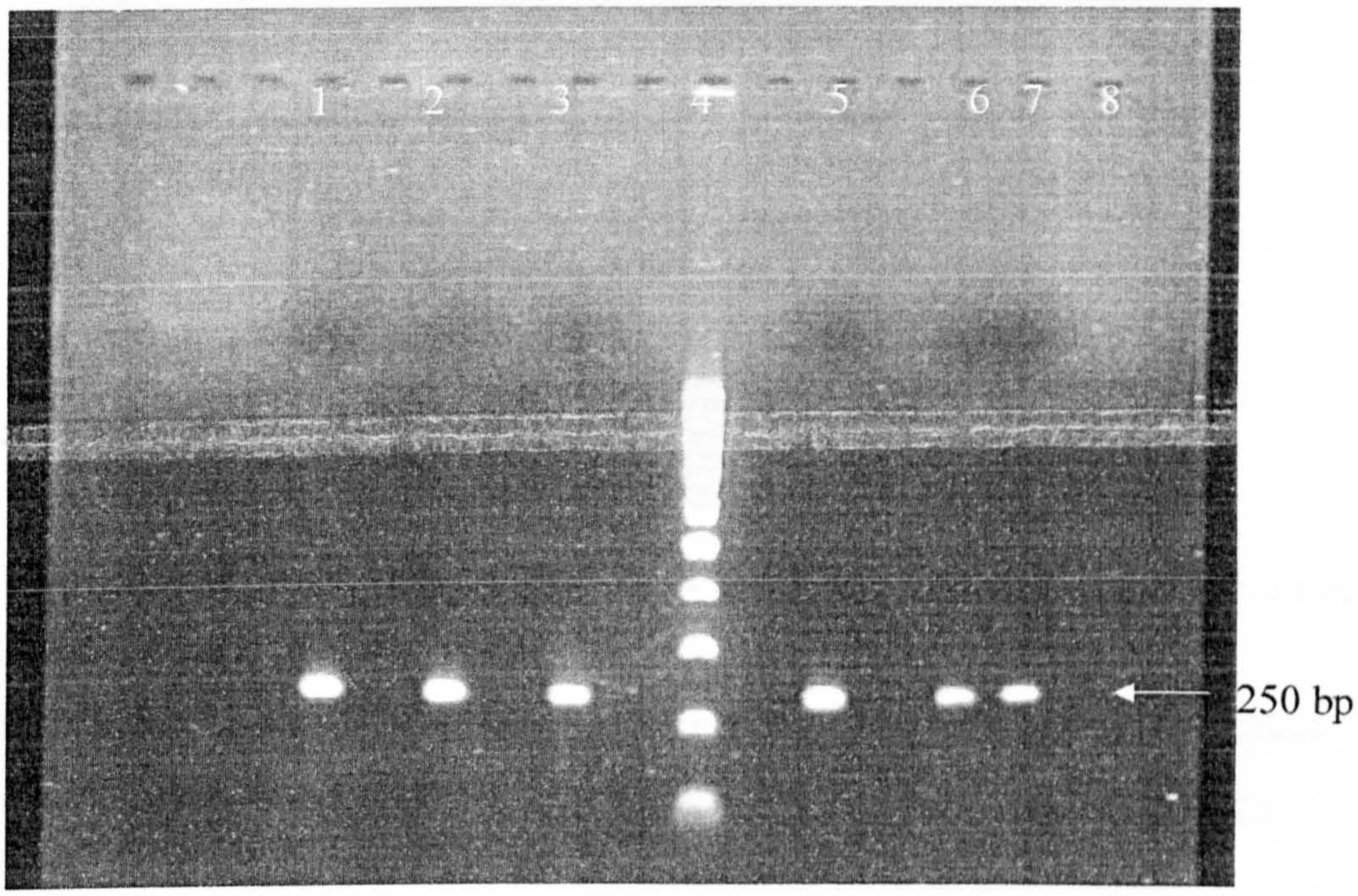


Figure 4.1 – Temperature gradient PCR for the *trh* gene using different annealing temperatures, ranging from 45-55°C. Test strain is *V. parahaemolyticus* NCTC 10903. Temperatures used for the PCR are –

Lane 1 = 46.6°C, Lane 2 = 49.2°C, Lane 3 = 50.6°C, Lane 4 = 100 bp ladder

Lane 5 = 52°C, Lane 6 = 53.2°C, Lane 7 = 54.9°C, Lane 8 = negative control

PCR bands were obtained for all annealing temperatures; the strongest band was seen at 46.6°C. However, such a low annealing temperature would encourage non-specific binding, therefore the temperature optima for the PCR was fixed at 50°C.

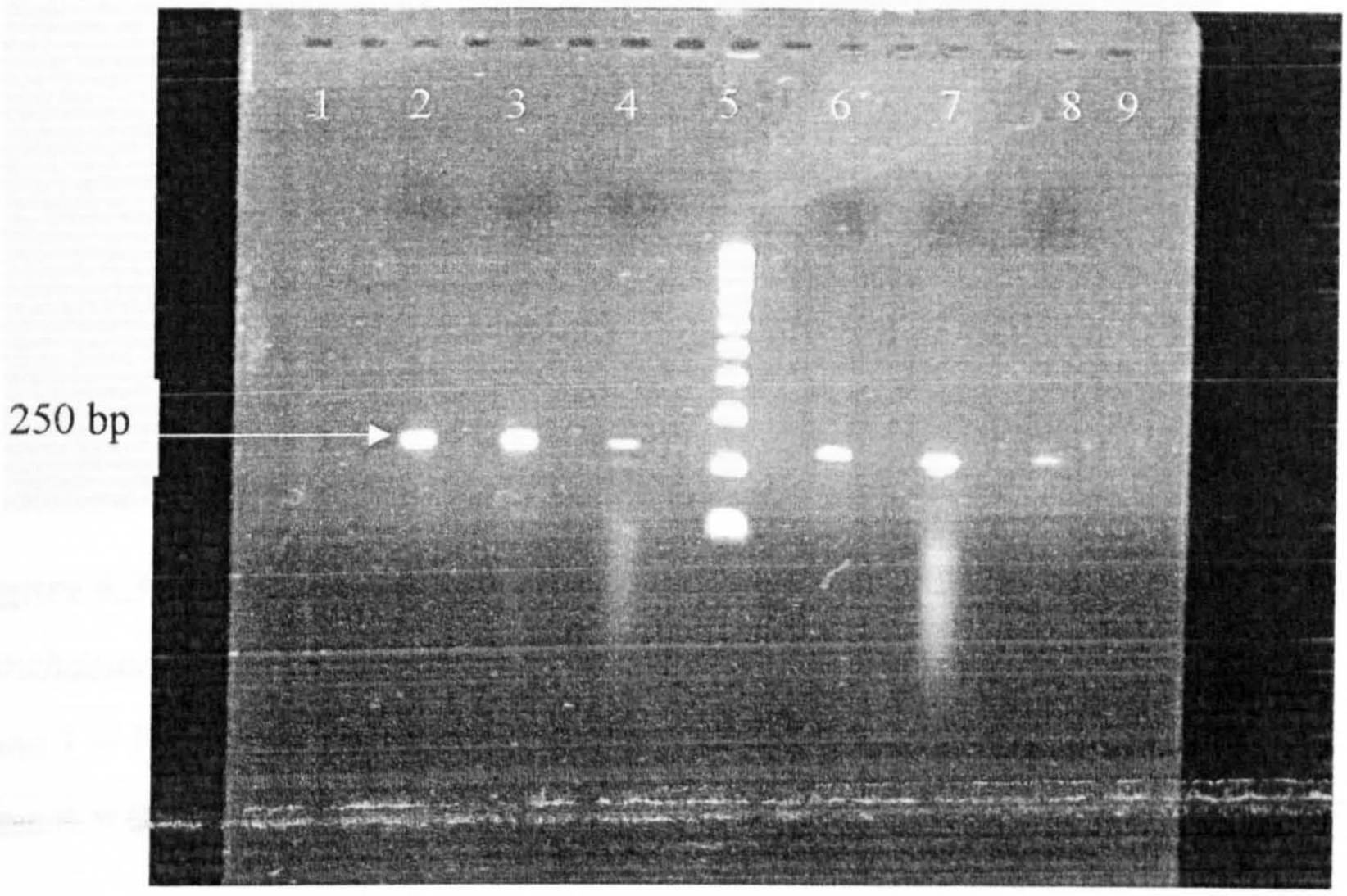


Figure 4.2 – PCR of the *trh* gene using different species of *Vibrio*. The numbers in the lane correspond to the following strains:

Lane 1 = *V. alginolyticus* NCTC 12160, Lane 2 = *V. parahaemolyticus* NCTC 10903 Lane 3 = *V. parahaemolyticus* NCTC 10441, Lane 4 = *V. parahaemolyticus* ATCC 27969, Lane 5 = 100 bp ladder, Lane 6 = *V. parahaemolyticus* NCTC 10884, Lane 7 = *V. mimicus* NCTC 11435, Lane 8 = *V. parahaemolyticus* ATCC 17802, Lane 9 = negative control.

The PCR was positive for all strains of *V. parahaemolyticus.* However, *V. mimicus* NCTC 11435 showed a false positive amplicon with this primer.

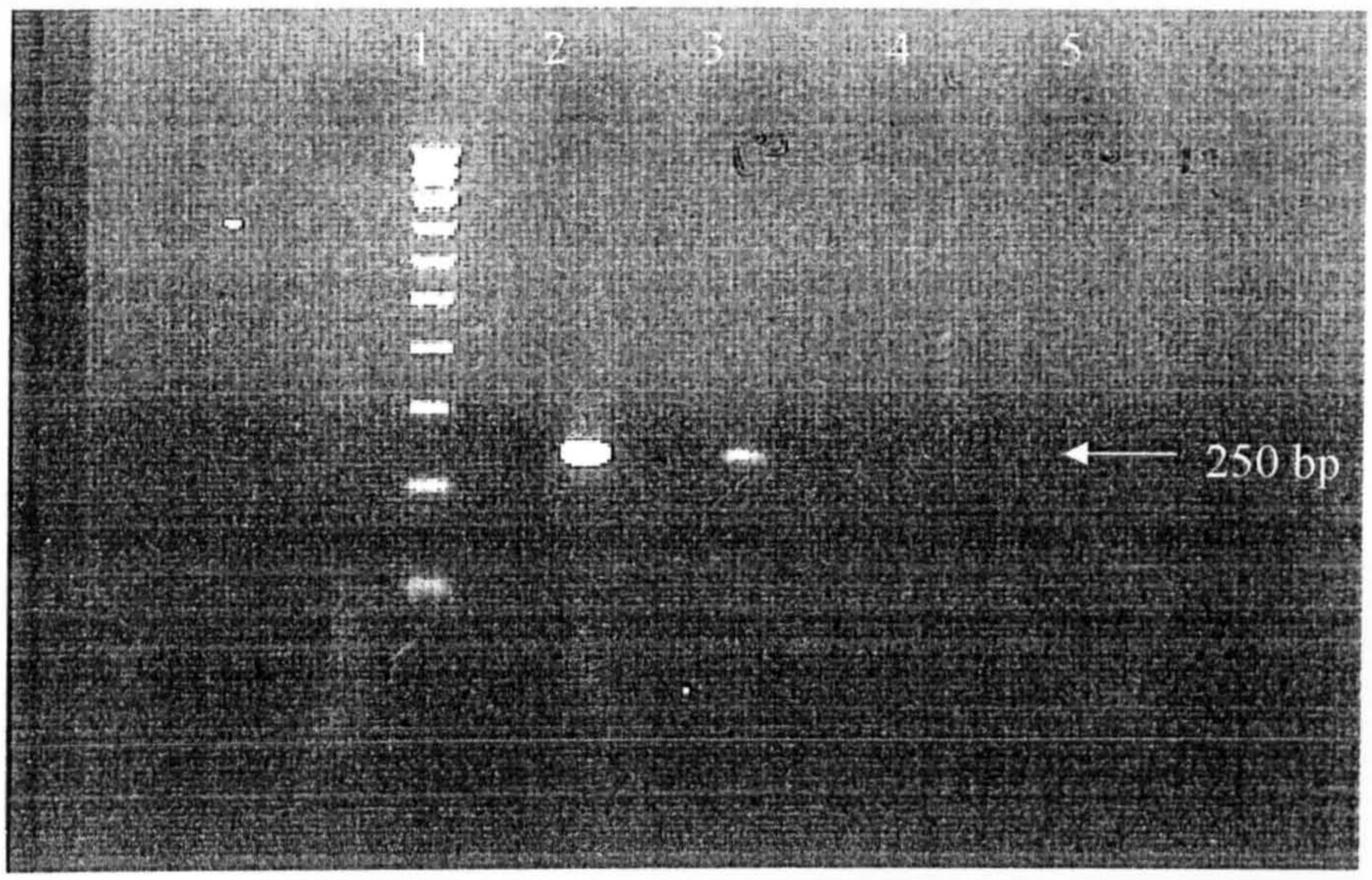


Figure 4.3: PCR of the *trh* gene using different concentrations of DNA from *V. parahaemolyticus* NCTC 10903. Lane numbers correspond to the following: Lane 1 = 100 bp ladder, Lane 2 = 115 µg DNA, Lane 3 = 57.2 µg DNA Lane 4 = 27.8 µg DNA, Lane 5 = negative control.

The detection limit was between 28 and 57 µg of DNA.

4.4 *toxR* gene:

With the *toxR* primers, an amplicon of 386 bp was generated after PCR. The effect of different primer concentrations on PCR is shown in Figure 4.4. Annealing temperature and DNA concentration were standardised as described in **section 2.3.4**. Magnesium concentration was also standardised using different concentrations of $MgCl_2$ - 1, 1.5, 2 and 2.5mM. After standardization, optimum primer concentration was fixed at 1 mM and optimum magnesium concentration at 2mM. The specificity of the primers was tested against a range of *V. parahaemolyticus* and non-parahaemolyticus species. The PCR was able to detect all strains of *V. parahaemolyticus* . There have been reports of non-specific amplicons with *V.vulnificus* (Kim *et al.*, 1999); which are supposed to be larger and less evident than the specific amplicons generated by *V. parahaemolyticus*. However, in our study, this strain did not show any false positive reaction. Sensitivity

of the assay was checked by using whole cells (Figure 4.5) and 10-fold dilutions of DNA samples from different strains of *V. parahaemolyticus*. The lowest detection limit for the *toxR* gene was around 20 ng.

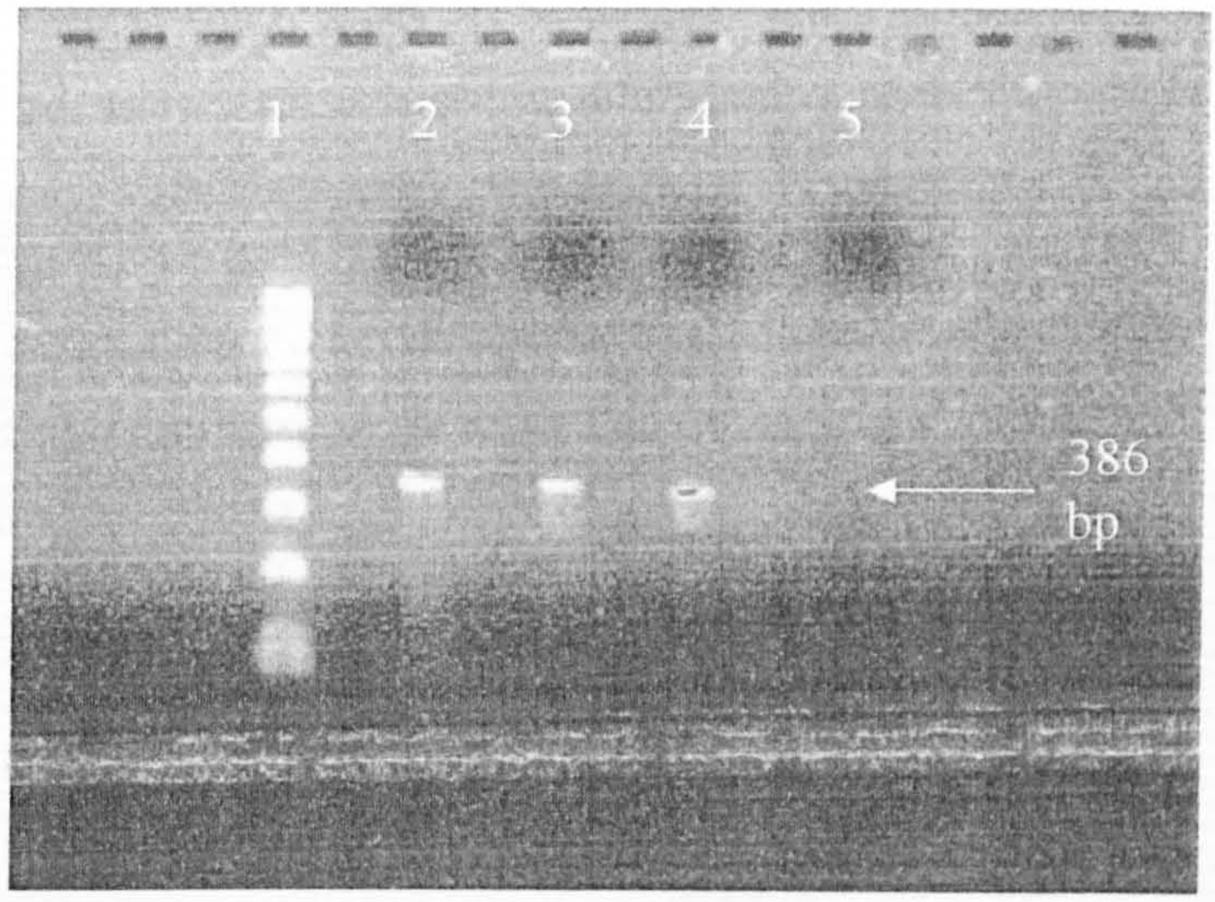


Figure 4.4: PCR of the *toxR* gene using different concentrations of the *toxR* primer. Test strain is *V. parahaemolyticus* NCTC 10903. The primer concentrations ranged from 1-100 mM. The lane numbers correspond to the following :

Lane 1 = 100 bp ladder, Lane 2 = 100 mM primer , Lane 3 = 10 mM primer
Lane 4 = 1mM primer, Lane 5 = negative control.

Amplicons were obtained for all the primer concentrations, therefore a concentration of 1 mM could be used for the PCR assay.

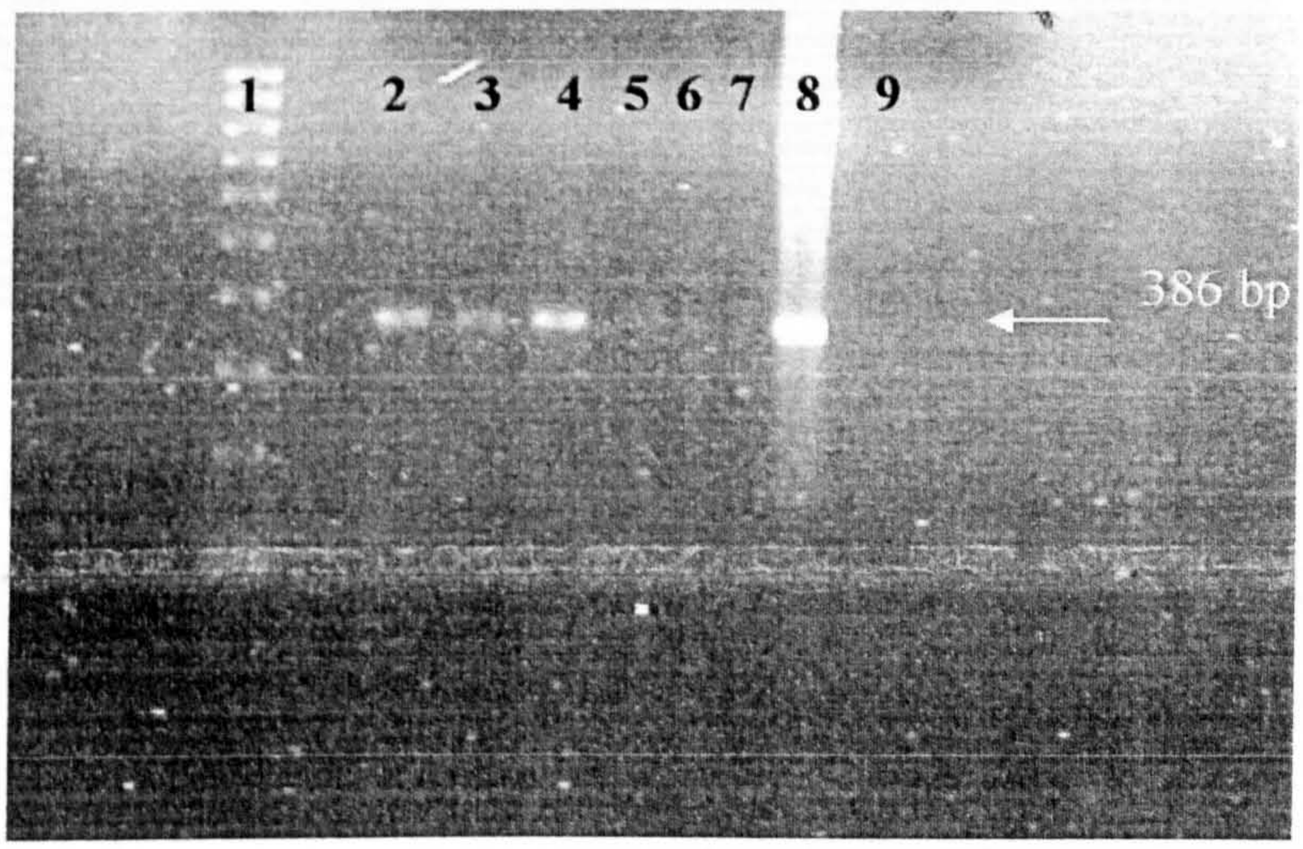


Figure 4.5: PCR of different culture densities of *V. parahaemolyticus* NCTC 10903 using the *toxR* primer. Culture densities are shown below. Lanes 1-6 were loaded with culture densities in a decreasing order, and lane 8 was loaded with the undiluted culture.

Lane 1 = 100 bp ladder, Lane 2 = 5×10^7 cfu/ml

Lane 3 = 2.5×10^7 cfu/ml, Lane 4 = 10^7 cfu/ml, Lane 5 = 5×10^6 cfu/ml

Lane 6 = 2.5×10^6 cfu/ml, Lane 7 = 10^6 cfu/ml, Lane 8 = 10^8 cfu/ml

Lane 9 = negative control.

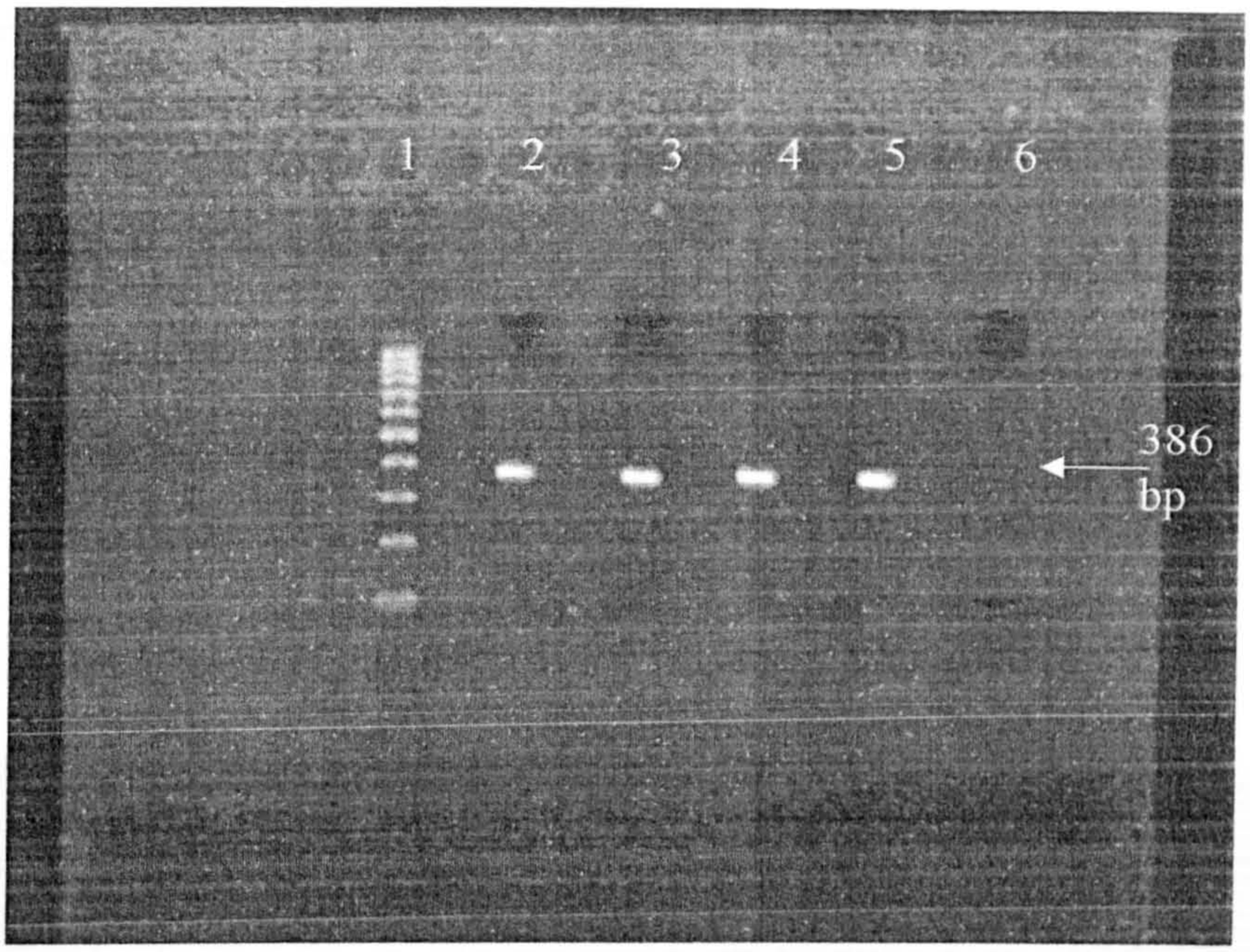


Figure 4.6: PCR of the *toxR* gene using different strains of *V. parahaemolyticus*. Lane 1 = 100 bp ladder,Lane 2 = *V. parahaemolyticus* NCTC 10903 Lane 3 = *V. parahaemolyticus* NCTC 10884, Lane 4 = *V. parahaemolyticus* NCTC 10441, Lane 5 = *V. parahaemolyticus* NCTC 27969, Lane 6 = Negative control.

All the *V. parahaemolyticus* strains showed a positive PCR with the *toxR* primers.

4.5 *gyrB* gene:

Using *V. parahaemolyticus* NCTC 10903, the conditions of primer and magnesium optimum were standardized (**section 2.3.4**). The optimum primer concentration for the *gyrB* gene was found to be 10 mM and the magnesium concentration at 2mM. A temperature gradient PCR was carried out to optimise annealing temperature, which was determined to be 60°C.

Specificity of the primers was determined by testing against different strains of *Vibrio* spp. and non-*parahaemolyticus* strains. The results are shown in the Figure 4.7. Non-specific amplicons were observed with two, *V. mimicus* NCTC 11435 and *V. vulnificus* ATCC 27562 (data not shown). The amplicon generated by *V. mimicus*

NCTC 11435 (at 400 bp) can be clearly differentiated from the amplicon of *V. parahaemolyticus* but not the non-specific amplicon of *V. vulnificus* NCTC 27562.

Two fold dilutions of the DNA extracted from *V. parahaemolyticus* NCTC 10903, NCTC 10441 and ATCC 27969 were used to determine the detection limits of the assay (Figure 4.8). Purified DNA at a concentration of 50 ng could be detected by this PCR assay.

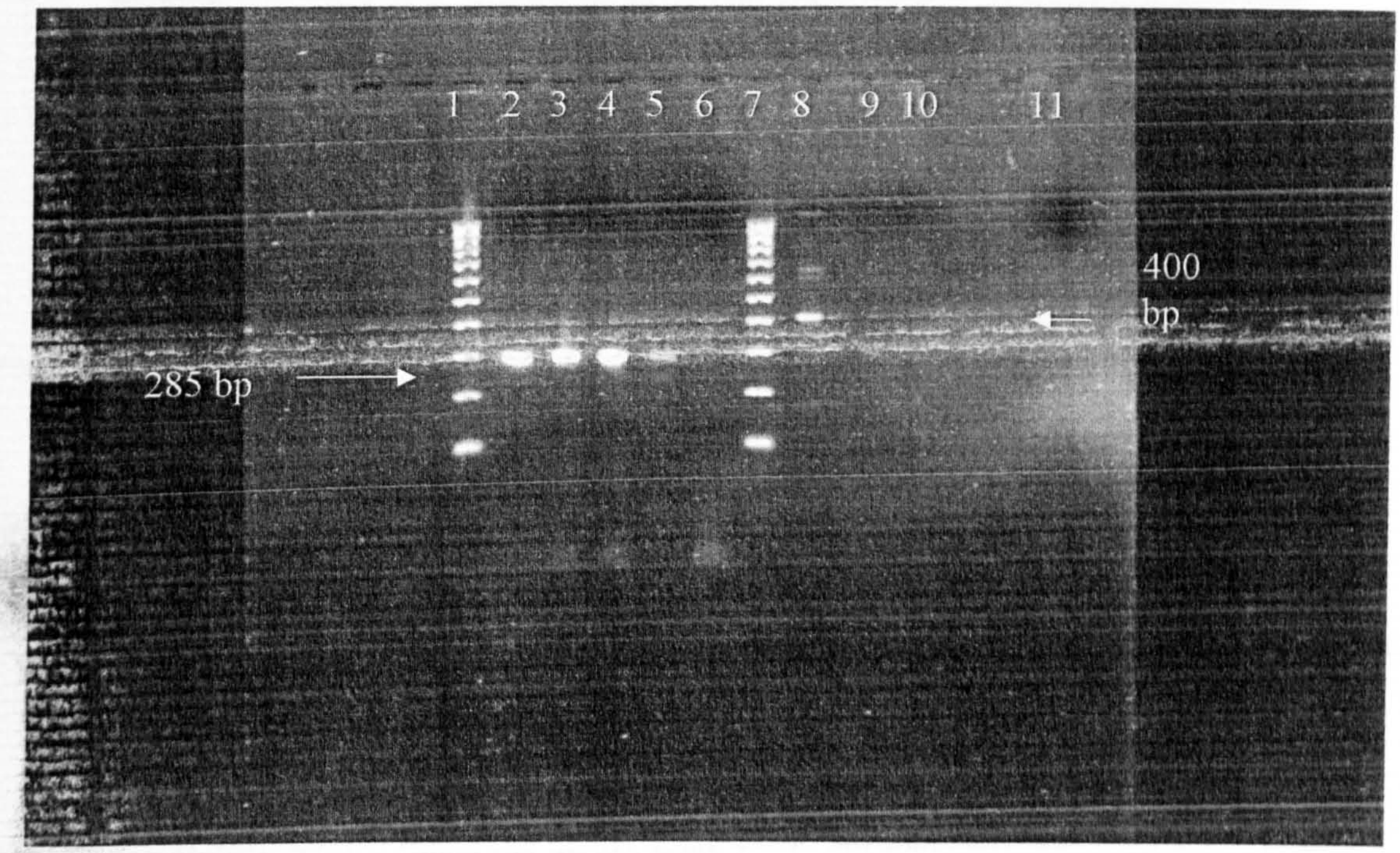


Figure 4.7: PCR of the *gyrB* gene using different strains of *Vibrio* spp. The lane numbers correspond to the following:

Lane 1 = 100 bp ladder, Lane 2 = *V. parahaemolyticus* NCTC 10903, Lane 3 = *V. parahaemolyticus* NCTC10804, Lane 4 = *V. parahaemolyticus* NCTC 10441, Lane 5 = *V. parahaemolyticus* ATCC 27969, Lane 6 = *V. parahaemolyticus* ATCC 17802, Lane 7 = 100 bp ladder, Lane 8 = *V. mimicus* NCTC 11435, Lane 9 = *V. alginolyticus* NCTC 12160, Lane 10 = *V. harveyi* NCTC 11346, Lane 11 = negative control.

V. mimicus NCTC 11435 shows a non-specific amplicon at 400 bp, which can be clearly distinguished because of its larger size.

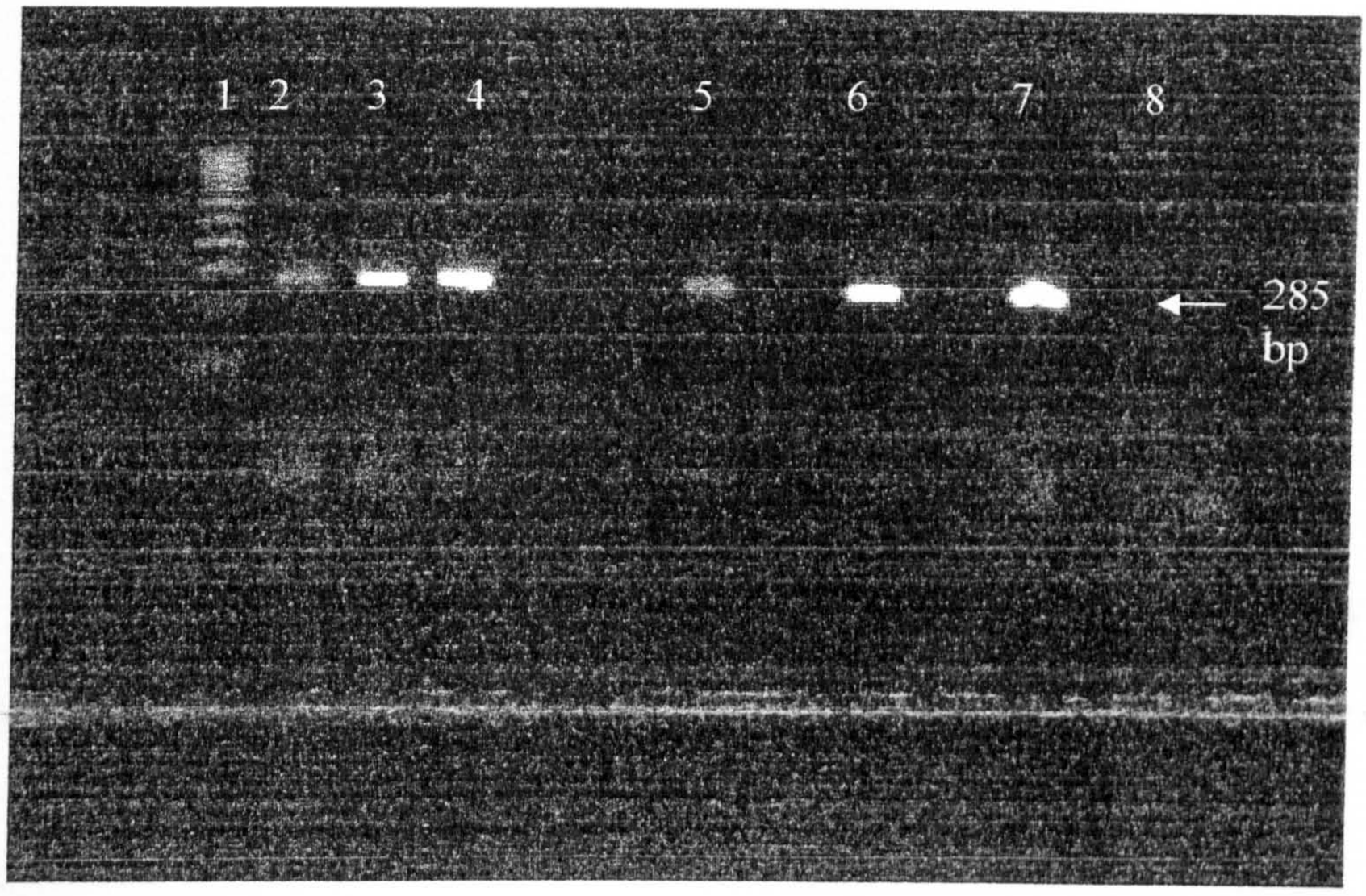


Figure 4.8– PCR of the *gyrB* gene using different DNA concentrations of *V. parahaemolyticus*. The lane numbers correspond to the following:

Lane 1 = 100 bp ladder, Lane 2 = *V. parahaemolyticus* NCTC 10903 (60 ng/µl), Lane 3 = *V. parahaemolyticus* NCTC 10884 (60 ng/µl), Lane 4 = *V. parahaemolyticus* NCTC 10441 (60 ng/µl), Lane 5 = *V. parahaemolyticus* NCTC 10903 (50 ng/µl), Lane 6 = = *V. parahaemolyticus* NCTC 10884 (50 ng/µl), Lane 7 = *V. parahaemolyticus* NCTC 10441 (50 ng/µl), Lane 8 = negative control.

The three strains showed a positive reaction at both the DNA concentrations.

Chapter 5

Results: Detection of *V. parahaemolyticus* by phage antibody display technology

5.1 Panning:

For selecting phage specific for *V. parahaemolyticus*, five rounds of panning were carried out. A negative panning step was incorporated in the second round, to eliminate cross-binding phage. The eluted phage were titred at the end of each round, using *E. coli* TG1 **(section 3.1.3)**. The titres are shown in Table 5.1.

Table 5.1: Titre of eluted phage after panning with *V. parahaemolyticus*.

