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# **Recombinant antibodies against the K99 colonisation factor of *E. coli***

**Mehdi Golchin DVM**

**A thesis in fulfilment of the requirement for the degree of  
Doctor of philosophy of the University of Glasgow**

**September 2004**

**Division of Infection and Immunity  
Faculty of Biomedical and Life Science  
University of Glasgow**

This thesis is dedicated to my mother, my wife, Najmeh,  
my son Ali and my daughters, Fatemeh and Faezeh,  
always source of support and my inspiration for my future



## Declaration

This thesis is the original work of the author except where otherwise stated.

Mehdi Golchin



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## Abstract

Most strains of enterotoxigenic *Escherichia coli* (ETEC) isolated from neonatal cattle with diarrhoea express the colonization factor antigen, K99. K99 (F5) pili are necessary for the bacteria to bind to a receptor, *N*-glycolylneuraminic acid-GM3 on the cells of the bovine small intestine. Bacterial growth in the intestine and secretion of toxins triggers diarrhoea that can be fatal due to the loss of water and electrolytes. When the attachment of ETEC to host cells is inhibited, bacteria do not accumulate sufficiently in the gut to cause disease. Anti-K99 antibodies in the colostrum of vaccinated dams can mediate protection against ETEC infection in the neonate through this mechanism, a finding demonstrated by several vaccine trials using a variety of preparations containing K99 including whole cell bacterins and crude cellular extracts. Passive oral administration of antibody against K99 antigen also prevents severe fatal enteric colibacillosis in newborn calves. Therefore, the K99 adhesin is confirmed as an important virulence factor in bovine colibacillosis induced by ETEC strains.

K99 was chosen as a model target for this study, to explore the application of recombinant antibody technology to livestock infection. Our aims were to isolate and characterise single-chain Fv antibodies against K99 using phage display. *Escherichia coli* B41, a clinical isolate that expresses K99 fimbriae, was grown and fimbriae were extracted by heat-shock treatment and then precipitated by ammonium sulphate. The major K99 subunit (Fan C) was purified from crude fimbriae extract by an ion-exchange chromatography method using SP-XL columns. The method successfully purified a single protein of 16.5 kDa with high yield. This purified protein had high activity with monoclonal anti-K99 antibody in ELISA, dot blotting and immunoprecipitation. It also showed the ability at high dilutions to agglutinate sheep red blood cells. This purification protocol proved to be faster, more convenient and cheaper than other previous reported methods.

The purified Fan C was coated to immunotubes and a large human semi-synthetic library (Griffin. 1library, Center of Protein Engineering, Cambridge, UK) was screened by four rounds of biopanning to extract scFvs able to recognise the fimbrial protein. Phage antibodies were isolated and screened by monoclonal phage ELISA. This revealed some clones isolated from the library had activity against K99 but the background in this assay was generally high. Also PCR analysis of these clones showed that only about one third of them carried a full-length scFv insert. Expression of soluble scFv antibodies was attempted by transfection of phage into the non-suppressor *E. coli* strain HB2151. Expression was not

successful. Recloning the inserts into a vector designed to stabilise scFvs and other strategies failed to overcome problems in the expression of these soluble fragments. Sequencing showed that stop codons occurred commonly in the CDRs or framework regions. Therefore other libraries were used to achieve the aims of the project.

The semi-synthetic Tomlinson I and J libraries (Center of Protein Engineering, Cambridge, UK) were used to select phage antibodies against K99 using immunotubes coated with the major fimbrial subunit. After three rounds of selection, phage were transfected into *E. coli* HB2151 for the expression of soluble scFvs. Fifteen scFv clones with high activity against K99 fimbriae were identified by ELISA and sequenced. Of these, six scFvs carried sequences that were reasonably diverse. These proteins were purified for further characterization. The recombinant antibodies were shown to react with fimbriae present at the surface of *E. coli* B41 using immunofluorescence microscopy and immunogold electron microscopy. Some of the purified scFv antibodies were also able to inhibit the agglutination of sheep erythrocytes by *E. coli* B41 grown at 37 °C. To pursue this observation, attempts were made to use the scFvs in an *in vitro* model of bacterial colonisation in which bacteria were tested for attachment to isolated bovine intestinal villi. Although bacteria could be observed adhering to the brush border, the scFvs appeared unable to prevent this attachment. Further experiments with this *in vitro* model or a mouse model of ETEC infection, allied with epitope mapping studies should determine if anti-colonisation activity is attributable to binding of scFvs to the receptor-recognition site on the major subunit of the adhesin.

Overall, phage display thus offers a rapid and flexible approach to isolate reagents for a better understanding of microbial pathogenesis. Potentially, these reagents may also be of value in diagnosis and therapy.

## Abbreviations

BSA: bovine serum albumin  
C<sub>H</sub>: constant region of heavy chain  
C<sub>L</sub>: constant region of light chain  
CDNA: complementary DNA  
CDR: complementary determining region  
FR: framework region  
H chain: heavy chain  
HRP: horse radish peroxidase  
Ig: immunoglobulin  
IPTG: Isopropyl- $\beta$ -D-thiogalactopyranoside  
L chain: light chain  
LB: luria broth  
MAB: monoclonal antibody  
MPBS: milk in phosphate buffered saline  
PBS: phosphate buffered saline  
PCR: polymerase chain reaction  
RT-PCR: reverse transcriptase polymerase chain reaction  
scFv: single chain antibody fragment  
V: variable region  
V<sub>H</sub>: variable region of heavy chain  
V<sub>L</sub>: variable region of light chain  
HABA: 4'-hydroxyazobenzene-2-carboxylic acid

# **Chapter 1**

## **General Introduction**

# 1. General Introduction

## Antibodies for diagnosis and therapeutics

Antibodies are a component of the immune system, which constitutes a versatile system to defend the body against intruders. The main purpose of antibodies is to bind pathogens specifically and thereby mark them for attack by other protein components of the immune system or its cells (Breitling and Dübel, 1999). Antibodies are capable of highly specific interactions with a wide variety of ligands including tumor-associated markers, viral coat proteins, and lymphocyte cell surface glycoproteins. They are potentially very useful agents for diagnosis and treatment of diseases (Riethmuller *et al.*, 1993). In recent years, antibodies have become increasingly accepted as therapeutic reagents, particularly for cancer but also for numerous other disorders. An indication of the emerging significance of antibody-based therapeutics is that over a third of the proteins currently undergoing clinical testing in the United States are antibodies (Maynard and Georgiou, 2000).

Animals naturally produce antibodies as part of the protective response against agents that are encountered during life. This property is extensively exploited by vaccination either to protect the host in advance of infection or to raise antibody responses for passive transfer to other animals, for example the neonate. The great variety of antibodies, as well as their high specificity and affinity for antigens, makes it possible to use them as tools for *in vivo* and *in vitro* diagnostics. Antibodies may also have natural enzymatic activities, which can be optimised by mutations (Fletcher *et al.*, 1998). These antibodies, known as abzymes, might become a new source of enzymes. Further more, antibodies could be used to manage environmental pollution by neutralizing toxic substances (Harris, 1999). Antibodies can also mediate protection against intracellular pathogens (Casadevall, 1998). In this respect, they could provide a major line of defence against biological attacks (Casadevall, 2002).

Antibodies neutralize pathogens via different mechanisms. It is conventional to view the activation of complement or antibody-dependent cellular cytotoxicity (Gura, 2002) as the main mechanisms by which antibodies lead to the elimination of pathogens. However, simple binding of the antibody to a key molecule of the pathogen may be sufficient to inactivate it (for example, by steric obstruction of interaction with a receptor) and thereby

provide protection. In this context, recent studies have shown that the simultaneous use of monoclonal antibodies directed against several different conformational epitopes of the human immunodeficiency virus envelop which binds CD4 in human lymphocytes, prevented infection by various mutants of virus (Ferrantelli and Ruprecht, 2002). Antibodies can specifically block the action of natural factors *in vivo*, and are used to inhibit rejection mechanisms after organ transplantation. Antibodies can also be used as vehicles to target biologically active molecules to specific cells. For instance, radioactive ions can be bound to antibodies that specifically recognize tumour cells (Quadri *et al.*, 1993; Webber, 1998). Toxins bound to antibodies can have similar effects (Baluna *et al.*, 2000).

As can be seen, antibodies have a vast range of uses both *in vivo* and *in vitro*, all of which require different forms of these molecules. .

### ***Antigen-binding sites – structures and generation***

The antigen-binding site of an antibody is created by the non-covalent association of the N-terminal “variable” domains of the heavy and light chains. Structural studies of antibodies complexed with antigen (Wilson and Stanfield, 1994; MacCallum *et al.*, 1996) have shown that the antigen-binding site is formed from the solvent-exposed side chains of amino acids in 6 loops, 3 from VH and 3 from VL. These emerge from an underlying structure formed by the framework regions of the variable domains. The H3 loop, the most variable in sequence and length, and L3, occupy the center of the binding site and tend to make most of the key contacts with antigen. These 6 loops are substantially, but not exactly, similar in extent to the previously defined complementarity determining regions (CDRs) (Wu and Kabat, 1970). The CDRs were initially identified as regions within the variable domains that exhibited even greater variability between different antibodies. For 5 of the 6 loops, it has been possible to identify a small number of canonical conformations that each can adopt depending on sequence, length and the identity of key framework residues which contact the loops (Chothia and Lesk, 1987; Chothia *et al.*, 1989; Tramontano *et al.*, 1990). H3 remains too variable in sequence and length for such rationalization. Using this information, predictions can be made about the conformation of a newly derived antibody sequence. Some estimate of the likely structure of the antigen-binding site, based upon sequence analysis, other calculations or structural determination, is important for many antibody engineering projects (*eg* humanization or affinity modification). The sequences of H3 and L3 are generated during the process of genomic recombination and somatic mutation which occurs during B cell development.

The coding sequence of H3 is formed when one member from each of 3 genetic segments, the VH minigenes, DH (diversity) segments and JH (joining) segments, is brought together during the maturation of the Ig heavy chain. The coding sequence for L3 is formed by recombination of one each from a set of VL minigenes and JL segments during the formation of the light chain. H1 and H2, and L1 and L2 are derived from the coding sequence in the VH and VL minigenes, respectively, but can also be altered later during the somatic mutation process (Tramontano *et al.*, 1990). The number of these sequences in humans, primates and rodents, their organization into sequence subfamilies and their relative utilization in the immune response is of interest to antibody engineering projects. Heavy chain recombination occurs before light chain reorganization and the heavy chain variable domain appears on the surface of pre-B cells as a membrane bound protein associated with a surrogate light chain. Later in B cell development, rearrangement of the  $\kappa$  locus occurs, and if no productive  $\kappa$  chain is made at either of the  $\kappa$  alleles then  $\lambda$  rearrangement occurs until a functional light chain is formed. A functional light chain then appears on the B cell surface with a heavy chain. Exposure to antigen and selection for B cells expressing binding sites with reasonable affinity for antigen then takes place. Within a species, the V minigene sequences can be grouped into a number of families according to amino acid or nucleotide sequence homology.

## Antibody engineering

Antibody engineering became possible with the development in 1975 of hybridoma technology, the fusion of a myeloma with B-cells from an immunised animal to create cells able to produce monospecific antibody in culture (Kohler and Milstein, 1975). For the production of hybridomas, a laboratory animal (usually a mouse or rat) is immunised to raise a high titre of circulating antibodies against the immunising antigen. B lymphocytes are then isolated from the spleen. Although each lymphocyte produces antibody with affinity for a single antigenic unit or epitope, the proteins cannot be obtained directly from isolated B lymphocytes in culture as the life time of these cells is too short *in vitro*. To solve this problem, Kohler and Milstein fused B lymphocytes with plasmacytoma cells, descendants of a plasma-cell-tumour. The resulting “hybridoma cells” possess the properties of both fusion partners: they are immortalised and produce the monoclonal antibody originally secreted by the B lymphocyte partner. Monoclonal antibody technology remains one of the core technologies of biotechnology, and thousands of medically and diagnostically relevant hybridomas have been developed. In 1980 two monoclonal antibodies were subjected to clinical trials (Reichert, 2001), but this number



has increased considerably over the past 20 years. Currently, approximately 200 antibodies and their derivatives are in clinical trials for the treatment of disease (van Dijk and van de Winkel, 2001; Brekke and Sandlie, 2003). The majority of monoclonal antibodies produced by hybridoma technology are murine antibodies, which are immunogenic when used as therapeutic agents in humans (Monzavi-Karbassi and Kieber-Emmons, 2001). The induced human anti-mouse antibody (HAMA) response quickly reduces the effectiveness of therapy by clearing the murine antibody from the bloodstream. The generation of human monoclonal antibodies (Humab) may be performed either by immortalization of human B-lymphocytes through fusion with neoplastic cells or by transformation with Epstein-Barr virus. Human hybridoma technology has been restricted by the inefficiency of these immortalization procedures and the ethical constraints on immunization of humans (Monzavi-Karbassi and Kieber-Emmons, 2001).

A revolution in antibody engineering began in the late 1980s when efficient systems for the cloning and expression of antibody genes in bacteria were developed (Winter and Milstein, 1991). Cloning the genes of monoclonal antibodies from murine hybridomas was greatly simplified by the discovery of the polymerase chain reaction (PCR), and bacterial expression systems allowed rapid and straight-forward production of functional recombinant antibody fragments for analysis (Pluckthun and Skerra, 1989).

To clone antibodies, mRNA is isolated from hybridoma, spleen, or lymph cells, reverse transcribed into cDNA, and antibody genes are amplified by PCR. This strategy requires oligonucleotide primers that can recognise any antibody gene. Numerous primer sets have been derived from the N-terminal sequence of purified antibodies (Benhar *et al.*, 1994), rapid amplification of cDNA ends (Ruberti *et al.*, 1997), antibody leader sequences (Larrick *et al.*, 1989) and most popularly, primers based on known variable region framework amino acid sequences.

An artefact of the process used to generate hybridomas is that myeloma cell lines may express irrelevant heavy or light chains, in addition to the desired monoclonal antibodies (Maynard and Georgiou, 2000). The general cloning strategy outlined above will amplify all antibody genes present at the mRNA level, and multiple heavy and light chains, in addition to the desired ones, may thus be cloned (Krebber *et al.*, 1997a). Once the correct genes encoding VH and VL domains have been identified, they can be assembled in a number of forms suitable for expression or future manipulation (Maynard and Georgiou, 2000).

Whilst these methods can be applied to the isolation and expression of human antibodies, engineering techniques can also overcome the immunogenicity of rodent monoclonal antibodies in human patients.

## ***Humanization of monoclonal antibodies***

### **Chimeric antibodies**

Chimeric antibodies represented the first attempt to reduce the immunogenicity of heterologous monoclonal antibodies. Chimeric monoclonal antibodies are 60-70% human and are obtained by exchanging the variable regions (Fv) of human antibody heavy and light chain genes for those derived from the rodent monoclonal, thereby imparting the desired antigenic specificity. They are constructed by joining the DNA segments encoding the mouse variable regions, specific for a certain antigen, to segments of DNA encoding human constant regions. This retains variable regions that are of murine sequence, but in spite of the possible immunogenicity of these structures, four chimeric monoclonal antibodies have reached the market as therapeutic agents (Van den Broeck et al., 1999; Brekke and Sandlie, 2003).

### **CDR-Grafted antibodies**

CDR-grafted antibodies, developed in the early 1990s, represented a step closer to the complete humanization of murine monoclonal antibodies. Interaction between antibody and antigen is typically limited to the CDRs (complementary-determining regions) present on each variable domain of the immunoglobulin ( $V_H$  and  $V_L$ ), the overall conformation of the domains being determined by the flanking framework regions (Rees *et al.*, 1994). Mouse CDRs for a specific antigen can be grafted onto a human framework, resulting in an antibody that is 90-95% human in sequence, and that retains the affinity and specificity characteristics of the original murine monoclonal antibody (McCafferty and Glover, 2000). To date, five CDR-grafted antibodies have reached the market for therapeutic application (Reichert, 2001).

## ***Transgenic Antibodies***

A further strategy to obtain fully human antibodies involves the genetic engineering of the immune system of the mouse. The basic idea of this technology, introduced in the mid-1990s, is to generate an immune response in "knock-out" mice that have been engineered

to produce human antibodies and then use hybridoma methods to obtain immortalised cell lines secreting antibodies. One way to achieve this has been to inactivate the endogenous mouse Ig system and subsequently introduce human Ig gene segments, enabling the mouse to produce entirely human antibody molecules (including isotype switching and affinity maturation) when immunized with a specific target. These animals have been termed "HuMab" mice (Neuberger, 1996; Green, 1999). Another strategy is to engineer mice to carry human minichromosomes derived from human chromosomes 14 and 2, containing the complete germ-line gene clusters for heavy and  $\kappa$  light chains. In these "trans-chromosomal" mice, the environment around the human immunoglobulin genes is very similar to that in the original host (Tomizuka *et al.*, 2000). A further approach is to introduce human lymphocytes from immune donors or cancer patients into SCID (Severe Combined Immunodeficiency) mice or into "Trimera" mice (mice lethally irradiated and then reconstituted by transplantation of bone-marrow cells from SCID mice) (Reisner and Dagan, 1998). Following antigen immunization, mouse spleen cells are fused to myeloma cells, as described above, and the resulting hybridomas screened for human antibodies of the desired specificity. All these approaches have been used with varying degrees of success to produce human antibodies but such is their relative complexity, their application has not extended to other mammals (*eg* livestock animals).

## Recombinant antibodies

With the development of recombinant antibodies, a new approach for the production of antibodies has recently emerged. The antibodies are no longer produced in laboratory animals, but in bacteria or in cell culture, *in vitro*. The focus of attention in these technologies is the antigen-binding portion of the antibody.

In order to obtain higher yields in bacteria, the rest of the antibody molecule is usually relinquished. The resulting immunoglobulin fragments can no longer perform all the functions of a naturally produced antibody but they can be fused with enzymes or other antibodies with comparative ease. In this way recombinant antibodies acquire a completely new set of properties given the numerous possibilities of recombination with other gene fragments (Breitling and Dübel, 1999).

The smallest component of an antibody that retains antigenic specificity is the minimal recognition unit (MRU), derived from the peptide sequence of a single CDR (Ritter Mary *et al.*, 1995). The binding affinities of these sequences is often low and is only elevated by a protein able to bring several MRUs together, *eg* a single  $V_H$  domain. These suffer different limitations - isolated  $V_H$  domains are very unstable and prone to aggregation *in*

*in vivo* because the hydrophobic area that normally forms the interface with the V<sub>L</sub> domain is exposed to the solvent.

In general, the presence of both of V<sub>H</sub> and V<sub>L</sub> domains is needed for high stability and antigen affinity. V<sub>H</sub> and V<sub>L</sub> chains can be expressed as separate peptides in bacteria where they spontaneously assemble into Fv fragments.

In these dimeric proteins, the two peptide chains are held together by noncovalent interactions and are therefore prone to dissociation and aggregation. The two chains can be covalently assembled by engineering an interchain disulfide bond, to give a dsFv antibody. This design is more stable than an Fv fragment, but it is difficult to produce by bacterial fermentation, and the disulfide bond can be reduced under mild conditions. A more popular strategy is to introduce a short polypeptide linker to fuse the V<sub>H</sub> and V<sub>L</sub> chains together into a single chain (sc) Fv antibody fragment. ScFvs are relatively small (26-27 kDa), generally quite stable, and are encoded by a single gene which simplifies later manipulations. The most common linker is a flexible (Gly<sub>4</sub>Ser)<sub>3</sub> decapentapeptide (Huston *et al.*, 1988). The two variable domains can be connected either as V<sub>H</sub>-linker-V<sub>L</sub> or V<sub>L</sub>-linker-V<sub>H</sub>, with the former being more common. The order of the two domains can affect expression efficiency, stability, and the tendency to form dimers in solution (Tsumoto *et al.*, 1994; Merk *et al.*, 1999).

In addition to scFvs, the other commonly used recombinant antibody fragments are Fabs. Fabs consist of two polypeptide chains, one containing the light chain variable and constant domains (V<sub>L</sub>C<sub>κ</sub> or λ), the other a truncated heavy chain containing the variable domain and one constant domain (V<sub>H</sub>C<sub>H1</sub>). Just as in intact IgG immunoglobulins, the two chains are linked together by a disulfide bond. The more extensive interface between the two chains and the presence of the disulfide bond confer increased stability. Although the expression of Fab requires the association of two chains, it often occurs quite efficiently in bacteria (Carter *et al.*, 1992; Skerra, 1994). On the other hand, the presence of the two chains somewhat complicates genetic manipulations, and the larger size of these portions may limit their bioavailability for certain therapeutic applications. Several Fab fragments are more poorly expressed in functional form in *E. coli* than Fv-fragments and their derivatives (Skerra and Pluckthun, 1991). It appears that a higher tendency to aggregate can cause this problem.

The production of whole antibodies in *E. coli* suffers several limitations. Firstly, the proteins lack normal glycosylation in the Fc region that contributes to stability and biological functions (Shin *et al.*, 1992). Yields of these large proteins are often poor. In order to preserve the bivalency that is an important feature of native immunoglobulins and a very effective means of increasing the functional affinity (avidity) (Crothers and

Metzger, 1972) another strategy can be used: the linking of scFv fragments by a small modular dimerization domain in the form of one or two amphipathic helices (Pack and Pluckthun, 1992; Pack *et al.*, 1993). These “miniantibodies” (Pack and Pluckthun, 1992; Pack *et al.*, 1993) assemble in dimeric form in *E. coli*, and the binding performance of the best of them is indistinguishable from a whole antibody in avidity terms.

Antibody fragments such as F(ab')<sub>2</sub>, Fab, and scFvs, may have particular advantages over intact antibodies. F(ab')<sub>2</sub> is the largest proteolytic fragment that retains the bivalent binding sites of an antibody and through its size advantage, remains in the blood much longer than smaller antibody fragments, such as Fab or scFv (Chamow and Ashkenazi, 1999). Some attempts have been made to alter the stability of these smaller antibody fragments (Worn and Pluckthun, 2001) and increase their circulation time *in vivo* by chemical linkage to polyethylene glycol (PEG; "pegylation") (Wang *et al.*, 1998; Chapman *et al.*, 1999; Chapman, 2002). However, scFvs or Fabs may be preferred in situations such as tumour imaging where tissue penetration and clearance from the circulation is important and the effector functions of the Fc portion are dispensable. Once a Fab or scFv fragment with high affinity and specificity for a target antigen has been obtained, it may prove useful to genetically reconstruct these fragments into an intact fully human antibody (Huls *et al.*, 2001). The overall advantages of scFv antibodies are their easier and faster production, the ability to isolate them from libraries to generate reagents against an enormous number of antigens (foreign antigens, self antigens, non-immunogenic antigens and toxic antigens), their lower retention times in non-target tissues, their reduced immunogenicity, and the ability to fuse them to other proteins and peptides (Little *et al.*, 2000).

### ***Fusion and Bispecific Antibodies***

Several approaches have been developed in order to increase the efficiency of antibodies or antibody fragments, particularly in regard to their effector functions. Chemical conjugation of antibodies to effector compounds such as bacterial or plant toxins or cytotoxic drugs allows increased antibody efficiency but can inactivate antibody-binding sites or alter the effector agents as a result of the chemical manipulation. Some of these problems can be overcome by genetic fusion of the antibody or fragment to the intended ligand. In doing this, the ligand must leave the antigen-binding site free. Ligands that have been used to improve effector functions are as toxins (commonly used to target cancer cells), cytokines, and enzymes (functioning as a drug or pro-drug converting system). Fusion proteins can also bring other properties to antibodies such as the introduction of a lipid anchor to produce a membrane-bound scFv fusion (Laukkanen *et al.*, 1993).

An alternative method to improve effector functions is to produce bispecific antibodies - molecules that have two different specific antigen-binding sites. If one component carries a target-binding arm and the other, an effector-binding arm (*eg* an antibody against a cell marker), conventional and novel effector mechanisms can be induced. Bispecific antibodies can be generated from scFvs and Fabs by somatic cell hybridization, chemical conjugation (heteroconjugates), or genetic engineering. For example, conjugation of Fab fragments can give rise to bispecific F(ab')<sub>2</sub> molecules or trimeric Fab molecules. These proteins can be used in immunoassays, immunodiagnostics, and therapy (Kriangkum *et al.*, 2000; Schmiedl *et al.*, 2000; Zuo *et al.*, 2000). Recently, fusion proteins comprising scFvs or Fabs and peptides that possess antibody effector functions (pepbodies) have been expressed in bacteria. These fusion proteins were able to initiate antibody effector functions (Lunde *et al.*, 2002).

### **Antibody-Like Structures**

Antibodies can also be engineered into compact and multivalent structures, such as diabodies, triabodies, tetrabodies, and minibodies (Adams and Schier, 1999). Changing the linker length between V domains of a scFv creates new types of Fv modules (Hudson and Kortt, 1999). If the linker is 5-12 residues long, a scFv molecule is constrained, forcing association with a second scFv to form a dimer called a diabody (Arndt *et al.*, 1998; Volkel *et al.*, 2001). When the linker length is reduced to less than 3 residues, three scFvs are forced to associate into trivalent (triabody) or a tetravalent structures (tetrabodies), depending on the linker length, its composition and the orientation of the V domains (Kortt *et al.*, 1997; Atwell *et al.*, 1999; Hudson and Kortt, 1999; Kortt *et al.*, 2001). According to the specificity of the variable domains used in construction, it is possible to produce bivalent and bispecific antibody fragments (Holliger *et al.*, 1996), bivalent monospecific diabodies (Kortt *et al.*, 1997), non-functional triabodies (Pei *et al.*, 1997), monospecific trivalent triabodies or tetravalent tetrabodies (Kortt *et al.*, 1997; Kortt *et al.*, 2001), and trispecific and tetraspecific triabodies or tetrabodies (Hudson and Kortt, 1999). The use of multimeric antibodies in cancer imaging (Power and Hudson, 2000), therapeutics, targeting (Holliger *et al.*, 1996; FitzGerald *et al.*, 1997; Kortt *et al.*, 2001), and recruitment of effector functions (Holliger *et al.*, 1997; Kontermann *et al.*, 1997; Cochlovius *et al.*, 2000) has been explored.

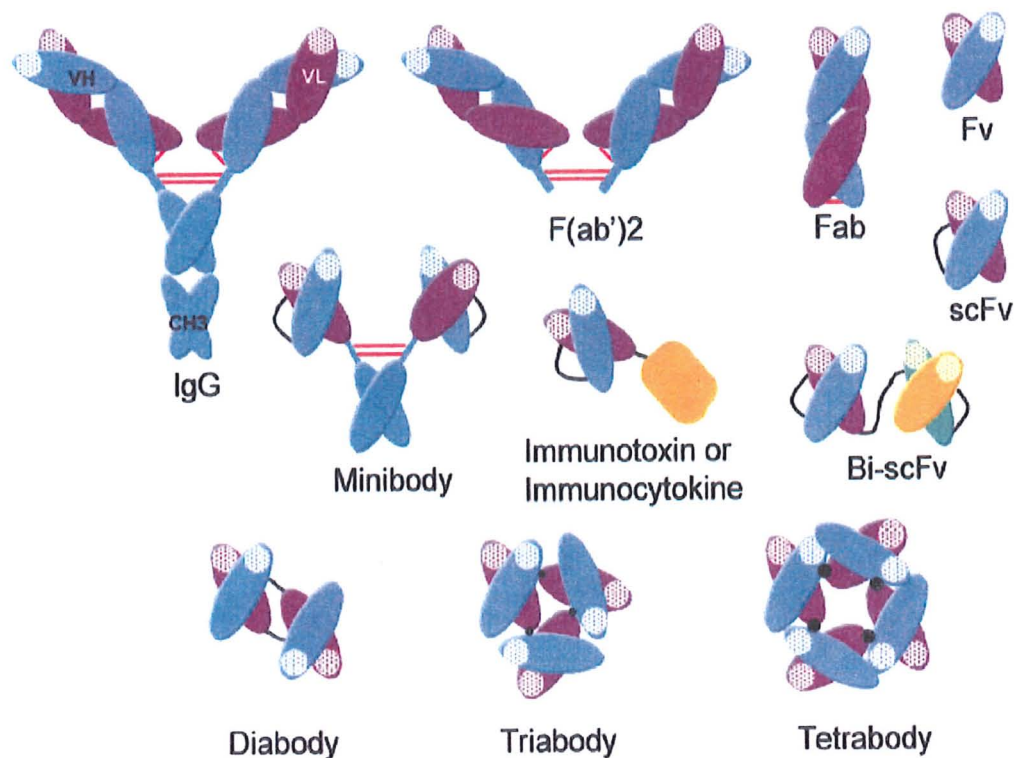
The various formats of engineered antibodies are illustrated in Figure 1.