Round of panning	Titre* of phage added (ptu/ml)	Titre* of phage eluted (ptu/ml)
1 Positive selection	1×10^{12}	5×10^{3}
2 + 3 (in parallel) Negative selection followed by positive selection	1×10^{12}	1×10^{6}
4 Positive selection	1×10^{12}	5×10^{6}
5 Positive selection	1×10^{12}	3×10^{7}

*Titres are expressed in phage titre units/ml

The titre of the phage increased from 10^3 in the first round to 10^7 by the end of the 5^{th} round. The 2^{nd} round was a negative panning step and it was carried out parallel with the positive panning round. The increase in titre suggests that phage are being selectively enriched in each round of panning.

5.2 PCR of the phage clones:

After completion of the five panning rounds, clones were screened for their CDR3 insert size. PCR was carried out on the naïve phage library and after the last round of selection. The PCR of the initial library showed considerable diversity in CDR3 size, as expected. However , the PCR of phage clones at the end of the last (5th round) showed that a single clone seems to be dominant.

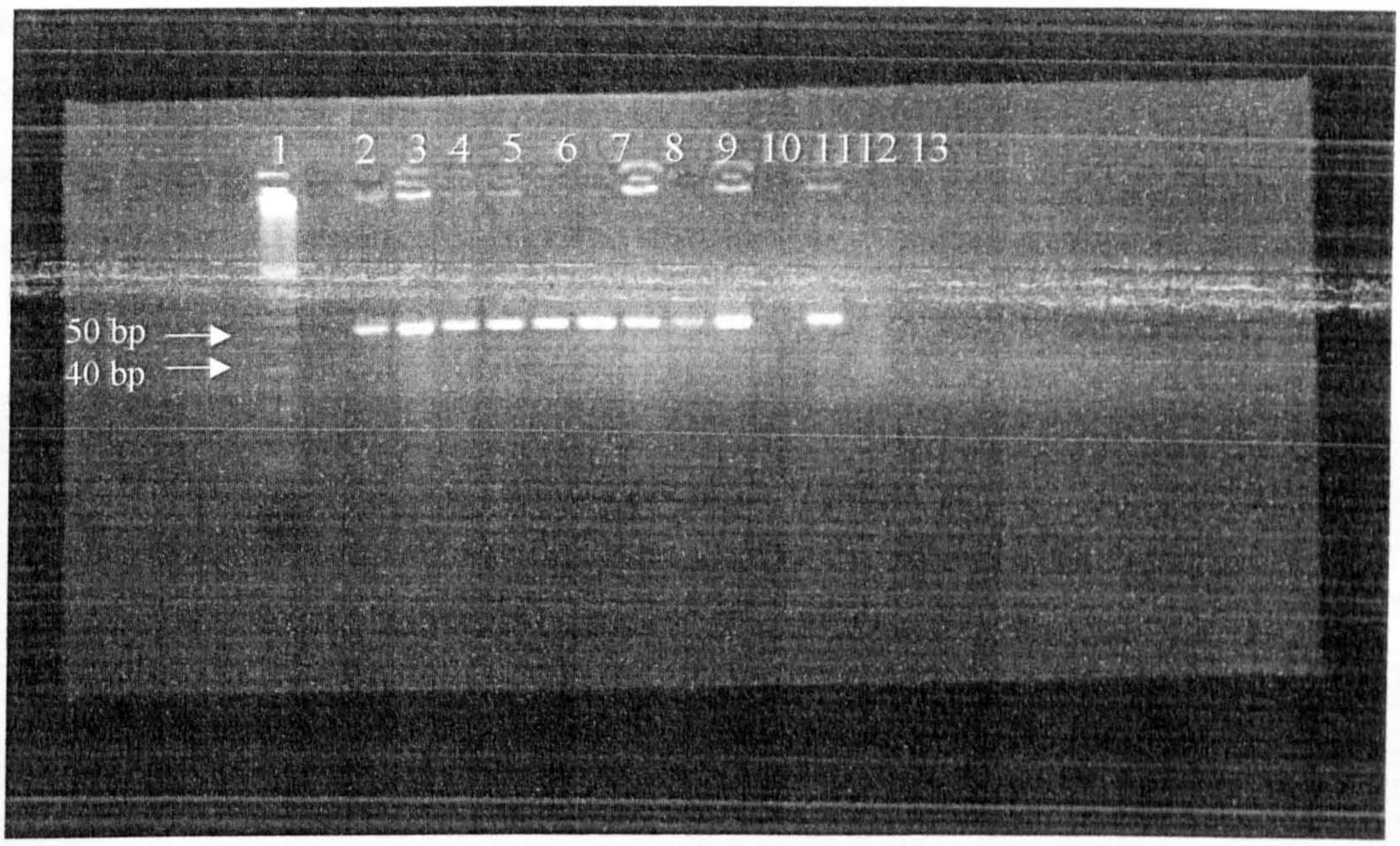


Figure 5.1: PCR of the CDR3 region of the clones, picked at random, after the 5th round of panning. PCR products were separated on a 5% (w/v) metaphor agarose gel. The figure shows the clones have the same insert size.
Lane 1 = 10 bp ladder, Lane 2 -12 = phage clones picked at random from the 5th round of panning, Lane 13 = negative control

To check the stage at which a single clone predominates, we performed PCR on clones from the earlier rounds of panning. The results are shown in Figure 5.2a .and b.

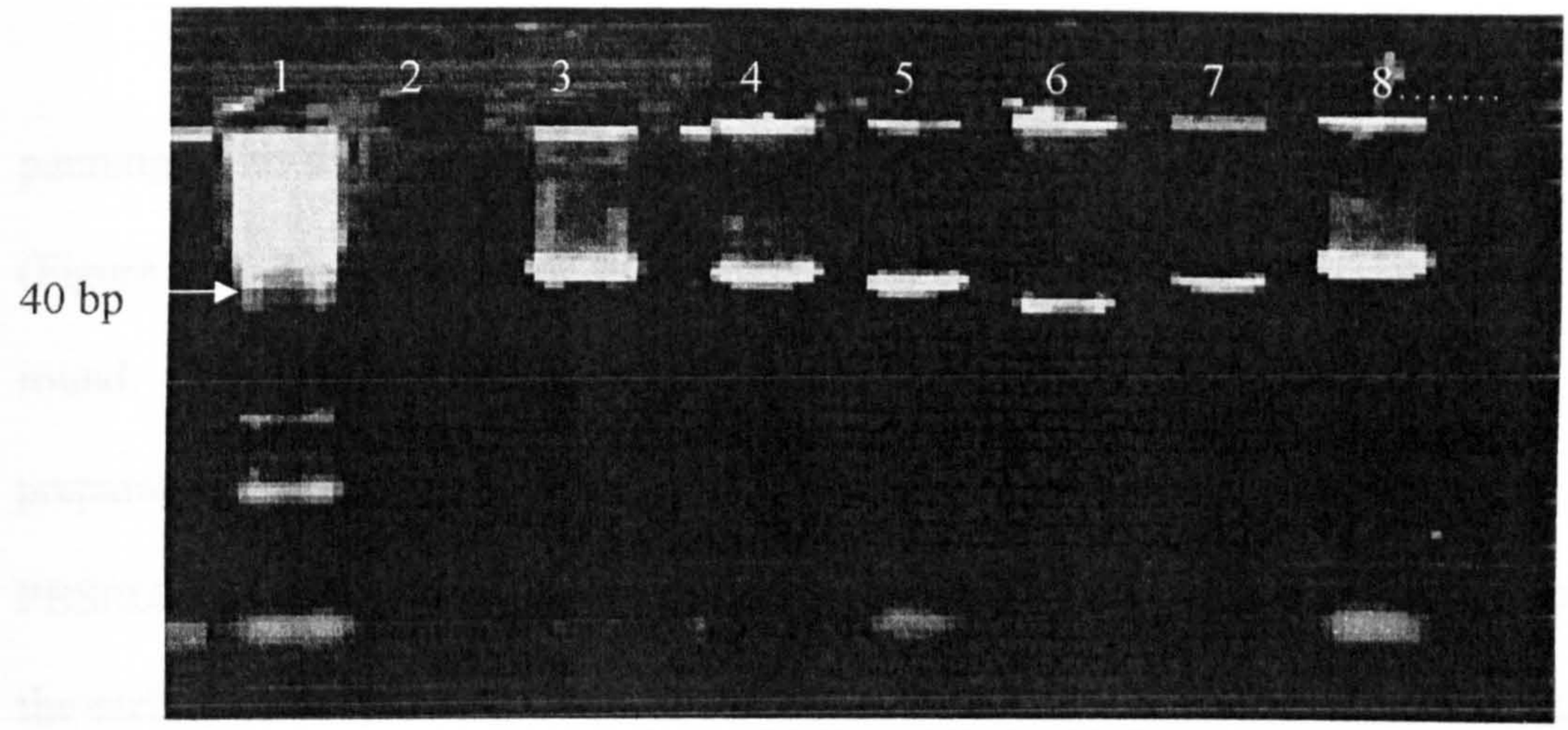


Figure 5.2a: PCR of the CDR3 region of clones after the 4th round of panning. Clones were picked at random after the 4th round of panning. PCR products were separated on a 5% metaphor agarose gel. The figure shows the clones have a different insert size. Lane 1 = 10 bp ladder, Lane 2 -7 = phage clones picked at random from the 4th round of panning, Lane 8 = negative control

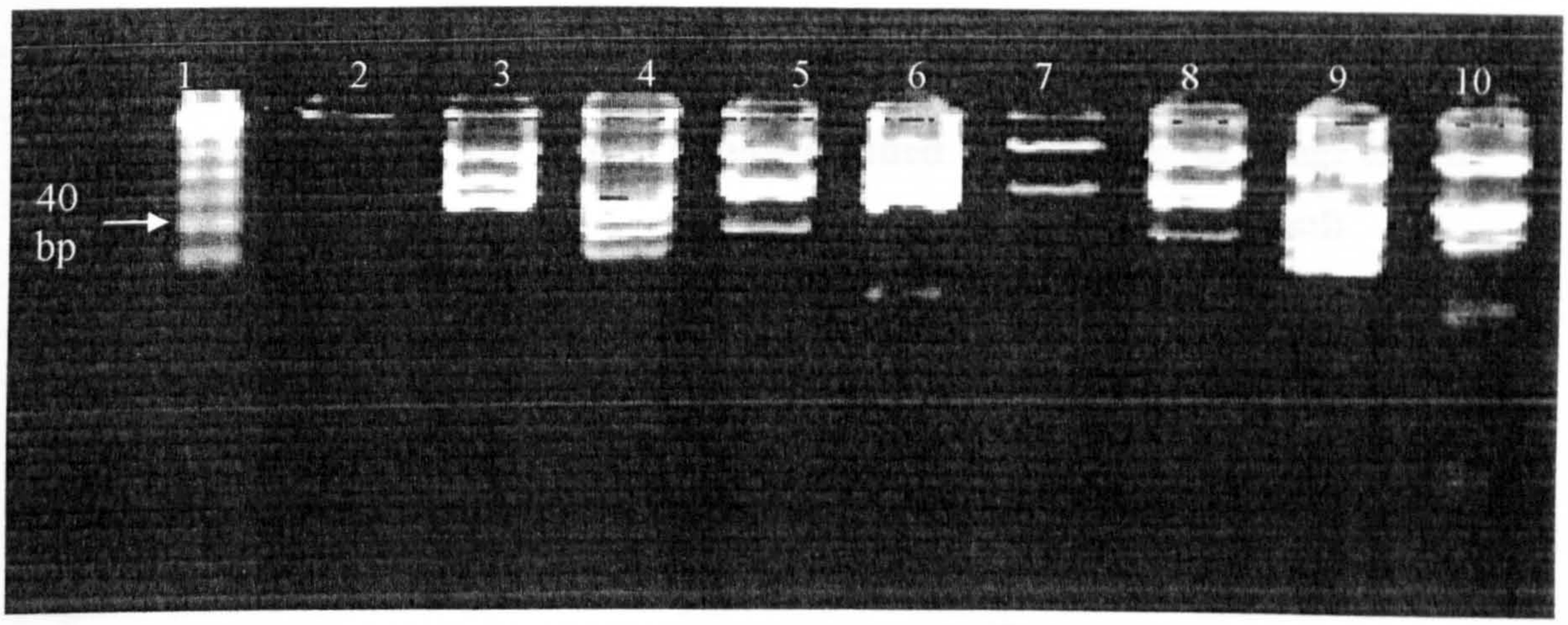


Figure 5.2b: Restriction digest of the scFv region of phage clones from the 4th round of panning. The phage clone was first amplified by scFv PCR and then digested with the restriction enzyme,*BstN1*. PCR products were separated on a 2.5% (w/v) metaphor agarose gel.

Lane 1 = 10 bp ladder, Lane 2= negative control, Lane 3 -10 = phage clones, picked at random at the end of round 4 of panning.

As shown in Figure 5.2a, the clones exhibit diversity up to the 4th round of panning. This diversity seems to have been lost after the 5th round (positive panning) (Figure 5.1). Only one single strong binding clone dominates at the end of the 5th round. Hence the last two rounds of panning were repeated again, using a phage preparation from round 3. During this round of panning,the cells were washed in PBS/0.05% (v/v) Tween solution instead of the PBS/0.1% (v/v)Tween solution used for the earlier rounds panning. This would select for clones,which are strong binding, but are eluted by the higher concentration of Tween. The results of the new panning round are shown in Table 5.1a. The titres for round 4 and 5 were from the second set of panning.

Table 5.1a: Titre of eluted phage after a new round 4 and 5.:

Round of panning	**Titre* of phage added (ptu/ml)**	**Titre* of phage eluted (ptu/ml)**
1 Positive selection	1×10^{12}	5×10^{3}
2 + 3 (in parallel) Negative selection followed by positive selection	1×10^{12}	1×10^{6}
4 Positive selection	1×10^{12}	4×10^{8}
5 Positive selection	1×10^{12}	6×10^{9}

PCR of the CDR3 region was carried out after the new rounds of panning and CDR3 diversity was observed. The results can be seen in Figure 5.3.

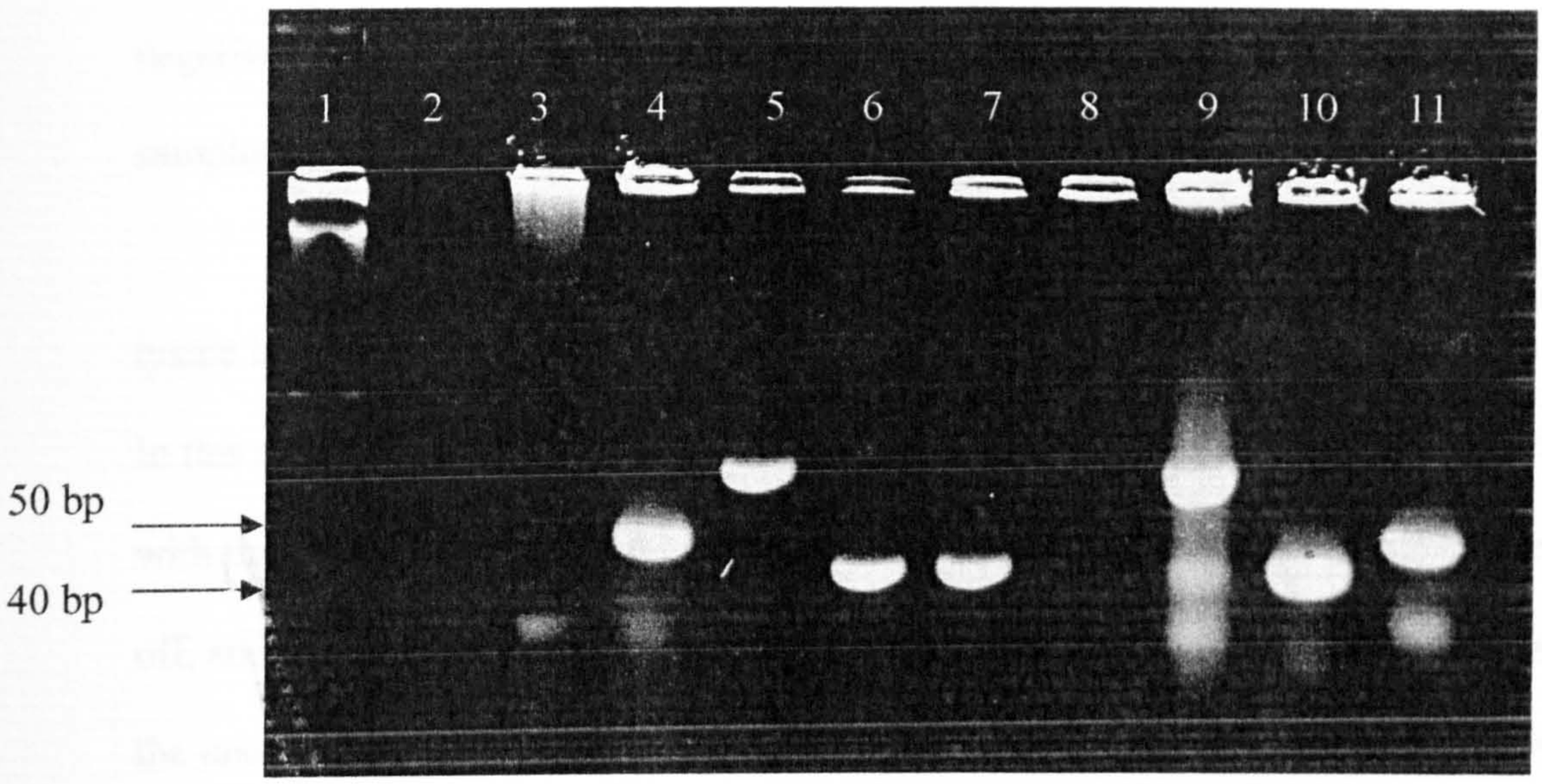


Figure 5.3 : PCR of the CDR3 region of clones , after repeating panning round 4 and 5. The PCR shows that the clones show diversity in the CDR3 region.
Lane 1 = 10 bp ladder, Lane 2 = negative control, Lane 3 -11 = phage clones picked at random from the (new) 5th round of panning.

5.3 ELISA:

ELISA was used as the initial screening step to determine the binding capacity of the phage antibodies. One advantage of ELISA is that a large number of clones can be screened in a short time period.

Randomly selected 192 clones were screened by ELISA from the enriched library at the end of the 5th round. They were then grown overnight with helper phage . For ELISA, the bacterial cultures were blocked and then incubated with the phage antibody for two hours. The unbound antibody was washed off and anti-M13-HRP antibody was added. After an hour, this was washed off, and colour reagent was added. Once the colour developed, it was measured by an ELISA reader.

ELISA was carried out using each of the individual cultures (both *V. parahaemolyticus* and non-*V. parahaemolyticus* strains) in duplicate, and also after

pooling the cultures together. ELISA was also carried out by pooling cultures of *V. parahaemolyticus* with a non-*parahaemolyticus* strain, *E. coli* (which is a Gram negative bacterium belonging to the same family, but not likely to be found in seafood samples).

ELISA was carried out using both poly-L-lysine and uncoated plates. Poly-l-lysine is used to help the target antibody or antigen bind to the plate by making it sticky. In this case, the target antigen was *V. parahaemolyticus* cells. They were incubated with the coated plates and left overnight. Next day, the unbound antigen was washed off, and the plate used for ELISA. The poly-L-lysine plates gave a higher reading than the uncoated plates. However, repeated testing showed that some clones on the coated plates showed non-specific binding, inspite of use of the blocking buffer. There was also very high background reading on the coated plates. The two sets of readings have been compared in Table 5.2a and 5.2b. Non-specific binding is shown in Table 5.2c. Based on these results, all further ELISAs were carried out using uncoated plates.

Table 5.2a – ELISA of phage clones binding to *V. parahaemolyticus* NCTC 10441 (Poly-l-lysine coated plates). ABTS was used as a dectection reagent. Absorption was measured at A_{630}-A_{405}, using anti-NIP antibody as the negative. Blank wells indicate that the absorbance was ≤ 0.2

Clones	1	2	3	4	5	6	7	8
A	0.831		0.303		1.703		0.776	
B	1.665		0.669		0.616			
C		1.028	1.749	0.642				
D			0.698				0.229	*0.106 (anti-NIP)
E	0.199			0.282				
F	0.451	0.459	0.803		0.390	0.404		

Table 5.2b – ELISA of phage clones binding to *V parahaemolyticus* NCTC 10441 (Uncoated plates). The same clones were used as in Table 5.2a. ABTS was used as a detection reagent. Absorption was measured at A_{630}-A_{405}, using anti-NIP antibody as the negative. Blank wells indicate that the absorbance was ≤ 0.2

Clones	1	2	3	4	5	6	7	8
A			0.231		0.358		0.865	0.763
B			0.461		0.357			
C				0.263				
D			0.218					*0.030 (anti-NIP)
E	0.553							
F	0.512	0.236		0.317				

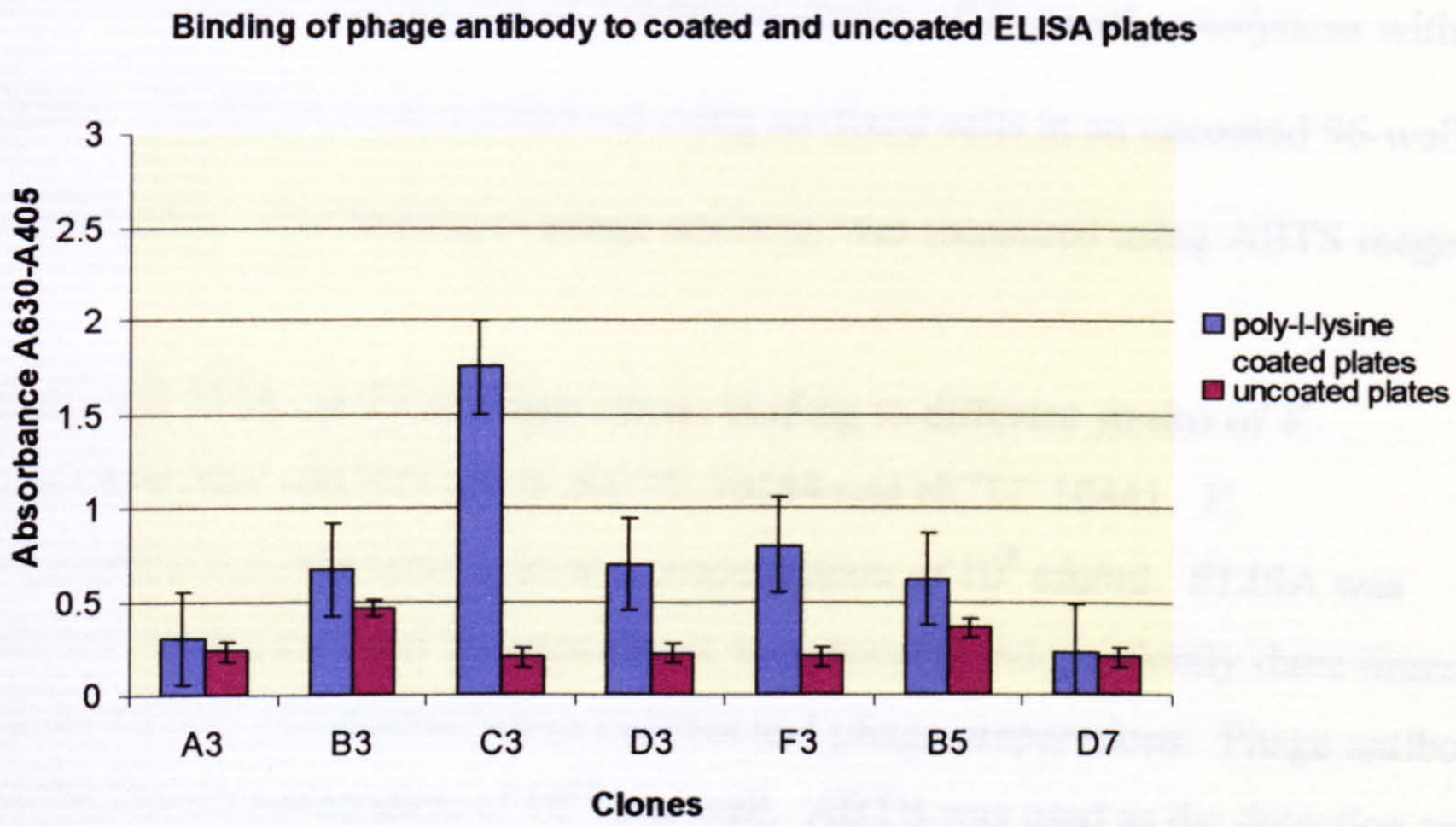


Figure 5.4 – The chart compares the binding of phage antibody clones in poly-l-lysine coated plates and uncoated plates in ELISA, using *V. parahaemolyticus* NCTC 10441. Plates were done in triplicate. The table shows that several of the clones in the coated plate show non-specific binding to cells of *V .parahaemolyticus* NCTC 10441.

Another comparative study was done using free (un-fixed) and fixed (air-dried or fixed using methanol) cells of *V. parahaemolyticus.* Overnight cultures of *V. parahaemolyticus* were fixed in methanol for 10 min and then used for ELISA. This was then compared with ELISA readings from un-fixed cells. Higher readings were obtained using the un-fixed cells. When the cell numbers of *V. parahaemolyticus* were counted using a haemocytometer at the end of the ELISA, it was observed that fewer cells were present in the plate with fixed cells than the unfixed ones, though both had the same initial numbers. This was because of better retention of cells if they were washed by centrifugation (un-fixed cells ELISA plate) than washing by inverting the plates. The fixed cells tend to get disloged easily during the washing steps.

Table 5.3 compares the binding of 3 different strains of *V. parahaemolyticus* with phage antibody. The ELISA was carried out using un-fixed cells in an uncoated 96-well microtitre plate. The binding of phage antibody was measured using ABTS reagent.

Table 5.3: ELISA results of phage clones binding to different strains of *V. parahaemolyticus* –NCTC 10903, NCTC 10884 and NCTC 10441. *V. parahaemolyticus* cells were used at a concentration of 10^8 cfu/ml. ELISA was performed in duplicate and the experiment was repeated independently three times, using the same *V.parahaemolyticus* cultures and phage preparations. Phage antibody was added at a concentration of 10^{11} ptu/ well. ABTS was used as the detection reagent and the absorbance was measured at A_{630}-A_{405}.Anti-NIP was used as the blank.

Clone	NCTC 10903	NCTC 10884	NCTC 10441
B11	+	+	+
C9	+	-	+
C11	+	+	+
C12	-	+	+
D1	+	-	+
D5	-	+	+
D9	+	-	+
F4	+	+	+
G4	+	+	+
G10	+	+	-
G11	-	+	+
H11	+	+	+

Key: + = absorbance ≥0.500 ; - = ≤0.500

Based on these results, it was found that four clones - C11, F4, G4 and H11 show strong binding to *V. parahaemolyticus* strains and do not cross-react with non-*parahaemolyticus* strains.

5.4 Flow ctyometry:

Flow cytometry (FACS) analysis was carried out to study the binding profiles of the clones. The results of ELISA showed four strong binding clones - C11, F4, G4 and H11. These were further analysed by FACS, in addition another 11 clones showing good binding on ELISA were tested.

In flow cytometry the cells are incubated with phage antibody and then tagged with a fluorescent dye using a secondary antibody. Flow cytometry was used as a second screening step after ELISA. Binding profiles were studied for each of the individual clones with all the *parahaemolyticus* and non-*parahaemolyticus* clones. In total 192 clones were screened by ELISA , and out of these 15 clones were screened using FACS. Clones were selected on the basis of strong binding to *V. parahaemolyticus* strains in ELISA and low or non-binding to other species (non-parahaemolyticus and non-*Vibrio*). CDR3 PCR was carried out for these clones to check for diversity.

Phage concentrations were also standardized for the assay, using *V. parahaemolyticus* NCTC 10903 and NCTC 10884. Phage concentrations of 10^9 and 10^{10} ptu/ml resulted in a poor binding profile for the positive phage clones. Good binding profiles were obtained if the sample contained at least 10^{11} ptu/ml phage clones. *V. parahaemolyticus* cells are 3-5 μm in length and therefore not many phage may be able bind to one cell. Table 5.4 shows the binding percentages of the 6 clones to *V. parahaemolyticus* NCTC 10903, at two different cell concentrations.

Similar analysis was carried out using three different concentrations of *V. parahaemolyticus* cells. Cells were used at a concentration of 10^7,10^8 and 10^9 cfu/ml. A concentration of 10^8 cfu/ml was found to be optimum for the flow cytometry assay.

Table 5.4: Assessing binding of phage clones to *V. parahaemolyticus* NCTC 10903 at two different concentrations of phage using flow cytometry. The percentage binding was determined for the positive clones using an M1 marker (where total cell population, n= 10,000). Percentage binding of anti-NIP irrelevant phage was set at 5% on each experiment. The percentage of cells positive with each phage clone was determined using this marker. Analysis was carried out in duplicate, and the experiment was repeated independently three times.

Clone	Binding percentage (%) at two different phage concentrations	
	10^{10} ptu/ml	10^{11} ptu/ml
9A	8.4	11
9B3	19.1	23.9
9B5	25.5	28.7
9C	25.5	32.7
9E	14.6	17.4
9F	15.9	16.5

Once optimum cell numbers and phage concentration were determined, the remaining clones were tested for binding using flow cytometry. Briefly, cells of *V. parahaemolyticus* were incubated with the phage clones for 2 h, and then the unbound antibody was washed off. Anti-M13 antibody was added to the cells and incubated for

1 h. After another washing step to remove unbound antibody, a rabbit anti-mouse FITC (fluorescein) conjugated reagent was added and the cells analysed with a flow cytometer. The results of flow cytometry analysis are shown in Figure 5.5 to 5.9 below.

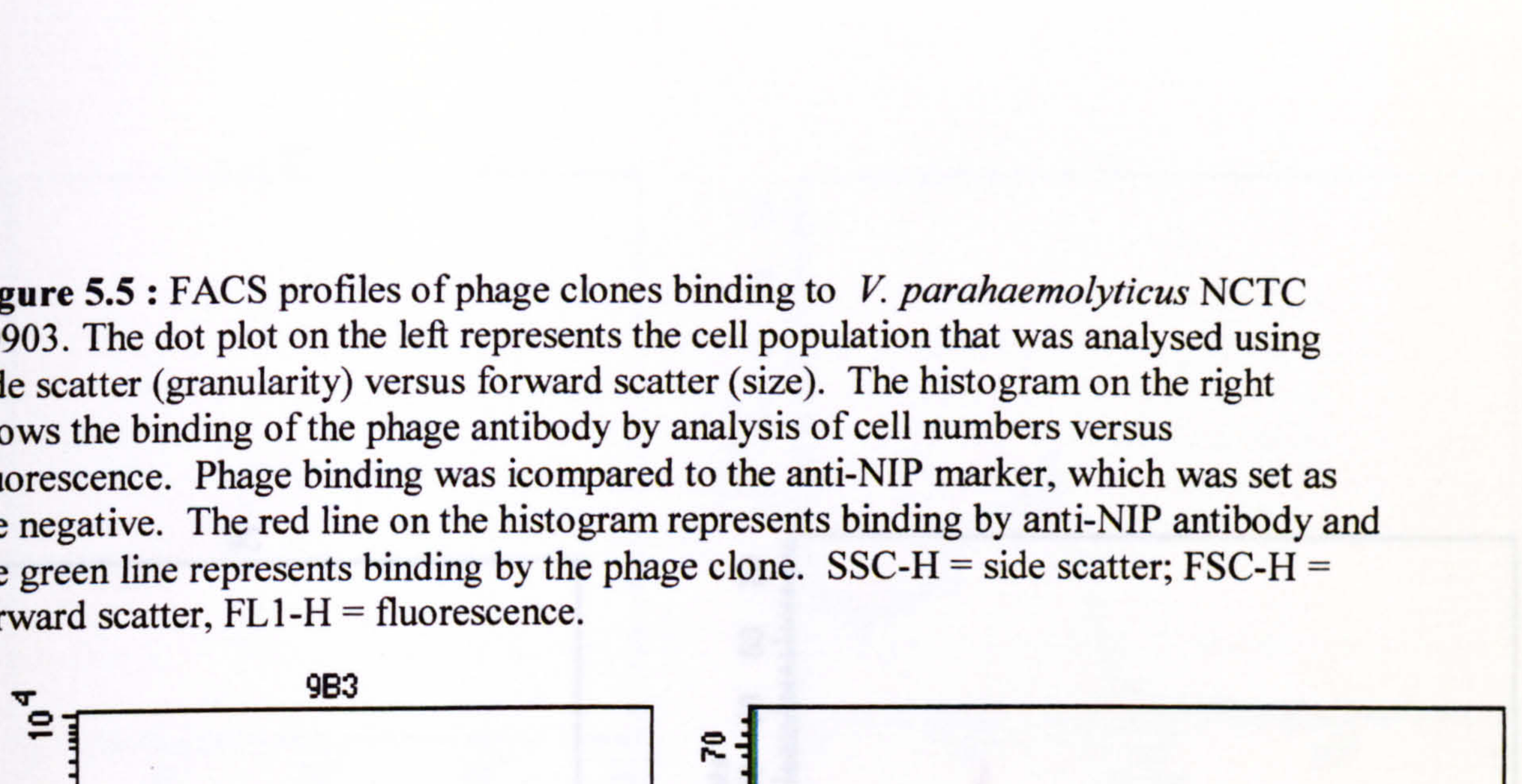
Table 5.5: Assessing binding of selected phage antibodies against *V. parahaemolyticus* NCTC 10903 and NCTC 10884 using flow cytometry. The percentage binding was determined for the positive clones using an M1 marker (where total cell population, n= 10,000). Percentage binding of anti-NIP irrelevant phage was set at 5% on each experiment. The percentage of cells positive with each phage clone was determined using this marker. Analysis was carried out in duplicate, and the experiment was repeated independently three times.

Clone	*V. parahaemolyticus* NCTC 10903 (% binding)	*V. parahaemolyticus* NCTC 10884 (% binding)
A1	11.3	7.7
B3	21.3	21.9
B5	27.6	27.8
C2	33.0	30.5
E1	16.6	12.5
F2	16.9	8.15

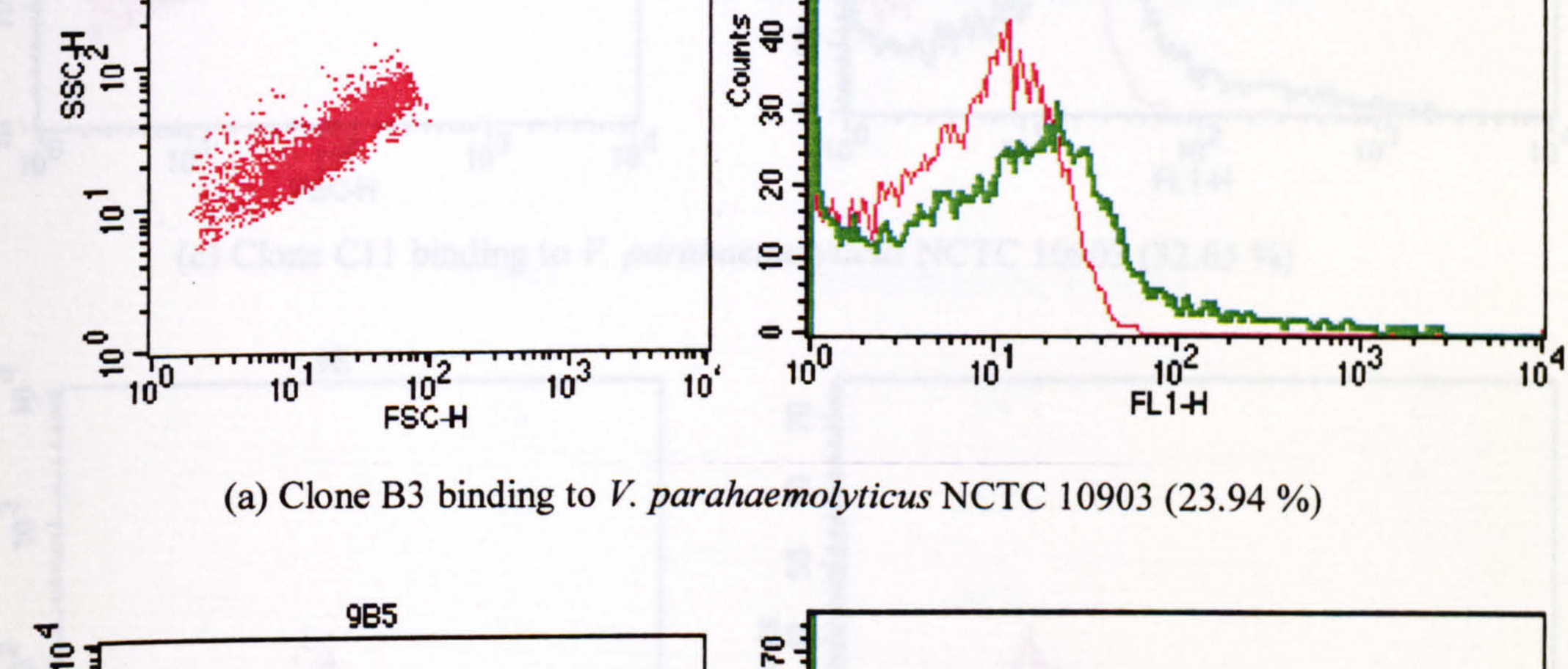
5.4.1- *Flow cytometry profiles*

The histograms below show the binding of phage antibody clones to *V. parahaemolyticus*. Binding was determined by analysing cell counts (X-axis) versus fluorescence (Y-axis). Total cell population (n) was equal to 10,000 cells. The percentage binding was determined using an M1 marker with anti-NIP as the negative (irrelevant) phage clone.