## ***Expression systems for production of recombinant antibodies***

Several systems for production of recombinant antibody fragments have been described using bacteria, yeast, plant, insect, and mammalian cells. Each system has potential advantages and limitations. Bacteria are particularly suitable for the production of antibody fragments but cannot assemble whole glycosylated antibodies. Full-length antibodies have been expressed in yeast, but glycosylation tends to introduce highly mannose and multiply-branched oligosaccharides. These antibodies were shown to be defective in effector functions such as complement-mediated lysis. Full-length antibodies have also been produced in insect cells via baculovirus vectors. These also contained carbohydrate structures that were very different from those produced in mammalian cells. Native antibodies with full functionality have been expressed in myeloma and non-lymphoid mammalian cells where mechanisms required for correct assembly, post-translational modification, and secretion all exist. The production of antibodies and antibody fragments in different expression systems has been reviewed by Kipriyanov and Little (Kipriyanov and Little, 1999).

### **Expression of antibody fragments in *E. coli***

One trigger to the rapid growth of antibody engineering was the discovery that functional antibody fragments could be secreted into the periplasmic space of *E. coli* by fusion of a bacterial signal peptide to the antibody N-terminus (Better et al., 1988; Skerra and Pluckthun, 1988). After this, the production of antigen-specific antibodies in bacteria was attempted (Fuchs et al., 1992). The goal of capturing large antibody repertoires from the immune system was accomplished when it was shown that immunoglobulin families could be PCR-amplified from the lymphocytes of immunised mice, cloned into plasmids and transformed into bacteria. Immunogen-reactive antibodies were then selected by an ELISA using the supernatant of cultured bacteria (Ward et al., 1989). By inserting the antibody operon into bacteriophage  $\lambda$ , this procedure was improved. Selection systems of various formats have been designed to ease the identification of clones of potential interest based upon retroviral (Russell et al., 1993), baculoviral (Boublik et al., 1995), yeast (Kieckhefer et al., 1997), and cell free ribosome display (Mattheakis et al., 1994; He and Taussig, 1997). The most successful selection and expression system remains that founded upon filamentous bacteriophages of the M13 family (Smith and Petrenko, 1997).



**Figure 1.1 The various formats of engineered antibodies**

IgG is composed of two identical heavy chains (VH+CH1+CH2+CH3) and two identical light chains (VL+CL). Both chains are organized as domains containing about 110 amino acids with each domain possessing an intra-disulfide bond (not shown). Inter-disulfide bonds (red lines) link the light chain to the heavy chain and the two heavy chains. The variable domains (VH and VL) contain the complementary determining regions (CDRs) which bind to the antigen (CDR represented by small dots). The Fv fragment (VH and VL domains) possesses the binding activity. The scFv corresponds to the VH linked to the VL by a flexible peptide linker (black lines). Dia-, tri- and tetrabodies can be obtained by using short linkers (represented by black lines or dots). The Figure has been taken from the work of Chames and Baty (Chames and Baty, 2000).



## Antibody phage display

The principles of phage display are simple and elegant. DNA encoding millions of variants of certain ligands (*eg* peptides, proteins or fragments thereof) is batch-cloned to create fusions to one of the genes encoding a phage coat protein (pIII, pVI or pVIII). Upon expression, the fusion protein will be incorporated into new phage particles that are assembled in the bacterium. This results in the ligand being presented on the phage surface, while its genetic material resides within the phage particle. This connection between ligand genotype and phenotype allows the enrichment of specific phage using selection on an immobilized target. Phage that display a relevant ligand will be retained, while non-adherent phage will be washed away. Bound phage can be recovered from the surface, reinfected into bacteria and re-grown for further rounds of selection.

The filamentous phage contains a single strand DNA genome that is enclosed in a tubular array of approximately 2700 molecules of the major coat protein pVIII (encoded by gene 8 or g8p). At the tips of the phage, four minor proteins (g3p, g6p, g7p, g9p) are found. Protein pIII or g3p is present in about five copies per virion and initiates infection of *E. coli* by binding to the F pilus. The pIII protein has a three domain structure. The two N-terminal domains appear to be involved in the infection process but peptides and proteins can be introduced at the N-terminus or between the second and third domains without destroying this function (Smith, 1985; Parmley and Smith, 1988; Riechmann and Holliger, 1997). An important regulatory element of the genome, the intergenic region (IR), carries an origin of replication and a DNA packaging signal. A plasmid that carries IR, is known as a phagemid because the IR allows packaging of the phagemid into phage particles as single-stranded closed circular DNA if other viral functions are brought to the system by co-infection with a helper phage. Packaged phagemids can infect bacteria and are known as transducing particles since on entry to the bacterial host, phagemid functions (*eg* antibiotic resistance) are expressed by the replicating plasmid.

Filamentous phage are not lytic to the bacterial host - they leave the cell by extrusion through the membrane leaving the cell unharmed. Assembly begins with the coating of the single-strand of genomic (or phagemid) DNA by g5p. This nucleoprotein complex then migrates to the inner membrane. Coat proteins then replace g5p in a vectorial fashion: g7p and g9p bind first forming one tip of the viral particle, followed by polymerisation of g8p on to the DNA rod, and finally g6p and g3p cap the particle. G3p and g8p are exported

through the cytoplasmic membrane of the bacterial host and become anchored on the periplasmic face from where they are assembled to form the surface of the phage. These abilities make them ideal targets for fusion with antibody domains not least because secretion into the periplasmic space is required for the correct folding and assembly of antibodies produced in *E. coli*.

For successful phage display, the properties of the helper phage are also important. Helper phage such as M13K07 or KM13 are ideal because they form the viral structure but package their own DNA into the filament less efficiently than the phagemid. Superinfection is usually triggered by addition of 10 helper phage per bacterium. This infection process could be by the binding of pIII fusion proteins translated from the phagemid to the pilin subunit of F-pili. This is limited by the accumulation of the protein in the cytoplasmic membrane. According to Kay *et al.* (Kay *et al.*, 1996), the most important aspects of filamentous phage biology for display methods are: (a) the phages can infect special strains of *E. coli* containing an F-plasmid because they attach to the F-pilus and reach the membrane by depolymerization of the F-pilus. (b) depolymerization of the F-pilus is immediately after infection, resulting in infection of *E. coli* with only one phage. Thus cloning of phage is possible *via* cloning of *E. coli*. (c) The size of DNA packed into a filamentous phage particle is adapted to the size of the packaged DNA during extrusion of the phage from *E. coli*.

In antibody phage display, combinations of  $V_H/V_L$  able to interact with the target of interest are selectively enriched through a series of immunoaffinity steps referred to as "library panning". By washing away non-specific phage, the remaining antigen-specific phages are extracted and then recovered using acid or alkali (Marks *et al.*, 1991). These treatments break the interaction between phage and the target ligand, but fail to prevent the infection process when phage are mixed with bacteria. Other elution methods have been described. Proteolytic cleavage sites for trypsin (Breitling *et al.*, 1991) or for Genenase I (Ward *et al.*, 1996) have been introduced between the antibody domain and pIII. Also the competitive elution of bound phage with a monoclonal antibody has been described (Meulemans *et al.*, 1994). The presence of an amber stop codon between the DNA coding for the antibody and pIII facilitates the characterization of selected antibodies. In suppressor strains of bacteria, the antibody-pIII fusion will be translated but on transfer to nonsuppressor strains of *E. coli*, only the antibody domain is synthesised (Hoogenboom *et al.*, 1991). Since the recombinant antibodies lack the features often recognised by species-specific reagents, marker peptides are often present either at the C-terminus (Ward *et al.*, 1989) or N-terminus (Parmley and Smith, 1988) or within the linker peptide between

heavy and light chain variable domains (Parmley and Smith, 1988) of a scFv. In a recent report, several of these features were incorporated into an improved expression phagemid (Hayashi *et al.*, 1995). This vector also provides the antibodies with a hexahistidine sequence for purification by metal chelate chromatography and a C-terminal cysteine to facilitate conjugation with other molecules.

## ***Phage display antibody libraries***

Various methods have been used for the construction of phage display libraries to clone antibody repertoires from humans, mice, rabbits, chickens, camels and other animal species. These libraries have usually been generated in one of four formats: immune, naïve, semi-synthetic and synthetic libraries.

### **Immune libraries**

In this strategy, antibody genes are isolated using PCR and sets of primers homologous to the heavy chain and light chain ( $\kappa$  and  $\lambda$ ) using RNA from immunised donors. A set of primers designed from the amino acid sequence of human immunoglobulins has been described by Welschhof *et al* (Welschhof *et al.*, 1995). One advantage of immune libraries is that they are particularly rich in antibody chains that bind to the immunogen with high affinity, since IgGs will have undergone affinity maturation during the development of plasma B cells. Clinically useful antibodies against several diseases have been isolated from this kind of library by using phage display selection systems.

### **Naïve libraries**

Phage display libraries may be produced from human or animal lymphocytes following RT-PCR amplification of  $V_H$  and  $V_L$  genes. These libraries called “naïve libraries” because they are composed of  $V_H$  and  $V_L$  genes from non-immune donors who do not have ongoing immune responses against a particular antigen. Naïve libraries provide a readily available resource of antibody fragments with binding activities against many different antigens (Marks *et al.*, 1991; Marks *et al.*, 1993). It is estimated that about  $10^6$ - $10^8$  antibodies with different binding properties are present in the human immune system at any time. This number is enough to bind most antigens including synthetic compounds that the immune system has never encountered. Since IgMs are expressed mostly on the surface of non-activated B-lymphocytes, one approach has been to amplify

and randomly combine chains of the naïve IgM repertoire (Marks *et al.*, 1991). Naïve libraries are constructed by two or three cloning steps. In the two-step strategy, the amplified repertoire of light chain genes is cloned into the phage-display vector in the first step, because the heavy chain contributes more to diversity owing to its highly variable CDR3 region. In the second step, the heavy chain repertoire is cloned into the amplified light chain library (Johansen *et al.*, 1995; Welschof *et al.*, 1997). In the three-step strategy, separate heavy and light chain libraries are engineered, and the repertoire of the  $V_H$  library is then excised and cloned into the phage-display vector containing the  $V_L$  library (Welschof *et al.*, 1997). Another common method that has been used to clone naïve scFv phage display libraries is assembly PCR.  $V_H$  and  $V_L$  genes are amplified separately, connected in a subsequent PCR step and then cloned into the vector (McCafferty *et al.*, 1994; Vaughan *et al.*, 1996).

The affinity of antibodies selected from a naïve library is relative to the size of the library. Affinities in the range of  $10^6$ - $10^7$  /M are typical for a small library with  $3 \times 10^7$  clones (Marks *et al.*, 1991; Griffiths *et al.*, 1993; de Wildt *et al.*, 1996), whereas  $10^8$ - $10^{10}$  /M has been achieved for a very large library with  $10^{10}$  clones made by brute force cloning (Vaughan *et al.*, 1996). Several problems may impact upon the use of naïve libraries. Firstly, there is very little information on the exact nature of the V-gene repertoire contained in the library. Poor expression and toxicity to the host bacteria can also arise.

### **Semi-synthetic and synthetic libraries**

This approach is founded upon the assembly *in vitro* of V-gene segments that carry a predetermined level of randomisation in CDR regions and (in some libraries) bordering framework-regions. Diversity can be introduced into germline V-gene segments (Hoogenboom and Winter, 1992), or rearranged V-genes (Barbas *et al.*, 1992). The regions and degree of diversity may be chosen to correspond to areas of highest natural diversity of the antibody repertoire. Most natural structural and sequence diversity is found in the loop most central to the antigen combining site, the CDR3 of the heavy chain, while the five other CDRs have limited variation. In some cases, the framework of a well-known antibody has been used as a scaffold on which to integrate new, randomly created CDR3 of the heavy chain and CDR3 of the light chain regions (Barbas *et al.*, 1992; Desiderio *et al.*, 2001). Jirholt *et al.* (Jirholt *et al.*, 1998) and Söderlind *et al.* (Jirholt *et al.*, 1998) amplified all CDRs derived from B cells and then shuffled them into a single antibody framework in an assembly PCR step. Fully synthetic libraries are constructed from different or unique  $V_H$  and  $V_L$  germline frameworks combined with six synthetically created CDR cassettes

(Knappik *et al.*, 2000). As an example of a completely synthetic library, Knappik *et al.* (Knappik *et al.*, 2000) used seven different V<sub>H</sub> and V<sub>L</sub> germline frameworks combined with six synthetically created CDR cassettes. In one of the largest synthetic libraries (Griffiths *et al.*, 1994), 49 human heavy chain segments were combined with a collection of 47 human kappa and lambda light chain segments with partially randomized CDR3 regions. The heavy and light chain V-gene repertoires were combined on a phage vector in bacteria using the *lox*-Cre site-specific recombination system to create a large  $6.5 \times 10^{10}$  clone repertoire of Fab fragments. Although some good antibodies were isolated from this library that had nanomolar affinities for their targets (Griffiths *et al.*, 1994; de Wildt *et al.*, 1996), the library proved to be difficult to re-propagate without significant loss of diversity. Ultimately, there must be a physical limitation to the enrichment that may be achieved in selection, and this may limit value of extremely large synthetic libraries. The source of antibody genes for naïve, semi-synthetic and synthetic libraries - sometimes referred to as "single pot libraries" because antibodies against virtually any antigen can be extracted - are presented in Table 1.1 (Hust and Dubel, 2004).