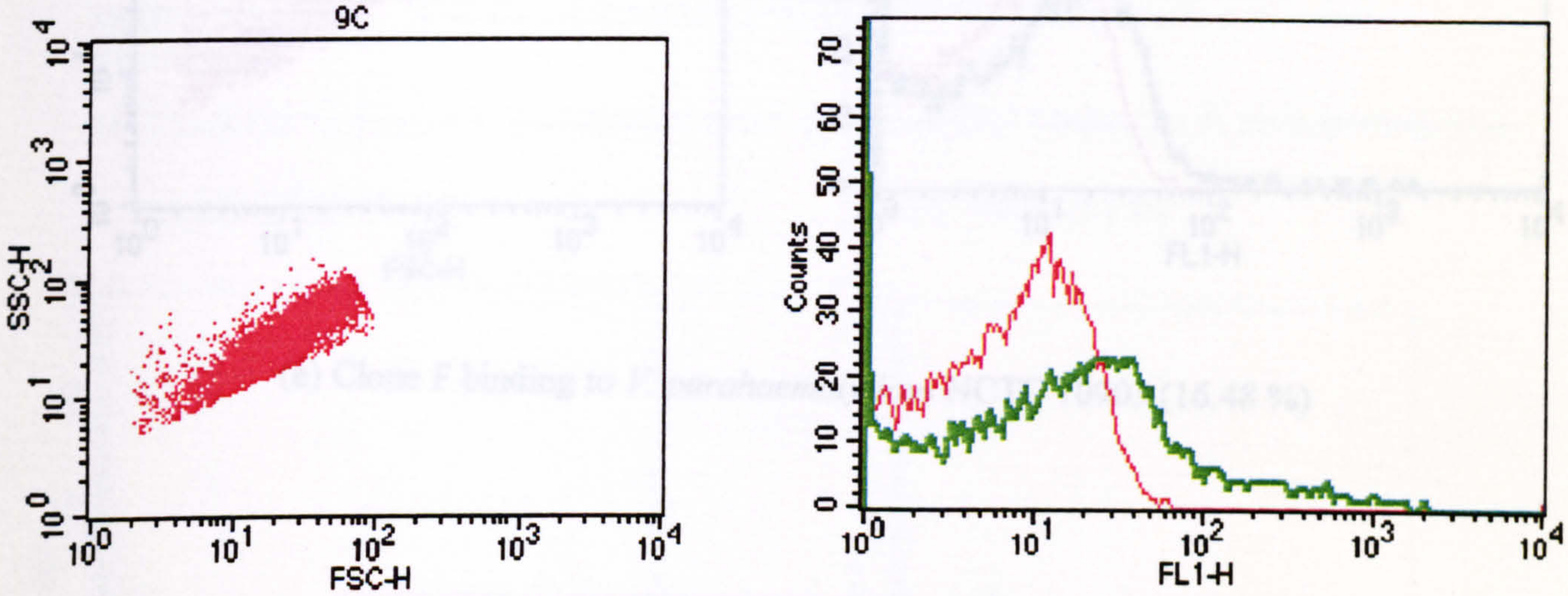
Figure 5.5 : FACS profiles of phage clones binding to *V. parahaemolyticus* NCTC 10903. The dot plot on the left represents the cell population that was analysed using side scatter (granularity) versus forward scatter (size). The histogram on the right shows the binding of the phage antibody by analysis of cell numbers versus fluorescence. Phage binding was icompared to the anti-NIP marker, which was set as the negative. The red line on the histogram represents binding by anti-NIP antibody and the green line represents binding by the phage clone. SSC-H = side scatter; FSC-H = forward scatter, FL1-H = fluorescence.



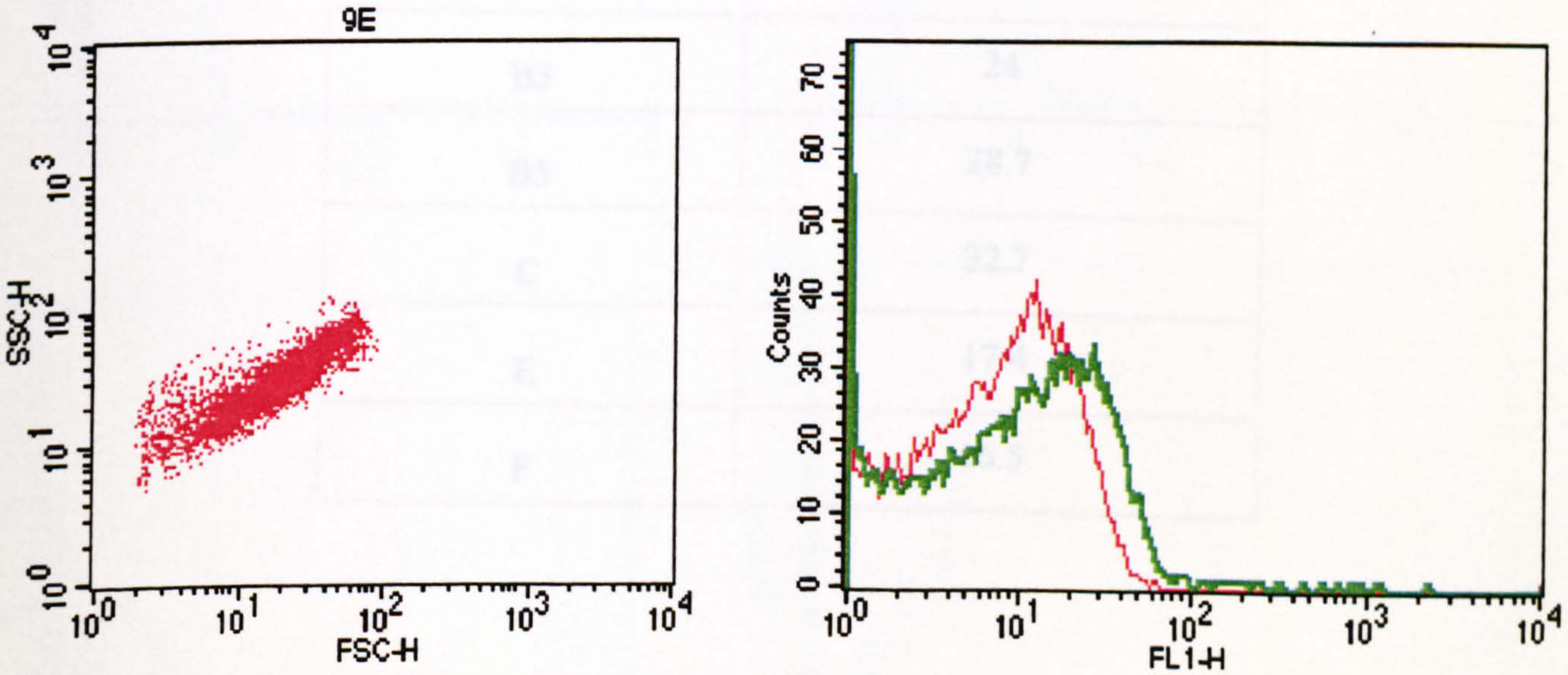
(a) Clone B3 binding to *V. parahaemolyticus* NCTC 10903 (23.94 %)



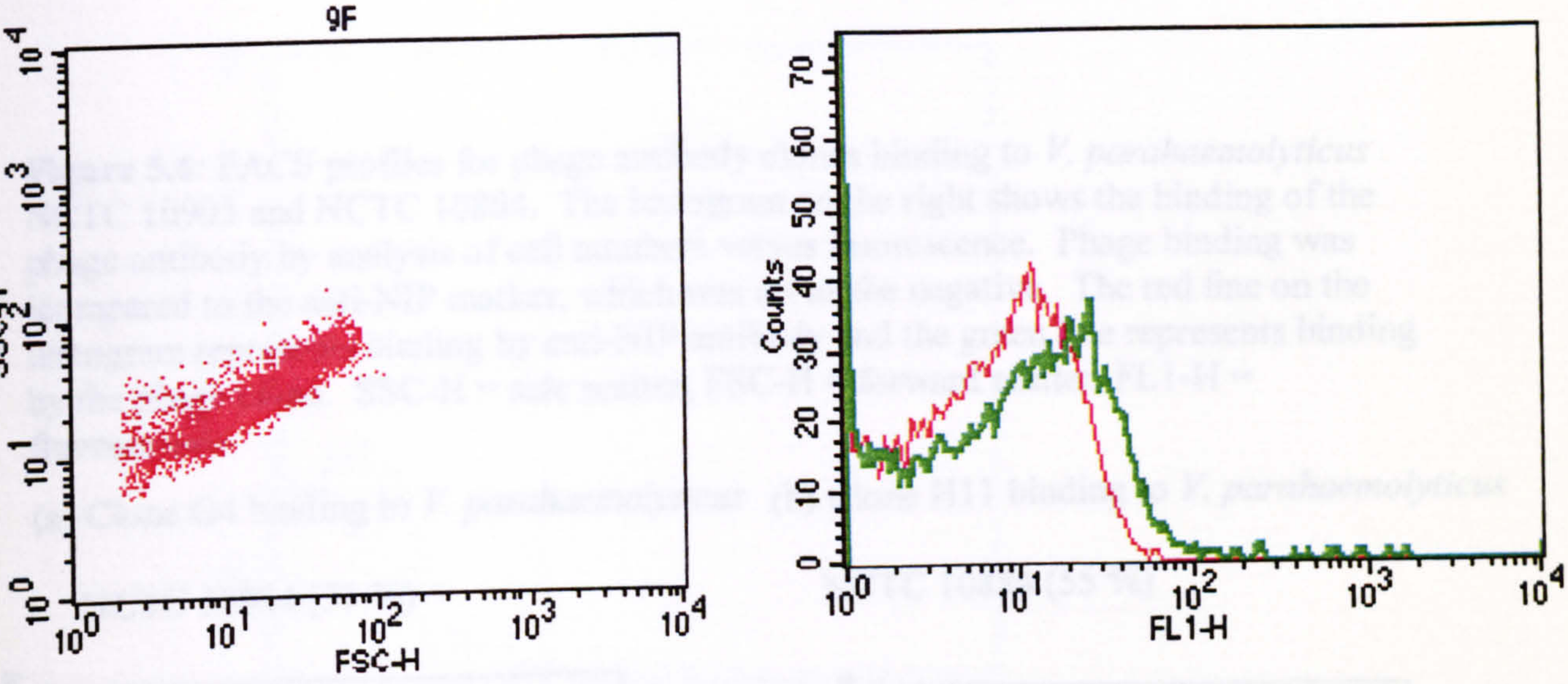
(b) Clone B5 binding to *V. parahaemolyticus* NCTC 10903 (28.71 %)



(c) Clone C11 binding to *V. parahaemolyticus* NCTC 10903 (32.65 %)



(d) Clone E binding to *V. parahaemolyticus* NCTC 10903 (17.37 %)

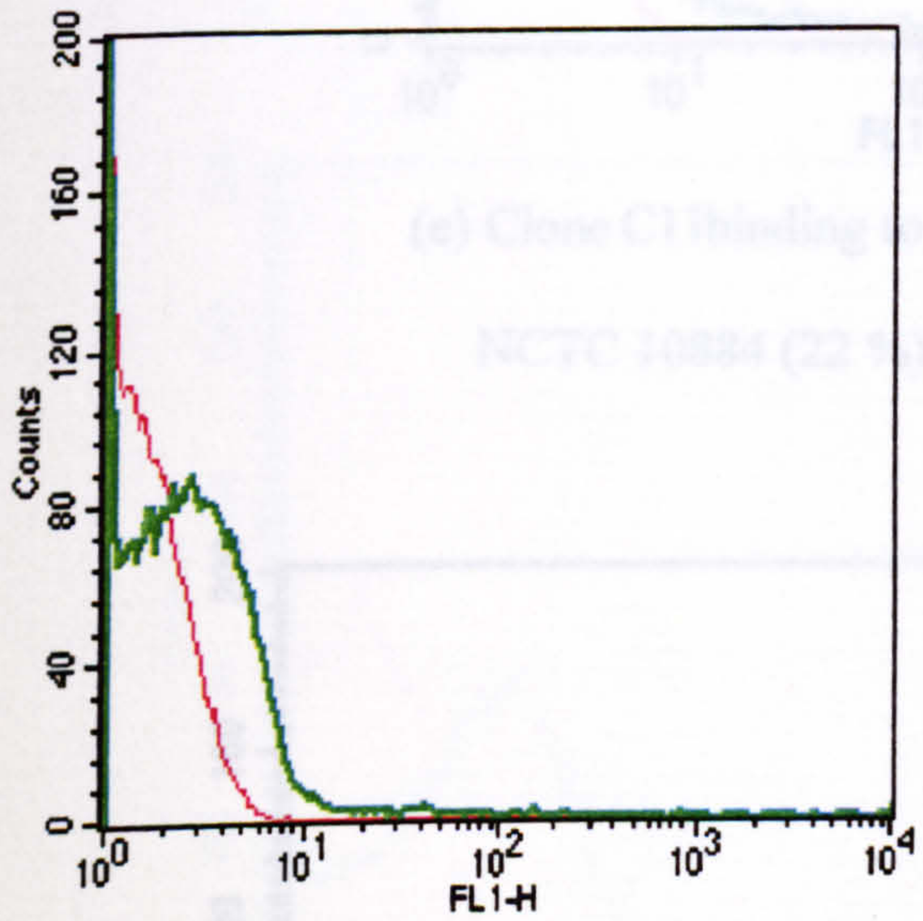


(e) Clone F binding to *V. parahaemolyticus* NCTC 10903 (16.48 %)

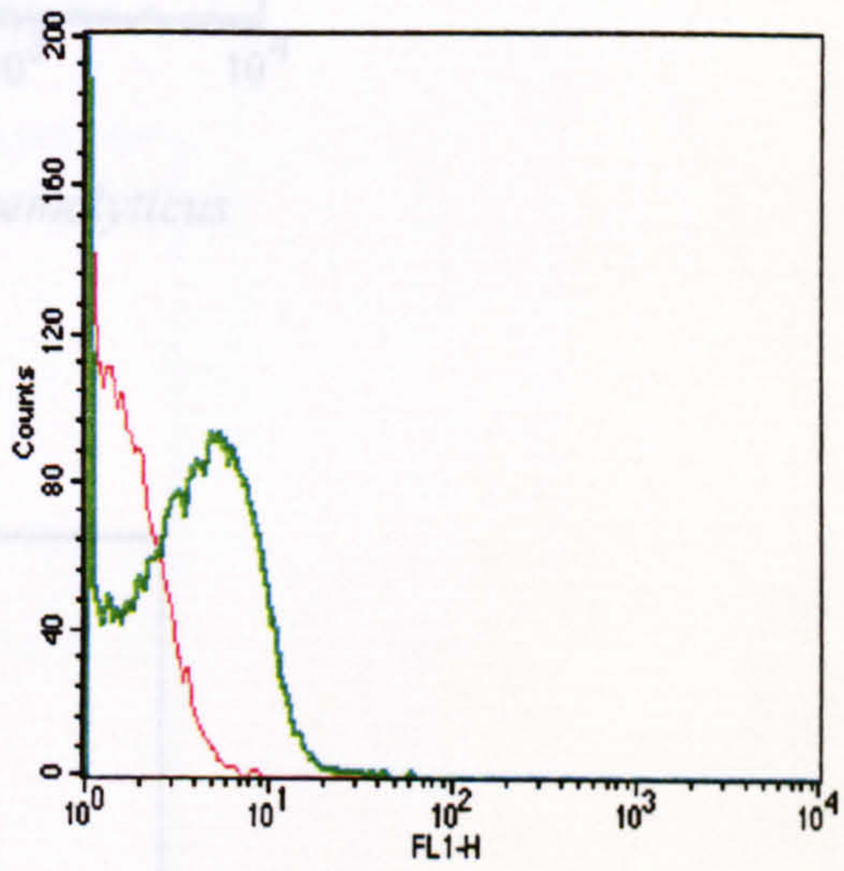
Clone	Percentage(%) binding
B3	24
B5	28.7
C	32.7
E	17.4
F	16.5

Figure 5.6: FACS profiles for phage antibody clones binding to *V. parahaemolyticus* NCTC 10903 and NCTC 10884. The histogram on the right shows the binding of the phage antibody by analysis of cell numbers versus fluorescence. Phage binding was icompared to the anti-NIP marker, which was set as the negative. The red line on the histogram represents binding by anti-NIP antibody and the green line represents binding by the phage clone. SSC-H = side scatter; FSC-H = forward scatter, FL1-H = fluorescence.

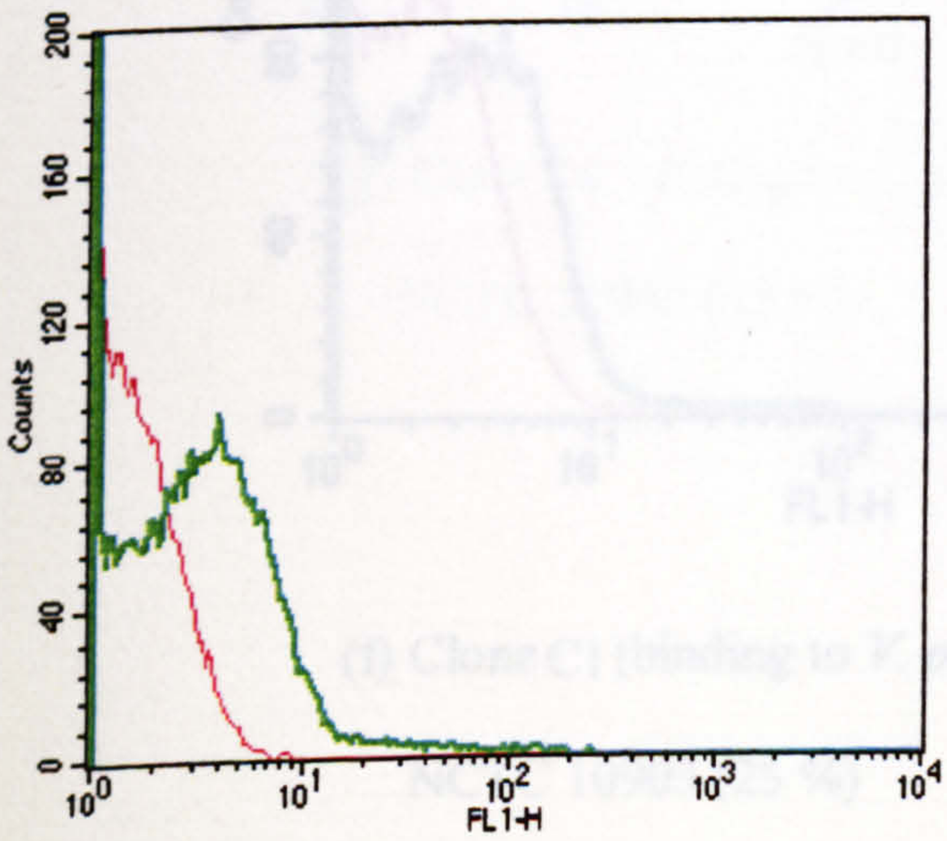
(a) Clone G4 binding to *V. parahaemolyticus* NCTC 10884 (33 %)



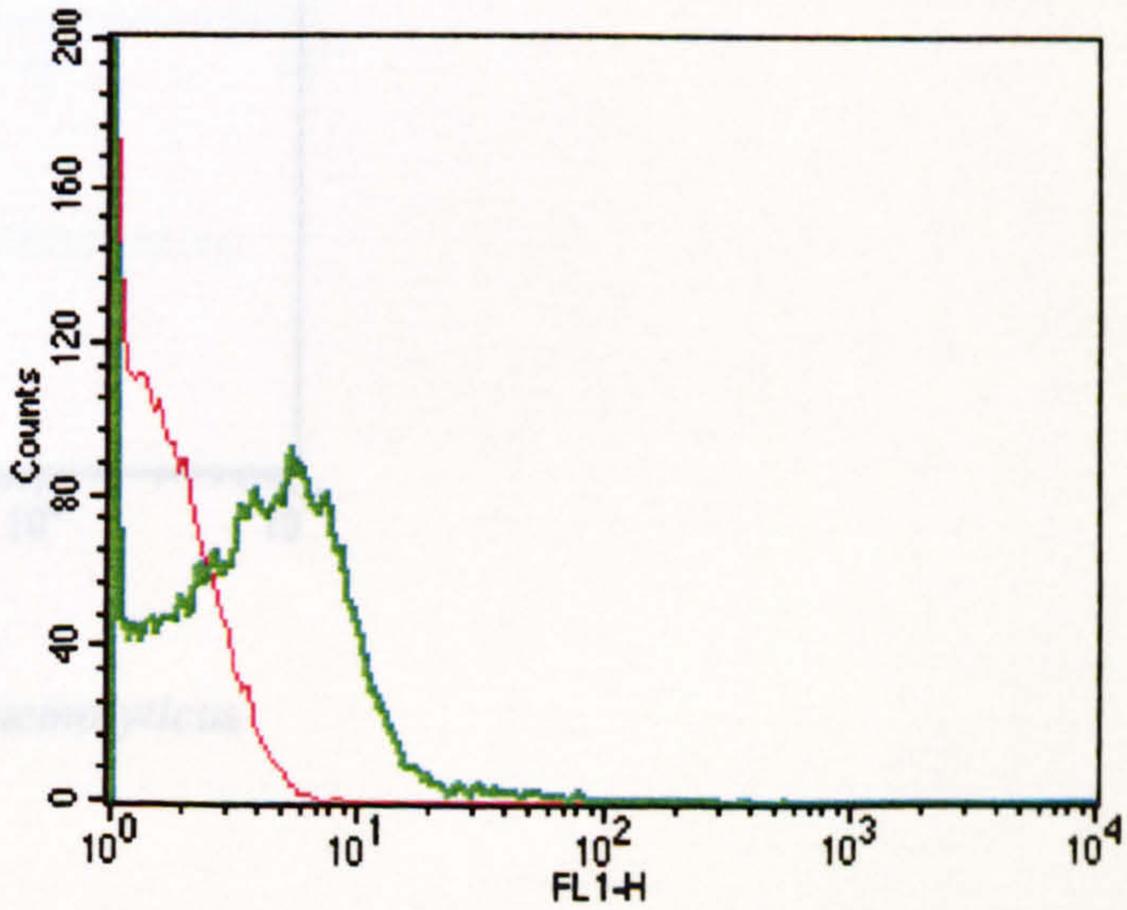
(b) Clone H11 binding to *V. parahaemolyticus* NCTC 10884 (55 %)

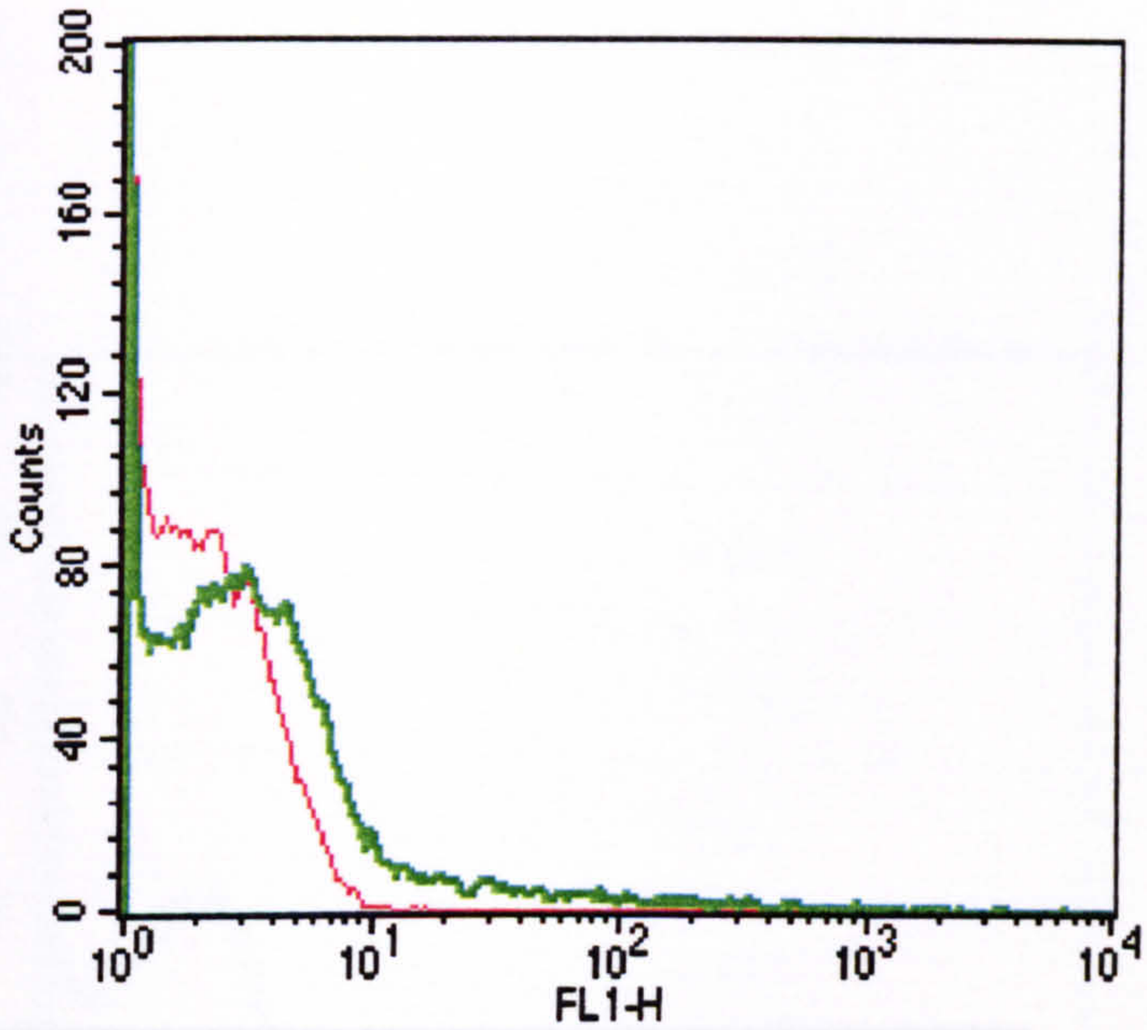


(c) Clone H11 binding to *V. parahaemolyticus* NCTC 10903 (46 %)



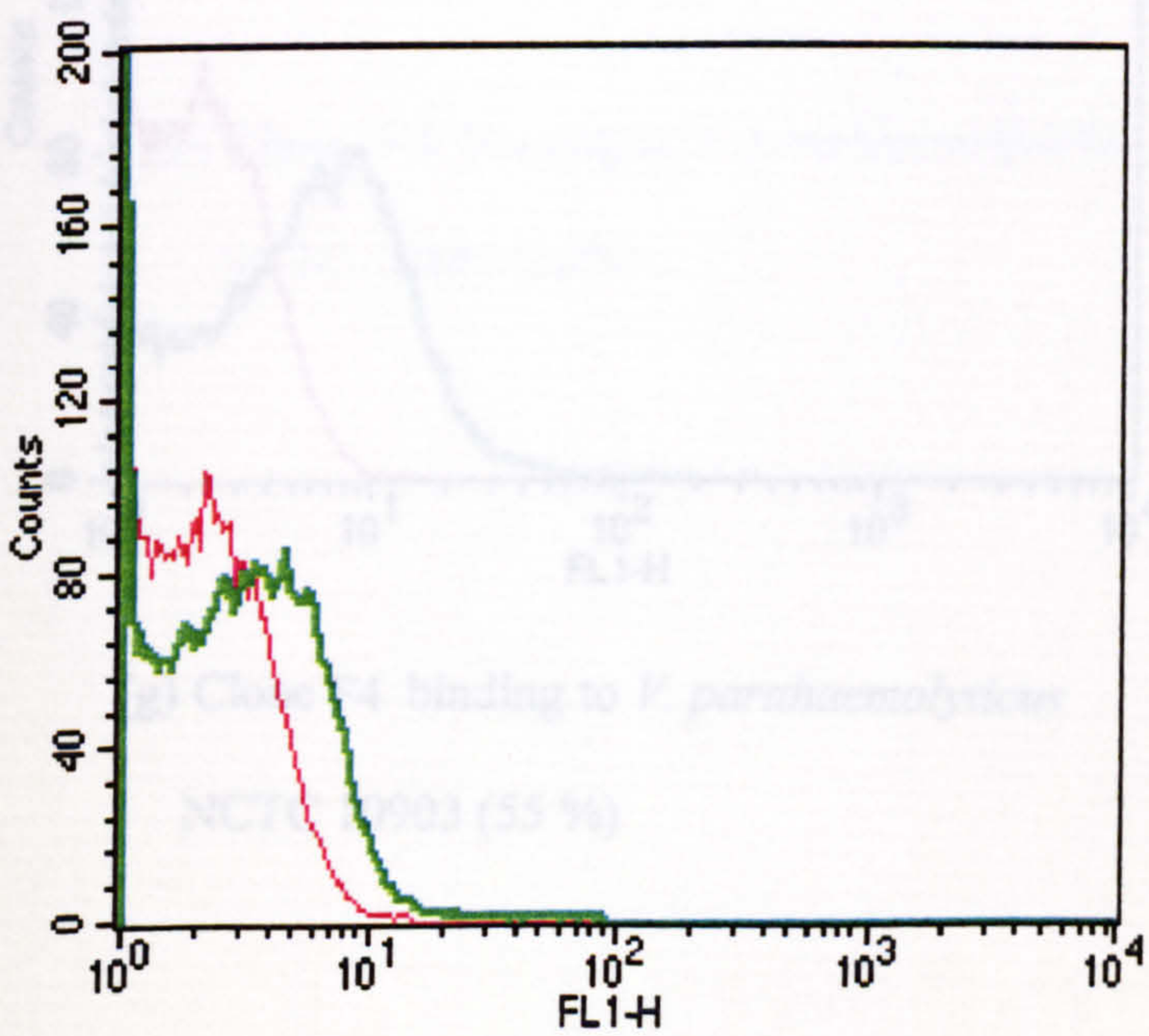
(d) Clone G4 binding to *V. parahaemolyticus* NCTC 10903 (55 %)





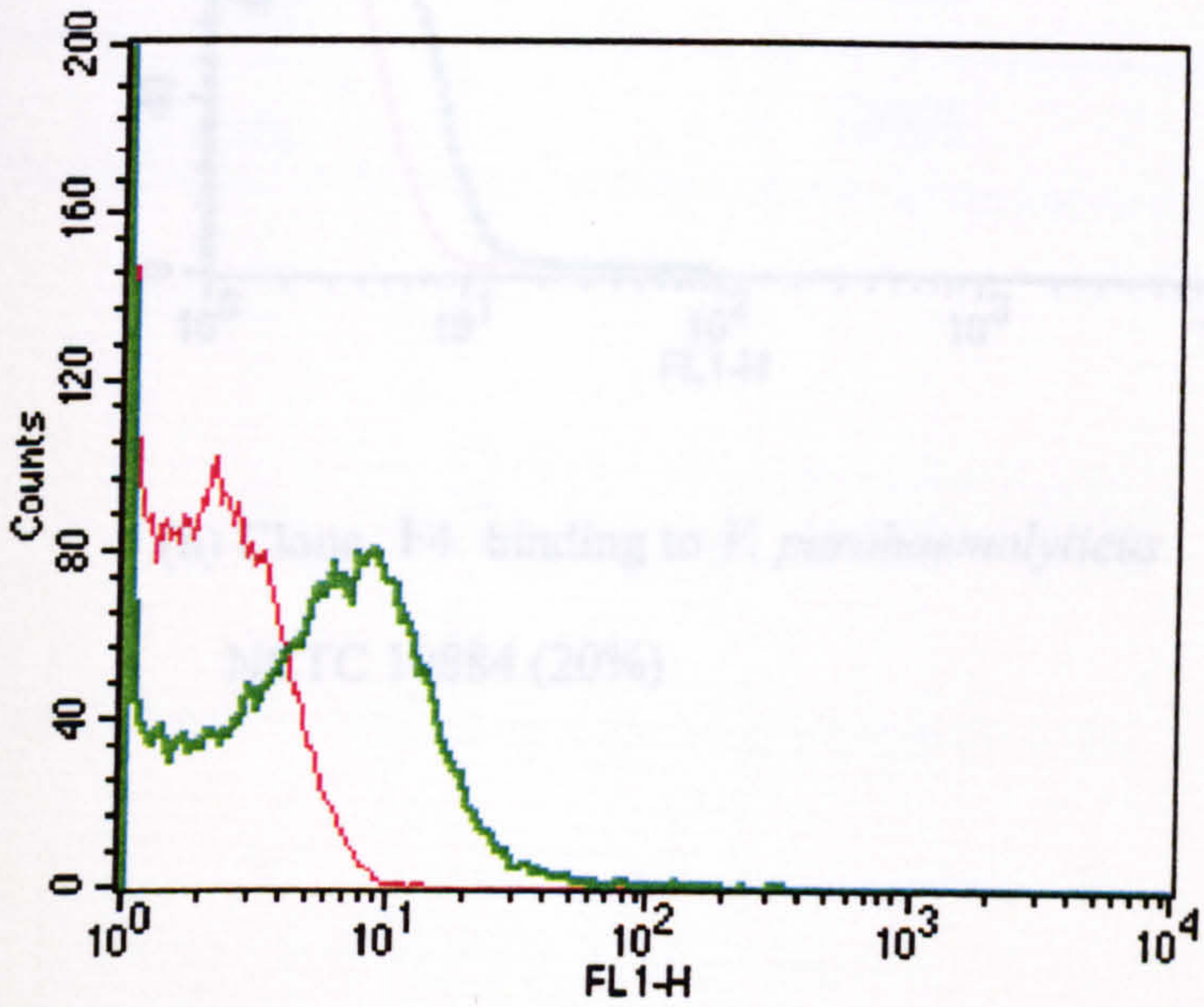
(e) Clone C11binding to *V. parahaemolyticus*

NCTC 10884 (22 %)



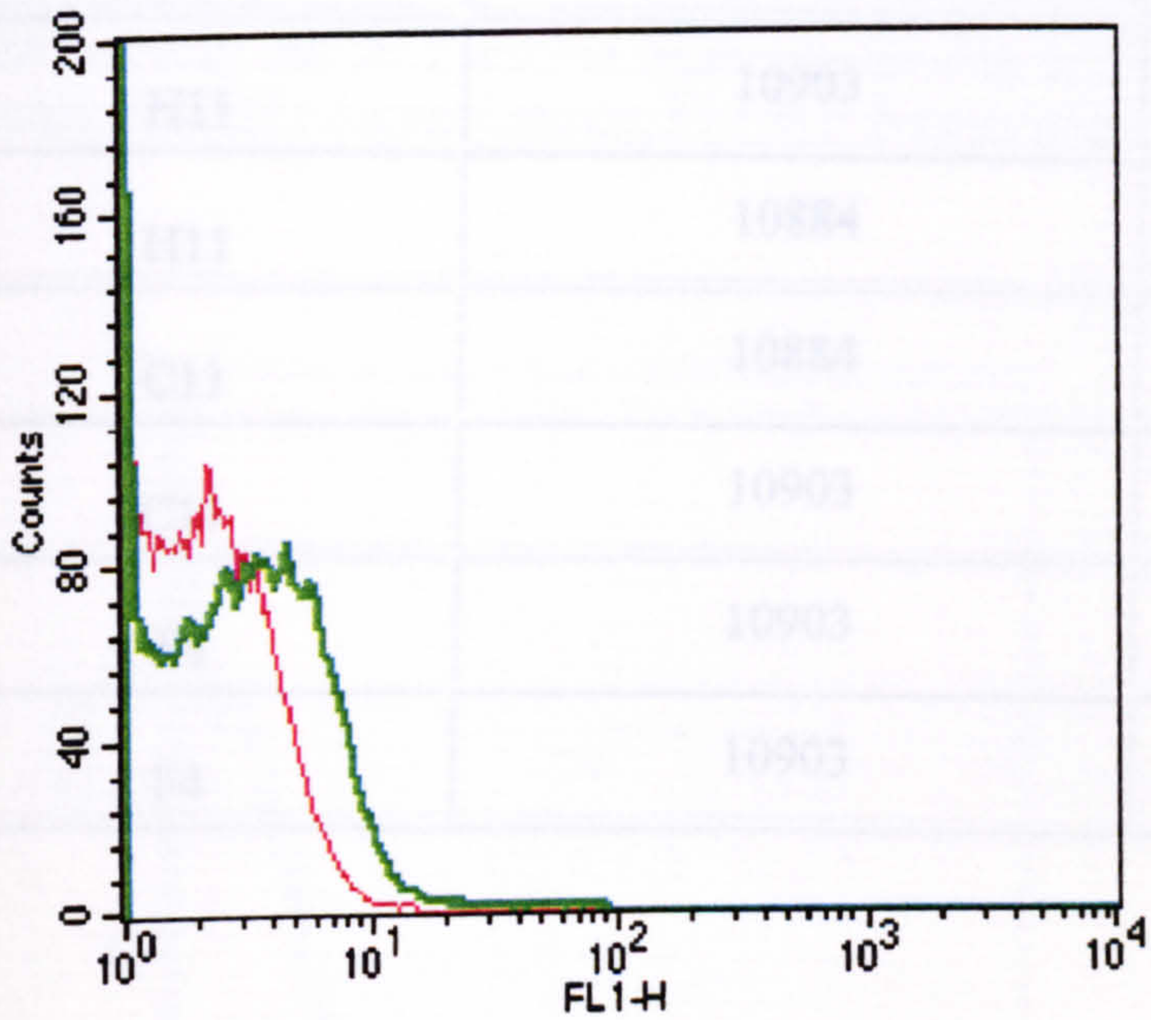
(f) Clone C11binding to *V. parahaemolyticus*

NCTC 10903 (25 %)



(g) Clone F4 binding to *V. parahaemolyticus*

NCTC 10903 (55 %)

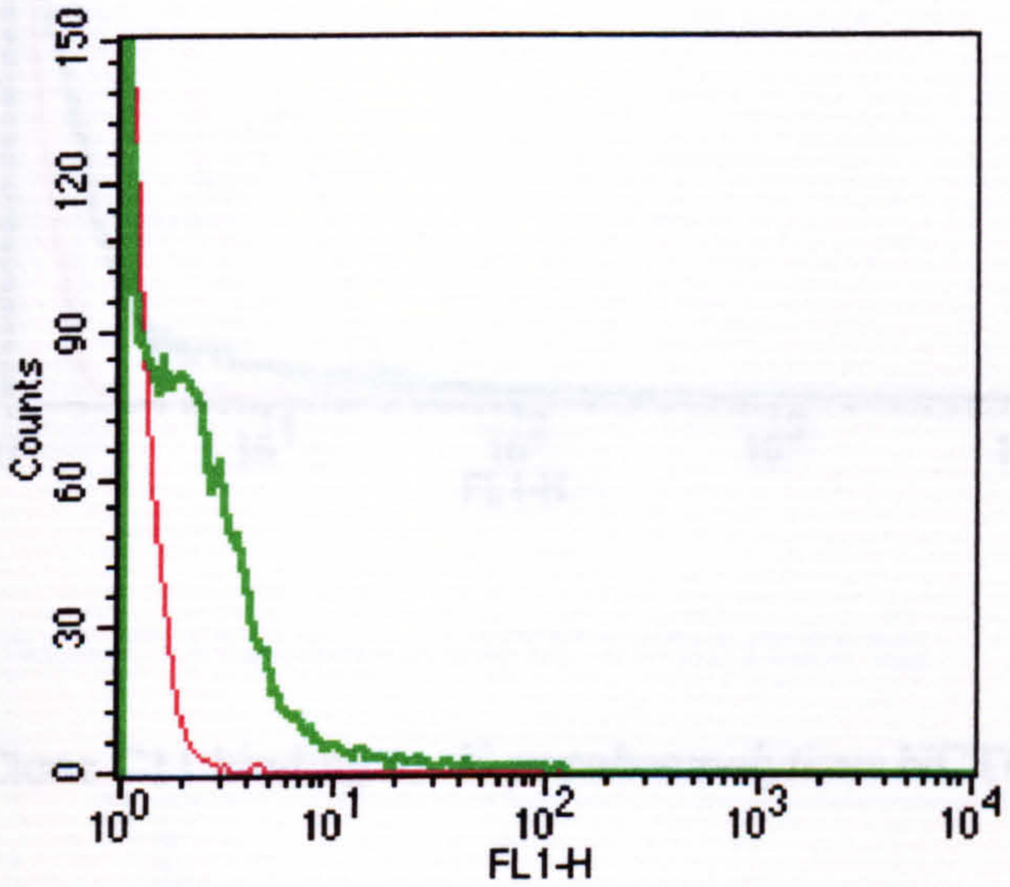


(h) Clone F4 binding to *V. parahaemolyticus*

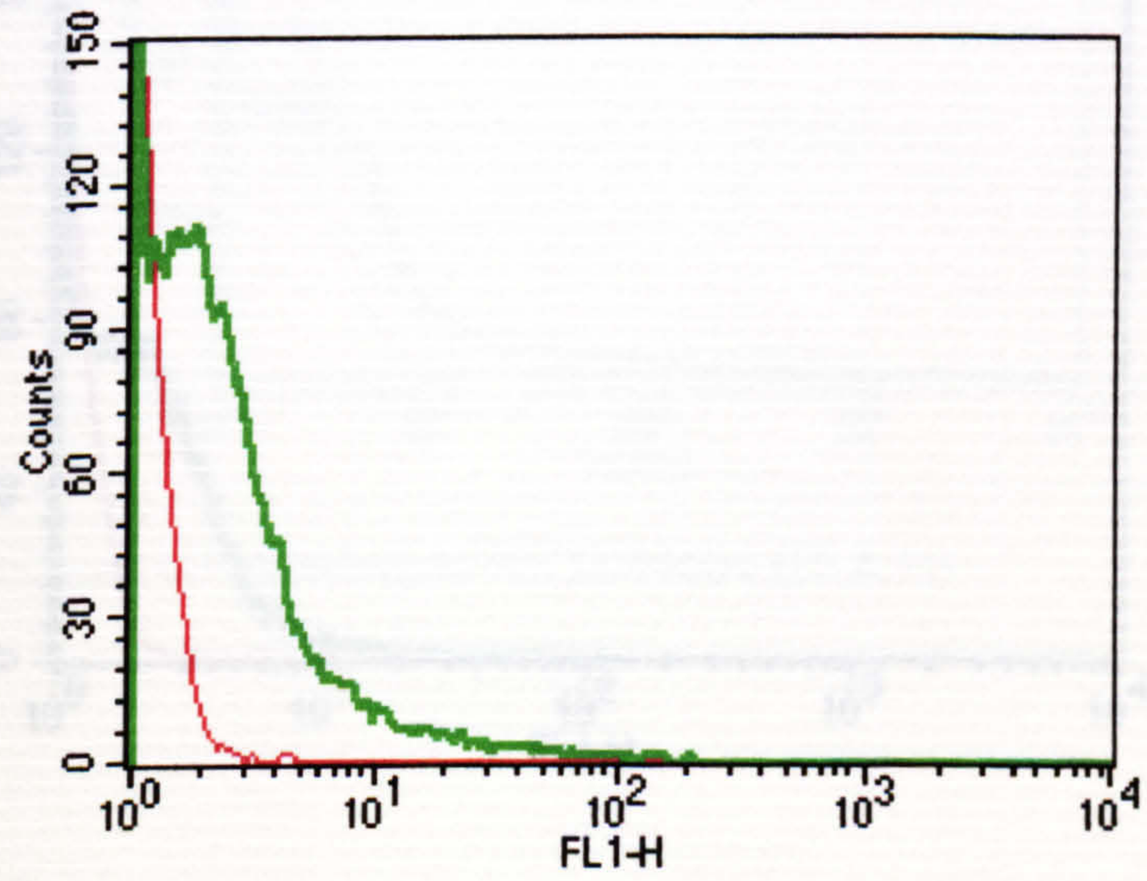
NCTC 10884 (20%)

Clone	*V. parahaemolyticus* strain	Percentage(%) binding
G4	10884	33
G4	10903	55
H11	10903	46
H11	10884	55
C11	10884	22
C11	10903	25
F4	10903	55
F4	10903	20

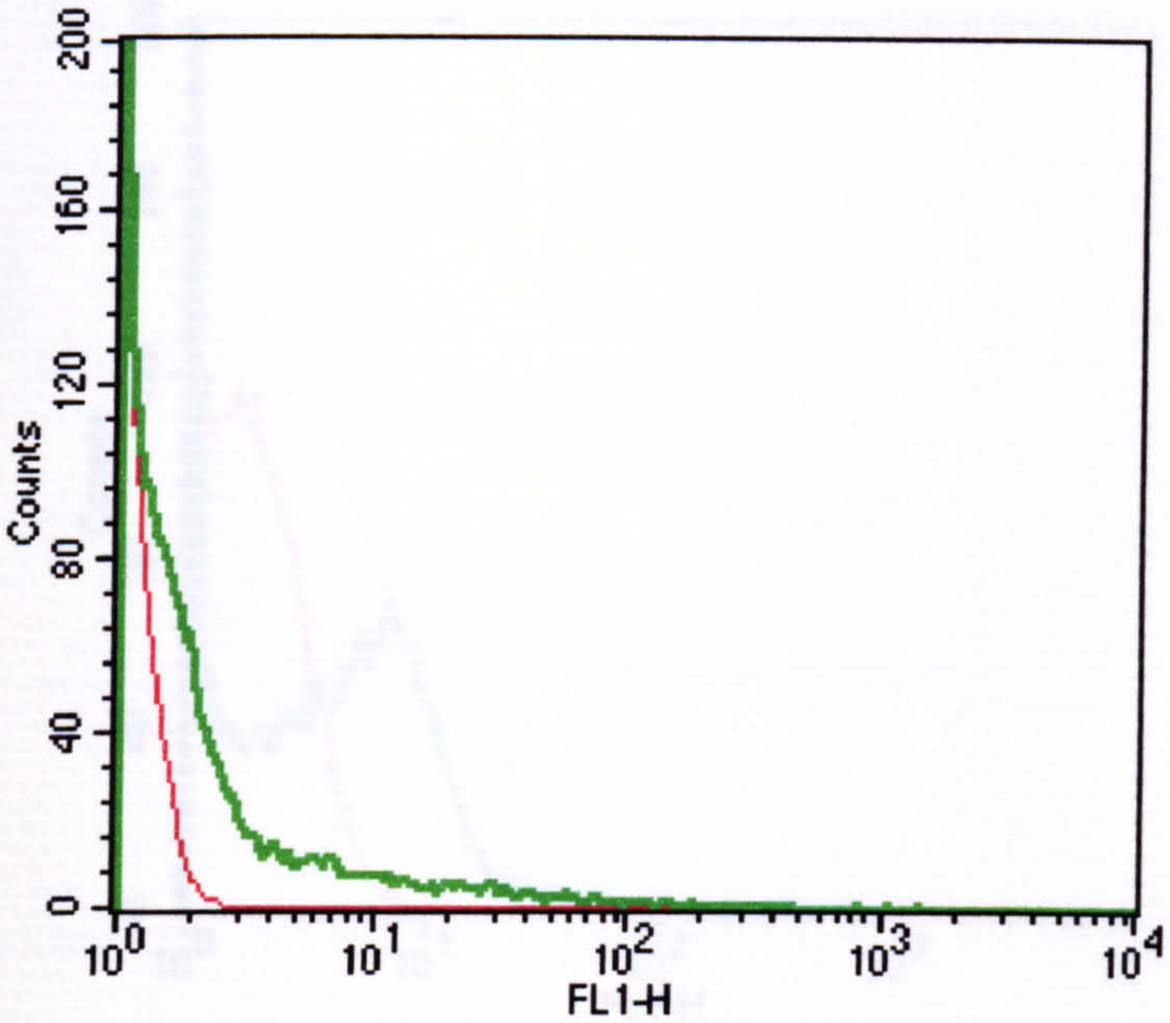
Figure 5.7: FACS profiless of clone C11 binding to strains of *V. parahaemolyticus*. As before, percentage binding was determined using M1 marker. Anti-NIP antibody was used to set the negative marker at 5% for each experiment. The percentage of cells positive with each antibody was determined using this marker. This is shown below the histogram in brackets (%). The red line on the histogram represents binding by anti-NIP antibody and the green line represents binding by the phage clone. SSC-H = side scatter; FSC-H = forward scatter, FL1-H = fluorescence.



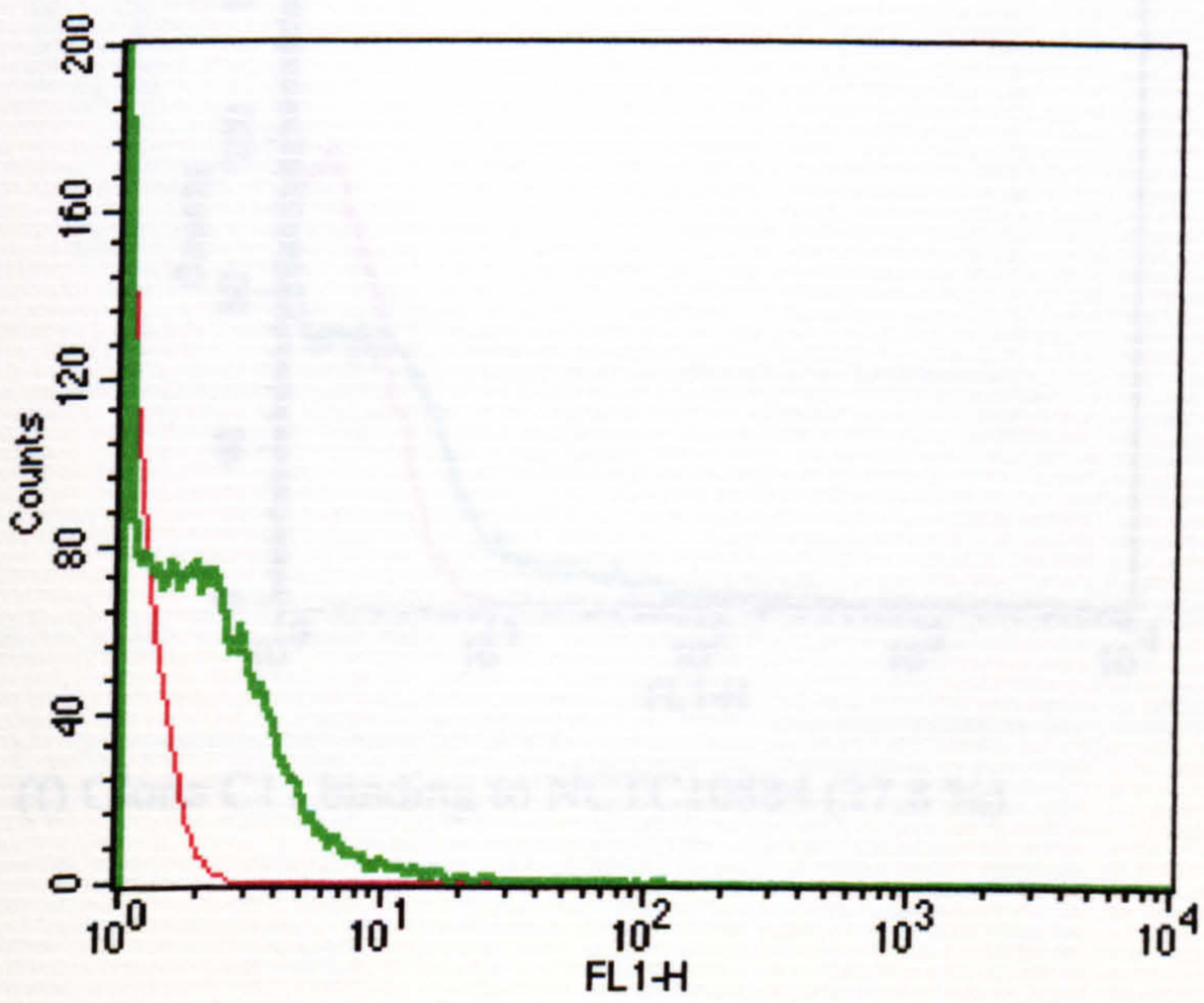
(a)Clone C11 binding to *V. parahaemolyticus* ATCC 27969 (43.5%)



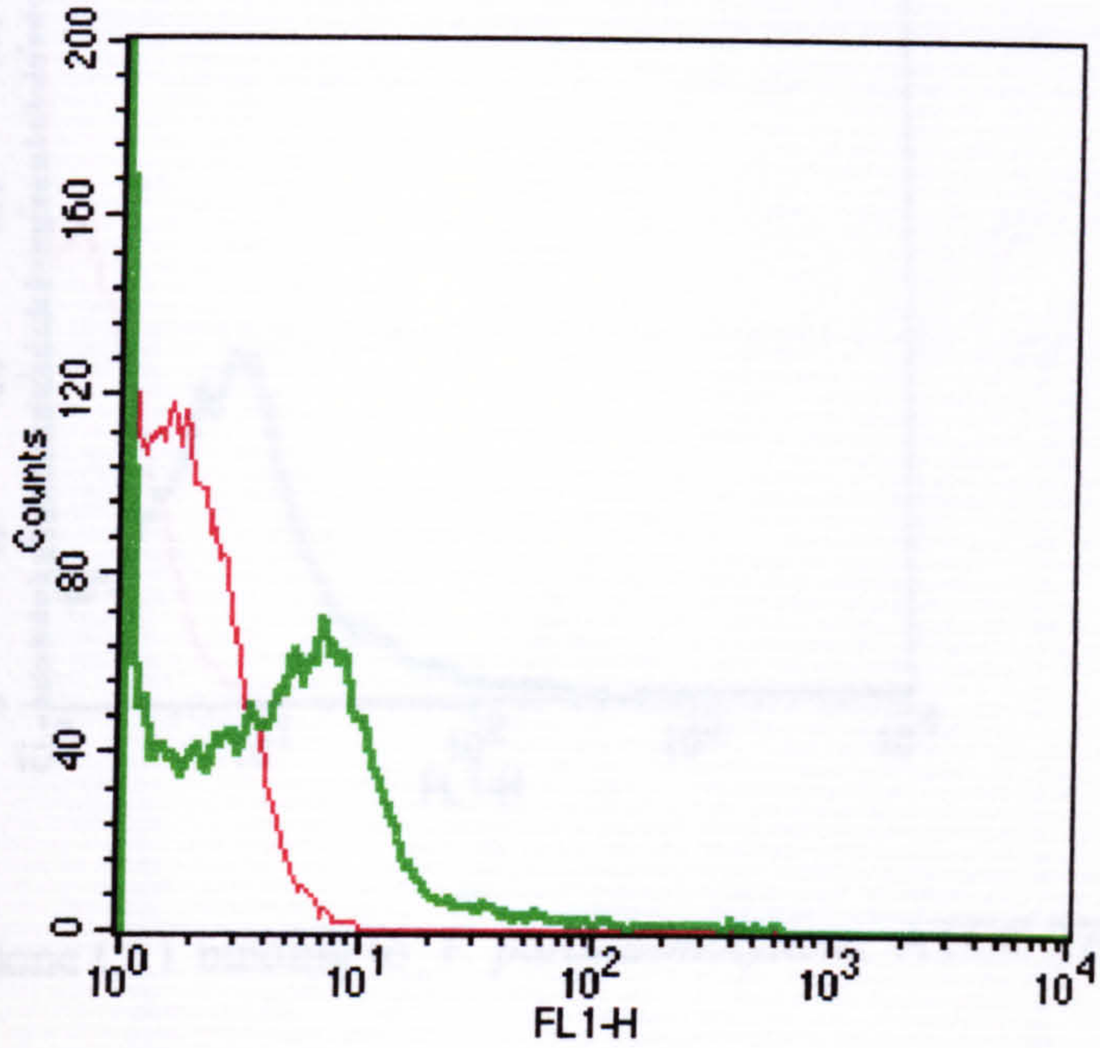
(b) Clone C11binding to *V. parahaemolyticus* NCTC 10441(59.2%)



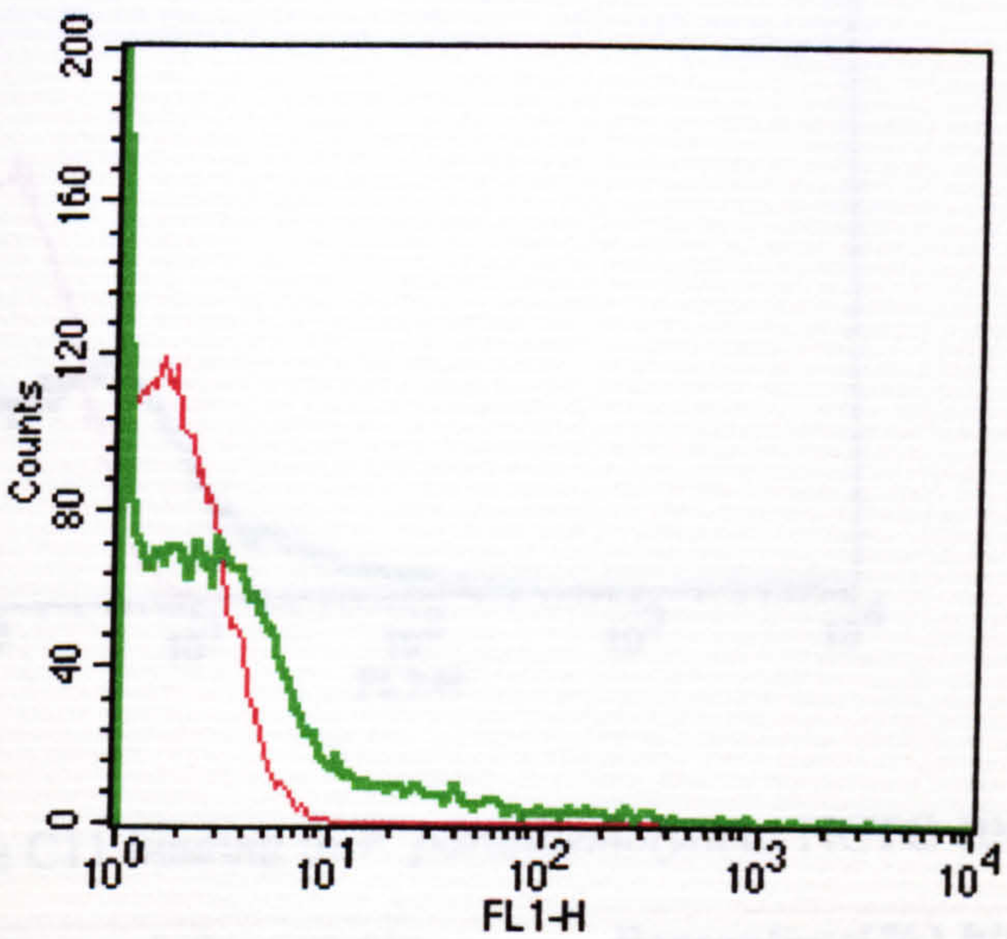
(c) Clone C11 binding to *V. parahaemolyticus* NCTC 10884(36.5 %)



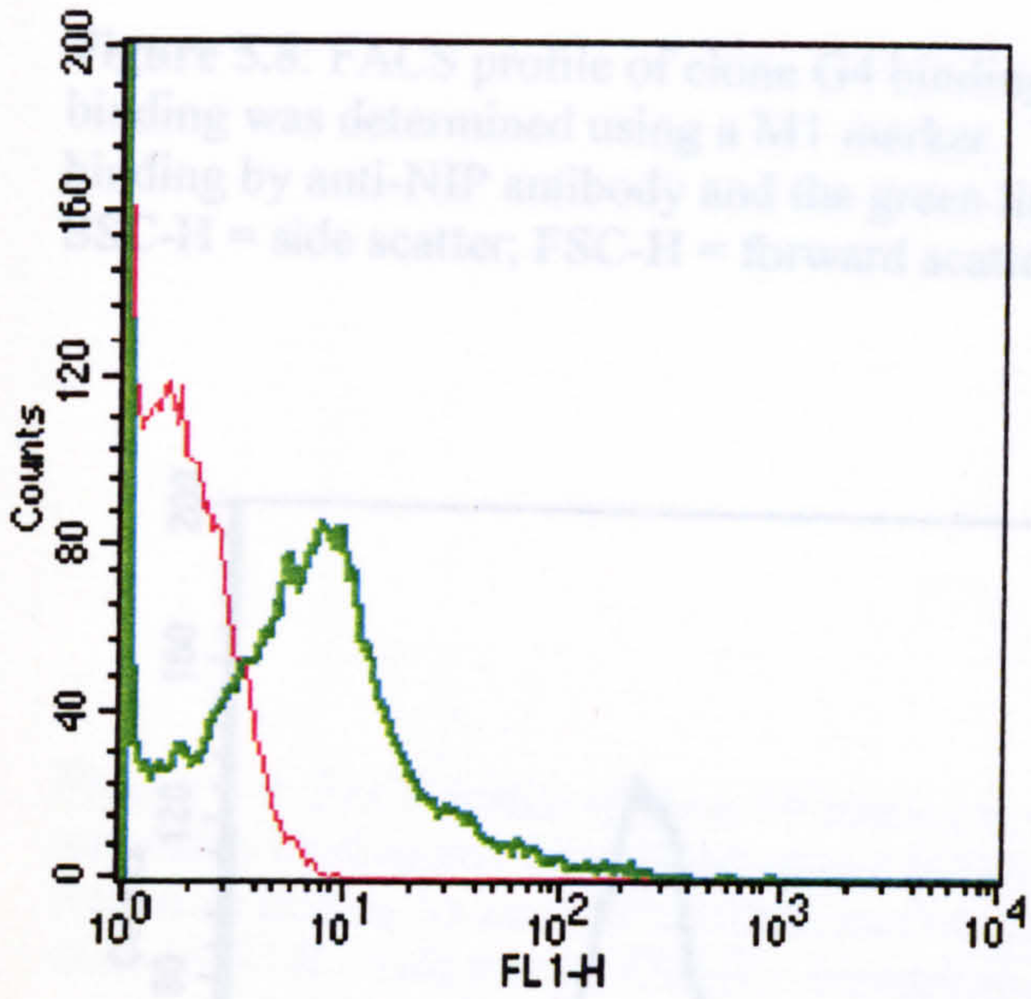
(d) Clone C11 binding to NCTC 10903 (46.1 %)



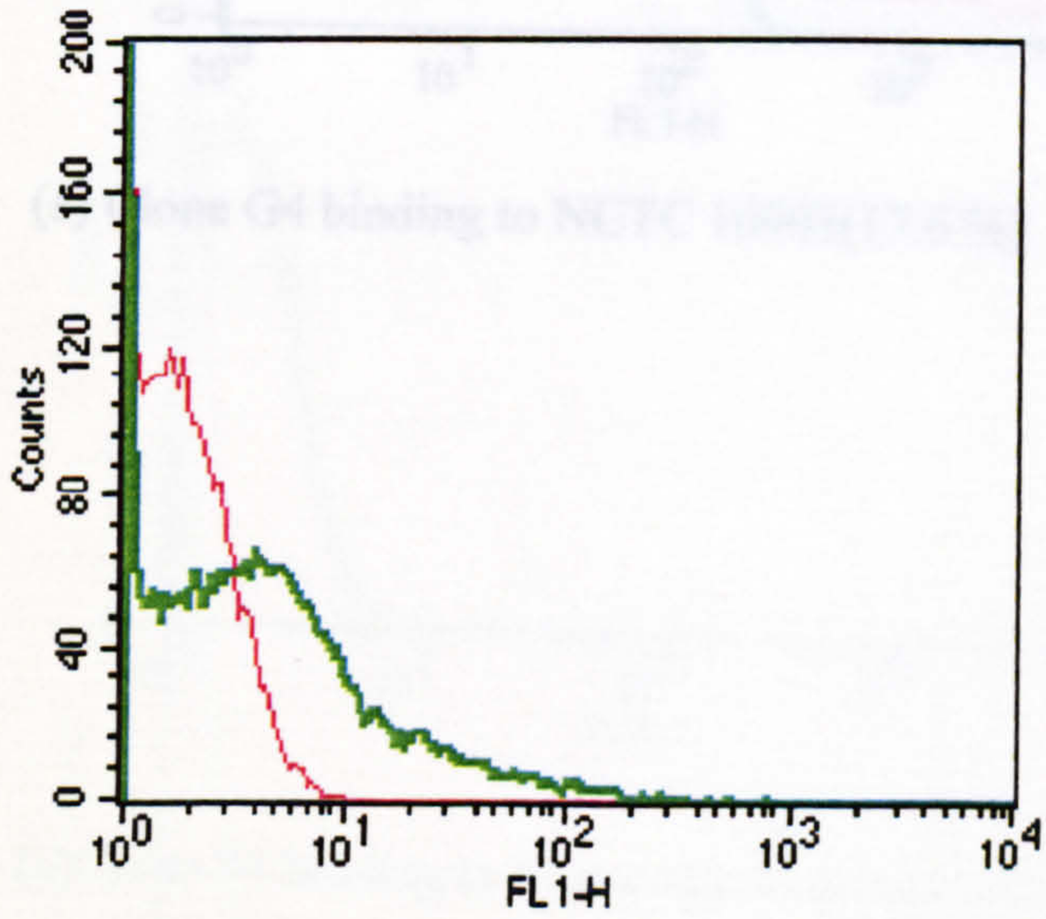
(e) Clone C11 binding to NCTC 10903 (40.9 %)



(f) Clone C11 binding to NCTC10884 (27.8 %)



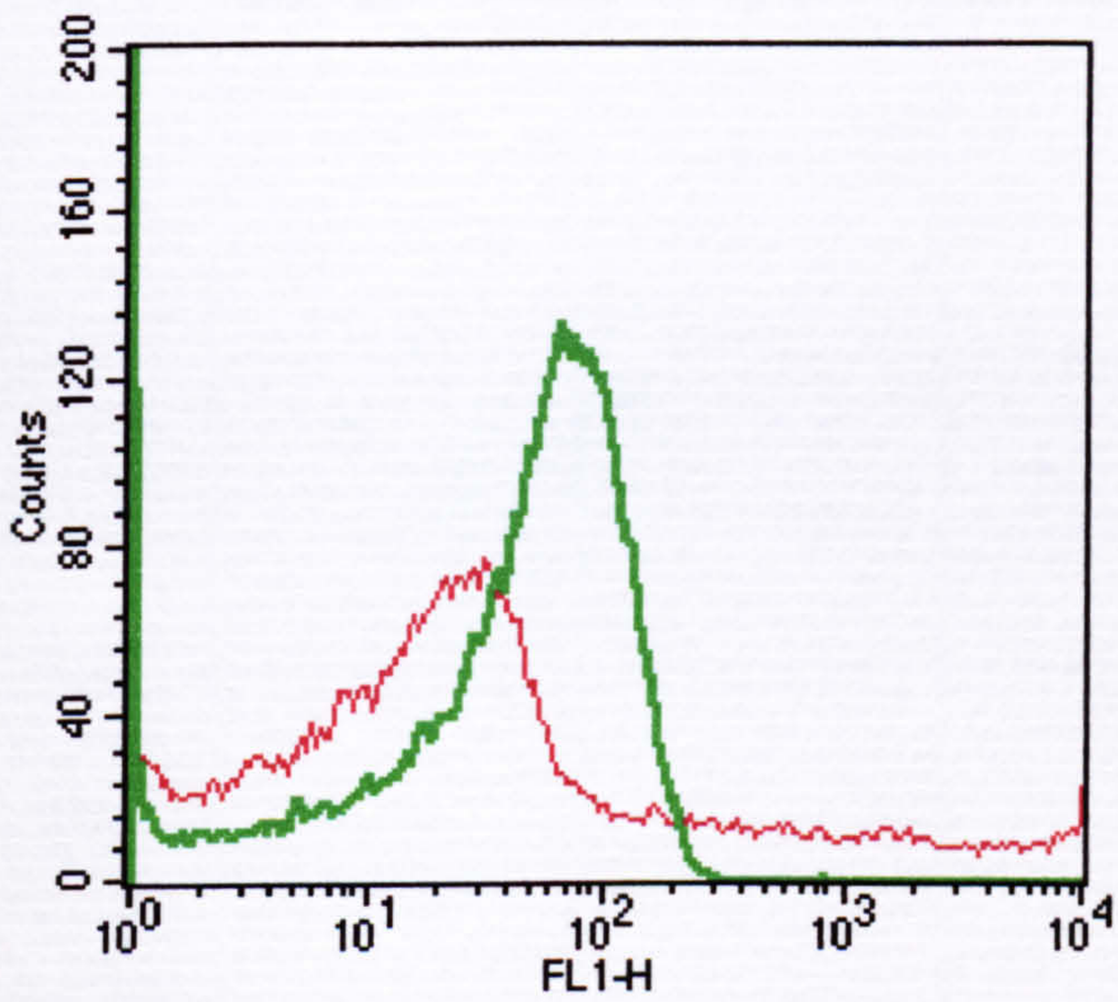
(g) Clone C11 binding to *V. parahaemolyticus* ATCC 27969(65.5 %)



(h) Clone C11 binding to *V. parahaemolyticus* NCTC 10441 (40.4 %)

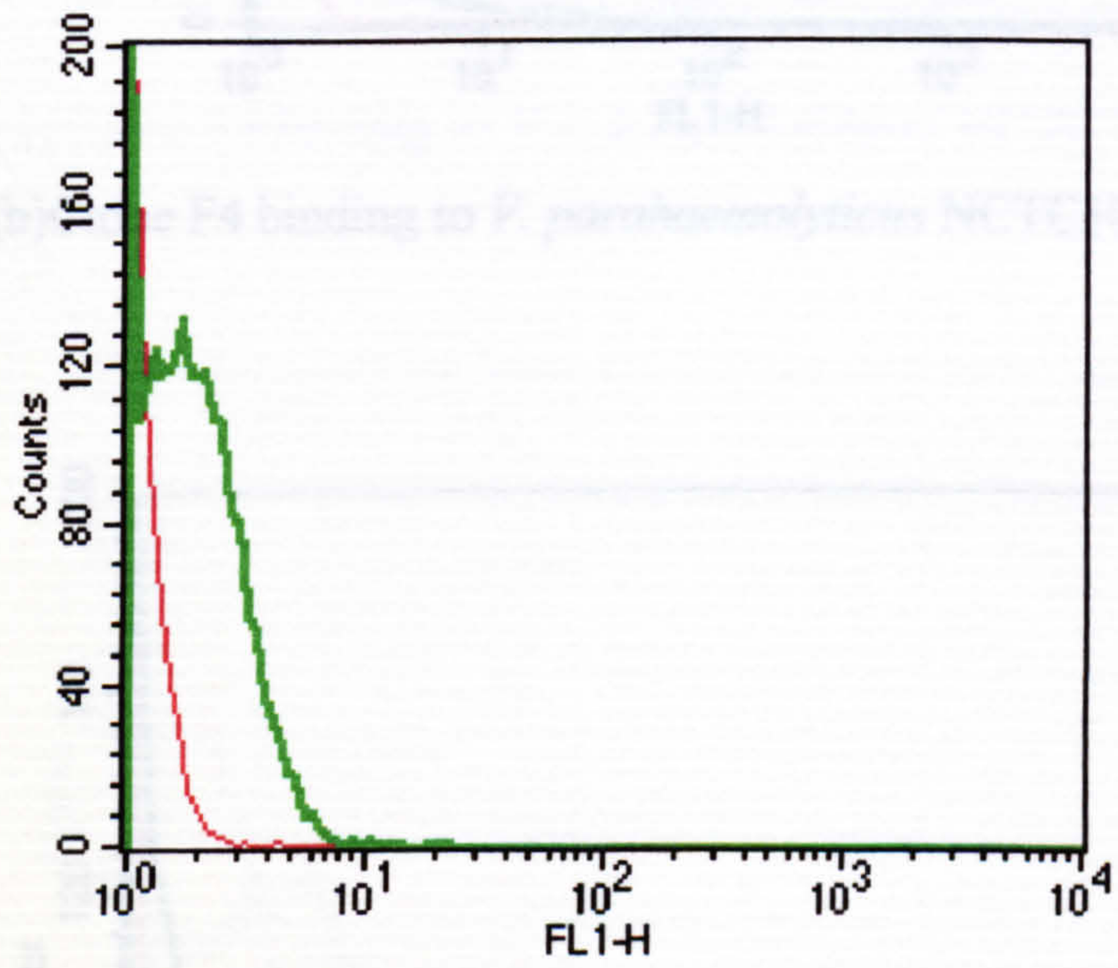
***V. parahaemolyticus* strain**	**Percentage(%) binding**
27969	43.5
27969	65.5
10441	59.2
10441	40.4
10884	36.5
10884	27.8
10903	46.1
10903	40.9

Figure 5.8: FACS profile of clone G4 binding to *V. parahaemolyticus* .The percentage binding was determined using a M1 marker. The red line on the histogram represents binding by anti-NIP antibody and the green line represents binding by the phage clone. SSC-H = side scatter; FSC-H = forward scatter, FL1-H = fluorescence.