Synthetic antibody libraries have a significant advantage over naïve libraries that use naturally rearranged V-genes. Importantly, the choice of V-gene segments may be guided by factors that will increase the overall performance of the library, such as good expression and folding and low toxicity in *E. coli*. This will increase the functional library size. Large differences in V-gene usage both *in vivo* and in phage repertoires (Griffiths *et al.*, 1994) also suggest that some frameworks may be better suited to form antigen-binders than others. Second generation synthetic antibody libraries have been built using 'master' frameworks representing each of the Kabat subclasses. In principle, this should make it possible to choose only well expressed frameworks. To overcome the problem of introducing stop-codons during *in vitro* diversification, V-genes were assembled with oligonucleotides made from trinucleotides instead of from single bases (Virnekas *et al.*, 1994). To improve the performance of synthetic libraries, pre-selection of amplified and displayed synthetic V-domains on Ig-domain binding proteins (protein A for V<sub>H</sub>, protein L for V<sub>K</sub>, etc.) (Akerstrom *et al.*, 1994) enables elimination of clones that carry stop codons and frameshifts, and selection of correctly folded V-domains (Tomlinson, unpublished).

**Table 1.1. The source of antibody genes for construction of different antibody libraries**

Library vector	Library type	Antibody type	Library cloning strategy	Library size
pHEN1	Naive	scFv	Assembly PCR	$2 \times 10^5/2 \times 10^6$
pCANTAB3his	Naive <sup>a</sup>	scFv	Assembly PCR	$1.2 \times 10^8$
pSEX81	Naive	ScFv <sup>b</sup>	Four-step cloning	$1.6 \times 10^7/1.8 \times 10^7/4 \times 10^7$
pSEX81	Naive	scFv <sup>b</sup>	Two-step cloning	$4 \times 10^7$
pCANTAB 5	Naive <sup>a</sup>	scFv	Assembly PCR	$4.5 \times 10^7$
pHEN1	Naive	scFv	Assembly PCR	$10^7-10^8$
pFAB60	Naive <sup>a</sup>	Fab	Two-step cloning	$>10^8$
fdTet	Naive	scFv	Recloning of a naive library <sup>c</sup>	$5 \times 10^8$
pSEX81	Naive	scFv <sup>b</sup>	Two-step cloning	$4 \times 10^9$
pHEN1	Naive	scFv	Assembly PCR	$6.7 \times 10^9$
pCANTAB 6	Naive	scFv	Assembly PCR	$1.4 \times 10^{10}$
pCES1	Naive	Fab	Three-step cloning (L chain, V <sub>H</sub> )	$3.7 \times 10^{10}$
pDAN5	Naive	scFv	Cre-lox	$3 \times 10^{11}$
pAP-III <sub>0</sub> scFv	Naive	scFv	Assembly PCR	ND <sup>d</sup>
pEXmide5	Semi-synthetic (germline V <sub>H</sub> DP47 and V <sub>λ</sub> DPL3 framework)	scFv	Assembly PCR, CDR shuffling	$9 \times 10^6$
pHEN1-V <sub>λ</sub> 3	Semi-synthetic (V <sub>λ</sub> 3 anti-BSA Ab light chain)	scFv	PCR with random CDR H3 primers	$10^7$
pScUAGDcp3	Semi-synthetic	scFv connected to C <sub>κ</sub>	Three-step cloning with random CDR3 primers	$1.7 \times 10^7$
pDN322	Semi-synthetic (anti-AMCV CP ab framework)	scFv	Random CDR3 primer, assembly PCR	$3.75 \times 10^7$
pComb3	Semi-synthetic (anti-tetanus Ab framework <sup>e</sup> )	Fab	PCR with random CDR H3 primers	$5 \times 10^7$
pComb3	Semi-synthetic (anti-tetanus Ab framework <sup>e</sup> )	Fab	PCR with random CDR H3 primers	$>10^8$
pHEN1-V <sub>λ</sub> 3	Semi-synthetic (V <sub>λ</sub> 3 anti-BSA Ab light chain)	scFv	PCR with random CDR H3 primers	$>10^8$
pIT2 (Tomlinson I/J)	Semi-synthetic (3 V <sub>H</sub> and 4 V <sub>κ</sub> genes)	scFv	PCR with random CDR2 and CDR3 primers	$1.47 \times 10^8/1.37 \times 10^8$
pAALFab	Semi-synthetic (anti-hen egg white lysozyme Ab framework)	Fab	PCR with random CDR primers, assembly PCR	$2 \times 10^8$
pLG18	Semi-synthetic (anti-HER2 Ab framework)	Fab	PCR with random CDR primers, two-step cloning	$2-3 \times 10^8$
pDN322	Semi-synthetic (V <sub>H</sub> DP47 and V <sub>κ</sub> DPK22 V genes)	scFv	Random CDR3 primer, assembly PCR	$3 \times 10^8$
pHEN1-V <sub>λ</sub> 3	Semi-synthetic (V <sub>H</sub> )/naive (V <sub>L</sub> )	scFv	Three-step cloning, PCR with random CDR H3 primers	$3.6 \times 10^8$
Pdnek (ETH2)	Semi-synthetic (V <sub>H</sub> DP47, V <sub>λ</sub> DPL16 and V <sub>κ</sub> DPK 22 V genes)	scFv	Random CDR3 primer, assembly PCR	$5 \times 10^8$
pHEN2 (Griffin 1)	Semi-synthetic	scFv	Recloning of the lox library in scFv format <sup>f</sup>	$1.2 \times 10^9$
pFAB5c-His (n-CoDeR)	Semi-synthetic (germline V <sub>H</sub> DP47 and V <sub>λ</sub> DPL3 framework)	scFv	Assembly PCR, CDR shuffling	$2 \times 10^9$
fdDOG-2lox, pUC19-2lox	Semi-synthetic	Fab	PCR with random CDR3 primers, Cre-lox	$6.5 \times 10^{10}$
pMorph series (HuCAL)	Synthetic	scFv	Two-step cloning plus two-step CDR3 replacement	$2 \times 10^9$

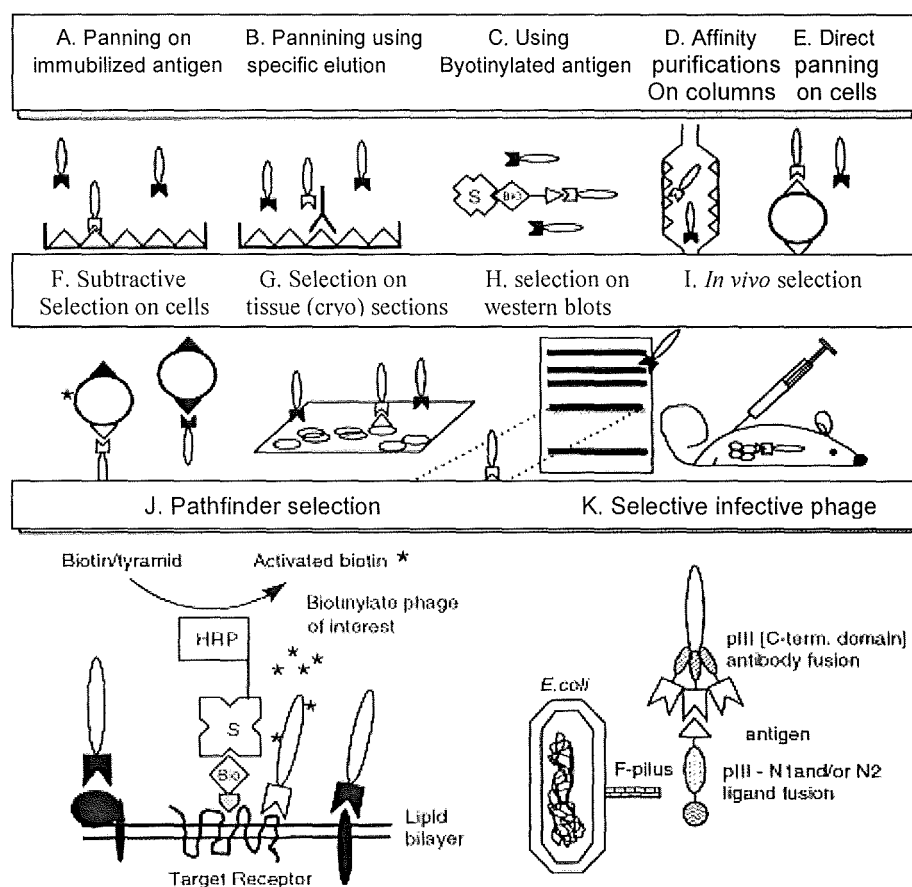
<sup>a</sup>Mouse library.<sup>b</sup>with N-terminus of C<sub>H</sub>1 and C<sub>L</sub>.<sup>c</sup>Sheets *et al.* [36].<sup>d</sup>Not determined.<sup>e</sup>Persson *et al.* [81].<sup>f</sup>Griffiths *et al.* [38].<sup>g</sup><http://www.mrc-cpe.cam.ac.uk>.

The table has been taken from the work of Hust and Dubel (2004).

## ***Phage antibody selection procedure***

The selection of recombinant antibodies from phage libraries consists of two main steps: panning and screening. During panning, library phage are incubated with the antigen of choice, unbound phage are discarded during several washing steps and remaining phage are recovered by elution to retrieve those with specific binding activity. Any method that separates clones that bind from those that do not, can be used as a selection method, and as such, many different selection methods have been used. In the top panel of Figure 2, the most popular procedures are shown. These include biopanning on immobilized antigen coated onto solid supports, columns or BIAcore sensorchips (Clackson *et al.*, 1991; Marks *et al.*, 1991; Griffiths *et al.*, 1994; Malmborg *et al.*, 1996), selection using biotinylated antigen (Hawkins *et al.*, 1992), panning on fixed prokaryotic cells (Bradbury *et al.*, 1993) and on mammalian cells (Cai and Garen, 1995), subtractive selection using sorting procedures (de Kruif *et al.*, 1995b; Van Ewijk *et al.*, 1997), enrichment on tissue sections or pieces of tissue (Van Ewijk *et al.*, 1997), and, in principle, selections using living animals, as reported for peptide phage libraries (Pasqualini and Ruoslahti, 1996). The selection methods described in Figure 2, panels A–F, have been extensively described and have been reviewed elsewhere (Winter *et al.*, 1994; Hoogenboom, 1997).

The simplest approach to selecting specific phage is to use purified antigen coated directly onto a plastic surface, incorporated in an affinity column (McCafferty *et al.*, 1990; Marks *et al.*, 1991) or biotinylated and subsequently captured onto a streptavidin-coated solid surface (Hawkins *et al.*, 1992). While the purification of soluble proteins in a functional form may be achievable, many membrane proteins are modified by purification leading to loss of biological activity. Leaving these proteins *in situ* and using whole cells as the target for selection may be of some value but the biochemical complexity of the cell surface may bring its own problems. A variety of techniques have been developed to direct phage selection to particular proteins on the cell surface and other complex substrates. The simplest technique is to use a combination of negative and positive selection, whereby phage libraries are pre-absorbed on cells lacking antigen (negative selection) prior to selection on cells expressing the antigen (positive selection). When variation between individuals is a consideration, pre-absorption of a phage is best achieved by repeated rounds of absorption on cells from a number (5–10) of antigen-negative donors prior to the positive selection (Watkins and Ouwehand, 2000).



**Figure 1.2 Selection strategies for obtaining specific phage ligands.**

Affinity selection of phage (antibody) libraries by (A) panning on antigen adsorbed onto a solid support. After washing, specific phage are eluted with acid or basic solutions; (B) alternatively elution with antibody or an excess of the antigen is possible. (C) To avoid conformational changes during coating, selection of specific antibodies to biotinylated antigen in solution is preferable. Bound and unbound phage antibodies are separated using streptavidin-coated magnetic beads. (D) Antigen can be immobilized onto a column for affinity selection. (E) Selection on cells can be done directly by panning on cell monolayers or cells in suspension. (F) Subtraction via FACS: the cells of interest are fluorescently labeled and separated from the others by cell sorting. (G) Tissue and organ specific phage antibodies might be obtained by selection on tissue slides. (H) Non-purifiable or unknown antigens may be separated on SDS-PAGE and blotted onto membranes for selection. (I) *In vivo* selection. (J) The procedure of 'Pathfinder' selection, and (K) infection-mediated selection. Specific phage carry a white ligand, irrelevant phage a black one. The Figure has been taken from the work of Hoogenboom, *et al.* (Hoogenboom *et al.*, 1998).



Phage antibodies bound to antigen can be eluted in different ways: with a single aliquot or gradients of an acidic solution such as HCl or glycine buffers (Kang *et al.*, 1991; Roberts *et al.*, 1992), with basic solutions like triethylamine (Marks *et al.*, 1991), with chaotropic agents, with DTT (dithiothreitol; Cleland's reagent) when biotin is linked to antigen by a disulphide bridge (Griffiths *et al.*, 1993), by enzymatic cleavage of a protease site engineered between the antibody and gene III (Ward *et al.*, 1996), or by competition with excess antigen (Clackson *et al.*, 1991) or antibodies to the antigen (Meulemans *et al.*, 1994).

The power of these method is evident from the observation that a single round of selection can enrich for specific phage by 20 to 1000-fold and repeating the selection procedure increases further the enrichment (McCafferty *et al.*, 1990; Marks *et al.*, 1991). Many factors affect the selection efficiency of the panning process. For instance, the selection of antibodies with low binding affinities is encouraged by fewer washes, multivalent display or a high coating level of antigen. In contrast, the isolation of antibodies with a high affinity for antigen is favored by exhaustive washing, a low level of antigen coating and monovalent display (Winter *et al.*, 1994). Phage antibodies with higher affinity may be enriched during successive rounds of selection by decreasing the concentration of antigen. This is similar in concept to during B-cell selection *in vivo* (Hawkins *et al.*, 1992). In one of the most complete studies on antibody affinity maturation carried out (Schier *et al.*, 1996), it was necessary to empirically determine the antigen concentration to be used for selection, as well as the elution condition for phage retrieval (Schier and Marks, 1996).

### **Phage antibody screening**

After selection, a mixture of binding ligands with differing properties are likely to be recovered. It may be necessary to screen large numbers of antibodies to identify those variants with the most optimal characteristics. The best screening assays are fast, strong, amenable to automation (*eg* 96-well format), and able to use unpurified phage antibodies, or the soluble antibody fragments from the bacterial supernatant. The screening assay should test as closely as possible the functional requirements of the ligand. Binding of polyclonal or monoclonal phage antibodies to the antigen has been tested with different assays, ranging from a simple ELISA (Marks *et al.*, 1991), to bioassays that screen for direct neutralization upon binding (Zaccolo *et al.*, 1997), and whole cell ELISA or flow cytometry. Normally, for a first screen, ELISA-based assays are used in combination with restriction-fingerprinting of the antibody-DNA to identify different clones (Marks *et al.*,

1991). Further, the specificity of antibodies may be tested (Carnemolla *et al.*, 1996; de Wildt *et al.*, 1996; Van Ewijk *et al.*, 1997).

To improve the speed of screening, phagemid vectors that incorporate dual function have been developed. These allow both monovalent display of antibody fragments and the production of soluble antibody fragments for screening without the necessity to subclone the antibody V-genes. In such systems, an amber codon is positioned between the antibody and pIII genes (Hoogenboom *et al.*, 1991). A variety of tags have been described that can be appended to the antibody fragment for detection, including the myc-derived tag recognized by the antibody 9E10 (Marks *et al.*, 1991), and the Flag sequence (Lah *et al.*, 1994; Lindner *et al.*, 1997). This set-up allows the use of unpurified phage antibodies or antibody fragments derived from crude supernatant or periplasmic extracts. Another useful modification is the incorporation of a histidine tag between the antibody and gIII, for purification of antibodies using immobilized metal affinity chromatography (Hochuli *et al.*, 1988; McCafferty *et al.*, 1994).

### ***Recloning of selected phage antibodies***

Recombinant antibodies isolated by phage display can be recloned for expression in other hosts. Expression levels in *E. coli* are dependent on the primary sequence of the individual antibody, and can be extremely variable (from 10 µg to 100 mg/l). When yields in this host are low, recloning may increase the expression level (Strachan *et al.*, 2002). Unless yields are sufficient, it is worth recalling that phage display vectors are rarely designed with high-level expression as a primary purpose (Pluckthun and Pack, 1997). Eukaryotic expression vectors have been described that may be used for one-step recloning of V-genes derived from any phage repertoire for expression as Fab fragments or whole antibodies, and for targeting to different intracellular compartments (Persic *et al.*, 1997a; Persic *et al.*, 1997b). This facilitates either transient or stable expression in mammalian cells. By carefully choosing restriction sites that are rare in human V genes, immunoglobulin genes may be batch-cloned into these expression vectors. All the important elements in the vectors (promoter, leader sequence, constant domains and selectable markers) are flanked by unique restriction sites, allowing simple substitution of elements and further engineering.

## **Production of recombinant antibodies**

When yields permit, recombinant antibodies can be conveniently expressed in microorganisms, particularly in *E. coli*. ScFv antibodies contain two disulfide bonds, whereas Fabs contain five disulfides that must all form for stable folding. Normally, the oxidation of cysteine thiols into disulfides occurs only after a protein has been exported from the highly reduced environment of the cytoplasm to a more oxidizing secretory compartment. Therefore, in order for antibody fragments to fold into their native, functional state in *E. coli*, they must be exported into the periplasmic space. This can be accomplished by in-frame fusion of a well-characterized bacterial leader peptides (PelB, Pho A, Omp A) to the N terminus of the antibody fragment. Proteins located within the periplasm can be recovered by osmotic shock or from total cell lysates. Prolonged high-level expression of antibodies at 37 °C often renders the outer membrane permeable, and the protein can be recovered from the culture media (Skerra and Pluckthun, 1988; Skerra, 1993). Very high levels of scFv, exceeding 1-2 g/L, have been achieved in a fermenter by periplasmic expression (Kipriyanov, 2002) but levels of expression can vary widely on a case by case basis. Antibodies, especially scFvs, often fail to fold properly and aggregate within the bacterial periplasm, or they can be toxic to the host cell. Toxicity most likely occurs because the export of these proteins interferes with the normal function of the secretory machinery of the cell. Protein misfolding and aggregation can be reduced by growing the cells at 30 or 25°C (Somerville *et al.*, 1994), using tightly regulated promoters such as the pBAD arabinose-inducible promoter (Clark *et al.*, 1997), or by co-expression of proteins that assist folding. Such proteins include enzymes that catalyse disulfide bond formation, isomerization or chaperones such as Skp (Skerra and Pluckthun, 1991; Humphreys *et al.*, 1996; Bothmann and Pluckthun, 1998; Hayhurst and Harris, 1999). Addition of non-metabolized additives that induce osmotic stress, such as sucrose or sorbitol and glycine betaine, can increase the proportion of correctly folded antibodies (Kipriyanov *et al.*, 1997; Kipriyanov *et al.*, 1999). Amino acid replacements can have dramatic effects on protein solubility, and therefore on the levels of functional antibody expressed (Duenas *et al.*, 1995; Forsberg *et al.*, 1997). Also CDR grafting to the framework of well-expressed antibodies has been used to generate scFvs with improved stability that result in better production (Jung and Pluckthun, 1997; Worn and Pluckthun, 1999).

High-level production of antibody fragments in the cytoplasm of *E. coli* often results in extensive aggregation and the formation of inclusion bodies. These can be solubilized by

adding strong detergents and a reducing agent, and correctly folded antibody fragments have been recovered by removal of the denaturant and refolding. This topic has been reviewed (Rudolph and Lilie, 1996; Clark, 1998; Lilie *et al.*, 1998).

### ***Improving affinities***

Selection from very large libraries with more than  $10^{10}$  antibody variants can yield antibodies with nanomolar affinities or less (Griffiths *et al.*, 1994; Vaughan *et al.*, 1996). The affinities of antibodies from more modest-sized naïve, synthetic or immune libraries are typically sufficient for use as research reagents but often too low for therapeutic applications such as tumour targeting and virus neutralization. *In vivo*, antibodies are matured stepwise; by the progressive introduction of mutations that each causes a small incremental improvement of the affinity. Mutations may affect affinity indirectly by influencing the positioning of side chains contacting the antigen, by providing new contact residues or by replacing repulsive or low-affinity contact residues with more favourable interactions. This process can be imitated *in vitro* to improve the properties of recombinant antibodies by: (1) introducing diversity in the antibody V-genes, thereby creating a secondary library; (2) selection of the higher-affinity variants; (3) screening to discriminate variants with differences in affinity or kinetics of binding. The selection may be chosen to favour kinetic parameters (Hawkins and Winter, 1992) such as off-rate or affinity. This hinges on the use of limited and decreasing amounts of antigen, and on performing the selections in solution rather than by avidity-prone panning. For instance, antibodies of the highest affinity can be preferentially selected by using the antigen concentration at or below the desired dissociation constant (Hawkins and Winter, 1992).

The best area for mutagenesis will differ for each individual antibody. Diversity may be introduced more or less randomly in the V-genes by error-prone PCR, using mutator strains of bacteria that are deficient in DNA repair functions, chain shuffling or by DNA shuffling. Alternatively, the CDR regions may be targeted for oligonucleotide-directed or random mutagenesis. Another way to raise the affinity is to artificially extend the regions that make contact with the antibody. Other strategies for improving the affinity of antibodies have been reviewed by Maynard and Georgiou (Maynard and Georgiou, 2000) and Hoogenboom (Hoogenboom, 1997; Hoogenboom *et al.*, 1998).

## ***Storage of purified recombinant antibodies***

The long-term storage of some forms of recombinant antibody can be problematic. IgG molecules and Fab or F(ab')<sub>2</sub> fragments present the fewest difficulties and the addition of antibacterial agents, such as thiomersal or sodium azide, is recommended in every case. However, for long-time storage, the antibodies should be frozen at -70 °C in aliquots. Care must be taken that the solutions are not too dilute when frozen (not less than roughly 0.1 g/l total protein). ScFvs are a special case because they aggregate at high protein concentrations. This aggregation can be attributed to two properties. First, a portion of the surface of Fv fragments comprises a surface that is not solvent exposed in the intact immunoglobulin molecule. This can mediate increased non-specific protein interactions. Secondly, the interaction between V<sub>H</sub> and V<sub>L</sub> is of low affinity, enabling the assembly of oligomers and finally aggregates. For Fabs, the constant domains assist the stability of the heterodimer. Having said this, some scFv fragments have proved to be very stable and have been stored in a functional form for more than one year at 4 °C without protective protein. For scFvs without this advantage, the addition of a protective protein in high concentrations (BSA at 10-20 mg/ml) is recommended followed by storage in aliquots at -70 °C. When the addition of protective protein is not possible, recombinant antibody fragments can be stored at -20 °C after the addition of cryoprotectants such as glycerine (Breitling and Dübel, 1999).

## ***Application of phage display antibodies to studies of infectious diseases***

Although widely used for the isolation of antibodies for cancer biology, phage display has yet to take a prominent role in the identification of antibodies against antigens and virulence factors from pathogens other than viruses. A selection of examples in which phage display has been used are discussed briefly in the following sections.

### **Parasites and fungi**

#### ***Haemonchus contortus***

An immune scFv phage antibody library was constructed from the abomasal lymph node of a sheep immunized with *Haemonchous contortus* (White *et al.*, 2001). The phage antibodies were selected by panning the library against a purified outer membrane protein. The result of screening of 960 clones showed that only low affinity antibodies were

isolated. The authors attributed this outcome to limitations in their selection strategies. Given the success of other investigators in generating ovine phage display libraries and isolating high affinity scFvs, this seems the most likely explanation of the findings of the above work.

### ***Candida albicans***

A combinatorial human phage display scFv library was used to identify antigens specific for the filamentous form of *Candida albicans* (Bliss *et al.*, 2003). Two isolated single-chain antibody variable fragments (scFv) were characterized in detail. Filament-specific antigen expression was detected by an indirect immunofluorescence assay. Using these antibodies antigen epitopes were detected on clinical specimens obtained from infants with thrush and urinary candidiasis without passage of the organisms on laboratory media, confirming epitope expression in human infection. The availability of a monoclonal reagent that recognizes filaments from both *C. albicans* and *C. dubliniensis* and another specific only to *C. albicans* adds to the repertoire of potential diagnostic reagents for differentiation between these closely related species.

### **Viruses**

The isolation of recombinant antibodies with antiviral activities have been reviewed (Parren and Burton, 2001). In the last few years, numerous investigations have used phage display to study a number of viruses of importance in human and animal disease. Two examples of recombinant antiviral antibodies are presented below.

#### ***HIV-1***

A phage display antibody library was prepared from patients with lupus and scFv were isolated with the ability to bind gp120 and the conserved gp120 determinant composed of residues 421-436. The scFv neutralized R5 and X4-dependent HIV-1 strains from clades B, C, and D. The authors attributed the lupus repertoire may be useful as a source of neutralizing antibodies to HIV (Karle *et al.*, 2004).

#### ***FMDV***

To study the bovine antibody response at a molecular level, phage display technology was used to produce Fabs against bovine foot-and-mouth disease virus (FMDV). Heavy

chains with FMDV specific binding were isolated after selection from a library made from vaccinated cattle. Some of these proteins possessed viral neutralizing activity and showed homologies with integrin  $\beta 6$  chain in the CDRs. Though the involvement of these antibodies in the bovine immune response to FMDV remains to be established, the selected VH domains could shed light on the basis of FMDV type receptor specificities (Kim *et al.*, 2004).

## **Bacteria**

Considering the range of bacterial pathogens of importance to human and animal health, the detailed understanding of the process of pathogenesis and the rapid accumulation of genomic data on these organisms, it is surprising that phage display technology has yet to make substantial impact in this area. The following examples illustrate the scope of what has been accomplished and the potential in this area.

### ***Clostridium perfringens***

A scFv antibody against alpha-toxin of *Clostridium perfringens* type A, has been assembled from a hybridoma cell line producing a mouse monoclonal antibody against this toxin. The scFv gene was amplified, inserted into the expression vector pHOG21 and transformed into *E. coli* XL1-Blue. The scFv was highly expressed in recombinant bacteria and a neutralization assay showed that the expressed protein could neutralize the phospholipase C activities of alpha-toxin (Zhao and Xu, 2001).

### ***Bordetella pertussis***

A scFv phage display library was constructed from the peripheral blood of two patients recently recovered from pertussis infection. Ten scFv were isolated by panning the library against pertussis toxin. One scFv accounted for 33% of clones after panning. Six of the panned scFv bound to pertussis toxin. The ability of the scFv to neutralise pertussis toxin was assessed using the Chinese hamster ovary cell assay. The three scFvs were able to neutralise the pertussis toxin (Williamson and Matthews, 1999).

### ***Bacillus anthracis***

To isolate antibodies against spores of the genus *Bacillus*, a naive, human scFv phage display library was used in panning against live, native spores of *Bacillus anthracis*. Direct

*in vitro* panning and ELISA-based selection isolated a panel of nine scFv-phage clones of which two were chosen for further study. These clones differed in their relative specificity and affinity for bacillus spores. A variety of ELISA protocols indicated these scFv-phage clones recognized different spore epitopes. A Fab chain-shuffled sub-library was constructed from the nine positive clones and a subtractive panning strategy was used to remove cross-reactivity with *B. licheniformis* 5A24. The study provided a foundation to discover human antibodies specific for native spores of *B. anthracis* that can be developed as diagnostic and therapeutic reagents (Zhou *et al.*, 2002).

### ***Clostridium botulinum***

Several groups have attempted to use phage display antibodies against the botulinum toxin which is the most potent natural toxin known. In one recent example, the immune response to the binding domain (HC) of botulinum neurotoxin serotype A was studied at the molecular level using immune and non-immune human scFv phage libraries. A large panel of serotype specific phage expressing botulinum-binding scFv were selected from both libraries. Neutralizing activity of the isolated scFvs was evaluated using a mouse hemidiaphragm assay revealed that only a limited number of scFv from the immune library had toxin-neutralizing activity at biologically significant concentrations (Amersdorfer *et al.*, 2002).