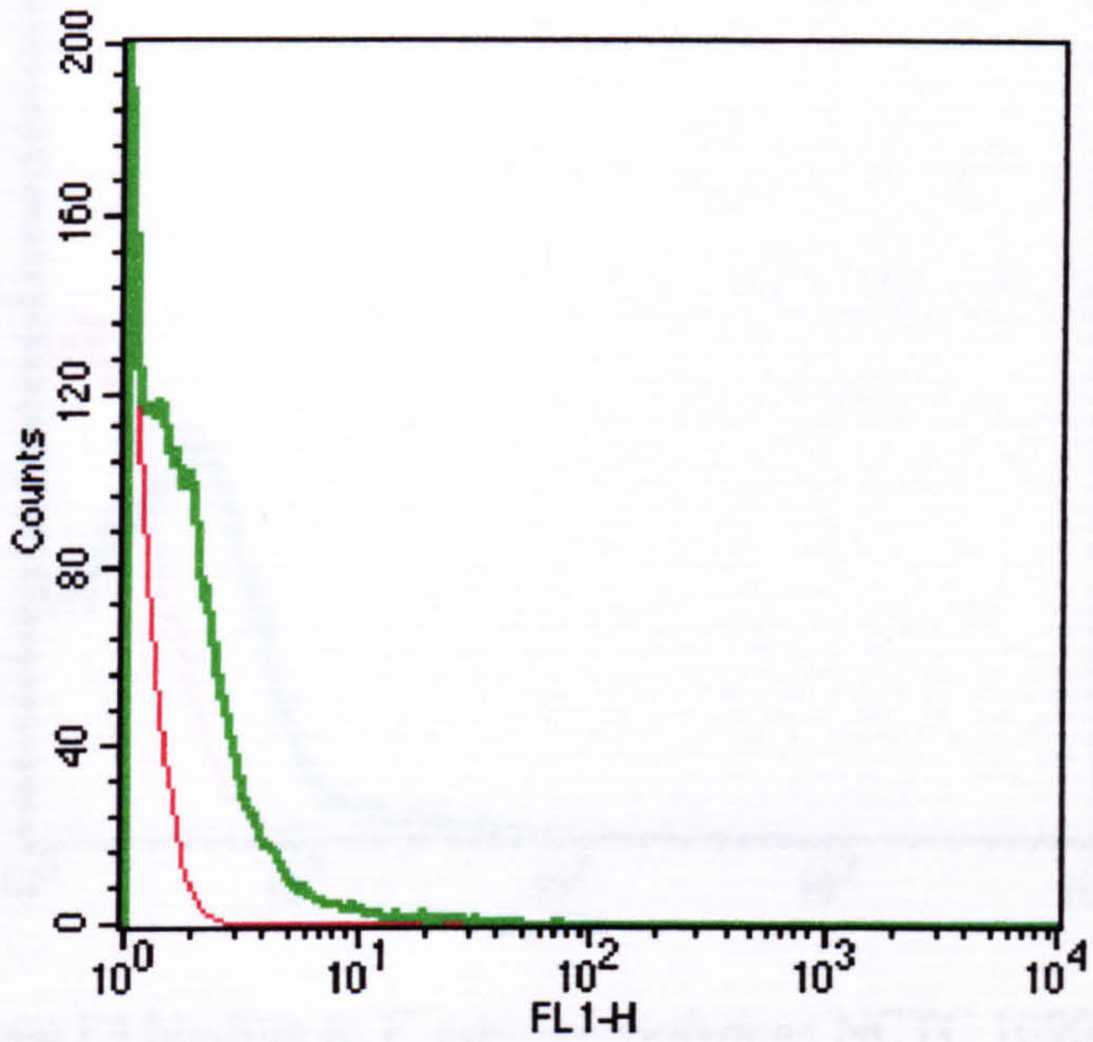


(a) Clone G4 binding to NCTC 10903(17.6 %)

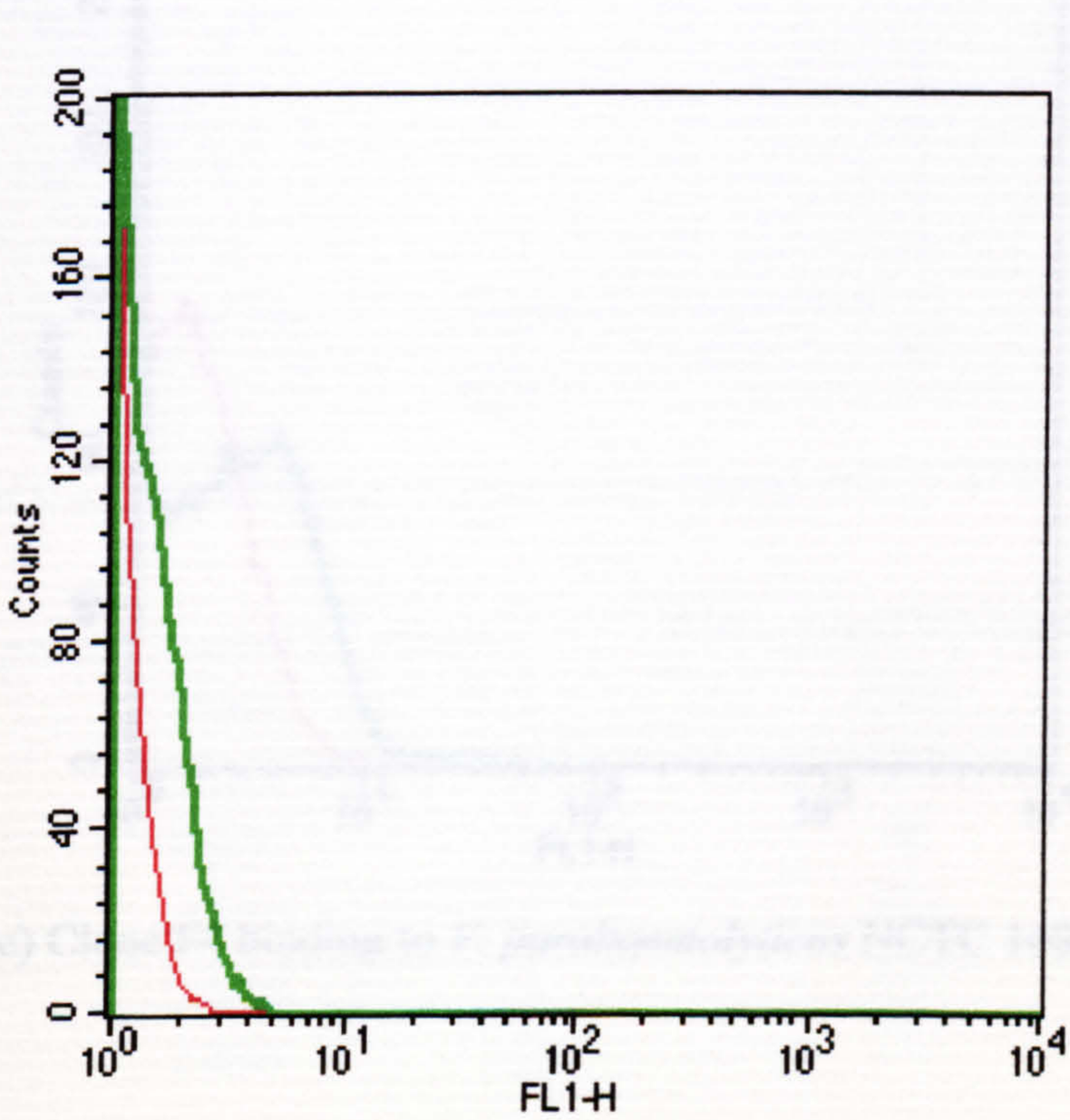
Figure 5.9: FACS profile of clone F4 binding to strains of *V. parahaemolyticus*. The percentage binding was determined using a M1 marker. The red line on the histogram represents binding by anti-NIP antibody and the green line represents binding by the phage clone. SSC-H = side scatter; FSC-H = forward scatter, FL1-H = fluorescence.



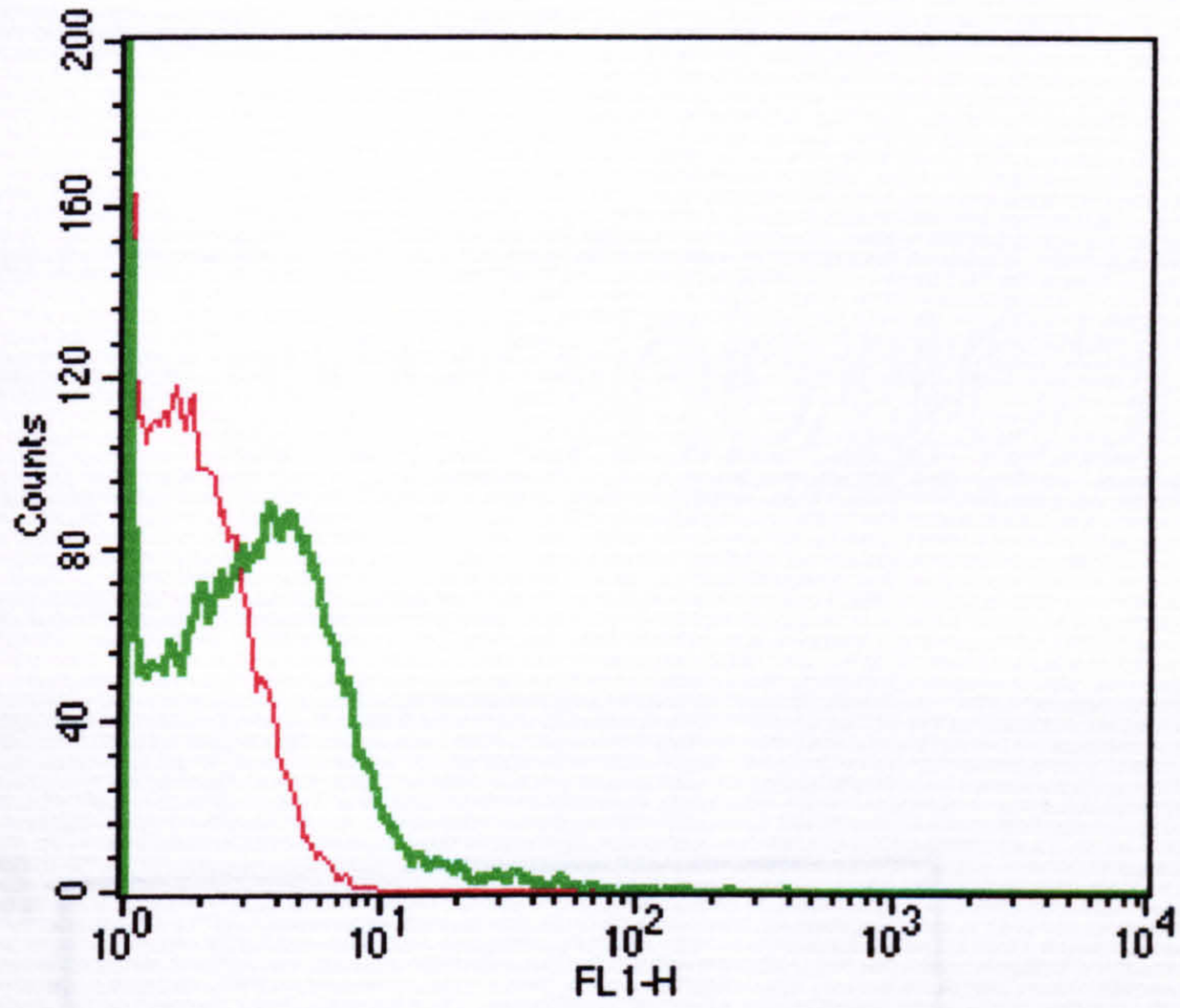
(a)Clone F4 binding to *V .parahaemolyticus* ATCC 27969 (53.5%)



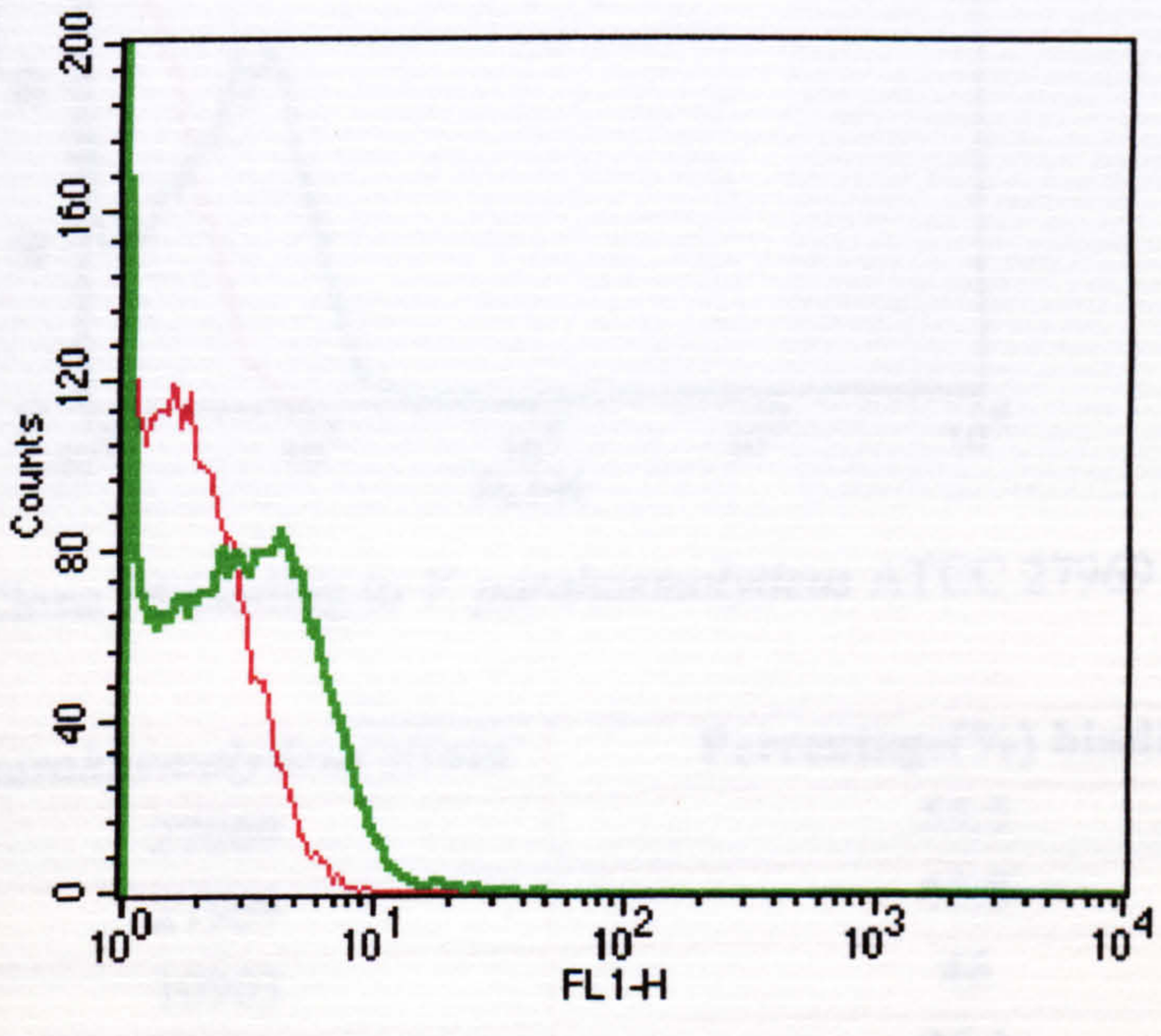
(b)Clone F4 binding to *V. parahaemolyticus* NCTC10903 (46 %)



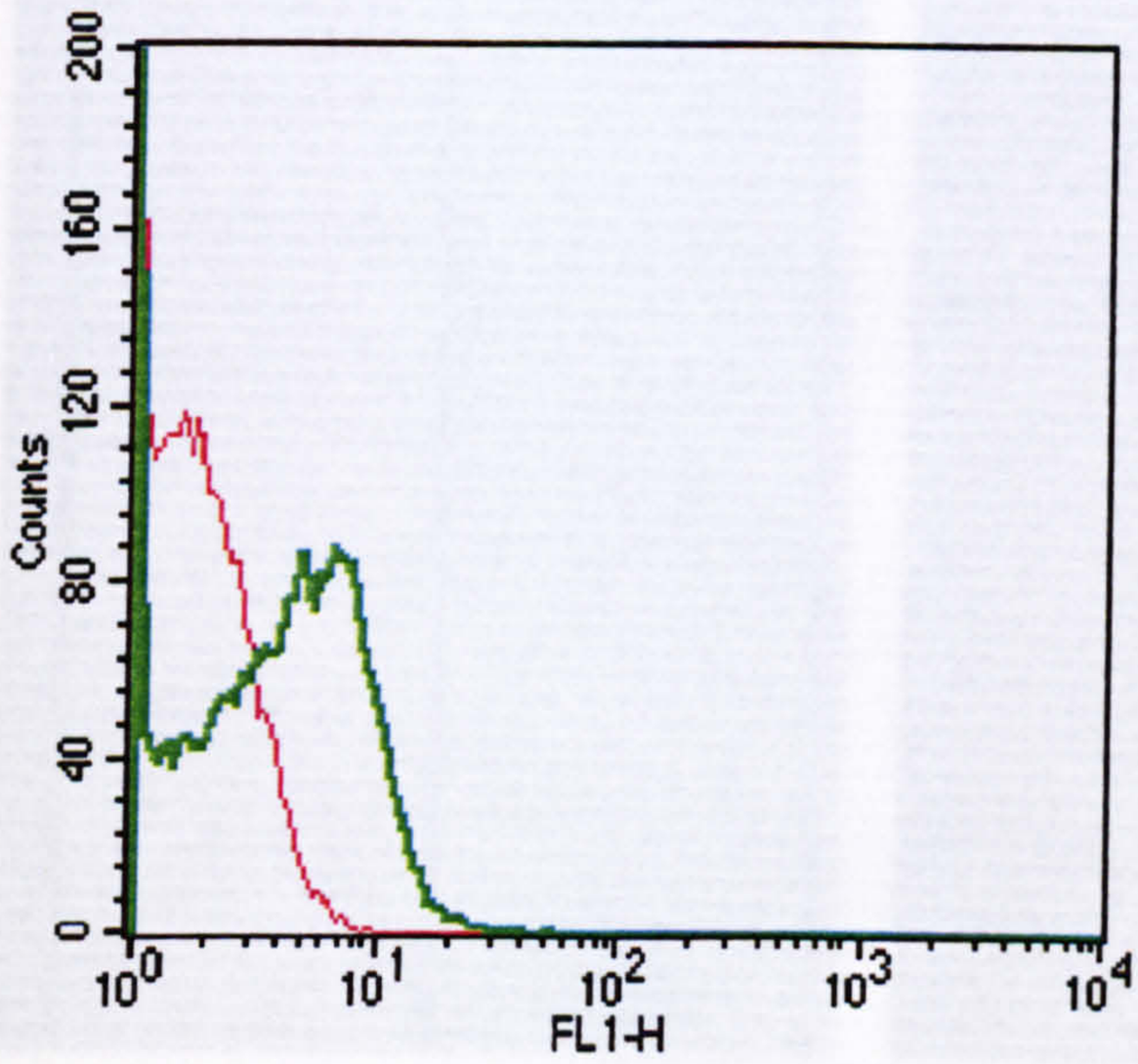
(c) Clone F4 binding to *V. parahaemolyticus* NCTC 10884(27.64%)



(d) Clone F4 binding to *V. parahaemolyticus* NCTC 10903(36.1%)



(e) Clone F4 binding to *V. parahaemolyticus* NCTC 10884 (27.2 %)

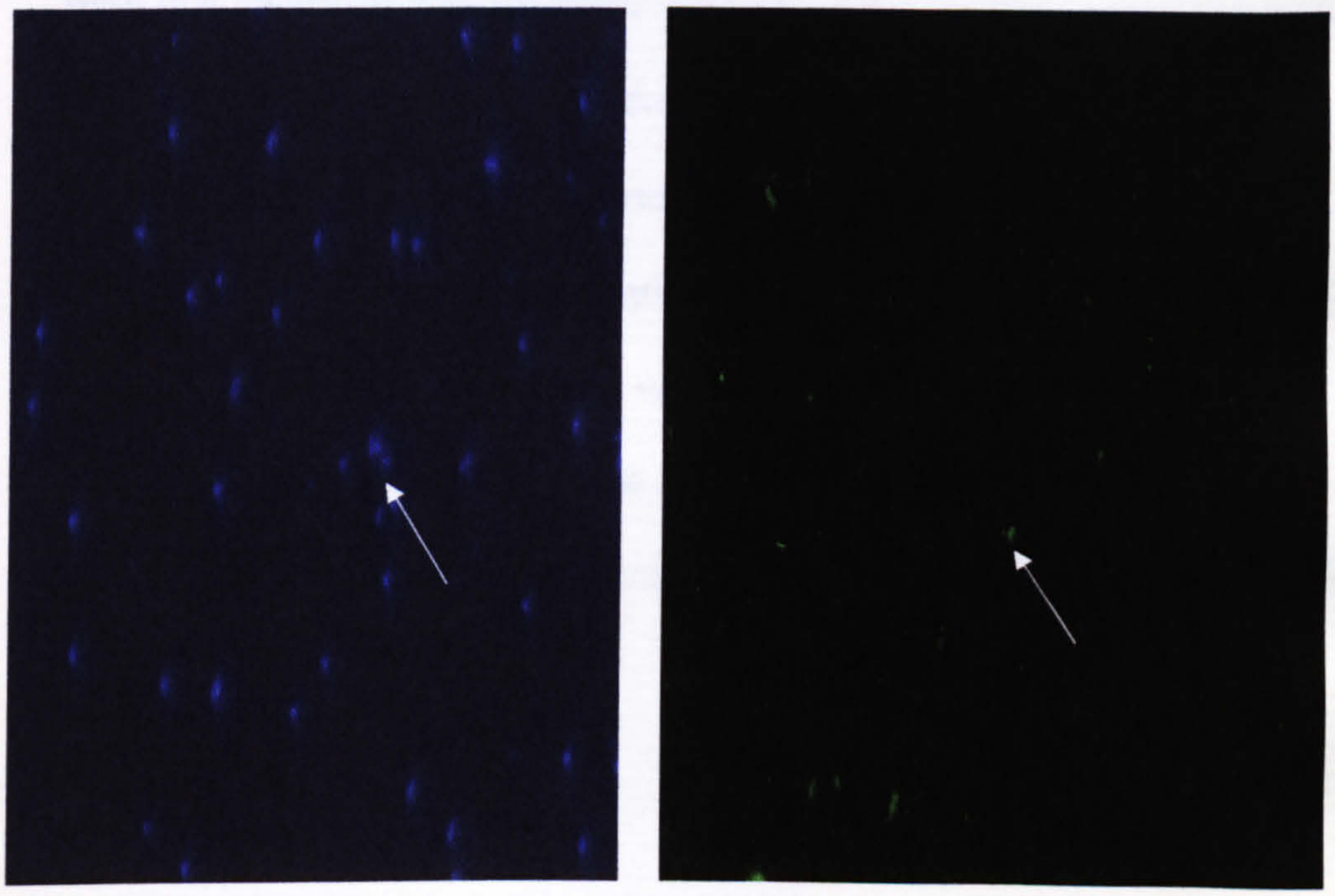


(f)Clone F4 binding to *V. parahaemolyticus* ATCC 27969 (45.8 %)

***V. parahaemolyticus* strain**	**Percentage(%) binding**
27969	53.5
27969	45.8
10903	46
10903	36.1
10884	27.6
10884	27.1

5.5 Fluorescent microscopy:

The clones which showed good binding on FACS were analysed by fluorescent microscopy. The procedure was the same as for flow cytometry. The cells were double stained with FITC and DAPI and observed under a fluorescent microscope. The results are shown in figure 5.10.



(A) (B)

Scale bar - —— = 10 μm

Figure 5.10: Fluorescent microscopy images of phage antibody binding to *V. parahaemolyticus.*

(A) *V. parahaemolyticus* cells stained with the DNA-specific dye, DAPI (1:1000), observed under UV illumination at 455 nm

(B) *V. parahaemolyticus* cells stained with the FITC conjugated phage antibody, F4, specific for *V. parahameolyticus*. The fluorescence was observed under UV illumination at 514 nm.

5.6 Chemiluminescent assay:

Binding of phage to *V. parahaemolyticus* was further analyzed using a chemiluminscent assay and X-ray film. The advantage of using a chemiluminescence assay is that it is much more sensitive than ELISA or fluorescent assay. In addition, it is more feasible to be used as a routine laboratory method than flow cytometry. In this assay, the cells were first incubated with the phage antibody. After washing, secondary antibody (anti-M13-HRP) was added. This was then followed by addition of reagents from the ECL detection kit. The luminescence was recorded using either an X-ray film or a Gel Cam (Polaroid). Plates were done in duplicate and each experiment was repeated three times.

The X-ray film was able to record the luminescence, this is shown in the Figure 5.11 and 5.12 below. The Gel Cam system, however, failed to detect any chemiluminescence, which may be due to the low sensitivity of the Polaroid film used in the system.

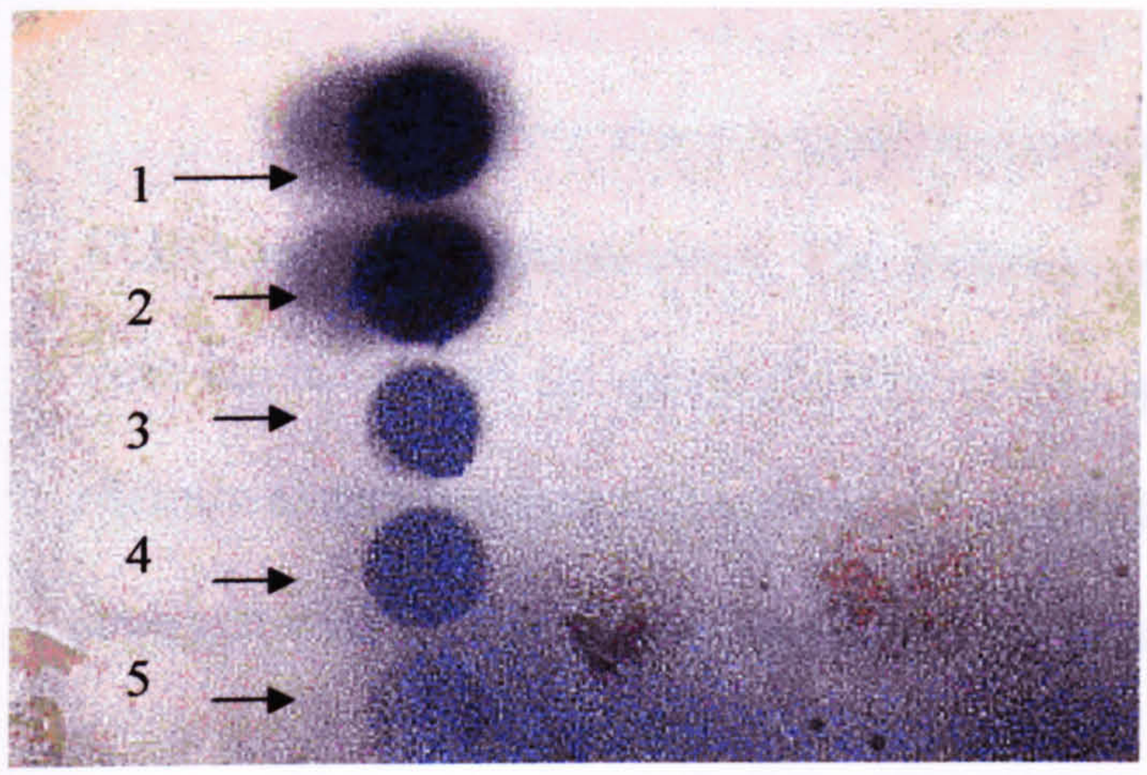


Figure 5.11: Binding of phage antibody clone C11 to *V. parahaemolyticus* and detection using ECL kit and X-ray film. The film was exposed for 5 min at room temperature. Lane numbers represent decreasing concentrations of *V. parahaemolyticus* NCTC 10903.

Lane 1 = 8 x10^8 cfu/ml, Lane 2 = 8 x 10^7 cfu/ml, Lane 3 = 8 x 10^6 cfu/ml

Lane 4 = 8 x 10^5 cfu/ml,Lane 5 = 8 x 10^4 cfu/ml

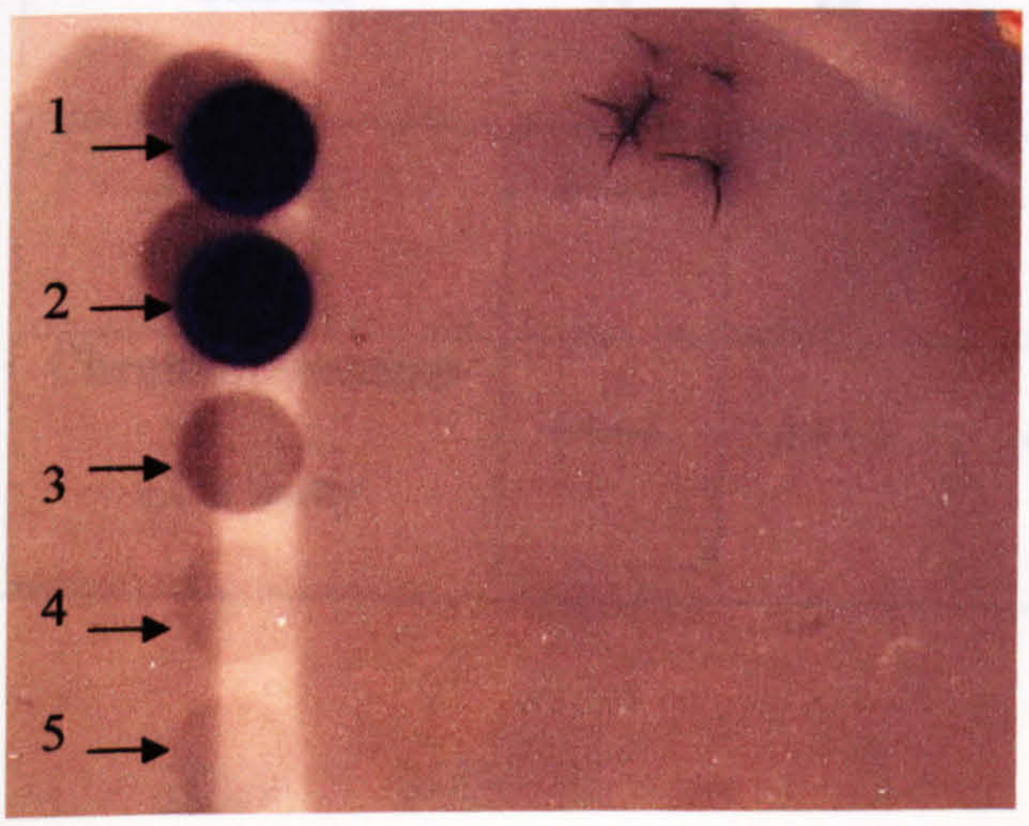


Figure 5.12: Binding of phage clone F4 to *V. parahaemolyticus* and detection using ECL kit and X-ray film. The film was exposed for 5 min at room temperature. Lane numbers represent decreasing concentrations of *V. parahaemolyticus* NCTC 10903.

Lane 1 = 8 x10^8 cfu/ml,Lane 2 = 8 x 10^7 cfu/ml,Lane 3 = 8 x 10^6 cfu/ml

Lane 4 = 8 x 10^5 cfu/ml,Lane 5 = 8 x 10^4 cfu/ml

The table below shows a qualitative comparative analysis of binding using ELISA and chemiluminescence. Five different cell densities of *V. parahaemolyticus* were incubated with 10^{11} phage clones and then analysed by ELISA and chemiluminescence assay. An arbitrary value of four(++++) was assigned to the strongest binding clones, whereas one (+) represents weak binding.

Table 5.6 – Comparative table of ELISA and chemiluminescence. The results below are from incubating *V. parahaemolyticus* NCTC 10903 with 10^{11} ptu/ml clone F4. The plates were done in duplicate and each experiment repeated three times. It shows that the chemiluminescent assay is more sensitive than ELISA in detection of lower numbers of cells.

Cfu/ml	**10^8**	**10^7**	**10^6**	**10^5**	**10^4**	**10^3**
ELISA	++++	+++	++	+	+	-
Chemiluminescene ECL assay	++++	++++	+++	++	++	-

5. 7 Sequencing:

After screening by ELISA, flow ctyometry and fluorescent microscopy, four strong binding clones were obtained. These clones, C11, F4, G4 and H11 were sequenced in the laboratory. However the method did not yield very high quality DNA fragments, and therefore the sequence obtained was fragmented. Thefore, the same samples were purified from PCR products and sent for commercial sequencing (Invitrogen). The results of sequencing for clone G4 and H11 are shown below.

The clones were sequenced with both the forward (LMB3) and the reverse (fd-Seq) primers for the entire scFv region. The DNA sequence was converted to a protein sequence using a translation tool (Expasy translation tool, http://us.expasy.org/tools/dna.html). This was then compared to known antibody exon sequences for the V_H region at: http://www.mrc-cpe.cam.ac.uk/vbase-ok.php?menu=901 . From the data, the DNA encoding for the CDR3 region could be determined.

Figure 5.13: Sequencing data for the CDR3 region of clone G4 and H11. The blue region corresponds to the V_H3 heavy chain and the pink region corresponds to the CDR3 chain.

(A)DNA sequence of the scFv insert of clone G4 with LMB3 primer(5'-3'):

5'
CTATCGCCAGCTTGCATGCAATTCTATTTCAAGGAGACAGTCATAATGAAATACCTATTG
CCTACGGCAGCCGCTGGATTGTTATTACTCGCGGCCCAGCCGGCCATGGCCCACGTGCAG
CTGGTCATGCTGGGGCTGAGGAGAAGGATCCTCTGTCCTCAAAGAAGGTCTCCTTCGAGG
CTTCTGGATACACCTTCACCAACAACTTTATGCACCGTGGTGTGACACGCCCCTGGACGA
GGACTTGAGTGGATGGGATGGATCAATGCTGGCGATGGTAACACAACATATGCACAGAA
CTTCCAGGGAAGAGTCACCATAACTAAGGACACCTCCATGACCACAGACAACGCGGAGC
TGAGCATCCTGAGATCTGAGGACACCGCCGATTATTACTGTGCAAAAAAGGAGGACTGTT
TGGGGCCAAGGAACCCTGGTCACCGTGTCAAGAGGTGGAGGCGGTTCAGGCGGAGGTGG
TTCTGGCGGTGGGCGGATCGCTCTGAGCTGACTCAGGGACCCTGTTGTGGTCTGTGAGCC
TTGGGACAGACACAATTCAAGGAATACTACTTGCCCAGGGAAACAGCCTTCGACAAAGCT
TTTGATGACGAGCGGGGTGACCAGCGAAAAGGCGCAGGAACAAGGCGCCGCTGGAGTAA
CTATTTCAGTATACGATAATTGAGGTAAAAAACACAACCGAGGCACCCTCGATAGGCGG
ATCCCCACGAACACCGACAGTATCTCTTCTGGCTTACGCGCGCGTATCGAAGAAAAGAAC
AACCTGTTCTTTGTGACCAGCAGTCACTATGATGGGCGTGTGCTGGGGCGAGAGAGATAG
AAAGGTGTGAATTTGAGTTGAGTGAGGTAGAGCTACTCTCGCCGCGCAGCGGGAGAGAA
GGTTGATACGCATGTGTGGCATATAGCGCTGCGTCGAGGGAGAAGATCACAGCGTGCTG
GCGCGTCGACTGCACATGCGAGTGGCGACGCGCGCCATAGAAGAAAGAAGACTCCCGCT
CTCTGTGCAAACAGAGAGTACGAGTCGCGGTCGAGTCGCGCGGCACGAGAACGCATATG
TGTATAGAGGTGTGTGTGACGTAGAACACCACTCGCGCACAGAGTAAATGCTTGTGTTGT
CACAGAAGCTCGCGGAGCACGAGAGTGAG-3'

B) DNA sequence of the scFv insert of clone H11 with LMB3 (5'-3'):

ATATCGCAGCTTGCATGCAATTCTATTTCAGGAGACAGTCATAATGATATACCTATTGCCT
ACGGCAGCCGCTGGATTGTTATTACTCGCGGCCCAGCCGGCCATGGCCCAGGTGCAGCTG
AGGAGTCGGGCCCAGGACTGGTGAAGCCTTCGGAGACCCTGTCCCTCACCTGCGTTGTCTC
TGGTGGCTCCATCAGCAGTAGTAACTGGTGGAGCTGGGTCCGCCAGCCCCCAGAGGAAGG
GGCATGGAGCTGGATTGGGGTAAATCTATCATCACGTGGGAGCCCCAACTACTAAGCCCG
GGATACCGCTCAAGAGCTACGAGTCTACCATCATCAGCTAGCACATGATGTTGCAGGTAA
CCGAGGGTTTCTCCCTGGAGAGCGTGTACTCTCTTGTGTACGCTGCCCGCGAACAACATCG
CGGTCGCACGCTGGTATATATCTGTGCGAACGAAAGATATAGGTATGAGGCTAGCTACGA
CAGCCGACACGCAGCGCGATAGCTCGACCTCGTAGATGTGCTACGGGCTCGCTCATGAGA
AGATGTGTATATAGATCGATAGCATATGATGACTCGAGAGACGAATTGCTTGTAGCCATC
GCGCTCACGAGCAGTGCGCAGGCGGCGCACATGGAGGGGGAGGTAGAGAGACTGTGTAC
ATGATGGATGCGAGATGGGGCGCTGCACACGCAGAATGTGCATGAGAATATCCAGCATGC
CACAATCTACCGCACGACGACACGATGCGCATTATTACGTGAGACTCGTGAGTACGCGAT
CGAGTGAGTAGCGCGAACATTATCATGTATGACGCACGATAGCGTTAGTGTACGCAAGCA
GTGGACGGATGTGCGGAGACTGAGGAGATAAAGATGTAAGCTTGAGAGAGATGACGTAT
GAAGTAATAGTACAGAGTC

Chapter 6

Results: Detection of V. *parahaemolyticus* in seafood

6.1 Introduction :

V. parahaemolyticus is frequently associated with gastroenteritis resulting from the ingestion of undercooked shellfish, particularly oysters (Doyle, 1998). One of the aims of this study was to develop a rapid method to detect this organism in contaminated shellfish. *Crassostrea gigas*, the Pacific oyster, was used in a series of experiments carried out to determine recovery of *V. parahaemolyticus* from *in vitro* contaminated oyster tissue. The recovery of cells tested by both PCR analysis and phage display method. In addition, the sensitivity and specificity of both methods were compared.

6.2 Detection by PCR:

Oyster tissue is known to contain several inhibitors (enzymes) (Lewis *et al.*, 2000) of the PCR reaction. Therefore additional steps have to be used to purify the DNA samples before it can be used in PCR.

In this experiment, cultures to be tested were inoculated into oyster tissue. They were then recovered by centrifugation and used for testing by PCR. The detailed protocol has been explained in **section 2.4.** DNA extraction from *V. parahaemolyticus* cells, which were recovered from the seeded oyster tissue, was carried out initially using proteinase K/lysozyme extraction, chelex extraction and the boiling method (**section 2.3**). The PCR was carried out using the *toxR* primers. The results of PCR are shown below in Figure 6.1.