### ***Mycobacterium tuberculosis***

An immune mouse library was generated and scFvs reactive with mycobacterial proteins were isolated by phage display. None of the extracted antibodies showed reaction with the immunodominant 65 kDa *M. tuberculosis* antigen, highlighting the potential of phage display systems to generate a diverse population of reagents that may prove useful for investigating the immune response to mycobacterial infection and vaccination (Cummings *et al.*, 1998)

### ***Meningococci Group B***

Using phage display, anti-idiotypic scFvs were obtained from mice immunized with the Seam 3 monoclonal antibody. Two Seam 3-specific scFvs competed with Group B capsular polysaccharide for binding to either the Seam 3 monoclonal or rabbit antibodies present in typing sera. Moreover, the scFvs could in turn act as an antigen in mice and rabbits, eliciting the production of antibodies that were reactive with both capsule and



whole meningococci, but not with human polysialic acid. The predominance of IgG2a in these scFv-induced responses indicated a Th1 type response. In addition, passive transfer of sera from scFv-immunized animals partially protected neonatal mice from experimental infection with group B meningococci (Beninati *et al.*, 2004).

### ***Streptococcus suis***

In this example, a human semi-synthetic library was used to select recombinant antibodies directed against surface components and the extracellular factor (EF) of a pathogenic strain of *Streptococcus suis* serotype 2. Whole cells and purified EF were used as targets for panning of the library. Clones were identified in each panning which bound to the antigen of interest (de Greeff *et al.*, 2000).

### ***Helicobacter pylori***

Human scFv antibodies against proteins present in a *H. pylori* cell lysate and the *H. pylori* urease were isolated from an immune phage display library, constructed from peripheral blood lymphocytes of an *H. pylori*-infected patient. After selection, 23% of the scFv clones tested showed binding activity against a lysate from the *H. pylori* Sydney strain in ELISA and 9% bound the urease. Further characterization by PCR-fingerprint analysis and sequencing revealed that two closely related *H. pylori* binders and one anti-urease scFv could be identified. The selected scFvs were highly specific as analysed by ELISA and immunoblots using various bacterial lysates and recombinant proteins (Reiche *et al.*, 2002).

### ***Cyanobacterial hepatotoxin***

A naïve human semi-synthetic phage display library was used to isolate recombinant antibody fragments against the cyanobacterial hepatotoxin microcystin-LR. The scFvs that emerged from selection were modified by fusion to a human kappa constant domain to create single-chain antibodies (scAbs). The scAbs with the best properties were capable of detecting microcystin-LR at levels below the World Health Organization limit in drinking water (1 µg/ lit) and cross-reacted with three other purified microcystin variants (microcystin-RR, -LW, and -LF) and the related cyanotoxin nodularin. These antibodies were used for purification of the toxins by immobilizing the scAbs on columns *via* a hexahistidine tag. The authors suggested that these antibodies could be used as biosensors

and components in on-line monitoring systems for the cleanup and concentration of these toxins from environmental samples (McElhiney *et al.*, 2002).

### ***Bacillus thuringiensis***

A synthetic scFv library was used to map the epitopes on cadherin-like receptors involved in the interaction between *Bacillus thuringiensis* Cry1A toxin and insect intestinal cells. After isolating Cry1A-binding phages, one purified scFv showed affinities to Cry1Aa, Cry1Ab, and Cry1Ac toxins in the range of 20-51 nM. Analysis showed this scFv molecule has a CDR3 sequence with significant homology to part of the cadherin-like protein from *M. sexta* (Bt-R<sub>1</sub>), *Bombyx mori* (Bt-R<sub>175</sub>), and *Lymantria dispar*. By doing further experiments, the investigators identified the amino acid region of Bt-R<sub>1</sub> and Bt-R<sub>175</sub> involved in Cry1A toxin interaction (Gomez *et al.*, 2001).

### ***Clostridium difficile* (Toxin B)**

Phage display technologies were used to produce a scFv against *Clostridium difficile* toxin B. The starting material was the mouse B cell hybridoma line 5A8, which secretes a monoclonal antibody against the toxin. The sensitivity of one of the scFvs was reported to be significantly higher than the original monoclonal antibody and could detect a minimum of 10 ng of toxin B/well in ELISA. Competitive ELISA established that the affinity of the 5A8 parent antibody and the best representative of the scFvs were similar and in the range of 10<sup>-8</sup> M (Deng *et al.*, 2003).

### ***Pseudomonas aeruginosa***

A Fab was constructed from a conventional monoclonal antibody against the O antigen of *Pseudomonas aeruginosa*. The parent antibody bound to the O antigen of the most clinically important serotypes. Immunotherapy with the antibodies against the LPS of *Pseudomonas aeruginosa* might form an alternative to LPS-based vaccines and antibiotics. The recombinant Fab antibody was shown to be specific for the LPS of most serotype O6 isolates (Tout and Lam, 1997).

### ***Moraxella catarrhalis***

Using a synthetic scFv phage library (de Kruif *et al.*, 1995a), specific antibodies were isolated that recognised the high molecular weight outer membrane protein (HMW-OMP)

of complement-resistant strains of *Moraxella catarrhalis*. HMW-OMP is not found on complement-sensitive strains, a property which was exploited in the selection procedure to deplete the library of scFvs against surface components common to complement-sensitive and complement-resistant strains (Boel *et al.*, 1998).

### ***Listeria monocytogenes***

In order to find a species-specific antibody against *Listeria monocytogenes* strains, a pool of random scFvs expressed on the surface of bacteriophage were used for biopanning. Panning against *L. monocytogenes* was used to enrich for phage clones with the desired binding affinity, and negative selection using *L. innocua* and *L. ivanovii* was used to remove phage expressing cross-reactive antibody fragments. After several stages of screening, the authors reported the isolation of a species-specific antibody for viable cells of *L. monocytogenes* (Paoli *et al.*, 2004).

### ***Goals of this project***

The studies outlined above show that phage display is gradually gaining acceptance as a technology for the understanding of microbial infection. Many of these investigations have isolated recombinant antibodies against virulence factors or surface proteins and then used these reagents for the specific identification of the pathogens concerned; rather few have used the isolated scFvs or Fabs as tools to define the structure, location or mode of action of the target and thereby aid an understanding of its contribution to disease. The general goal of this project was to address these issues using as a model, the bacterial colonisation factor K99. As described in the following chapter, much is known about the role of K99 fimbriae in the pathogenesis of *Escherichia coli* infection. The aims of this project were to purify this virulence factor, to isolate recombinant antibodies against it, and to determine the extent to which these reagents could be used in confirming the identity of the target, its location and its mode of action.

## **Chapter 2**

### **K99 Fimbriae**

## 2. K99 Fimbriae

### Introduction

#### ***Escherichia coli* infection**

*Escherichia coli* is a Gram-negative, rod-shaped bacterium. It belongs to the *Enterobacteriaceae*. Most members of this family are 'normal' members of the intestinal flora of mammals. Colonisation of the intestinal tract by *E. coli* takes place soon after birth. Pathogenic *E. coli* strains have the general ability to form a persistent interaction with the epithelium whereas non-pathogenic strains would only bind transiently or proliferate free in the lumen (Finlay and Falkow, 1989; Mol and Oudega, 1996). The pathogenic strains of *Escherichia coli* recovered from the intestinal tract of animals fall into four categories that are designated enterotoxigenic (ETEC), enteropathogenic (EPEC), enterohemorrhagic (EHEC), and necrotoxigenic (NTEC). Two other categories described in human infection - enteroinvasive (EIEC) and enteroaggregative (EAEC) have not been reported in animals (DebRoy and Maddox, 2001; Rodriguez-Angeles, 2002). The enterotoxigenic *Escherichia coli* (ETEC) strains cause neonatal diarrhoea in piglets and calves and travellers diarrhoea in humans. They appear less common in dogs and cats and so far, little information has been published about disease caused by ETEC strains in birds or reptiles (Nagy and Fekete, 1999). In humans, millions of people are affected by ETEC every year following ingestion of contaminated water or food (Organization, 1999). The subject of ETEC in farm animals has always attracted much interest because it can be related to human disease in many aspects and causes significant losses and animal welfare problems.

Enterotoxigenic *Escherichia coli* strains remain one of the leading causes of diarrhoea in calves and piglets. The expression of the K99 fimbrial colonisation factor (or F5<sup>+</sup> ETEC) is noted in nearly all ETEC infection found in newborn calves, sometimes accompanied by co-expression of F41 fimbriae (Gaastra and de Graaf, 1982). The so-called F17 (described in the earlier literature as FY and Att 25) adhesin is found less commonly (Lintermans *et al.*, 1988). ETEC strains responsible for diarrhoea in newborn calves often produce the heat-stable STaP enterotoxin and usually belong to the O8, O9, O20 and O101 serogroups. Heat-labile toxin is also commonly produced. These strains often produce an acidic polysaccharide K (A) antigen (K25, K28, K30, K35), making the colonies of such strains more compact and less transparent. It seems that these capsular

polysaccharide antigens increase colonisation of the intestinal epithelium mediated by K99 (Hadad and Gyles, 1982).

## ***Aetiology and pathology***

The common characteristics of ETEC strains isolated from different species are that the bacteria adhere to the epithelial cells of the small intestine (overwhelmingly in newborn or very young animals), thereby colonising the gut. They also secrete proteins or peptides (enterotoxins), which stimulate increased water and electrolyte secretion and/or decreased fluid absorption in the small intestine. The ability of ETEC to adhere to intestinal epithelial cells is mostly due to production of fimbriae or pilli. With the help of these adhesins, the bacteria are able to attach themselves to the microvilli and thereby deliver the enterotoxins that cause disease to the target cells. The pathogenic process is therefore highly dependent upon synthesis by the host of receptors for adhesion and enterotoxins released by the bacteria. In this way, ETEC are limited in their host range, illustrating the co-evolution of ETEC strains and their hosts is illustrated (Nagy and Fekete, 1999). The characteristics of fimbriae and enterotoxins from ETEC strains isolated from farm animals are summarised in Tables 2.1 and 2.2.

## ***Structure and formation of K99***

K99 is a filamentous polymeric protein structure located at the surface of ETEC. It has a diameter of 5 nm and is made up of monomers of major subunit of 16.5 kDa and minor subunits (Altmann *et al.*, 1982; Nagy and Fekete, 1999). The genes responsible for the biogenesis of the K99 adhesin are present on an 87.8 kb non-conjugative plasmid (Isaacson and Start, 1992; Lee and Isaacson, 1995). The K99 genes have been cloned and shown to reside on a 7.1 kb *Bam*HI fragment (de Graaf *et al.*, 1980b; van Embden *et al.*, 1980; de Graaf *et al.*, 1984). This fragment encodes eight gene products (Fan A to Fan H), and each is required for the successful biosynthesis of K99 (van Embden *et al.*, 1980; de Graaf *et al.*, 1984; Roosendaal *et al.*, 1987; Lee and Isaacson, 1995). The genetic organisation of the K99 gene cluster is shown in Figure 2.1 with brief descriptions of the role contributed by the different proteins to the synthesis and assembly of fimbriae (Mol and Oudega, 1996).

Previous investigations have demonstrated that K99 expression is dependent on variety of factors, including growth rate (van Verseveld *et al.*, 1985), growth phase

**Table 2.1** Designation and characterisation of fimbriae of enterotoxigenic *E. coli* isolated from farm animals.

Fimbriae	Mean diameter (nm)	Major subunit size (kDa)	Mannose sensitivity	Associated O serotype	Location of Genes
F4 (K88)	2.1	27.6	R	O8, O141, O149	plasmid
F5 (K99)	5	16.5	R	O8, O20, O101	plasmid
F6 (987P)	7	17.2	NH	O9, O20	plasmid
F17 Family	3.4	20			chromosome
F18			R	O139, O141, O147, O157	plasmid
F41	3.2	29	R	O101	chromosome
F42		32	R	?	plasmid
F165	4-6	17.5, 19	R	O115	chromosome

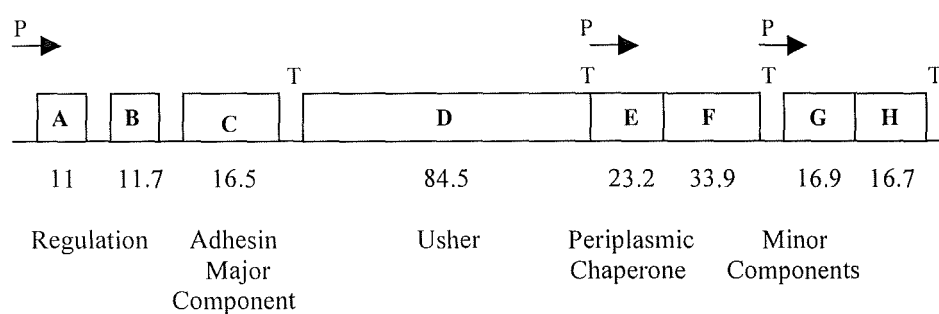
R: mannose resistant

S: mannose sensitive

NH: non-haemagglutinating

**Table 2.2** Designation and characterisation of toxins of enterotoxigenic *E. coli* isolated from farm animals.

Enterotoxin	Variant	Molecular size	Action	Receptor
LT-I LT-II	LTh-I, LTp-I LTIIa, LTIIb	1A (28kDa) 5B (11.5 kDa)	Hypersecretion: Stimulation of adenylate-cyclase system	LTI: GM1 LTIIa: GD1b LTIIb: GD1a
STa	STaH, STaP	18 or 19 aa. Peptide (2 kDa)	Hypersecretion: Stimulation of guanylate-cyclase system	PGCc
STb	-	48 aa. Peptide (5 kDa)	Malabsorption: Opening G-protein linked Ca <sup>++</sup> plasma membrane channel	G protein linked Ca <sup>++</sup> channel



**Figure 2.1 Genetic organisation of the K99 gene cluster.**

Genes are represented by boxes. The designations of the genes are given in the boxes and the relative molecular masses of the mature proteins are given in kDa. A short description of the function of different proteins is given. T, terminator; P, promoter. Arrows indicate direction of transcription.



(Isaacson, 1983), temperature (de Graaf *et al.*, 1980a), alanine concentration (de Graaf *et al.*, 1980a; Isaacson, 1983), and cellular levels of cyclic AMP, cAMP receptor protein (CRP) (Isaacson, 1983; Inoue *et al.*, 1993) and leucine-responsive protein (LRP) (Braaten *et al.*, 1992). K99 production is clearly highly regulated reflecting its critical contribution to virulence. Northern blot analyses have shown that the transcriptional organisation of K99 is complex and that it appears to be divided into three separately regulated gene clusters, regions I to III (Inoue *et al.*, 1993). Roosendaal *et al.* (Roosendaal *et al.*, 1989) detected a promoter at the 5' end of *fanA* and a second promoter at the 5' end of *fanB*. They presumed that these promoters were responsible for transcription of *fanA*, *fanB*, *fanC* and *fanD*, the region I genes. They also identified a strong transcription terminator between *fanC* and *fanD*. The results from Northern blots suggest that additional promoters exist in other regions of the K 99 genes and are responsible for the transcription of *fanE* to *fanH* (Inoue *et al.*, 1993).

The receptor for K99 fimbriae was first isolated from equine erythrocytes and was identified as Neu5Gc- $\alpha$ (2-3)Galp- $\beta$ (1-4)Glc- $\beta$ (1-1)-Ceramide (de\_Graaf *et al.*, 1981; Roosendaal *et al.*, 1984; Smit *et al.*, 1984; Teneberg *et al.*, 1990). This ganglioside receptor molecule was also detected in mucosal scrapings of piglet and calf small intestine. Investigations strongly suggest that this ganglioside represents the *in vivo* receptor for K99 fimbriae (Teneberg *et al.*, 1990). The major subunit FanC was found to be required for interaction with the receptor. Detailed analysis revealed that the residues Lys-132 and Arg-136 of Fan C are essential for receptor binding and most probably constitute a part of the receptor binding domain (Jacobs *et al.*, 1987).

From the nucleotide sequence of the FanC gene it can be predicted that it is composed of 159 amino acids preceded by an N-terminal signal sequence of 22 residues (Roosendaal *et al.*, 1984). Taking published data (Roosendaal *et al.*, 1984; Jacobs *et al.*, 1987; Simons *et al.*, 1990a) Genebank deposition (<http://www.ncbi.nih.gov/entrez/query.fcgi?db=nucleotide&cmd=search&term=M35282&doptcmdl=GenBank>) and ExPASy data (<http://ca.expasy.org/cgi-bin/niceprot.pl?P18103>) the amino acid sequence of Fan C is presented in Figure 2.2.

### **Binding of K99 fimbriae to receptor**

Fimbriae commonly confer adhesive properties upon pathogenic strains of *Escherichia coli*. For K99 fimbriae, recognition of the NeuGc $\alpha$ 3Gal $\beta$ 4Glc $\beta$  motif of the ganglioside N-glycolyl-GM3 appears responsible for this property. For some colonisation factors (eg P

Key	From To	Length	Description
SIGNAL	1 - 22	22	
CHAIN	23 - 181	159	K99 fimbrial protein.
DISULFIDE	38 - 79		Probable.
SITE	154 - 154	1	REQUIRED FOR RECEPTOR BINDING.
SITE	158 - 158	1	REQUIRED FOR RECEPTOR BINDING.
SITE	180 - 180	1	REQUIRED FOR STABILITY AND TRANSPORT.

102030405060

| | | | | |

MKKTLLAIL GGMAFATTNA SANTGTINFN GKITSATCTI DPEVNGNRTS TIDLGQAAIS

708090100110120

| | | | | |

GHGTVVDFKL KPAPGSNDCL AKTNARIDWS GSMNSLGFNN TASGNTAAKG YHMTLRATNV

130140150160170180

| | | | | |

GNGSGGANIN TSFTTAEYTH TSAIQSFNYS AQLKKDDRAP SNGGYKAGVF TTSASFLVTY

181

|

M

Figure 2.2 The amino acid sequence of Fan C

fimbriae) pili (Lindberg *et al.*, 1987) adhesion is mediated by minor protein subunits of the structure but in K88, K99 and colonization factor antigen I, it is the major fimbrial subunit that acts as the adhesive component (Jacobs *et al.*, 1987; Simons *et al.*, 1990b; Simons *et al.*, 1991; Bakker *et al.*, 1992a; Bakker *et al.*, 1992b). This has been confirmed by the observation that these fimbriae still display adhesive properties after deletion of their minor fimbrial components either by mutation or treatment with 2M urea (Bakker *et al.*, 1992b). Although the major subunit of different types of fimbriae acts as an adhesin, differences in the number of adhesin sites per fimbria have been noted. For structures like colonization factor antigen I, it is only the binding site carried by the major subunit at the very tip that is exposed and available for interaction with host receptor molecules (Buhler *et al.*, 1991). In contrast, the major subunit of K99 fimbriae (FanC) acts as an adhesin at numerous positions along the fimbrial structure, resulting in a multivalent interaction of fimbriae and receptors (Willemsen and de Graaf, 1993). Electron microscopy studies of the binding of gold particles coated with *N*-glycyl-L-GM<sub>3</sub> to K99 fimbriae has shown that receptor interaction is predominantly lateral and spaced at approximately 20 nm intervals along the length of fimbriae and occasionally at the tip (Willemsen and de Graaf, 1993). Studies of the immunological properties of the structure suggest that B-cell epitopes of FanC are likely to be continuous, with a potentially immunodominant epitope at the carboxy-terminus (Ogunniyi *et al.*, 2002).

### **Other fimbriae**

It seems that K99 and F41 are frequently produced concurrently by bacteria of the same ETEC strain. There are different receptors for K99 (sheep and horse haemagglutinin) and F41 reflected by their agglutination of sheep and horse, or guinea pig and human erythrocytes respectively. K99 and F41 also differ in that the genes for K99 are carried on a plasmid while those for F41 are chromosomal. K99, F41 and F17 fimbriae can, however, adhere to the porcine small intestinal brush border and can induce porcine enterotoxigenic colibacillosis (Nagy and Fekete, 1999). The association of F17 expression with enterotoxin production is not clear. Original description reported co-expression of F17 with enterotoxin activities (Lintermans *et al.*, 1988) but studies in recent years have revealed that F17 fimbrial adhesins are somewhat heterogeneous, forming a so-called F17 family (F17a, F17b, F17c, F17d and g fimbriae) based on their receptor specificities (Le Bouguenec and Bertin, 1999) and toxin production across this wide group of pathogens is variable.

It should also be mentioned that a non-fimbrial surface protein (CS31A) has also been associated with calf diarrhoea (Girardeau *et al.*, 1988) but this colonisation factor is also detected on septicaemic strains of *E. coli* isolated from calves, in marked contrast to K99 or F41 (Nagy and Fekete, 1999).

### **STa toxin**

STa is a 2 kDa peptide comprising 11 to 18 amino acids that is secreted into the culture medium or the intestinal fluid by producing strains of *E. coli*. Receptors for STa toxin are thought to be heterogeneous, the leading candidates being glycoproteins at the epithelial cell surface and particulate, transmembrane form of the enzyme guanylate cyclase (pGC-c) (Gardner, 1979; Frantz *et al.*, 1984). The effects of STa on mammalian cells are reversible but this does not appear to result from loss of interaction between the toxin and its receptor(s), suggesting that this association is not a typical reversible binding system (Gardner, 1979). The biological activity of STa is exerted through stimulation of guanylate cyclase leading to accumulation of intracellular cGMP and reduced absorption of water and electrolytes ( $\text{Na}^+$  coupled with  $\text{Cl}^-$ ) at the tips of epithelial villus, and simultaneous elevation of secretion of  $\text{Cl}^-$  and  $\text{H}_2\text{O}$  in crypt cells (Forte *et al.*, 1992). STa toxins produced by human and animal strains of ETEC seems to differ in length and show differences in amino acid sequence (Moseley *et al.*, 1983). Depending on the origin of the enterotoxigenic strain and the properties of the peptide, the toxins are designated as STaH and STaP. However, human ETEC strains may produce STaP and STaH while ETEC from calves and pigs only appear to produce STaP. The genes encoding the STa toxins are located on plasmids and are part of a transposon (Tn1681) that is flanked by insertion sequences (So and McCarthy, 1980).

### **Protective capacity of vaccines against K99**

Conventional vaccines against bovine ETEC have been shown to provide varying levels of immunity (Moon and Bunn, 1993; Ascon *et al.*, 1998). Fimbrial vaccines are routinely given parentally to pregnant cattle, sheep and swine to protect suckling newborn calves, lambs and pigs against ETEC infections. Such vaccines are practical and effective because (a) most fatal ETEC infections in farm animals occur in the early neonatal period when the antibody titres in colostrum and milk are highest. (b) more than 90% of the ETEC strains that infect farm animals belong to a small family of fimbrial antigen types. (c) the fimbriae themselves are antigenic components of the bacterial surface and in this

location, are readily available to antibody. (d) fimbriae are required for a critical step early in the pathogenesis of the disease (Moon and Bunn, 1993). Neonatal calves cannot be directly vaccinated because their immune systems take several months to achieve maturity.

The vaccines for use in cattle all include fimbrial antigen F5 (K99) whereas those for swine all include F4 (K88), F5 and F6. Some vaccines also include F41. The vaccines themselves – bacterins and K99 preparations to varying degrees of purity - can be produced directly from field isolates but increasingly, *E. coli* strains are used that carry plasmids constructed by the use of recombinant DNA technology. Most products are designed for parental vaccination with two doses during the first pregnancy and a single booster during each subsequent pregnancy (Moon and Bunn, 1993).

The efficacy with which the vaccination of dams with fimbriae protects the suckling neonate against ETEC infection via passive lacteal immunity was first convincingly demonstrated by Rutter and Jones, using F4 fimbriae in swine (Rutter and Jones, 1973). Subsequent work confirmed the efficacy of the approach and generalised the concept by extending it to other ETEC fimbriae and other animal species (Morgan *et al.*, 1978; Nagy *et al.*, 1978; Acres *et al.*, 1979; Nagy, 1980). It also demonstrated that ETEC fimbriae apparently do not have cross-protective epitopes and therefore vaccines are most useful if they contain the fimbrial antigens prevalent in the target host species (Morgan *et al.*, 1978). Hence vaccination of dams with F5 protects calves and pigs against ETEC strains bearing both F5 and F41 (F5<sup>+</sup> F41<sup>+</sup>) (Runnels *et al.*, 1987; Moon, 1990) whereas vaccination with either F1 or F41 did not (Morgan *et al.*, 1978; To *et al.*, 1984; Runnels *et al.*, 1987). Interestingly, F41 vaccines are apparently more effective than F5 vaccines in protecting mice against F5<sup>+</sup> F41<sup>+</sup> ETEC (Runnels *et al.*, 1987; Duchet-Suchaux, 1988). In consequence, most of the vaccines in current use achieve best effect by including F5 in vaccines for cattle or by combining F4, F5 and F6 in those for swine. Although F1 and F41 fimbriae are prevalent among animal ETEC strains, their efficacy as protective antigens in cattle and swine is less well defined than for F4, F5 and F6 (Morris *et al.*, 1982; Wilson and Francis, 1986; Runnels *et al.*, 1987; Moon, 1990).

There is less uniformity with regard to the inclusion of other ETEC virulence factors in vaccines. The STa toxin has not been an effective protective antigen when used as a hapten in vaccines, and the stimulation of STa neutralising antibodies appears difficult (Moon *et al.*, 1983; Frantz *et al.*, 1987). Some vaccines include polysaccharide O (cell wall) and K (capsular) antigens from the most common porcine or bovine ETEC strains. However, the evidence on their value is contradictory. In one series of experiments, O

antigens were not effective, but a true capsular polysaccharide K antigen of the A (mucoid, heat stable) variety did appear to be effective. Regardless of their efficacy, the practical value of O or polysaccharide K antigens is limited by the multiplicity of O and K antigens that commonly occur among ETEC strains and it is therefore unreasonable to expect vaccines of limited valency to provide broad efficacy in the field. Furthermore, many ETEC lack true polysaccharide capsules (Moon and Bunn, 1993).

New approaches to these issues are starting to appear. A new attenuated *Salmonella* vaccine designed for oral delivery effectively delivered K99 fimbriae to mucosal sites resulting in sustained elevation of IgA and IgG antibodies and eliciting protective immunity (Ascon *et al.*, 1998). It has also been shown that three DNA vaccine formulations against human ETEC could elicit CFA/I-specific immune responses and hence DNA vaccines may also represent a promising approach against enteric bacterial pathogens (Alves *et al.*, 1999).

In conclusion, limited protection with purified K99 fimbriae or formalin-inactivated ETEC has been demonstrated but the need for an efficacious vaccine against bovine ETEC still exists (Moon and Bunn, 1993).

### ***Administration of anti-K99 antibodies***

Since colonization is considered to be an essential step in the pathogenesis of enteric colibacillosis, it has been postulated that prevention of bacterial adherence in the small intestine would reduce the severity of the disease. This could be achieved by introducing K99-specific antibody into the gut lumen of newborn calves. This concept is well-established in practice: ingestion of colostrum taken from cows vaccinated with purified K99 antigen, whole cell bacterins or crude fimbrial extracts protects newborn calves from fatal diarrhoea (Myers, 1978; Acres *et al.*, 1979; Myers, 1980; Nagy, 1980; Acres *et al.*, 1982). It is generally assumed that anti-K99 antibody prevents diarrhoea by acting locally in the small intestine to prevent colonization.