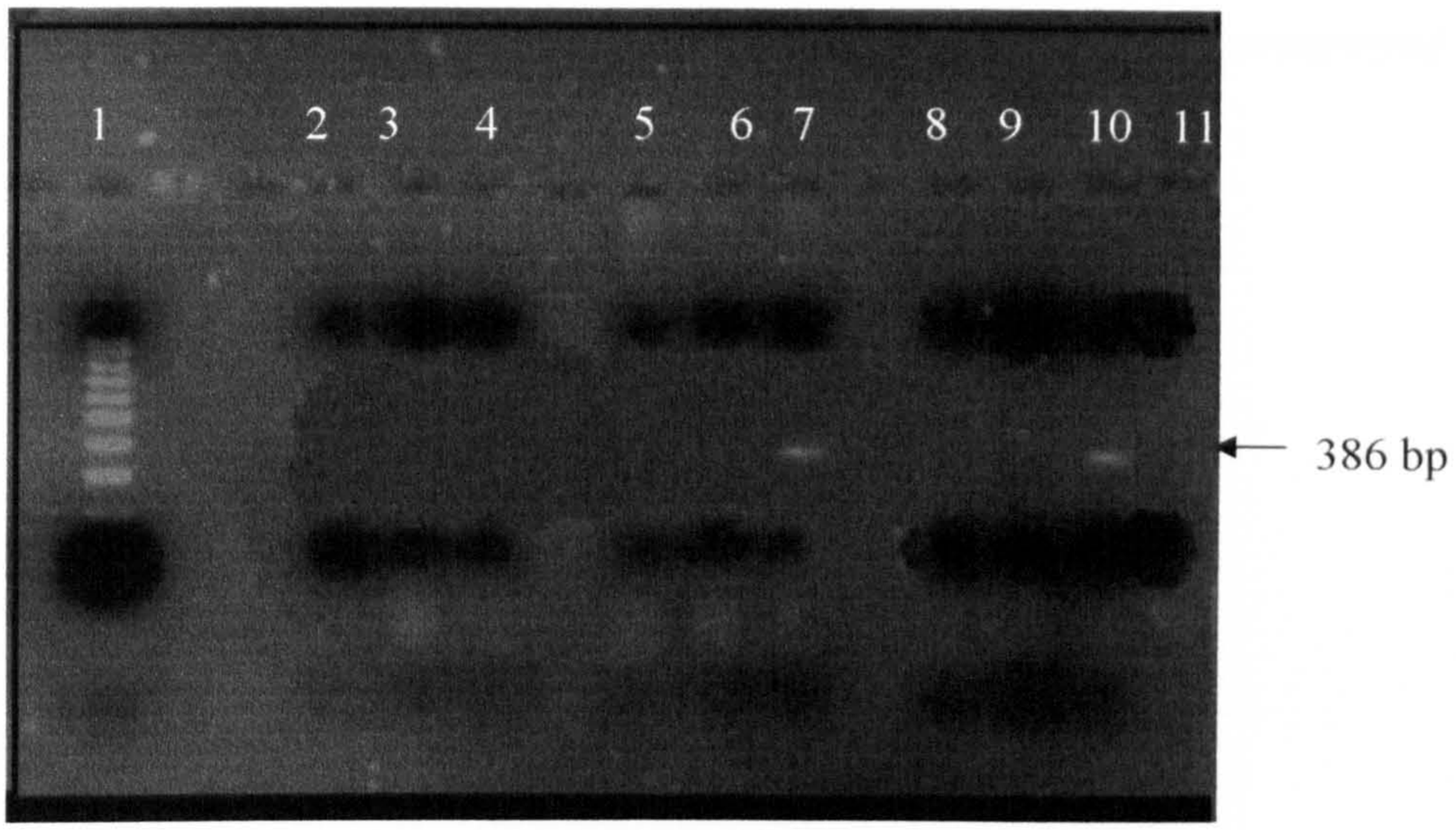


Figure 6.1: DNA extraction from cells of *V. parahaemolyticus* using three different extraction methods; followed by *toxR* PCR

Lane 1 = 100 bp ladder

Lane 2-4 = samples extracted by proteinase K/lysozyme method -Lane 2 = *V. parahaemolyticus* NCTC 10884, Lane 3 = *V. parahaemolyticus* NCTC 10441, Lane 4 = *V. parahaemolyticus* NCTC 17802.

Lane 5-7 = samples extracted by boiling method - Lane 5 = *V. parahaemolyticus* NCTC 10884, Lane 6 = *V. parahaemolyticus* NCTC 10441, Lane 7 = *V. parahaemolyticus* NCTC 17802.

Lane 8-10 = samples extracted by Chelex method - Lane 8 = *V. parahaemolyticus* NCTC 10884, Lane 9 = *V. parahaemolyticus* NCTC 10441, Lane 10 = *V. parahaemolyticus* NCTC 17802.

As can be seen from the results, the PCR was positive only by the boiling and chelex method for *V. parahaemolyticus* NCTC 17802 and did not work with the other cultures. As can be seen in Figure 4.6, **section 4.4** the *toxR* PCR is positive with all pure cultures of *V. parahaemolyticus*. The lack of amplification in PCR in this sample may be due to inhibitors of PCR found in the oyster tissue, which have not been completely removed by the methods used for DNA extraction. To overcome this effect,

DNeasy columns were used for purification of DNA once the cells had been recovered from the oyster tissue (**section 2.4.1**). The results are shown in Figure 6.2.

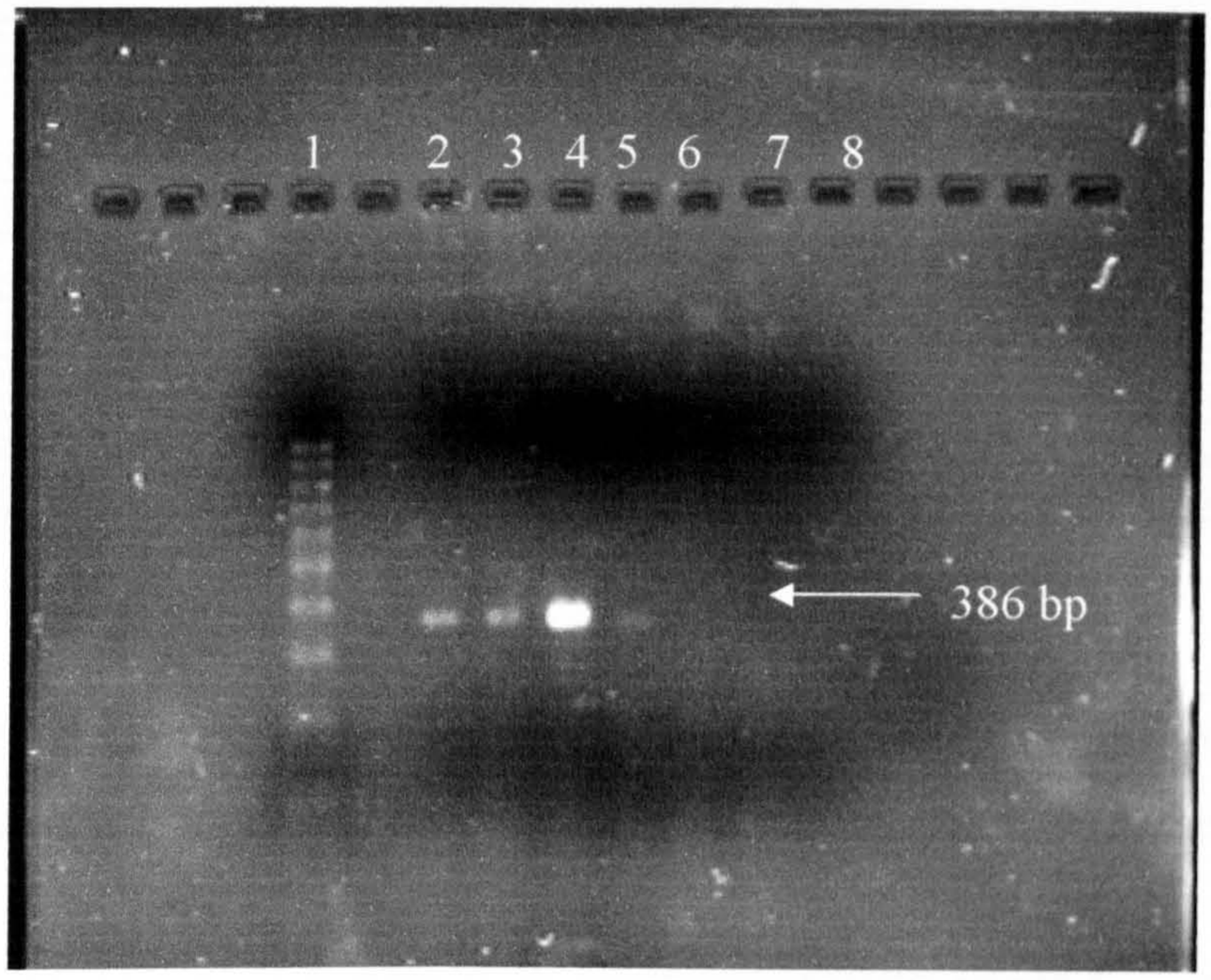


Figure 6.2: *toxR* PCR of DNA extracted using DNeasyn columns.
Lane 1 = 100 bp ladder, Lane 2 = *V. parahaemolyticus* NCTC 10903, Lane 3 = *V. parahaemolyticus* NCTC 10884, Lane 4 = *V. parahaemolyticus* NCTC 10441
Lane 5 = *V. parahaemolyticus* ATCC 27969, Lane 6 = *V. mimicus* NCTC 11435
Lane 7 = *V. alginoyticus* NCTC 12160, Lane 8 = negative control

After using the DNeasy columns, the PCR was positive for all the cultures of *V. parahaemolyticus*. To check the sensitivity of the method, the DNA sample was diluted 3-fold and the PCR was repeated again

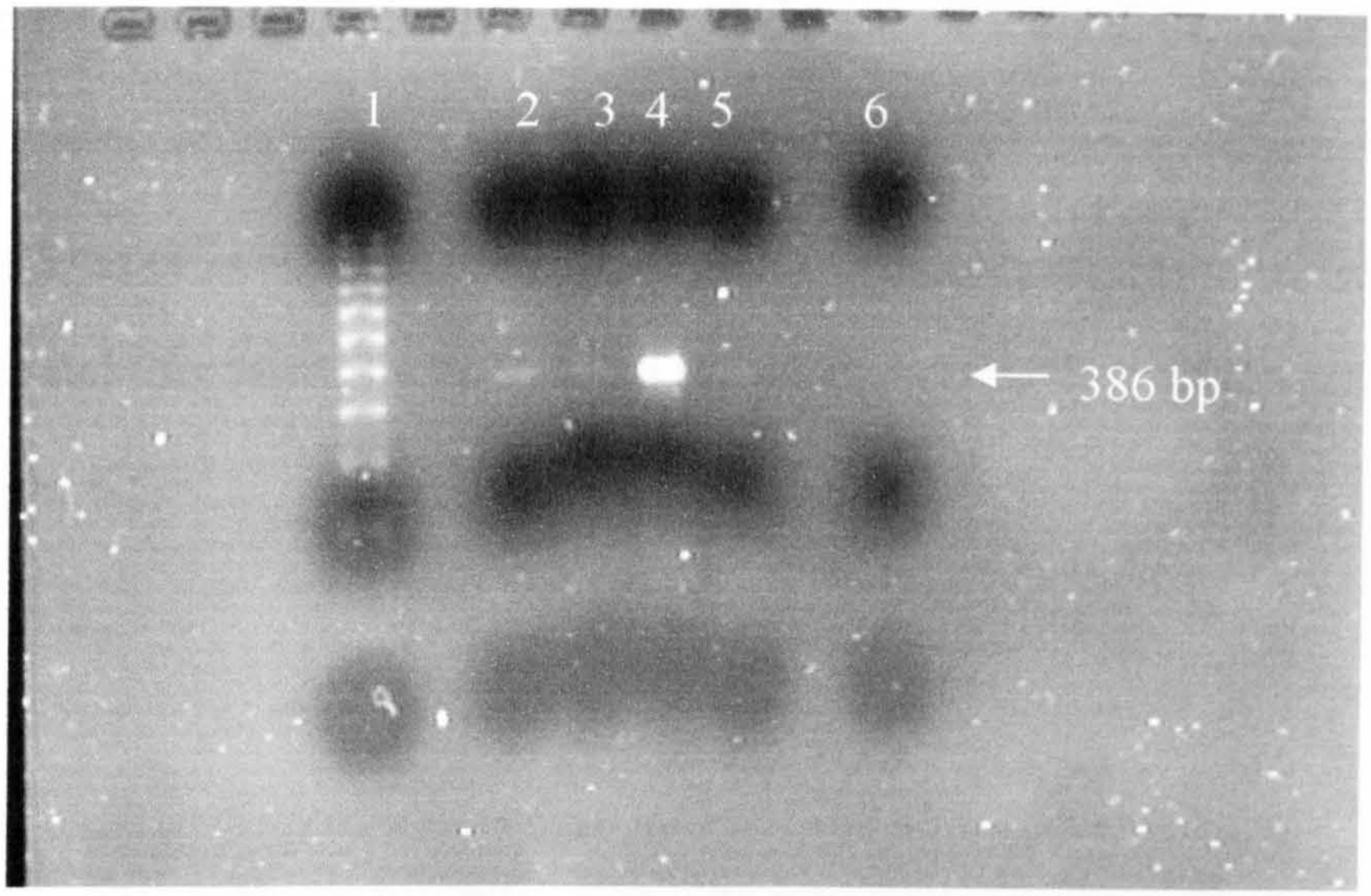


Figure 6.3: PCR after DNA extraction using DNeasy columns from cells of *V. parahaemolyticus* . The samples used were 3-fold dilutions from the samples used in Figure 6.2.

Lane 1 =100 bp ladder, Lane 2 = *V.parahaemolyticus* NCTC 10903,Lane 3 = *V. parahaemolyticus* NCTC 10884,Lane 4 = *V. parahaemolyticus* ATCC 27969 Lane 5 = *V. parahaemolyticus* NCTC 10441, Lane 6 = negative control.

The PCR was positive for all strains of *V. parahaemolyticus*. Though exact DNA concentrations were not determined, the results show that the Dneasy method can be used over a range of DNA concentration.

The subsequent experiments were carried out to find out the lowest cell density required for detection *in vitro*. Culture suspensions of *V. parahaemolyticus* NCTC 10903 of varying cell densities (from 10^1 to 10^8 cfu/ml) were incubated with the oyster homogenate and then tested by PCR, using the *toxR* primers. The results are shown in Figure 6.4 and 6.5.

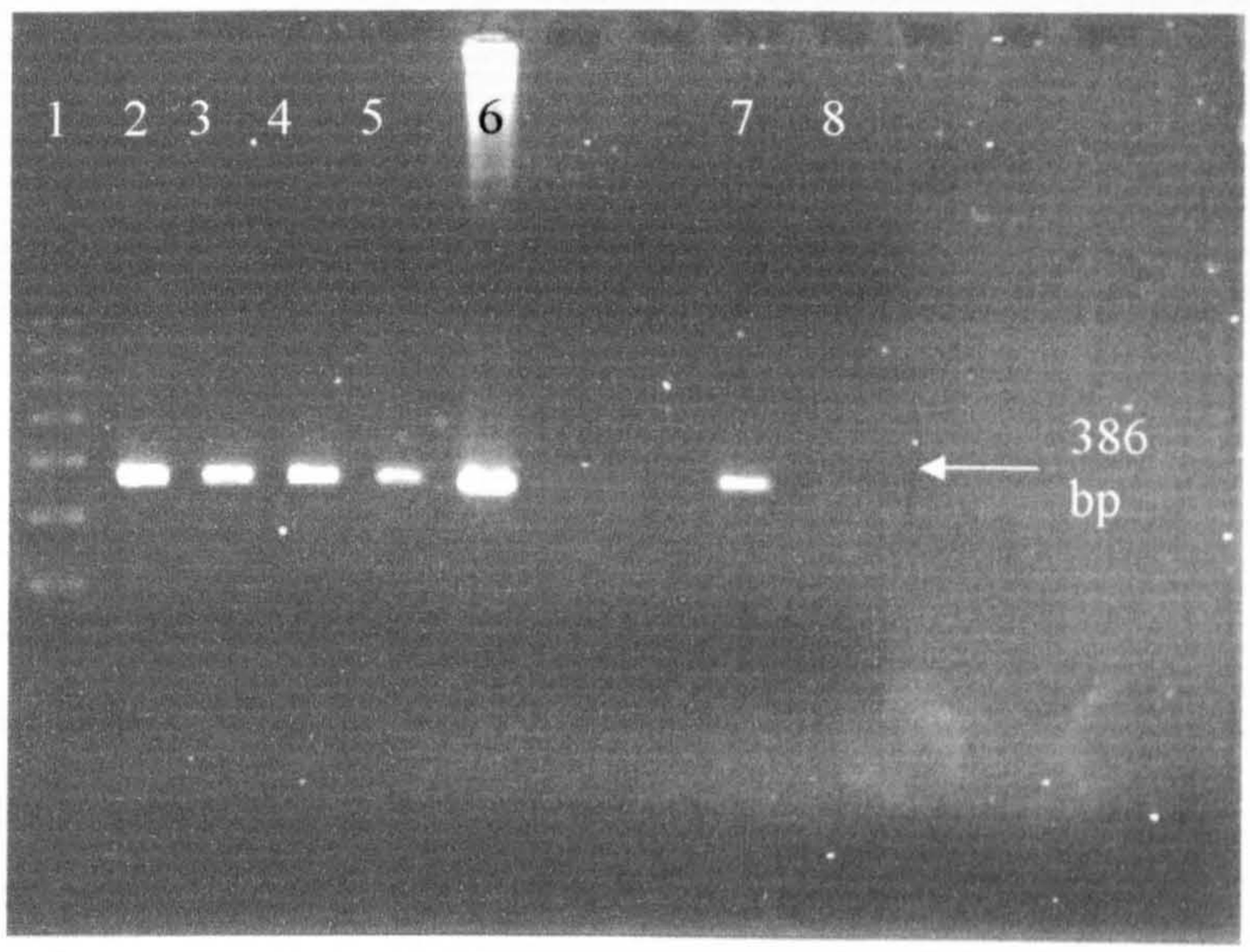


Figure 6.4: Seeding oyster tissue with different culture densities of *V. parahaemolyticus* NCTC 10903. DNA was extracted using DNeasy columns and cells detected by *toxR* PCR. Culture densities are shown below:

Lane 1 = 100 bp ladder,Lane 2 = 16×10^8 cfu/ml,Lane 3 = 8×10^8 cfu/ml

Lane 4 = 4×10^8 cfu/ml,Lane 5 = 2×10^8 cfu/ml,Lane 6 = 10^8 cfu/ml

Lane 7 = 5×10^7 cfu/ml,Lane 8 = negative control.

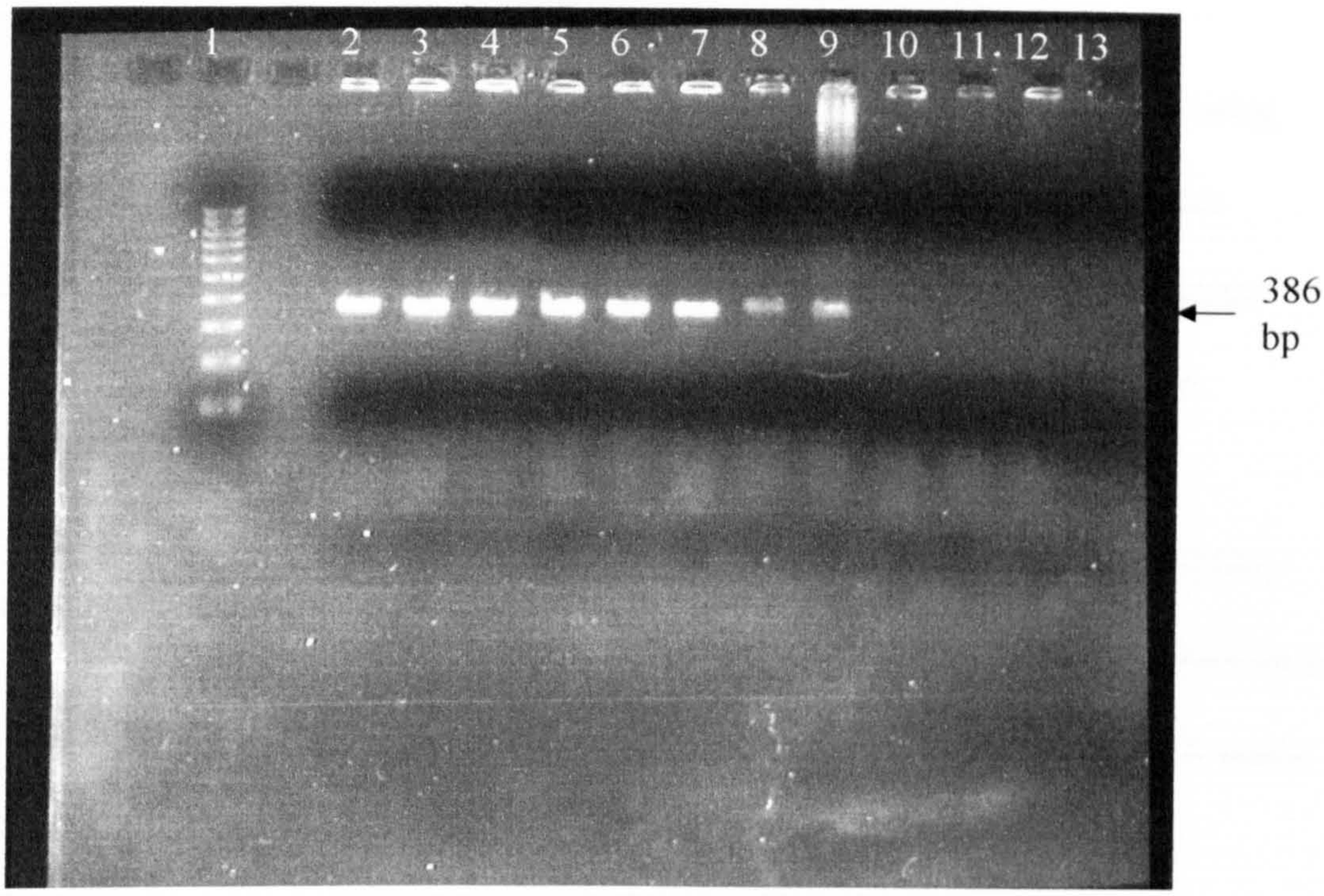


Figure 6.5: PCR using different culture densities of *V. parahaemolyticus* NCTC 10903. Culture densities are shown below.

Lane 1 = 100 bp ladder, Lane 2 = 2 x 10^7 cfu/ml, Lane 3 = 2 x 10^6 cfu/ml,
Lane 4 = 10^6 cfu/ml, Lane 5 = 5 x 10^5 cfu/ml,Lane 6 = 2.5 x 10^5 cfu/ml,
Lane 7 = 6 x 10^4 cfu/ml, Lane 8 = 3 x 10^4 cfu/ml, Lane 9 = 10^4 cfu/ml,
Lane 10 = 5 x 10^3, Lane 11 = 2.5 x 10^3 cfu/ml,
Lane 12 = 10^3 cfu/ml ,Lane 13 = negative control.

The PCR was positive for a cell density of 10^4 cfu/ml without enrichment. There is a report in literature that short enrichment period in liquid media can increase recovery of cells for PCR (Lee *et al.*, 1995). Therefore, the *V. parahaemolyticus* cells were incubated in liquid media for 1-12 h after recovery from oyster tissue. It was found that an enrichment step of 3 hours increased the sensitivity of the PCR assay to 10 cfu/ml.

6.2 Detection by Phage Antibody Display:

The recovery of *V. parahaemolyticus* from seeded oyster tissue was also tested using phage display method. ELISA was used to detect *V. parahaemolyticus* cells after recovering the organisms from oyster tissue, since it is a method which allows us to screen a large number of clones in a short period of time. The protocol has been outlined in **section 3.8.**

Similar to PCR, initial results did not show a strong positive signal for ELISA. This may be due to presence of ELISA inhibitors in the oyster homogenate. Therefore a pre-enrichment step was incorporated into the protocol, similar to that in the PCR assay, i.e., the cultures were incubated with the oyster homogenate and recovered by centrifugation. They were then incubated in liquid media for various time intervals, ranging from 1 - 12 h. The cells were recovered again by centrifugation and used for ELISA with the phage antibodies. The results are shown in Table 6.1.

Table 6.1: Recovery of V. *parahaemolyticus* NCTC 10903 (initial cell density = 8×10^8 cfu/ml) from seeded oyster tissue using ELISA as the detection method. Phage antibody was added at a concentration of 10^{11} ptu/well. ABTS was used as the developing reagent and the optical density was measured at A_{630}-A_{405}.Anti-NIP was used as the blank. Each clone was tested in duplicate and the experiment was repeated at least three times.

Clone	Pure culture	Culture incubated with oyster tissue			
		0 h	3 h	6 h	24 h
A9	+	+	++	++	++
A12	++	+	+++	+++	++
B5	+	+	++	++	+
C11	+++	+	+++	++	+
D6	++	+	++	++	+
F4	+	+	+++	+++	++
G4	+++	+	++	+++	+++
H11	+++	+	+++	+++	++
H12	+	+	++	++	+

Key: + = 0.25-0.5 OD, ++ = 0.5 -1.0 OD, +++ = >1.0 OD. OD= Optical density.

Optical density was measured at 600nm.

The table shows that all the clones show binding in the absence of oyster tissue. When cells are tested after being recovered from oyster tissue (0 h), the readings are lower than the initial binding. The binding increases again when cells are allowed to recover with an enrichment step. At a cell density of 8×10^8 cfu/ml,at 3 and 6 h

incubation there are sufficient cells to give a strong ELISA signal, which becomes weaker after a 24 h incubation.

Table 6.2: Recovery of V. *parahaemolyticus* NCTC 10441 (initial cell density = 8×10^8 cfu/ml) from seeded oyster tissue using ELISA as the detection method. Phage antibody was added at a concentration of 10^{11} ptu/well. ABTS was used as the developing reagent and the optical density was measured at A_{630}-A_{405}.Anti-NIP was used as the blank. Each clone was tested in duplicate and the experiment was repeated at least three times.

Clone	Pure culture	Culture incubated with oyster tissue			
		0 h	3 h	6 h	24 h
A2	+	+	+++	+	+
A4	++	+++	++	+++	++
B7	+++	++	+++	+	++
B9	+	+	+++	+	+
C11	+	+	+++	+	++
F4	+++	++	+++	+++	+++
F9	+	+	+++	+++	+
G4	+	+	+++	+++	+++
H11	+	+	+++	++	+

Key: + = 0.25-0.5 OD, ++ = 0.5 -1.0 OD, +++ = >1.0 OD. OD= Optical density.

Optical density was measured at 600 nm.

Table 6.3: Recovery of V. *parahaemolyticus* ATCC 27969 (initial cell density = 8×10^8 cfu/ml) from seeded oyster tissue using ELISA as the detection method. Phage antibody was added at a concentration of 10^{11} ptu/well. ABTS was used as the developing reagent and the optical density was measured at A_{630}-A_{405}.Anti-NIP was used as the blank. Each clone was tested in duplicate and the experiment was repeated at least three times.

Clone	Pure culture	Culture incubated with oyster tissue			
		0 h	3 h	6 h	24 h
A2	++	+	+++	++	++
B4	++	++	+++	++	++
C5	++	+	+++	++	++
C11	++	+	+++	+	+
D8	+	+	++	+	+
F4	++	+	+++	++	++
F7	++	++	+++	++	++
G4	+++	+	+++	+++	+++
H11	++	+	++	++	++

Key: + = 0.25-0.5 OD, ++ = 0.5 -1.0 OD, +++ = >1.0 OD. OD= Optical density.

Optical density was measured at A_{600}.

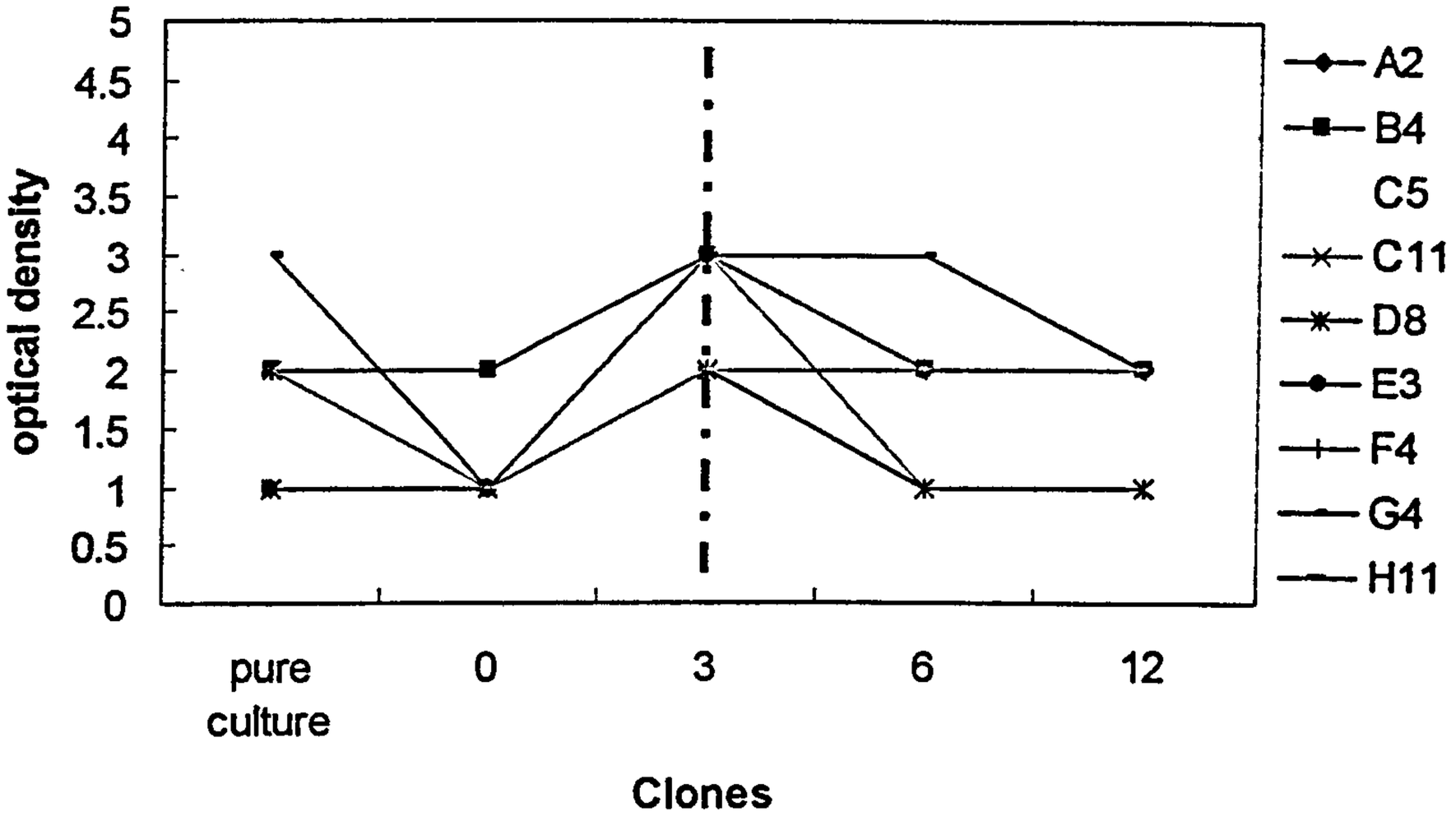


Figure 6.6: ELISA before and after enrichment of *V. parahaemolyticus* ATCC 27969. The cells were inoculated in oyster homogenate,then recovered by centrifugation. ELISA was carried out after 0 h to 12 h pre-enrichment step in liquid media. The data shows that the OD increases from 0 h to 3 h, then drops for some clones at 6 h and for most clones at 12 h, indicating that a 3 hr enrichment is optimum for this ELISA. The dashed line (‾ · ‾) indicates the period of maximum growth (3 h) for most strains.

Similar experiments were carried out for the other strains of *V. parahaemolyticus* and non-parahaemolyticus strains. It was seen that even a 3 h enrichment greatly improved the detection of the cells by ELISA.

In addition, the sensitivity of the assay was also tested by using different culture densities of *V. parahaemolyticus* NCTC 10903, 10884 and 10441 and ATCC 27969 and 17802. The same protocol was followed as outlined in **section 3.8.** The cultures were enriched in liquid media for different time periods from 0 h to 12 h. The results for *V. parahaemolyticus* NCTC 10903 are shown in the Table 6.4.

Table 6.4: Recovery of *V. parahaemolyticus* NCTC 10903 (initial cell density as outlined in the table) from seeded oyster tissue using ELISA as the detection method. Phage antibody was added at a concentration of 10^{11} ptu/well. ABTS was used as the developing reagent and the optical density was measured at A_{630}-A_{405}. Anti-NIP was used as the blank. Each clone was tested in duplicate and the experiment was repeated at least three times.

Clone	Cell density (cfu/ml)	Time of incubation		
		3 hours	6 hours	24 hours
A9	10^6	+	++	++
	10^5	+	+	++
	10^4	-	+	++
B5	10^6	+	++	++
	10^5	+	++	+
	10^4	-	++	+
C11	10^6	+++	++	+
	10^5	+++	++	+
	10^4	++	+++	++
D6	10^6	+	++	++
	10^5	+	+	++
	10^4	-	-	++
E1	10^6	+	++	++
	10^5	+	+	++
	10^4	+	+	+
F4	10^6	+++	++	++
	10^5	++	+++	++
	10^4	++	++	++
G4	10^6	+	++	+++
	10^5	+	+++	+++
	10^4	+	++	+++
H11	10^6	++	++	++
	10^5	++	+++	+++
	10^4	+	+++	+++

Key: - = ≤0.25 OD, + = 0.25-0.5 OD, ++ = 0.5 -1.0 OD, +++ = >1.0 OD. OD= Optical density. Optical density was measured at A_{600}.

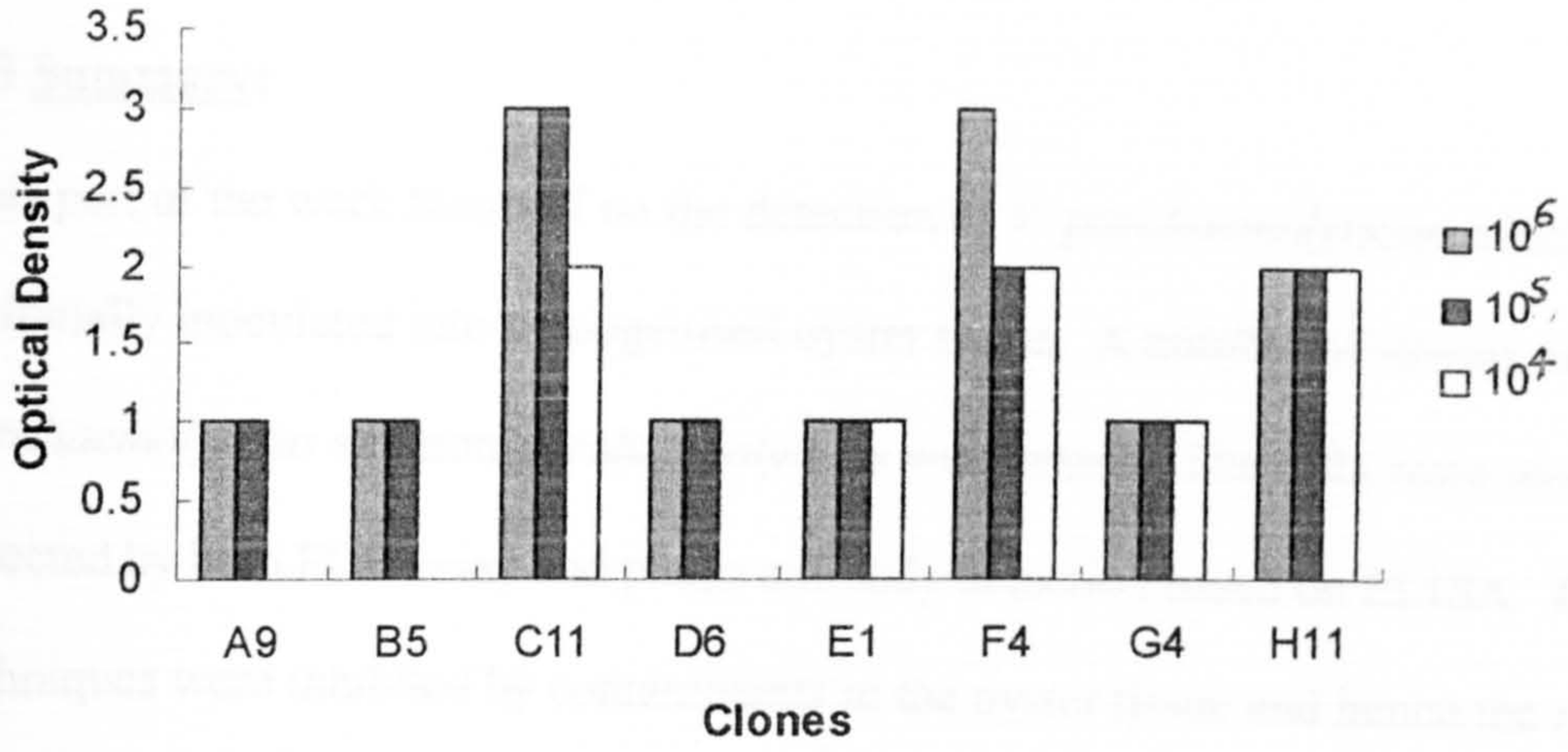


Figure 6.7: Readings of ELISA over different cell concentrations of *V. parahaemolyticus* NCTC 10903, from 10^6 to 10^4 cfu/ml, after recovery from seeded oyster sample. ELISA was carried out after 3 h enrichment step in APW.

The data shows that initial cell density has an influence on the outcome of ELISA. For example, clone A9, B5 and D6 do not detect *V. parahaemolyticus* when the initial inoculum is 10^4 cfu/ml. However, clones C11, E1, F4, G4 and H11 can detect *V. parahaemolyticus* cells at this density.

6.3 Summary:

This part of the work focussed on the detection of *V. parahaemolyticus* which was artificially inoculated into homogenised oyster tissue. A number of strains of *V. parahaemolyticus* and non-*parahaemolyticus* were tested. The cells were recovered and detected by both PCR assay and phage antibody detection based on ELISA. Both the techniques were inhibited by contaminants in the oyster tissue and hence the method had to be adapted specifically for oyster tissue. For PCR, DNA was purified using a DNeasy column and then used for the assay. In ELISA, the cells were first inoculated in liquid media for 3 h and then incubated with phage antibodies for detection. PCR was able to detect as low as 10^4 cfu/ml of *V. parahaemolyticus* without enrichment, and an enrichment of 3 h allowed the detection of numbers as low as 10 cfu/ml. The ELISA method allowed detection of *V. parahaemolyticus* numbers as low at 10^4 cfu/ml after a 3 h enrichement. Clones C11, F4, G4 and H11 retained their binding capacity in the presence of inhibitors from oyster homogenate. They also showed a positive ELISA result at a cell density of 10^4cfu/ml. Therefore, this method has the potential to be adapted in routine laboratory use for the detection of *V. parahaemolyticus* from contaminated oyster or shellfish samples.

Chapter 7

DISCUSSION

V. parahameolyticus has been implicated in cases of food-poisoning related to the consumption of raw oysters and shellfish (Alam *et al.*, 2002; Arakawa *et al.*, 1999; Bag *et al.*, 1999; Blake *et al.*, 1979 and Fujino *et al.*, 1951). A recent report by the Texas Department of Health (Disease Prevention News, 1999) suggests that much of the morbidity associated with *V. parahaemolyticus* could have been avoided by reducing delays in the surveillance system. Conventional methods of detection are generally time consuming and laborious and can take up to four days for positive identification (Elliot *et al.*, 1992 ; Kaysner *et al.*, 1992). This time delay is crucial in identifying contaminated sea food samples and preventing an outbreak. Therefore there is a need to develop a rapid and sensitive detection method for this organism.

The PCR technique, which is a rapid method, has been used with some success in the detection of *V. parahaemolyticus*. However, cross-reactivity with closely related strains is a drawback of PCR. The aim of this project was to develop a novel rapid and sensitive method, using bacteriophage antibody display, for the detection of *V. parahaemolyticus* from oyster tissue, and to compare this with the standard PCR detection methods. Bacteriophage display has been shown previously to distinguish between cell surface markers in different eukaryotic cell types (de Nardo *et al.*, 1999; Roovers *et al.*, 2001 ; Schier *et al.*, 1995) but few attempts have also been made to isolate antibodies that distinguish between surface markers on bacterial cells (Boel *et al.*, 1998 ; de Greef *et al.*, 2000; Srivastava, *et al.*, 2000). In the present study, phage clones were isolated, which could differentiate between *V. parahaemolyticus* and non-*V. parahaemolyticus* cell surface markers. These clones were also then tested successfully in seeded oyster tissue. This is the first report of its kind where bacteriophage antibody technology has shown to have a potentially diagnostic application in medical bacteriology.

Panning and screening of phage library by ELISA for *V. parahaemolyticus* specific clones yielded four strong clones -C11, G4, F4 and H11. Flow cytometry confirmed that these clones showed strong binding to *V. parahaemolyticus* and did not cross-react with other non-*parahaemolyticus* strains. An assay was carried out to determine if these clones retained their binding specificity in *in vitro* contaminated shellfish samples. Oyster tissue was seeded with the range of *Vibrio* spp. cultures and their recovery and detection tested using the four phage antibody clones. The results showed that, using the phage antibody technique, cells of *V. parahaemolyticus* could be detected by ELISA, after they had been seeded into oyster tissue. In addition, the ELISA was modified such that the secondary antibody was detected by a using a chemiluminescent dye, which increases the sensitivity of the assay. In literature it has been shown that the use of chemiluminescent dyes greatly increases the sensitivity of the assay (Kolbert *et al.* 1995; Okwumabua *et al.* 1992; Youmans *et al.*, 1993). In the present study, light emission was captured using a light-sensitive film, and an immediate comparison can be made between the standard and unknown cell densities, or between the presence and absence of *V. parahaemolyticus* cells. This technique is therefore more feasible to be used for routine laboratory analysis as it eliminates the need for an ELISA reader, while at the same time retaining the advantages of an ELISA assay. The detection method then becomes a two step process, with ELISA as the first stage, followed by exposure to some kind of capture film, like an X-ray film.

Different cell densities were also standardized for the chemilumenescent assay. In general, the sensitivity of the assay depends on the sensitivity of the capture film. A sensitive capture film can detect a weak signal over a longer time period. We used two types of capture films, HyperHCL which is an X-ray film, and a Polaroid black and white film. In our assays, *V. parahaemolyticus* cell densities of 10^4/ml gave a good

readable signal using the HyperHCL film, whereas the Polaroid film could not capture the emitted light. In standard ELISA technique, using a colour dye, at least 10^8 cfu/ml of *V.parahaemolyticus* was needed to give reproducible results. The use of a sensitive capture film thus enhanced the sensitivity of the assay. It is possible that if a more sensitive film is used, the detection limits for ELISA will be lowered. This range is lower than that of PCR using standard techniques; however, the use of chemilumenescene dyes eliminates the need for radioactive probes or expensive PCR enzymes.

It has been reported in literature that environmental conditions can impose a variety of stresses on bacteria (Baker *et al.*, 1983). In their natural environment, marine bacteria, including a number of marine *Vibrio* spp. and *V. parahaemolyticus*, are subjected to the stresses of nutrient starvation and low temperatures in the cold season. To adapt to these stresses, these bacteria enter a state known as the viable-but-nonculturable (VBNC) state (Jiang and Chai, 1996; Oliver *et al*, 1991). The role that these cells might play in pathogenesis has still not been fully elucidated. Cells in the VBNC state cannot form colonies on plates, but are still metabolically active (Colwell and Huq, 1994). When appropriate conditions are restored, these cells can revert back to actively growing cells (Colwell, 1996; Whitestides and Oliver, 1997). One of the ways in which resuscitation of these cells may be achieved is by a short enrichment period in nutrient rich media. Normally a 3-6 h period of enrichment is sufficient for recovery of these cells for subsequent culture work or PCR analysis (Lee *et al.*, 1995).

Niederhauser *et al.* (1992) proposed that, in contrast to fully viable food-borne bacterial pathogens seeded *in vitro* in food samples, food-borne bacteria in the natural food samples have reduced viability. In our study, *V. parahaemolyticus* and non-*parahaemolyticus Vibrio* spp. were seeded in oyster tissue and then recovered for analysis. A 3 h enrichment in APW was used before testing the recovery of cells by

PCR and ELISA. When testing naturally occurring contaminated seafood samples, this 3 h enrichment may be important for the recovery of stressed *Vibrio* spp. in the VBNC state. When seafood, including shellfish, is harvested from the ocean, they are normally stored at low temperature conditions till they reach the retail market. These storage conditions may induce the VBNC state in *Vibrio* spp (Oliver and Wanucha, 1989). Over reliance on conventional agar plating methods for routine analysis of seafood may mean that VBNC bacteria may not be detected, though they may still be potentially pathogenic (Colwell *et al.*, 1985). Further studies need to be carried out to determine the role, if any, of *V. parahaemolyticus* in the VBNC state in relation to food poisoning outbreaks.

In addition to the VBNC state, there are other problems associated with the direct detection of pathogenic bacteria in seafood. These are generally related to components of food which may inhibit the PCR or ELISA reaction. Several reports have outlined the presence of inhibitors in oyster samples during *in vitro* detection assays (Atmar *et al.*, 1993; Hill *et al.*, 1991; Jones *et al.*, 1993). In our study, we modified the DNA extraction method for PCR from the conventional proteinase K/lysosyme method, to the DNeasy column which resulted in a cleaner DNA sample. This greatly enhanced the recovery of *V. parahemolyticus* from oyster samples. For the PCR technique, a pre-enrichment step was not crucial for the detection of PCR products, but it greatly enhanced the sensitivity of the assay. This is probably due to the dilution of inhibitors and non-*parahaemolyticus* bacteria on selective enrichment in APW. For ELISA however, other modifications had to be made to the methods, since no DNA extraction is involved. The best solution for ELISA was to incorporate a pre-enrichment step of *V. parahaemolyticus* cells recovered from seeded oyster tissue. These were then enriched in APW for varying time intervals. For high inocula, a 3 h incubation was found to cause sufficient increase in cell numbers to give a stronger

However in cases where the initial inoculum seeded into the oyster tissue was low, greater enrichment times were required. Thus when culture densities were lower than 10^5 cfu/ml, results of a 6 h enrichment culture gave a stronger signal in ELISA than the 3 h enrichment.

The CDR3 sequence of the clones was determined as explained in **section 5.8** The determination of CDR3 sequence is of significance because it allows us to modify or alter the antibody without altering its binding properties (for example, tagging it with a dye). It also gives us a very target specific antibody, which can have many commercial applications. In addition it is an invaluable tool in determining the antigenic structure of the epitope on *V. parahaemolyticus*.

The sensitivity of the phage antibody technique was also compared with the PCR technique. The PCR is a rapid detection method that has been used for the detection of pathogenic bacteria in both environmental and pathogenic samples (Kadival *et al.*, 1995; Martineau *et al.*, 2000 and Waage *et al.*, 1999). PCR techniques have been developed for *V. parahaemolyticus* based on the toxin-producing genes. However, most of the primer sets used for PCR till date show cross-reactivity with closely related *Vibrio* spp. PCR was carried out using four different primer sets, using both whole cells and purified DNA. The quality of DNA plays an important role in the PCR assay. Of the methods tested, the DNeasy method showed the highest yields and the most pure DNA, while the boiling method showed the lowest yield, with the lowest $A_{260/280}$ ratios, indicating higher protein contamination The proteinase K/lysozyme method also gave good yields, but lower than the DNeasy method..

Three of the primers targeted the toxin-producing genes of *V. parahaemolyticus* - *tdh*, *trh* and the *toxR* genes. The fourth primer set was targeted to the, *gyrB* gene, which is involved in the production of DNA gyrase for cell reproduction. Initially it was believed that all toxin produced by *V. parahaemolyticus* must be TDH and

was believed that all toxin produced by *V. parahaemolyticus* must be TDH and therefore only *tdh* positive strains could be pathogenic. (Nishibuchi and Kaper, 1985 and Lee and Pan,1993). However, it was later shown that some strains could be pathogenic without TDH. The gene responsible for this toxin production was shown to be *trh* (Honda *et al.*, 1991 and Tang *et al.*,1994). With the *tdh* primers, amplicons were generated with most, but not all, strains of *V. parahaemolyticus*.. With the *trh* primers all strains of *V. parahaemolyticus* were positive but *V. mimicus* showed a non-specific amplicon in PCR. Both assays were sensitive and could detect a DNA concentration of 40 ng or higher.

With the *toxR* primers, a PCR amplicon was generated with all the strains of *V. parahaemolyticus*. In other studies, cross-reactivity has been observed with *V. vulnificus* (Kim *et al.*, 1999) but no cross-reactivity was found with our strain. It may be that some strains of *V. vulnificus* possess a closely related gene. This assay was also more sensitive than the *tdh* and *trh* primers, and showed detection limit of 20ng or higher.

The *gyrB* gene, which is a non-species-specific gene, is involved for the production of the β-subunit of DNA gyrase (topoisomerase II), which is essential for cell replication; therefore is it present in all bacterial strains. The primers used in our study targeted regions of the *gyrB* gene that were conserved in *V. parahaemolyticus*. Non-specific amplicons were generated with *V. mimicus* and *V.vulnificus*, however the amplicon of *V. mimicus* can easily be distinguished at 400bp from the specific amplicons of *V. parahaemolyticus* at 285 bp. The lowermost detection limit was 50 ng. Thus the four sets of PCR primers enabled us to establish the range of sensitivity and specificity of PCR.

Phage antibody display involved panning phage clones against strains of *V. parahaemolyticus*, obtained from clinical sources. One of the goals of phage display

was to isolate antibodies which were specific for *V. parahaemolyticus* and did not bind to (cross-react with) closely related *Vibrio* strains (a drawback of PCR assay). Five rounds of panning were carried out, four of these were positive panning rounds and one was a negative panning round. These steps of panning were carried out to enrich for phage binding to *V. parahaemolyticus* and to remove cross-reactive clones. Even though the strains of *V. parahaemolyticus* used for panning were all from food poisoning outbreaks and therefore potentially pathogenic, the panning process itself did not bias the antibodies towards only epitopes present in pathogenic strains. Thus the end result would ensure that the antibodies would be able to bind to environmental *V. parahaemolyticus* isolates as well, since they can also be potentially pathogenic. This is in contrast to PCR where most primer sequences are designed from genes carried by pathogenic strains. In addition, whole cells were used for the panning process, so as to mimic their natural state *in vivo* in the environment as closely as possible. A negative panning step was carried out concurrently with the second positive panning. This consisted of strains closely related to *V. parahaemolyticus- V. mimicus, V. alginolyticus* and *V. harveyi.* This was carried out to ensure that phage antibodies which recognise similar antigens will bind to these strains and then be eliminated, enriching the pool of antibodies binding to *V. parahaemolyticus..*

Before commencing panning, both fixed and free (unfixed) cells were tested to determine which would be better able to withstand the washing steps involved in the panning process. Free cells were better able to withstand the washes, without a drastic decrease in cell numbers at the end of washing, and were therefore used for the panning process.

At the end of the first round of panning, a PCR analysis of the CDR3 region showed that one single clone dominated at the end of the five rounds of panning. Investigative studies showed that this selection of dominant antibody had occurred in

the third round. The presence of a single dominant clone at the end of panning does not allow us to carry out the range of tests for further investigations. It is therefore important to have some diversity in the antibodies at the end of panning. Therefore repanning was carried out from the 3rd round onwards, and this resulted in a better diversity of clones, enriched for binding to *V. parahaemolyticus*.

At the end of panning, clones were picked randomly and each clone was seeded into a single well of two 96-well microtitre plates. These clones were then further screened by ELISA technique and then by flow cytometry. Flow cytometry gives a good indication of percentage binding and also eliminates false positive reactions from ELISA. Out of several clones picked for ELISA, 15 were screened by flow cytometry against a range of *V. parahaemolyticus* and non-*V. parahaemolyticus* strains. At least 8 of those clones showed positive results in flow cytometry, with a strong binding profile. To visually observe the binding the clones were tagged with a fluorescent dye and observed under a UV microscope. Phage clones which gave good binding in flow cytometry, showed a strong visual binding under the fluorescent microscope. To increase the sensitivity of the assay, the clones were tagged with a chemiluminescent dye as mentioned previously.

Production of scFv or Fab fragments is another important aspect of phage display technology. ScFv fragments are the antibody part of the single chain antibody, which is involved in binding to cellular epitopes. The advantages of producing scFv fragments are many, but primarily because these fragments can be used for a number of commercial applications, and in ELISA and FACS based assays, without the steric hindrance of being attached to a phage clone. ScFv fragments can either be purified by inserting the phage clones in sup^- *E. coli* cells (Krebs *et al.*, 2001) or purifying them from sup^+ *E. coli* cells with leaky cells membranes. The latter approach was used in our study. ScFv fragments were purified and concentrated by dialysis to remove media

particles; and further concentrated by lyophilization. However, the concentration of these scFv chains in the dialysed samples was not strong enough to be detected by Western blotting.

The detection of *V. parahaemolyticus* is important in the surveillance of contaminated seafood, both in seafood plants and in the shelf stock of retail markets. *V. parahaemolyticus* related infections are an increasing threat, especially in coastal areas. Surveillance systems generally concentrate on bacteria causing severe gastroenteritis leading to morbidity. In addition, they rely heavily on conventional agar based methods. Many infections by *V. parahaemolyticus* are not reported, since they generally run a self limiting course and rarely cause morbidity. However, they can spread very rapidly if the source of contamination is not detected in time and lead to epidemics and pandemics (Texas Department of Health Report, 1998). Culture techniques give results after 4 days, by which time a large proportion of the contaminated seafood may have been ingested. Therefore it is important to incorporate a rapid detection method for routine laboratory use and in routine surveillance of seafood samples.

Detection methods must be adequate for the specific requirements of the assay. The limits of detection are based on the risk factor, which in turn set the limits for the sensitivity and specificity of the assay. *V. parahaemolyticus* is mainly a seafood (shellfish) associated disease. In oysters, the current cut off limits for surveillance of *V. parahaemolyticus* has been set to 10,000 cfu/g of oyster meat. Any detection method must be able to detect this number to be used for routine surveillance. In addition, it must also be able to detect this organism in the presence of inhibitors and tissue from the shellfish. The specificity of our assay is excellent and can easily detect *V. parahaemolyticus* in oyster meat in the presence of other closely related bacteria. The sensitivity (both by PCR and phage display method) is within the range currently prescribed but evidence supports that lower numbers can also cause infection and it

maybe as low as 200 cfu/g oyster meat (CDC report, 1998). It is therefore important to carry out further work to increase the sensitivity of the assay system. This might also help in the detection of probable VBNC bacteria in the sample. No real evidence has been shown for the existence of VBNC state in marine *Vibrio* spp., but data suggests that in suitable conditions these bacteria might revert back to an infectious state. The method must therefore be suitably modified to include the detection of these VBNC, through the use of a suitable culture medium or pre-enrichment step. Both the PCR method and the phage antibody display method can be suitably modified to include this step.

Future work:

In the present study we compared the sensitivity and specificity of two methods for the detection of *V. parahaemolyticus* in pure cultures and in seafood samples. The first method, was a novel technique based on the use of bacteriophage antibody display for the selection of unique antibody clones that are able to distinguish between markers on *V. parahaemolyticus* and other bacteria. These antibody clones (displayed on the surface of the phage) were able to bind only to cells of *V. parahaemolyticus* in the presence of other bacteria, food material and various inhibitory substances. Thus these clones can be exploited to develop a rapid assay system for the detection of this organism in seafood and environmental samples. The technique has been standardised so that it is possible to perform a direct ELISA, with or without pre-enrichment, once a sample has been obtained for testing. Further, chemiluminescent dyes have been used to increase the sensitivity of the assay. Further work could concentrate on standardising the chemiluminescent assay system, which would lead to a rapid reliable identification method for these organisms. In addition, it is also possible to produce soluble scFv fragments by growing these clones in the appropriate vector. ScFv fragments are

smaller in size than the phage-scFv molecules, and thus less susceptible to steric hindrance. Thus they may be more adaptable to tagging by chemiluminescent dyes. The sequence of some of the CDR3 regions of the antibody clones has also been postulated. CDR3s are the antigen binding domains of the antibody. Since the CDR3 sequence is known, it may be possible to construct synthetic antibody constructs in the laboratory from scratch, without the need for *in vitro* or *in vivo* culture techniques.

Bibliography

Akeda, Y., Nagayama, K., Yamamoto, K. and Honda, T. (1997) Invasive phenotype of *Vibrio parahaemolyticus*. Journal of Infectious Diseases 176: 822-824.

Alam, M.J., Tomochika, K.I., Miyoshi, S.I. and Shinoda S. (2002) Environmental investigation of potentially pathogenic *Vibrio parahaemolyticus* in the Seto-Inland Sea, Japan. FEMS Microbiology Letters 208: 83-7.

Alcaide, E., Amaro, C., Todoli, R. and Oltra, R. (1999) Isolation and characterization of *Vibrio parahaemolyticus* causing infection in Iberian toothcarp *Aphanius iberus*. Diseases of Aquatic Organisms 35: 77-80.

Arakawa, E., Murase, T., Shimada, T., Okitsu, T., Yamai, S. and Watanabe, H. (1999) Emergence and prevalence of a novel *Vibrio parahaemolyticus* O3:K6 clone in Japan. Japanese Journal of Infectious Diseases 52: 246-7.

Aronson, S. M. (1998) The world's mine oyster. Medicine and Health, Rhode Island 81: 282-283.

Atmar, R. L. Metcalf, T. G. Neill, F. H. and Estes, M. K. (1993) Detection of enteric viruses in oysters by using the polymerase chain reaction Applied and Environmental. Microbiology 59: 631-635.

Ayers, P. A. and Barrow, G. I. (1978) The distribution of *Vibrio parahaemolyticus* in British coastal water: report of a collaborative study 1975-6. Journal of Hygiene (London) 80: 281-284.

Azzazy, H. M. and Highsmith, W. E., Jr. (2002) Phage display technology: clinical applications and recent innovations. Clinical Biochemistry 35: 425-445.

Baba, K., Shirai, H., Terai, A., Takeda, Y. and Nishibuchi, M. (1991) Analysis of the *tdh* gene cloned from a *tdh* gene- and *trh* gene-positive strain of *Vibrio parahaemolyticus*. Microbiology and Immunology 35: 253-258.

Bag, P.K, Nandi, S., Bhadra, R.K., Ramamurthy, T., Bhattacharya, S.K., Nishsibuchi M., Hamambata T., Yamasaki, S., Takeda, Y. and Nair G. B. (1999) Clonal diversity

among recently emerged strains of *Vibrio parahaemolyticus* O3:K6 associated with pandemic spread. Journal of Clinical Microbiology 37: 2354-2357.

Baker, R.M., Singleton, F.L. and Hood, M.A. (1983) Effects of nutrient deprivation on *Vibrio cholerae*. Applied and Environmental Microbiology 46: 930 -940.

Barban, V., Fraysse-Corgier, S., Paranhos-Baccala, G., Petit, M., Manin, C., Berard, Y., Prince, A. M., Mandrand, B. and Meulien, P. (2000) Identification of a human epitope in hepatitis C virus (HCV) core protein using a molecularly cloned antibody repertoire from a non- symptomatic, anti-HCV-positive patient. The Journal of General Virology 81: 461-469.

Baross, J. and Liston, J (1968) Isolation of *Vibrio parahaemolyticus* from the Northwest Pacific. Nature 217: 1263-1264.

Barski, P., Piechowicz, L.,Galinski, J. and. Kur. J. (1996). Rapid assay for detection of methicillin-resistant *Staphylococcus aureus* using multiplex PCR. Molecular and Cellular Probes 10: 471-475.

Barrow, G. I. and Feltham, R. K. A. (1993) Cowan and Steels's Manual for the Identification of Medical Bacteria, Third edition . Cambridge University Press, Cambridge, UK.

Barrow, G. I. and Miller, D. C. (1972) *Vibrio parahaemolyticus*: a potential pathogen from marine sources in Britain. Lancet 1: 485-486.

Bean, N. H., Maloney, E. K., Potter, M. E., Korazemo, P., Ray, B., Taylor, J. P., Seigler, S. and Snowden, J. (1998) Crayfish: a newly recognized vehicle for vibrio infections. Epidemiology and Infection 121: 269-273.

Blake, P.A., Merson, M. H., Weaver, R. E., Hollis, D. G. and Heublein, P. C. (1979). Disease Caused by a Marine Vibrio, Clinical Characteristics and Epidemiology. New England Journal of Medicine 300: 1-5.

Boel, E., Bootsma, H., de Kruif, J., Jansze, M., Klingman, K. L., van Dijk, H. and Logtenberg, T. (1998) Phage antibodies obtained by competitive selection on complement- resistant *Moraxella (Branhamella) catarrhalis* recognize the high-molecular-weight outer membrane protein. Infection and Immunity 66: 83-88.

Breitling, F., Dubel, S., Seehaus, T., Klewinghaus, I. and Little, M. (1991) A surface expression vector for antibody screening. Gene 104: 147-153.

Cann, D. C., Taylor, L. Y. and Merican, Z. (1981) A study of the incidence of *Vibrio parahaemolyticus* in Malaysian shrimp undergoing processing for export. The Journal of Hygiene (London) 87: 485-491.

Cassels, F. J., Odom, E. W. and Vasta, G. R. (1994) Hemolymph lectins of the blue crab, *Callinectes sapidus*, recognize selected serotypes of its pathogen *Vibrio parahaemolyticus*. Annals of the New York Academy of Sciences 712: 324-326.

Canadian Communicable Disease Report (1997) Outbreaks of *Vibrio parahaemolyticus* related to raw oysters in the British Columbia Canadian Communicable Disease Report, 23: 145-148.

CDC report (1999) Outbreak of *Vibrio parahaemolyticus* infection associated with eating raw oysters and clams harvested from Long Island Sound - Connecticut, New Jersey and New York,1998. Morbidity and Mortality Weekly report 48: 48-51.

CDC report (1998) Outbreak of *Vibrio parahaemolyticus* infections associated with eating raw oysters-Pacific Northwest, 1997. Morbidity and Mortality Weekly report 47: 457-462.

Chakraborty, S., Nair, G. B. and Shinoda, S. (1997) Pathogenic vibrios in the natural aquatic environment. Reviews on Environmental Health 12: 63-80.

Chan, T. Y. (1995) Shellfish-borne illnesses. A Hong Kong perspective. Tropical and Geographical Medicine 47: 305-307.

Colwell, R. R., Brayton, P. R., Grimes, D. J., Roszak, S. A., Huq, S. A. and Palmer, L. M. (1985). Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: implications for release of genetically engineered microorganisms. Bio/Technology 3: 817-820.

Colwell, R.R., Brayton, P., Herrington, D., Tall, B., Huq, A. and Levine, M.M. (1996) Viable but non-culturable *Vibrio cholerae* 01 revert to a culturable state in the human intestine. World Journal of Microbiology and Biotechnology 12: 28–31.

Colwell, R. R. and Green, J. R. (1986) Biotechnology and the sea. Ocean Development and International Law 17: 163-189.

Colwell, R. R. and Huq, A. (1994). Vibrios in the environment: Viable but nonculturable *Vibrio cholerae.* In: *Vibrio cholerae* and Cholera. Wachsmuth, I. K., Blake, P. A. and. Olsvik, O.(eds.) ASM Press, Washington, D.C. pp. 117-134.

Colwell, R.R., MacDonell, M.T. and De Ley, J. (1986) Proposal to recognize the family *Aeromonadaceae* fam. nov. International Journal of Systematic Bacteriology 36: 473-477.

Cook, D. W., Oleary, P., Hunsucker, J. C., Sloan, E. M., Bowers, J. C., Blodgett, R. J. and Depaola, A. (2002) *Vibrio vulnificus* and *Vibrio parahaemolyticus* in U.S. retail shell oysters: a national survey from June 1998 to July 1999. Journal of Food Protection 65: 79-87.

Daniels, N. A., Ray, B., Easton, A., Marano, N., Kahn, E., McShan, A. L., 2nd, Del Rosario, L., Baldwin, T., Kingsley, M. A., Puhr, N. D., Wells, J. G. and Angulo, F. J. (2000) Emergence of a new *Vibrio parahaemolyticus* serotype in raw oysters: A prevention quandary. JAMA: the Journal of the American Medical Association 284: 1541-1545.

Davis, J. W. and Sizemore, R. K. (1982) Incidence of *Vibrio* species associated with blue crabs (*Callinectes sapidus*) collected from Galveston Bay, Texas. Applied and Environmental Microbiology 43: 1092-1097.

de Greeff, A., van Alphen, L. and Smith, H. E. (2000) Selection of recombinant antibodies specific for pathogenic Streptococcus suis by subtractive phage display. Infection and Immunity 68: 3949-3955.

de Paola, A., Hopkins, L. H., Peeler, J. T., Wentz, B. and McPhearson, R. M. (1990) Incidence of *Vibrio parahaemolyticus* in U.S. coastal waters and oysters. Applied and Environmental Microbiology 56: 2299-2302.

de Nardo, S. J., de Nardo, G. L., DeNardo, D. G., Xiong, C. Y., Shi, X. B., Winthrop M. D., Kroger, L. A. and Carter, P. (1999) Antibody phage libraries for the next generation of tumor targeting radioimmunotherapeutics. Clinical Cancer Research 5(10 Suppl): 3213s-3218s.

Disease Prevention News (1999) *Vibrio parahaemolyticus* surveillance Disease Prevention News, 59: 1-4.

Dong, L., Chen, S., Bartsch, U. and Schachner, M. (2003) Generation of affinity matured scFv antibodies against mouse neural cell adhesion molecule L1 by phage display. Biochemical and Biophysical Research communications 301: 60-70.

Douet, J. P., Castroviejo, M., Dodin, A. and Bebear, C. (1996) Study of the haemolytic process and receptors of thermostable direct haemolysin from *Vibrio parahaemolyticus*. Research in Microbiology 147: 687-696.

Doyle, M. P. (1998) Extending the shelflife of refrigerated foods: For better or worse? Food Technology 52: 20.

Doyle, M. P. (1990) Pathogenic *Escherichia coli, Yersinia enterocolitica*, and *Vibrio parahaemolyticus*. Lancet. 336: 1111-1115.

Elliot, E.L., Kaysner C.A., Jackson L. and. Tamplin, M.L. (1998) Chapter 9 - *Vibrio cholerae, V. parahaemolyticus, V. vulnificus*, and other *Vibrio* spp. In: *Food and Drug Administration Bacteriological Analytical Manual*, 8th ed. (revision A). Merker, R.L. (ed.) AOAC International, Gaithersburg, MD.

FDA report (1999) Public health impact of *Vibrio parahaemolyticus* in Molluscan shellfish. Food and Drug Administration report Federal Register Notice 64.

Fujino, T., Miwatani, T., Yasuda, J., Kondo, M., Takeda, Y., Akita, Y., Kotera, K., Okada, M., Nishimune, H., Shimizu, Y., Tamura, T. and Tamura, Y. (1965) Taxonomic studies on the bacterial strains isolated from cases of shirasu food-poisoning (*Pasteurella parahaemolytica*) and related microorganisms. Biken Journal 8: 63-71.

Fujino, T., Okuno, Y., Nakada, D., A, A., Fukai, K., Mukai, T. and Ueho, T. (1951) On the bacteriological examination of Shirasu food poisoning. Journal of Japanese Association of Infectious Diseases 35: 11-21.

Fyfe, M.,Yeung, S. T., Daly, P., Schaillie, K., Kelly, M. T. and Buchanan, S. (1997) Outbreak of V*ibrio parahaemolyticus* related to raw oysters in British Columbia. Canadian Communicable Disease Reports 23: 145-148.

Givan, A. L. (1992) Chapter 3- Instrumentation: Beyond the black box. Wiley-Liss,Inc, New York.

Gram, H. (1999) Phage display in proteolysis and signal transduction. Combinatorial Chemistry and High throughput Screening 2: 19-28.

Griep, R. A., van Twisk, C., van der Wolf, J. M. and Schots, A. (1999) Fluobodics: green fluorescent single-chain Fv fusion proteins. Journal of Immunological Methods 230: 121-130.

Griffiths, A. D., Williams, S. C., Hartley, O., Tomlinson, I. M., Waterhouse, P., Crosby, W. L., Kontermann, R. E., Jones, P. T., Low, N. M., Allison, T. J., *et al.* (1994) Isolation of high affinity human antibodies directly from large synthetic repertoires. The EMBO Journal 13: 3245-3260.

Grothaus, M. C., Srivastava, N., Smithson, S. L., Kieber-Emmons, T., Williams, D. B., Carlone, G. M. and Westerink, M. A. (2000) Selection of an immunogenic peptide mimic of the capsular polysaccharide of *Neisseria meningitidis* serogroup A using a peptide display library. Vaccine 18: 1253-1263.

Hernandez-Lopez, J., Gollas-Galvan, T., Magallon-Barajas, F. and Vargas-Albores, F. (1997) Isolation of *Vibrio* and *Pseudomonas* from brown shrimp (*Penaeus californiensis Holmes*) intestine. Revista latinoamericana de microbiologia 39: 109-115.

Harrison J. L., Williams, S. C., Winter, G. and Nissim A. (1996) Screening of phage antibody libraries. Methods in Enzymology 267: 83-109.

Hill, W.E., Keasler, S.P.. Trucksess, M.W., Feng, P., Kaysner, C.A. and Lampel, K.A. (1991) Polymerase chain reaction identification of *Vibrio vulnificus* in artificially contaminated oysters. Applied and Environmental Microbiology 57: 707-711.

Hlady, W. G. (1997) *Vibrio* infections associated with raw oyster consumption in Florida,1981-1994. Journal of Food Protection 60: 353-357.

Ho, H., Amin, H. and Lee, H. R. (2002) Vibrio infections. At: http://www.emedicine.com/med/topic375.htm#sectionsbibliography. Accessed November 2003.

Holt, L. J., Enever, C., de Wildt, R. M, and Tomlinson, I.M. (2000) The use of recombinant antibodies in proteomics. Current Opinion in Biotechnology 11: 445-9.

Honda, T., Abad-Lapuebla, M. A., Ni, Y. X., Yamamoto, K. and Miwatani, T. (1991) Characterization of a new thermostable direct haemolysin produced by a Kanagawa-phenomenon-negative clinical isolate of *Vibrio parahaemolyticus*. Journal of General Microbiology 137: 253-259.

Honda, T. and Iida, T. (1993) The pathogenicity of *Vibrio parahaemolyticus* and the role of thermostable direct haemolysin and related haemolysins. Reviews in Medical Microbiology 4: 106-113.

Honda, T., Taga, S., Takeda, T., Hasibuan, M. A., Takeda, Y. and Miwatani, T. (1976) Identification of lethal toxin with the thermostable direct hemolysin produced by *Vibrio parahaemolyticus*, and some physicochemical properties of the purified toxin. Infection and Immunity 13: 133-139.

Hoogenboom, H. R., de Bruine, A. P., Hufton, S. E., Hoet, R. M., Arends, J. W. and Roovers, R. C. (1998) Antibody phage display technology and its applications. Immunotechnology 4: 1-20.

Hoogenboom, H. R., Lutgerink, J. T., Pelsers, M. M., Rousch, M. J., Coote, J., Van Neer, N., De Bruine, A., Van Nieuwenhoven, F. A., Glatz, J. F. and Arends, J. W. (1999) Selection-dominant and nonaccessible epitopes on cell-surface receptors revealed by cell-panning with a large phage antibody library. European Journal of Biochemistry (FEBS) 260: 774-784.

Huntley, J. S. and Hall, A. C. (1996) Nature of the cation leak induced in erythrocyte membranes by Kanagawa haemolysin of *Vibrio parahaemolyticus*. Biochimica et Biophysica Acta 1281: 220-226.

IASR report (1996) *Vibrio parahaemolyticus*, Japan, 1994-1995. Infectious Agent Surveillance Report 17: 1.

Ishihara, M., Kawashishi, A., Watanabe, H., Tomochika, K., Miyoshi, S. and Shinoda, S. (2002) Purification of a serine protease of *Vibrio parahaemolyticus* and its characterization. Microbiology and Immunology 46: 299-303.

Jiang, X., and Chai, T. J. (1996). Survival of *Vibrio parahaemolyticus* at low temperatures under starvation conditions and subsequent resuscitation of viable, nonculturable cells. Applied and Environmental Microbiology 62: 1300-1305.

Johnson, W. G., Jr., Salinger, A. C. and King, W. C. (1973) Survival of *Vibrio parahaemolyticus* in oyster shellstock at two different storage temperatures. Applied Microbiology 26: 122-123.

Jones, D.D., Law, R. and. Bej, A.K (1993) Detection of *Salmonella* spp. in contaminated oysters using polymerase chain reaction and gene probes. Journal of Food Science 58: 1191-1197.

Joseph, S. W., Colwell, R. R. and Kaper, J. B. (1982) *Vibrio parahaemolyticus* and related halophilic Vibrios. Critical Reviews in Microbiology 10: 77-124.

Kadival, G.V., D'Souza, C. D., Kolk, A. H. and Samuel, A.M. (1995) Polymerase chain reaction in the diagnosis of tuberculosis - Comparison of two target sequences for amplification. Zentralblatt fur Bakteriologie : International Journal of Medical Microbiology 282: 353-61.

Karunasagar, I. and Venugopal, M. N. (1984) Levels of *Vibrio parahaemolyticus* in Indian shrimp undergoing processing for export. Canadian Journal of Microbiology 30: 713-715.

Kasman, L. M., Lukowiak, A. A., Garczynski, S. F., McNall, R. J., Youngman, P. and Adang, M. J. (1998) Phage display of a biologically active *Bacillus thuringiensis* toxin. Applied and Environmental Microbiology 64: 2995-3003.

Kaysner, C. A., Tamplin, M. L., Wekell, M. M., Stott, R. F. and Colburn, K. G. (1989) Survival of *Vibrio vulnificus* in shellfish and shucked oysters (*Crassostrea gigas* and *Crassostrea virginica*) and effects of isolation medium on recovery. Applied and Environmental Microbiology 55: 3072-3079

Kelly, M. T. and Stroh, E. M. (1988) Occurrence of Vibrionaceae in natural and cultivated oyster populations in the Pacific Northwest. Diagnostic Microbiology and Infectious Diseases 9: 1-5.

Khan, N.A., Greenman, J., Topping, K.P., Hough, V.C., Temple, G.S. and Paget, T.A. (2000) Isolation of *Acanthamoeba* specific antibodies from bacterial phage display library. Journal of Clinical Microbiology 38: 2347-2377.

Kim, Y. B., Okuda, J., Takahashi, N., Hashimoto, S. and Nishibuchi, M. (1999) Identification of *Vibrio parahaemolyticus* strains at the Species Level by PCR Targeted to the *toxR* Gene. Journal of Clinical Microbiology 37: 1173-1177.

Knappik, A., Ge, L., Honegger, A., Pack, P., Fischer, M., Wellnhofer, G., Hoess, A., Wolle, J., Pluckthun, A. and Virnekas, B. (2000) Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. Journal of Molecular Microbiology 296: 57-86.

Kolbert, C. P., Connolly, J. E., Lee, M. J. and Persing, D. H. (1995) Detection of the Staphylococcal mecA gene by chemiluminescent DNA hybridization. Journal of Clinical Microbiology 33: 2179-82.

Kontermann, R. E., Wing, M. G. and Winter, G. (1997) Complement recruitment using bispecific diabodies. Nature Biotechnology 15: 629-631.

Krantz, G. E., Colwell, R. R. and Lovelace, E. (1969) Vibrio parahaemolyticus from the blue crab Callinectes sapidus in Chesapeake Bay. Science 164: 1286-1287.

Krebs, B., Rauchenberger, R., Reiffert, S., Rothe, C., Tesar, M., Thomassen, E., Cao, M., Dreier, T., Fischer, D., Hoss, A., Inge, L., Knappik, A., Marget, M., Pack, P., Meng, X. Q., Schier, R., Sohlemann, P., Winter, J., Wolle, J, and Kretzschmar, T. (2001) High-throughput generation and engineering of recombinant human antibodies. Journal of Immunological Methods 254: 67-84.

Kretzschmar, T. and von Ruden, T. (2002) Antibody discovery: phage display. Current Opinion in Biotechnology 13: 598-602.

Lee, C. Y., Cheng, M. F., Yu, M. S. and Pan, M. J. (2002) Purification and characterization of a putative virulence factor, serine protease, from *Vibrio parahaemolyticus*. FEMS Microbiology Letters 209: 31-37.

Lee, C. and Pan, S. (1993) Rapid and specific detection of the thermostable direct hemolysin gene in *Vibrio parahaemolyticus* by the polymerase chain reaction. *Journal of General Microbiology* **139**, 3225-3231.

Lee, K. J., Pan, S. F.and Chen , C. H. (2002) Phage-display selection of a human single-chain fv antibody highly specific for melanoma and breast cancer cells using a chemoenzymatically synthesized G(M3)-carbohydrate antigen. Journal of the American Chemical Society 124: 12439-12446.

Lee, K. J., Mao, S., Sun, C., Gao, C., Blixt, O., Arrues, S., Hom, L. G., Kaufmann, G. F., Hoffman, T. Z., Coyle, A. R., Paulson, J., Felding-Habermann, B. and Janda, K. D. (2002) Phage-display selection of a human single-chain fv antibody highly specific for melanoma and breast cancer cells using a chemoenzymatically synthesized G(M3)-carbohydrate antigen. Journal of the American Chemical Society 124: 12439-12446.

Lewis, G. D., Molloy, S. L., Greening, G. E. and Dawson, J. (2000) Influence of environmental factors on virus detection by RT-PCR and cell culture. Journal of Applied Microbiology 88: 633.

Lin, Z., Kumagai, K., Baba, K., Mekalanos, J. J. and Nishibuchi, M. (1993) *Vibrio parahaemolyticus* has a homolog of the *Vibrio cholerae toxRS* operon that mediates environmentally induced regulation of the thermostable direct hemolysin gene. Journal of Bacteriology 175: 3844-3855.

Ljungh, A., Popoff, M. and Wadstrom, T. (1977) *Aeromonas hydrophila* in acute diarrheal disease: detection of enterotoxin and biotyping of strains. Journal of Clinical Microbiology 6: 96-100.

Ljungh, A. and Wadstorm, T. (1982) *Aeromonas* toxins. Pharmacology and Therapeutics 15: 339-352.

Madigan, M. T.; Martinko, J. M. and Parker, J. (2000) Chapter 16: Prokaryotic Diversity: Bacteria. In: Brock, Biology of Microorganisms ,9th ed. Prentice Hall, New Jersey.

Marks, J. D., Griffiths, A. D., Malmqvist, M., Clackson, T. P., Bye, J. M. and Winter, G. (1992) By-passing immunization: building high affinity human antibodies by chain shuffling. Biotechnology (N Y) 10: 779-783.

Marks, J. D., Hoogenboom, H. R., Bonnert, T. P., McCafferty, J., Griffiths, A. D. and Winter, G. (1991) By-passing immunization. Human antibodies from V-gene libraries displayed on phage. Journal of Molecular Biology 222: 581-597.

Marks, J.D., Ouwehand, W.H., Bye, J.M., Finnern, R., Gorick, B.D., Voak, D., Thorpe, S., Hughes-Jones, N.C. and Winter, G. (1993) Human antibody fragments specific for human blood group antigens from a phage display library. Bio/Technology 11: 1145-9.

Martineau, F., Picard, F. J., Grenier, L., Roy, P. H., Ouellette, M.and Bergeron, M.G. (2000) Multiplex PCR assays for the detection of clinically relevant antibiotic resistance genes in staphylococci isolated from patients infected after cardiac surgery. The ESPRIT Trial. Journal of Antimicrobial Chemotherapy 46: 527-34.

Matsumoto, C., Okuda, J., Ishibashi, M., Iwanaga, M., Garg, P., Rammamurthy, T., Wong, H. C., Depaola, A., Kim, Y. B., Albert, M. J. and Nishibuchi, M. (2000) Pandemic spread of an O3:K6 clone of *Vibrio parahaemolyticus* and emergence of related strains evidenced by arbitrarily primed PCR and *toxRS* sequence analyses. Journal of Clinical Microbiology 38: 578-585.

Matte, G.R., Matte, M. H., Sato, M. I., Sanchez, P. S., Rivera, I. G. and Martins, M. T. (1994) Potentially pathogenic Vibrios associated with mussels from a tropical region on the Atlantic coast of Brazil. Journal of Applied Bacteriology 77: 281-287.

McCarthy, S. A., DePaola, A., Cook, D. W., Kaysner, C. A. and Hill, W. E. (1999) Evaluation of alkaline phosphatase- and digoxigenin-labelled probes for detection of the

thermolabile hemolysin (tlh) gene of *Vibrio parahaemolyticus*. Letters in Applied Microbiology 28: 66-70.

Miwatani, T. and Takeda, Y. (1976) Food poisoning due to *Vibrio parahaemolyticus* in Japan. In: *Vibrio parahaemolyticus*, a causative bacterium of food poisoning. Miwatani, T. and. Takeda, Y. (eds.) Saikon Publishing Co., Tokyo, Japan. pp. 22-25.

MMWR (1999) Outbreak of *Vibrio parahaemolyticus* Infection Associated with Eating Raw Oysters and Clams Harvested from Long Island Sound - Connecticut, New Jersey, and New York, 1998. Morbidity and Mortality Weekly Report 48: 48-51.

Niederhauser, C., Candrian, U., Hofelein, C., Jermini, M., Buhler, H. P. and Luthy, J. (1992) Use of polymerase chain reaction for detection of *Listeria monocytogenes* in food. Applied and Environmental Microbiology 58: 1564-1568.

Neri, D., Pini, A. and Nissim, A. (1998) Antibodies from phage display libraries as immunochemical reagents. Methods in Molecular Biology 80: 475-500.

Nishibuchi, M. and Kaper, J. B. (1995) Thermostable direct hemolysin gene of *Vibrio parahaemolyticus*: a virulence gene acquired by a marine bacterium. Infection and Immunity 63: 2093-2099.

Nishibuchi, M., Kumagai, K. and Kaper, J. B. (1991) Contribution of the *tdh*1 gene of Kanagawa phenomenon-positive *Vibrio parahaemolyticus* to production of extracellular thermostable direct hemolysin. Microbial pathogenesis 11: 453-460.

Nishibuchi, M., Taniguchi, T., Misawa, T., Khaeomanee-Iam, V., Honda, T. and Miwatani, T. (1989) Cloning and nucleotide sequence of the gene (*trh*) encoding the hemolysin related to the thermostable direct hemolysin of *Vibrio parahaemolyticus*. Infection and Immunity 57: 2691-2697.

Nissim, A., Hoogenboom, H. R., Tomlinson, I. M., Flynn, G., Midgley, C., Lane, D. and Winter, G. (1994) Antibody fragments from a 'single pot' phage display library as immunochemical reagents. The EMBO Journal 13: 692-698.

Nordstrom, J. L. and DePaola, A. (2003) Improved recovery of pathogenic *Vibrio parahaemolyticus* from oysters using colony hybridization following enrichment. Journal of Microbiological Methods 52: 273-277.

Nowak, A. and Kur, J. (1995) Genomic species typing of acinetobacters by polymerase chain reaction amplification of the recA gene. FEMS Microbiology Letters. 130: 327-32.

Okuda, J., Ishibashi, M., Hayakawa, E., Nishino, T., Takeda, Y., Mukhopadhyay, A. K., Garg, S., Bhattacharya, S. K., Nair, G. B. and Nishibuchi, M. (1997) Emergence of a unique O3:K6 clone of *Vibrio parahaemolyticus* in Calcutta, India, and isolation of strains from the same clonal group from Southeast Asian travelers arriving in Japan. Journal of Clinical Microbiology 35: 3150-3155.

Oliver, J. D., Nilsson, L. and Kjelleberg, S. (1991). Formation of nonculturable *Vibrio vulnificus* cells and its relationship to the starvation state. Applied and. Environmental Microbiology 57: 2640-2644.

Oliver, J. D., and Wanucha, D. (1989). Survival of *Vibrio vulnificus* at reduced temperature and elevated nutrient. Journal of Food Safety 10: 79-86.

O'Malley, S. M., Mouton, S. L., Occhino, D. A., Deanda, M. T., Rashidi, J. R., Fuson, K. L., Rashidi, C. E., Mora, M. Y., Payne, S. M. and Henderson, D. P. (1999) Comparison of the heme iron utilization systems of pathogenic Vibrios. Journal of Bacteriology 181: 3594-3598.

Okwumabua, O., Swaminathan, B,, Edmonds, P., Wenger, J., Hogan, J. and Alden M. (1992) Evaluation of a chemiluminescent DNA probe assay for the rapid confirmation of *Listeria monocytogenes.* Research in Microbiology 143: 183-9.

Peters, S., Baross, J. A. and Morita, R. Y. (1982) Partial purification and characterization of haemolysin from a psychotrophic Kanagawa-postivie marine vibrio. Applied and Environmental Microbiology 43: 39-49.

Raimondi, F., Kao, J. P., Fiorentini, C., Fabbri, A., Donelli, G., Gasparini, N., Rubino, A. and Fasano, A. (2000) Enterotoxicity and cytotoxicity of *Vibrio parahaemolyticus*

thermostable direct hemolysin in *in vitro* systems. Infection and Immunity 68: 3180-3185.

Roovers, R. C., van der Linden, E., de Bruine, A. P., Arends, J. W.and Hoogenboom, H. R. (2001) Identification of colon tumour-associated antigens by phage antibody selections on primary colorectal carcinoma. European Journal of Cancer 37: 542-9.

Sakurai, J., Matsuzaki, A., Takeda, Y. and Miwatani, T. (1974) Existence of two distinct hemolysins in Vibrio parahaemolyticus. Infection and Immunity 9: 777-780.

Sambrook, J., Fritsch, E. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual ,2nd edition. Cold Spring Harbor Press, Cold Spring Harbor, NY.

Sanyal, S. C. and Sen, P. C. (1974) Human volunteer study- the pathogenicity of *Vibrio parahaemolyticus*. In: International Symposium on *Vibrio parahaemolyticus*, Saikon Publishing Co., Ltd,Tokyo, Japan. pp 227-230.

Sblattero, D., Florian, F., Azzoni, E., Zyla, T., Park, M., Baldas, V., Not, T., Ventura, A., Bradbury, A. and Marzari, R. (2002) The analysis of the fine specificity of celiac disease antibodies using tissue transglutaminase fragments. European Journal of Biochemistry 269: 5175-5181.

Schier, R., Marks, J. D., Wolf, E. J., Apell, G., Wong, C., McCartney, J. E., Bookman, M. A., Huston, J. S., Houston, L.L. and Weiner, L. M., *et al.* (1995) *In vitro* and *in vivo* characterization of a human anti-c-erbB-2 single-chain Fv isolated from a filamentous phage antibody library. Immunotechnology 1: 73-81.

Shirai, H., Ito , H., Hirayama, T., Nakabayashi Y, Kumagai, K., Takeda, Y. and Nishibuchi, M. (1990) Molecular epidemiological evidence for association of thermostable direct haemolysin (TDH) and TDH-related haemolysin of *Vibrio parahaemolyticus* with gastroenteritis. Infection and Immunity 58: 3568-3573.

Sizemore, R. K., Colwell, R. R., Tubiash, H. S. and Lovelace, T. E. (1975) Bacterial flora of the hemolymph of the blue crab, *Callinectes sapidus*: numerical taxonomy. Applied Microbiology 29: 393-399.

Sowa, K. M., Cavanagh, D. R., Creasey, A. M., Raats, J., McBride, J., Sauerwein, R., Roeffen, W. F. and Arnot, D. E. (2001) Isolation of a monoclonal antibody from a malaria patient-derived phage display library recognising the Block 2 region of *Plasmodium falciparum* merozoite surface protein-1. Molecular and Biochemical Parasitology 112: 143-147.

Srivastava, N., Zeiler, J. L., Smithson, S. L., Carlone, G. M., Ades, E. W., Sampson, J. S., Johnson, S. E., Kieber-Emmons, T., Westerink, M. A. (2000) Selection of an immunogenic and protective epitope of the PsaA protein of *Streptococcus pneumoniae* using a phage display library. Hybridoma 19: 23-31.

Sun, J., Pons, J. and Craik, C. S. (2003) Potent and selective inhibition of membrane-type serine protease 1 by human single-chain antibodies. Biochemistry 42: 892-900.

Takahashi, A., Kenjyo, N., Imura, K., Myonsun, Y. and Honda, T. (2000) (a) Cl(-) secretion in colonic epithelial cells induced by the vibrio parahaemolyticus hemolytic toxin related to thermostable direct hemolysin. Infection and Immunity 68: 5435-5438.

Takahashi, A., Sato, Y., Shiomi, Y., Cantarelli, V. V., Iida, T., Lee, M. and Honda, T. (2000) (b) Mechanisms of chloride secretion induced by thermostable direct haemolysin of *Vibrio parahaemolyticus* in human colonic tissue and a human intestinal epithelial cell line. Journal of Medical Microbiology 49: 801-810.

Takeda, Y., Honda, T. and Miwatani, T. (1983) The use of the Biken test to detect enterotoxigenic "*Escherichia coli*" producing heat labile enterotoxin. Developments in Biological Standardization 53: 113-121

Takeda, Y., Taga, S. and Miwatani, T. (1978) Evidence that thermostable direct haemolysin of *Vibrio parahaemolyticus* is composed of two subunits. FEMS Microbiology Letters 4: 271-274.

Tang, G., Iida, T., Yamamoto, K. and Honda, T. (1997) Analysis of functional domains of *Vibrio parahaemolyticus* thermostable direct hemolysin using monoclonal antibodies. FEMS Microbiology Letters 150: 289-296.

Tang, G. Q., Iida, T., Yamamoto, K. and Honda, T. (1994) A mutant toxin of *Vibrio parahaemolyticus* thermostable direct hemolysin which has lost hemolytic activity but retains ability to bind to erythrocytes. Infection and Immunity 62: 3299-3304.

Taniguchi H, Ohta H, Ogawa M and Y., M. (1985) Cloning and expression in Escherichia coli of Vibrio parahaemolyticus thermostable direct hemolysin and thermolabile hemolysin genes. Journal of Bacteriology 162: 510-515.

TexasDepartment of Health report (1998) Galvestone Bay reopens for limited commercial oyster harvesting. TDH News Release: 1.

Topping, K. P., Hough, V. C., Monson, J. R. and Greenman, J. (2000) Isolation of human colorectal tumour reactive antibodies using phage display technology. International Journal of Oncology 16: 187-195.

Tuckey, C. D. and Noren, C. J. (2002) Selection for mutants improving expression of an anti-MAP kinase monoclonal antibody by filamentous phage display. Journal of Immunological Methods 270: 247-257.

Twedt, R. M. (1989) *Vibrio parahaemolyticus.* In: Foodborne Bacterial Pathogens. Doyle, M.P.(ed.) Marcel Dekker, Inc., New York, NY. pp. 543-568.

Vadivelu, J., Puthucheary, S. D., Mitin, A., Wan, C. Y., van Melle, B. and Puthucheary, J. A. (1996) Hemolysins and plasmid profiles of *Vibrio parahaemolyticus*. Southeast Asian Journal of Tropical Medicine and Public Health 27: 126-131.

Vanderzant, C. and Nickelson, R. (1972) Survival of *Vibrio parahaemolyticus* in shrimp tissue under various environmental conditions. Applied Microbiology 23: 34-37.

Venkatesh, N., Zaltsman, Y., Somjen, D., Gayer, B., Boopathi, E., Kasher, R., Kulik, T., Katchalski-Katzir, E. and Kohen, F. (2002) A synthetic peptide with estrogen-like activity derived from a phage- display peptide library. Peptides 23: 573-580.

Venkateswaran, K., Dohmoto, N. and Harayama, S. (1998) Cloning and nucleotide sequence of the *gyrB* gene of *Vibrio parahaemolyticus* and its application in detection of this pathogen in shrimp. Applied and Environmental Microbiology 64: 681-687.

Venkateswaran, K., Kiiyukai, C., Nakahishi, K., Nakano, H., Matsuda, O. and Hashimoto, H. (1990) The role of sinking particles in the overwintering process of *Vibrio parahaemolyticus* in a marine environment. FEMS Microbiology Letters 73: 159-166.

Waage, A. S., Vardund, T., Lund, V. and Kapperud, G. (1999) Detection of low numbers of pathogenic *Yersinia enterocolitica* in environmental water and sewage samples by nested polymerase chain reaction. Journal of Applied Microbiology 87: 814-21.

Wagatsuma, S. (1968) A medium for the test of the hemolytic activity of *Vibrio parahaemolyticus*. Media Circle 13: 159-163.

Whitesides, M.D. and Oliver, J. D. (1997) Studies on resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. Applied and Environmental Microbiology 63: 1002-1005.

WHO report (1999) *Vibrio parahaemolyticus*,Japan 1996-1998 Weekly Epidemiological Records 74: 361.

Winter, G., Griffiths, A. D., Hawkins, R. E. and Hoogenboom, H. R. (1994) Making antibodies by phage display technology. Annual Review of Immunology 12: 433-455.

Wong, H. C., Chen, M. C., Liu, S. H. and Liu, D. P. (1999) Incidence of highly genetically diversified *Vibrio parahaemolyticus* in seafood imported from Asian countries. International Journal of Food Microbiology 52: 181-188.

Wong, H. C., Ho, C. Y., Kuo, L. P., Wang, T. K., Lee, C. L. and Shih, D. Y. (1999). Ribotyping of *Vibrio parahaemolyticus* isolates obtained from food poisoning outbreaks in Taiwan. Microbiology and Immunology 43: 631-636.

Wong, H. C., Liu, S. H., Wang, T. K., Lee, C. L., Chiou, C. S., Liu, D. P., Nishibuchi, M. and Lee, B. K. (2000) Characteristics of *Vibrio parahaemolyticus* O3:K6 from Asia. Applied and Environmental Microbiology 66: 3981-3986.

Yeung, P. S., Hayes, M. C., DePaola, A., Kaysner, C. A., Kornstein, L. and Boor, K. J. (2002) Comparative phenotypic, molecular, and virulence characterization of *Vibrio parahaemolyticus* O3:K6 isolates. Applied and Environmental Microbiology 68: 2901-2909.

Yoh, M., Honda, T., Miwatani, T. and Nishibuchi, M. (1991) Characterization of themostbale direct haemolysin encoded by four representative tdh genes of *Vibrio parahaemolyticus*. Microbial Pathogenesis 10: 165-172.

Yoh, M., Miwatani, T. and Honda, T. (1992) Comparison of *Vibrio parahaemolyticus* hemolysin (Vp-TRH) produced by environmental and clinical isolates. FEMS Microbiology Letters 71: 157-161.

Yoh, M., Morinaga, N., Noda, M. and Honda, T. (1995) The binding of *Vibrio parahaemolyticus* 125I-labeled thermostable directhemolysin to erythrocytes. Toxicon : Official Journal of the International Society on Toxinology 33: 651-657.

Youmans, G.R., Davis, T.E. and Fuller, D. D. (1993) Use of chemiluminescent DNA probes in the rapid detection of oxacillin resistance in clinically isolated strains of *Staphylococcus aureus*. Diagnostic Microbiology and Infectious Disease 16: 99-104.

Appendix I

Media and Reagents

Phage titre units (ptu/ml)

Phage titre units is the number of phage particles produced per ml of culture.
For *E.coli TG1*, it is the number of colonies produced on an agar plate (containing ampicillin and glucose) after infection with M13 phage and incubation at 37°C for 24 h. Each colony represents infection by a single phage.

Media

Alkaline peptone water (APW), pH 8.4

Prepare peptone water (Oxiod) as per the manufacturers instructions. Adjust the pH to 8.4. Add 0.5 % (w/v) sodium chloride to bring the final concentration of sodium choloride to 1% (w/v) and sterilize by autoclaving at 121°C at 15 psi for 20 min.

Thiosulphate Citrate Bile Salts mediumTCBS

Prepare TCBS Cholera medium (Oxiod)according to the manufacturers instructions. Dissolve the medium by boiling , do not autoclave. Pour the plates without any further heating and allow to set at room temperature..

2X TY medium (per litre distilled water)

16 g Tryptone (Oxiod)

10 g Yeast extract (Oxiod)

5 g Sodium chloride

2X TYE Agar (per litre distilled water)

10 g Tryptone

5 g Yeast extract

8 g Sodium chloride

15 g agar powder

Antibiotic stock solutions

Ampicillin = 50 mg/ml in sterile deionised water, followed by filter sterilization through a 0.2 μm filter (Acrodisc filters, PAL laboratories). Stock solution are stored as aliquots at -20°C.

Kanamycin = 25 mg/ml in sterile distilled water, followed by filter sterilization through a 0.2 μm filter. Stock solutions are stored as aliquots at -20°C.

Both antibiotics are added to 2X TYE medium after it has been melted and cooled to 50°C.

Miscellaneous reagents:

Glucose

20% stock solution in sterile deionized water, followed by fitler sterilization through a 0.2 μm filter. Stock solutions are stored as aliquots at -20oC.

PBS/BSA/Azide

250 ml PBS

6.25 ml 10% BSA (final concentration 0.25%)

1.56 ml Sodium azide solution (1.6 M)

Sodium azide is a carcinogen and extreme care should be taken while handling this reagent.

Lysozyme

Prepare a stock solution of 10 mg/ml in sterile deionised water. Store as aliquots at -20°C.

Proteinase K

Prepare a stock solution of 25 mg/ml in sterile deionsed water. Store as aliquots at -20°C.

dNTPS

Dilute stock dNTPs (10 mM) if required with sterile deionised water , store at -20°C as aliquots.

Chloroform:Isoamyl alcohol (for DNA extraction)

Mix chloroform: Isoamly alcohol in a ratio of 24:1.

Store in a light tight container at 4°C

Phenol, buffered (Sambrook *et al.*, 1989)

Before use, phenol must be equilibriated to a pH greater than 7.8, because the DNA will partion into the organic phase at acid pH.

Liquified phenol must be stored at -20°C. Before use allow it to warm to room temperature and melt at 68°C. Add hydroxyquinoline to a final concentration of 0.1%. To the melted phenol add an equal volume of buffer (0.5 M Tris.Cl, pH 8.0) and mix on a magnetic stirrer for 15 minutes. Allow the two phases to separate completely, and aspirate as much as possible of the upper aqueous phase. Add an equal volume of buffer again and repeat the step two more times, till the pH of the phenolic phase it greater than 7.8 (measure using pH paper). Phenol is extremely corrosive to extreme care should be taken in its use.

After the phenol is equilibrated, and the final aqueous phase is removed, add 0.1 volume of 0.1M Tris.Cl buffer (pH 8.0) containing 0.2% b-mercaptoethanol. Store at 100 mM Tris.Cl (pH 8.0) in a light-tight bottle at 4°C until required, for up to one month.

1X MPBS Blocking buffer

2% Marvel skimmed milk powder

1% BSA

in sterile PBS. Prepare fresh for each use.

DNA ladder

Add equal quantities of the ladder, sterile deionised water and 6X bromophenol blue loading dye. Store at 4°C as aliquots.

PEG/NaCl

20%(w/v) polyethelyene glycol 8000 in 2.5M NaCl

PBS/Glycerol

15% (w/v) glycerol in PBS

Buffers

5X TBE for electrophoresis (per litre)

54 g Tris base(Trizma)

27.5 g Boric Acid

20 ml of 0.5 M EDTA

TE buffer

10 mM Tris, pH 8.0

1 mM EDTA, pH 8.0

TSE buffer

10 mM Tris-Cl, pH 7.4

5 mM NaCl

0.1 mM EDTA, pH 8.0

Reagents for Western Blotting:

10% Separating gel

	ml
Bis Acrylamide(40% w/v)	3.33
1.5 M Tris-HCl, pH 8.8	2.5
10% SDS	0.1
distilled water	4
TEMED	10 µl
10% ammonium persulphate	50 µl

3% Stacking gel

	ml
Bis Acrylamide(40% w/v)	0.65
1.5 M Tris-HCl, pH 8.8	0.62
10% SDS	0.05
distilled water	3.7
TEMED	10 µl
10% ammonium persulphate	25 µl

Running buffer

0.025 M Tris

0.192 M glycine

0.1% SDS

Adjust the pH of the buffer to 8.3.

Sample buffer

	Reducing	Non-reducing
4% Dithiothreitol in Tris buffer	13 ml	
1 M Tris-HCl, pH 6.8		13 ml
20 % SDS	6.5 ml	6.5 ml
Glycerol	5.2 ml	5.2 ml
0.5 % Bromophenol blue	0.26 ml	0.26 ml

Transfer buffer

Tris HCl	3 g
Glycine	14.4 g
Distilled water	800 ml
Methanol	200 ml

Refrigerate until use.

Coomassie Blue stain

Coomassie Blue	1.25 g
Methanol	455 ml
Distilled water	455 ml
Acetic acid	92 ml

Filter before use

Destaining solution for Coomassie blue dye

Glacial Acetic Acid	375 ml
Methanol	250 ml

Make up volume to 5 L using distilled water.

Tris-buffered saline

Tris HCl	2.42 g
Sodium chloride	8 g
1 M HCl	3.8 ml

Dilute to 1 L with distilled water and adjust pH to 7.6.

PBS-Tween (0.1 %)

To 100 ml of sterile PBS add 0.1 % Tween 20 and mix well.

Reagents for Sequencing:

10 X TBE

Tris HCl	108 g
Boric acid	55 g
0.5 M EDTA,	
disodium salt	40 ml

Add Ultra pure water to make the volume to 1 L. Filter the solution through 0.45 µl filter and store at room temperature. Discard if any precipitate forms.

Bind-Saline

Absolute Ethanol	200 ml
Bind-saline	40 µl
Glacial acetic acid	300 µl

Store at 4°C.

Ammonium persulphate

Make up as a 10% (w/v) solution in ultra pure water. Store in aliquots in the -20°C freezer.

Running buffer

0.025 M Tris

0.192 M glycine

0.1% SDS

Adjust the pH of the buffer to 8.3.

Sample buffer

	Reducing	Non-reducing
4% Dithiothreitol in Tris buffer	13 ml	
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Destaining solution for Coomassie blue dye

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Methanol	250 ml

Make up volume to 5 L using distilled water.

Detection of bacterial pathogens by phage antibody display

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1. Introduction

Vibrio parahaemolyticus is a halophilic Gram-negative facultative anaerobe commonly found in estuarine waters and in seafood like shellfish. It has been implicated in outbreaks of gastro-enteritis in several countries [1]. Food borne illness caused by *V. parahaemolyticus* results chiefly from the consumption of insufficiently heated or raw seafood, especially in the summer months. The standard method for detection of *V. parahaemolyticus* (Bacteriological Analytical Manual procedure) is a culture-based procedure which can take up to four days for positive identification.

Recent outbreaks of *V. parahaemolyticus* related illnesses have heightened the need to develop a rapid and reliable method to detect this pathogen in shellfish. PCR methods, based on the amplification of the toxin producing genes, unfortunately give false postive results with other Vibrio species [2]. In our studies we have used phage antibody display to differentiate between pathogenic *V. parahaemolyticus* and other pathogenic and non-pathogenic *Vibrio* sp.

2. Methods

2.1. Bacterial cultures

Cultures of *V. parahaemolyticus* and other *Vibrio* sp. were obtained from the National Collection of Type Cultures, London and the ATCC.

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2.2. Panning

The human synthetic scFv (NISSIM) library was used as a source of antibody fragments of different specificities (greater than 10^8) [3].

Five rounds of panning were used to select for antibodies specific for surface markers on *V. parahaemolyticus*. Pooled cultures of *V. parahaemolyticus* (cell density = 8×10^8 cells/ml at A_{600}) were used in 4 rounds of positive panning. One round of negative panning was carried out using pooled cultures of non-parahaemolyticus Vibrios (cell density = 8×10^8 cells/ml at A_{600}) to try and remove all cross-reactive phage.

The negative panning step was carried out after the first round of positive panning. The titre of eluted phage was checked after each round of panning.

2.3. ELISA

ELISA was used to determine binding specificity of phage clones. Phage clones after the 5th (and last) round of panning were grown up in 96-well round bottom ELISA plates overnight.

Flat bottom 96-well ELISA plates were coated with 10^8 cells/well of different strains of *V. parahaemolyticus* and strains of other non-parahaemolyticus sp. overnight. Phage supernatant was obtained by growing up individual phage clones overnight at 30°C and then spinning the cells at 2500 rpm for 10 minutes. Supernatant containing phage antibody was added to the wells and the phage were allowed to bind for 2 hours. Excess unbound phage was then washed off, and the bound phage detected by adding anti-M13 HRP conjugated antibody for 1 hour followed by the addition of tetramethylbenzidine (TMB) substrate. The absorbance was measured at 450 nm using an ELISA reader (MRX microplate reader, Dynex technologies). Irrelevant phage (anti-NIP) was used as a negative control.

Table 1
ELISA (Absorbance at 450nm) of phage clones binding to different strains of *Vibrio* sp

	1	2	3	4	5	6	7	8
A	*	*	*		*	*	*	
B	*	*	*		*	◇	*	
C	*	*	*	*	△	*	△	△
D		*	*	*				
E		*	*			*	*	
F	*	*	*	△		*	*	

Key: Readings greater than 1.000 at A_{450} have been considered positive.
◇ = *Vibrio parahaemolyticus* NCTC 10884.
* = *Vibrio parahaemolyticus* NCTC 10903 + NCTC 10884 (1 : 1 ratio).
△ = *Vibrio mimicus* NCTC 11346 and *Vibrio harveyi* NCTC 11435 (1 : 1 ratio) (cultures used for negative panning).

Table 2
Binding percentages of selected phage antibodies against *Vibrio* sp. (Percentage binding of anti-NIP was used as blank)

Clone	*Vibrio parahaemolyticus* NCTC 10903	*Vibrio parahaemolyticus* NCTC 10884
A1	11.3%	7.7%
B3	21.3%	21.9%
B5	27.6%	27.8%
C2	33%	30.5%
E3	16.6%	12.5%
F7	16.9%	8.15%

2.4. FACS analysis

Repeatedly strong binding clones were selected for screening their binding properties using a FACSCalibur flow cytometer. Single cultures of *V. parahaemolyticus* as well as pooled cultures were used for analysis.

3. Results

After the final round of panning, 48 clones were picked for screening by ELISA. ELISA was carried out using individual cultures of *V. parahaemolyticus* NCTC 10903 and *V. parahaemolytiucs* NCTC 10884 as well as pooled cultures mixed in a ratio of 1 : 1. Cell density was maintained at 10^8 cells/well. Out of 48 clones, 28 clones showed repeated strong binding to both strains of *V. parahaemolyticus*. Preliminary results have been shown in Table 1. Clone B6 was the only clone that was positive with *V. parahaemolyticus* NCTC 10884 and negative with NCTC 10903. It may therefore represent a clone that is strain specific.

ELISA was also carried out using pooled cultures of *V. mimicus* NCTC 11346 and *V. harveyi* NCTC 11435 in a 1 : 1 ratio. Only four clones of the 28 were cross reactive with the above cultures.

CDR3 insert size was determined using PCR as previously described [3]. BstN1 restriction digestion of a PCR fragment covering the whole scFv region was also carried out as previously described [4].

Six phage clones out of the 28 were picked at random and were analyzed for their binding specificities using flow cytometry. All 6 clones were positive for *V. parahaemolyticus* on FACS analysis. The percentage binding to each strain was also determined (Table 2).

4. Discussion

Using phage display, we have been able to isolate antibodies that bind to *V. parahaemolyticus*. We are now in the process of screening these antibodies against a broad range of *Vibrio* and other related sp., from different sources, to determine their specificity.

References

[1] CDC, Outbreak of *Vibrio parahaemolyticus* infections associated with eating raw oysters – Pacific Northwest, 1997, *MMWR Morbidity and Mortality Weekly Report* 47(27) (1998), 457–462.
[2] Y.B. Kim et al., Identification of *V. parahaemolyticus* strains at the species level by PCR targetted to the *toxR* gene, *Journal of Clinical Microbiology* 37(4) (1999), 1173–1177.
[3] A. Nissim, H.R. Hoogenboom and I.M. Tomlinson et al., Antibody fragments from a "single pot" phage display library as immunochemical reagents, *EMBO J.* 13 (1994), 628–629.
[4] J. Marks, H. Hoogenboom and T. Bonnert et al., Human antibodies from phage display libraries, *Journal of Molecular Biology* 222 (1991), 581–597.

A RAPID ASSAY FOR THE DETECTION OF *Vibrio parahaemolyticus* IN SEAFOOD

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Vibrio parahaemolyticus has been implicated in outbreaks of gastroenteritis resulting from the consumption of seafood. Recent outbreaks of *V. parahaemolyticus* food poisoning have heightened the need to develop a rapid and reliable method to detect this pathogen in seafood Current methods for detection include culture, DNA hybridization technology, PCR methods and ELISA techniques. However most of these methods show false positive reactions with closely related species. We have been using phage antibody technology to differentiate bacterial these pathogens. Phage-antibody display is a powerful tool for the generation of antibodies against immunogenic and non-immunogenic molecules. Thus the ability to raise phage antibodies against a broad range of surface molecules should allow closely related species or sub-species to be distinguished. Using the NISSIM phage display library, antibodies were selected against whole cells of *V. parahaemolyticus* and tested for their binding properties using ELISA, flow cytometry and fluorescence microscopy.

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A Novel Method For the Detection of *Vibrio parahaemolyticus in Seafood*

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Background: *Vibrio parahaemolyticus* is a halophilic Gram-negative facultative anaerobe, which has been implicated in outbreaks of gastroenteritis resulting from the ingestion of contaminated seafood. Recent outbreaks of *V. parahaemolyticus* food poisoning in Japan and the USA have heightened the need to develop a rapid and reliable method to detect this pathogen in seafood. Current methods in use are MPN-based, in addition, rapid methods such as DNA hybridization technology, PCR and ELISA have been used for detection but these can give false positive results with other *Vibrio* species. To overcome some of these problems, we have used phage antibody technology, which is an effective tool for the generation of antibodies against a range of immunogenic and non-immunogenic molecules. **Methods:** The NISSIM phage library was panned against whole cells of mixed strains of *V. parahaemolytyicus*. A negative panning step was incorporated, using closely related *Vibrio* species, to remove any cross-reacting antibodies. Phage clones specific for antigens on whole cells of *V. parahaemolyticus* were selected and cultured. The binding properties of these antibodies were further studied using ELISA, FACScalibur flow cytometry and fluorescence microscopy. Tests were also carried out with non-*parahaemolyticus* strains and other species to check for cross-reactivity. **Results:** We were able to isolate several clones which bound only to *V. parahaemolyticus* cells using ELISA. The specificity of binding was confirmed by further analysis using flow ctyometry and fluorescent microscopy. Work is ongoing to compare the efficacy of these antibodies for detection of *V. parahaemolyticus* in seeded oyster tissue as compared to standard PCR methods. In addition we are also in the process of characterizing the exact nature of the phage-antibody specific antigen. **Conclusion:** We have shown in our system that phage display antibodies have the potential to distinguish between species of *Vibrio*, in addition, they have the potential to be used as diagnostic or identification tools.

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A RAPID ASSAY FOR THE DETECTION OF *V. parahaemolyticus* IN SEAFOOD

Keywords: *Vibrio parahaemolyticus*, seafood, detection methods, phage display

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Vibrio parahaemolyticus is a halophilic Gram-negative facultative anaerobe found in estuarine waters and in shellfish. It has been implicated in outbreaks of gastroenteritis in several countries resulting chiefly from the consumption of insufficiently heated or raw seafood. Recent outbreaks of *V. parahaemolyticus* food poisoning have heightened the need to develop a rapid and reliable method to detect this pathogen in seafood. The standard method for detection of this organism is an MPN-based procedure and can take upto four days for positive identification. DNA hybridization technology, PCR methods and ELISA using monoclonal antibodies have been employed and although these methods are rapid and sensitive, they give false positive results with other *Vibrio* species.

We have been using phage antibody technology to differentiate bacterial pathogens. Phage-antibody display is a powerful tool for the generation of antibodies against immunogenic and non-immunogenic molecules; thus increasing our ability to distinguish closely related species of organisms. Using the NISSIM phage display library, antibodies were selected against whole cells of *V. parahaemolyticus*. ELISA, flow cytometry and fluorescence microscopy were also used to test binding properties of the phage antibodies and many of the clones bound to strains of *V. parahaemolyticus*

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