Other products are designed to protect against *E. coli* bacteraemia and septicaemia in hypogammaglobulinaemic neonates. Hypogammaglobulinaemia is common in calves because there is no passive Ig transfer from dam to foetus *in vivo* because of the structure of the bovine placenta. Also, independent Ig synthesis in young calves takes several weeks to establish and hence the neonate is heavily dependent upon antibody acquired passively from the dam. Under farm or ranch conditions, the levels of Ig transferred in this way are

frequently insufficient. Several of the products designed for oral administration to calves contain antibody against fimbriae and are designed to prevent ETEC infections. This approach is rational because a high proportion of fatal ETEC infection occurs during the first few days after birth and calves are only susceptible to colonisation by ETEC that express F5 during this period (Smith and Halls, 1967; Acres, 1985).

In all these studies, K99 antibody was administered to suckling calves through the colostrum of dams that were vaccinated before parturition. However, commercial vaccines have some recognizable disadvantages. Pregnant cows must be handled for vaccination twice during the first year and once during each following year of the vaccination programme. Some livestock owners are reluctant to accept the cost and inconvenience of preventative vaccination unless they have recently experienced an outbreak of enteric colibacillosis in their herds. Such outbreaks are difficult to predict because occurrence depends upon a variety of management and environmental factors as well as several different etiological agents. These concerns prompted investigation of an alternative method for direct passive immunization of newborn calves, namely, the oral administration of K99-specific antibodies shortly after birth. For example, it has been found that feeding 1 ml of mouse ascitic fluid containing K99-specific monoclonal antibody significantly reduced the degree of clinical dehydration and mortality rate in calves challenged with ETEC (Sherman *et al.*, 1983). Anti-K99 antibodies also have a broad range of investigative, diagnostic and clinical applications. Methods used for the detection of fimbrial expression in ETEC include agglutination, fluorescent-antibody tests (Arbuckle, 1970; Bertschinger *et al.*, 1972) and ELISAs (Mooi *et al.*, 1979; de\_Graaf *et al.*, 1981; Mills *et al.*, 1982). Some of these assays are applicable without the need for sophisticated equipment. For example it has been shown that slide agglutination tests with an anti-K99 monoclonal antibody can be highly specific and possess excellent stability (Angulo *et al.*, 1986). Capture ELISAs also enable direct detection of fimbriae in faecal specimens from calves with diarrhoea without the necessity for bacterial culture (Raybould *et al.*, 1987).

In conclusion, rapid detection of ETEC organisms in faecal specimens is possible via their fimbriae enabling specific diagnosis of the cause of diarrhoea outbreaks in calves. The antibodies that make this possible can also be applied in immunotherapy to improve the management of neonatal diarrhoea caused by ETEC strains.

## **Objectives**

The objectives of this aspect of the study were to isolate K99 fimbriae from B41 *E. coli* strain and purify its major subunit (Fan C). Subsequent objectives were to detect biological activities of the purified material and then to use for screening phage display the libraries of recombinant antibodies.



## Materials and methods:

### **Bacterial strain**

*Escherichia coli* strain B41, an O101 serotype of bovine origin produces the heat-stable toxin STa, K99 (F5) and F41 fimbriae. It has become established as the K99 reference strain (Orskov *et al.*, 1975). The bacterium was obtained as freeze-dried culture from the Central Veterinary Laboratory (New Haw, Addlestone, Surrey KT15 3NB, UK), and stored initially at room temperature, in the dark.

### **Growing bacteria**

To grow bacteria for expression of K99 fimbriae, a loopful of bacteria was taken from stock and plated onto sheep blood agar. The plate was incubated at 37 °C overnight. The following day, a single bacterial colony was added to 5 ml of Minca medium (KH<sub>2</sub>PO<sub>4</sub>, 1.36 g; Na<sub>2</sub>HPO<sub>4</sub> 2H<sub>2</sub>O, 10.1 g; glucose, 1 g; trace salts solution, 1 ml; Casamino Acids, 1 g; and distilled water, 1,000 ml. The pH is 7.5. The trace salts solution contained, per liter: MgSO<sub>4</sub>·7H<sub>2</sub>O, 10 g; MnCl<sub>2</sub> 4H<sub>2</sub>O, 1 g; FeCl<sub>3</sub>·6H<sub>2</sub>O, 0.135 g; and CaCl<sub>2</sub> 2H<sub>2</sub>O, 0.4 g) (Guinee *et al.*, 1976) and grown overnight at 37 °C with shaking at 200 rpm. For large-scale production of fimbriae, 1 ml of this culture was inoculated to 1 litre of Minca media and grown again overnight.

### **PCR confirmation of genes encoding K99 and F41 fimbrial subunits**

To confirm the presence in *E. coli* B41 of genes encoding K99 and F41 fimbriae, two PCR reactions were set up using gene-specific primers designed from published sequences (Franck *et al.*, 1998). The 40 µl PCR mixtures contained 0.5 µM concentrations of forward (5' TATTATCTTAGGTGGTATGG 3') and reverse primers (5' GGTATCCTTAGCAGCAGTATTTTC 3') for a fragment from the K99 operon, or forward (5' GCATCAGCGGCAGTATCT 3') and reverse primers (5' GTCCCTAGCTCAGTATTATCACCT 3') for isolation of part of the F41 gene. Also present were 1× AmpliTaq Mg free Gold buffer (Promega), 4µl of 25mM MgCl<sub>2</sub>, 5U of AmpliTaq Gold DNA polymerase (Promega UK), 0.2 mM concentrations of each dNTP (Promega), and 19 µl of a crude bacterial lysate. The lysate was prepared by suspending a colony of bacteria grown overnight on sheep blood agar in 50 µl of H<sub>2</sub>O and boiling for 10

min. After addition of all PCR reaction materials, samples were amplified in a Hybrid PCR Express thermal cycler under the following conditions: 25 cycles beginning with 30 s denaturation at 94 °C, primer annealing at 55 °C for 1 min, followed by extension for 1 min at 72 °C, and a final extension for 10 min at 72 °C. Products were analysed on a 1 % agarose gel, run for 1h at 100 V, stained with ethidium bromide, and photographed under UV light.

### ***Fimbrial extraction***

After growing the bacteria, fimbriae were extracted using a method based on that described previously (Vazquez *et al.*, 1996). Bacteria were harvested by centrifugation at 3300g for 10 minutes. The supernatant was discarded and the bacterial pellet was resuspended in 40 ml of 50 mM phosphate buffer pH 7.2 containing 2M urea (PU) as described earlier (de Graaf *et al.*, 1980b). Then, the bacterial suspension was heated at 65 °C for 30 min to release fimbriae followed by centrifugation at 5200g for 30 min to pellet bacterial cells. The supernatant was carefully recovered and fimbriae were precipitated by adding solid ammonium sulphate to 60% saturation (de Graaf *et al.*, 1981) and incubation for 1 hr on ice. The precipitate was collected by centrifugation at 5200 g for 1 h at 4 °C and dissolved in 2 ml of PU. The solution was then dialysed overnight at 4 °C, typically against phosphate buffered saline.

### ***Detection of K99 antigen in fimbrial extract***

#### **Detection by Fimbrex K99 kit**

The presence of K99 fimbriae in dialysed extracts was tested using a latex agglutination test (Fimbrex K99 kit, Veterinary Laboratories Agency, Surrey). The test is based upon the agglutination of latex beads coated with anti-K99 antibody in the presence of K99 fimbriae in solution or on the surface of bacteria. Two drops of dialysed extract were added to defined regions on the test card at room temperature. Reagents from the kit were added following the manufacturer's instructions, and the reaction was compared with controls.

#### **ELISA**

To assess the recognition of fimbriae extracts by anti-K99 antibodies, two rows of an ELISA plate (Iwaki, Japan) were coated with 100 µl of two-fold serial dilutions of fimbriae

extract in PBS. As negative control, PBS was coated added to other wells. The plate was incubated at 4 °C overnight to allow antigen binding. The following day, coating reagents were discarded by inverting the plate and shaking it, and wells were washed three times with PBS. The wells were then blocked by adding 200 µl of 0.1 % of BSA/PBS and incubating for 2 hrs at 37 °C. After washing three times with PBS, 100 µl of a 1:1000 dilution of sheep polyclonal anti-K99 antibody (Biogenesis, UK) in blocking buffer was added to half of the coated and control wells, and 100 µl of a 1:1000 dilution of mouse monoclonal anti-K99 (Biogenesis, UK) in blocking buffer was added to the remainder. Incubation was for 1 hr at 37 °C. Next, wells were washed three times with PBS containing 0.05 % Tween. To detect bound antibodies, 100 µl of 1:1000 dilutions of anti-sheep-HRP was added to the first series of wells and 100 µl of 1:1000 dilutions of anti-mouse-HRP was added to the second series. The plate was incubated for a further 1hr at 37 °C and then was washed three times with PBS containing 0.05 % Tween. The reaction was developed with 100 µl of substrate solution (1 mg/ml OPD in 0.1 M citrate buffer pH 4.5 and 0.012% (v/v) H<sub>2</sub>O<sub>2</sub>) and the absorbance was measured at 450 nm after 15 minutes.

### **SDS-PAGE gel electrophoresis**

Electrophoresis in SDS-PAGE was carried out by the method of Laemmli (Laemmli, 1970). Polyacrylamide gels were routinely cast with (5%) stacking and (12%) separating layers (Sambrook *et al.*, 1989). Twenty µl of fimbrial extract was boiled in 10 µl of 3 × sample buffer for 5 minutes. 10 ml of 3 × stock was made up with 2.4 ml of 1M Tris-HCl pH 6.8, 3 ml of 20% SDS, 3 ml of glycerol, 1.6 ml of β-mercaptoethanol and 0.006g of Bromophenol Blue. Twenty µl of each boiled sample was loaded to one lane of the polyacrylamide gel. Electrophoresis was carried out in 25 mM Tris, 0.2 M glycine buffer containing 0.1% (w/v) SDS at 200 volts for 1h. The gel was stained with Coomassie Brilliant Blue solution for 30 min. Then staining solution was discarded and gel was destained with destaining solution for overnight.

### ***Immunoblotting***

After electrophoresis, proteins were transferred from SDS gels to nitrocellulose membrane (Amersham Life Science, UK) following two blotting methods.

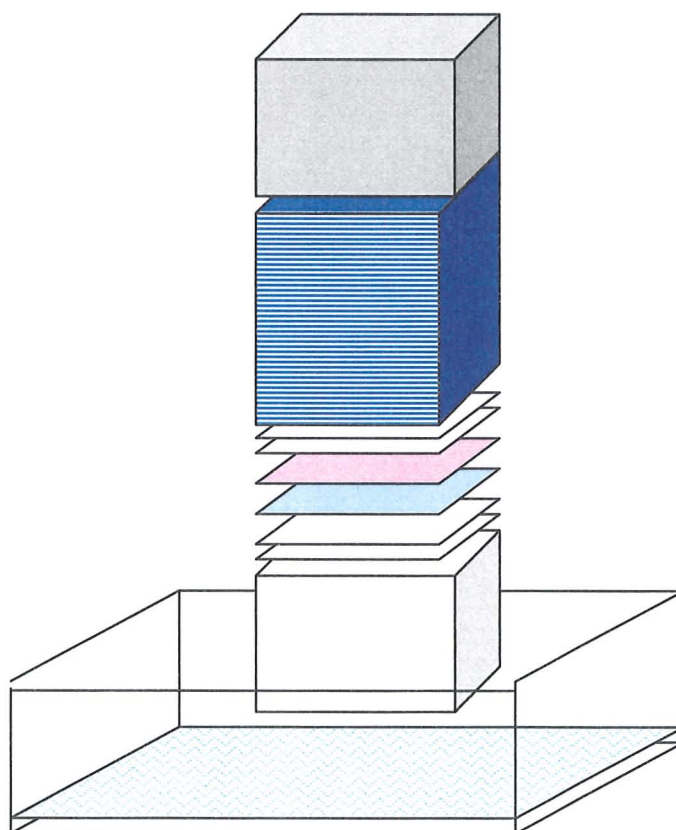
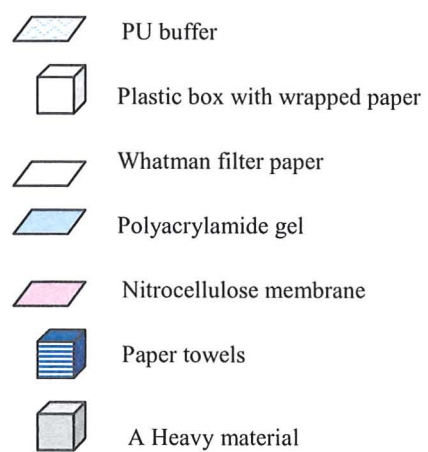
## Electroblotting

This method was as the same as that described as standard protocol for Western blotting (Sambrook *et al.*, 1989). A nitrocellulose membrane (Amersham Pharmacia Biotech, UK) and four pieces of Watman filter paper were cut to the same size as the gel and soaked with the gel in transfer buffer (25 mM Tris base, 192 mM glycine, 20 % (v/v) methanol) for 20 min. For transfer, a fibre pad was overlaid with two pieces of filter paper, the gel, nitrocellulose membrane, and the final two layers of filter paper. After addition of a second fibre pad, the sandwich was assembled into a blotting cassette and loaded into a BioRad electrophoresis tank. Transfer buffer was added along with a frozen insert to avoid overheating during transfer. Care was taken to ensure that the gel lay to the anode (-) side of the assembly and the membrane lay to the cathode (+) side. Proteins were transferred from the gel to the membrane for 1 hr at 100 volts. After transfer, the membrane was carefully separated, washed three times with PBS and stained with Ponceau red dye (Sigma, USA) by shaking for 5 min at room temperature to confirm that proteins had been transferred from the gel to the membrane. The membrane was then washed with distilled water to remove the dye. The membrane was blocked with 3% MPBS (Dried skimmed milk 3g and PBS up to 100 ml) for 1 hr at room temperature with shaking. After that, the membrane was washed and probed with a 1:1000 dilution of polyclonal anti-K99 for 90 minutes at 37 °C and 200 rpm shaking. The unbound antibody was washed out with three changes of PBS containing 0.05 % Tween. The membrane was then incubated in a 1:1000 dilution of anti-sheep-HRP for 90 minutes at 37 °C and 200 rpm shaking. Then the membrane was washed as before and developed using diaminobenzidine substrate (2mg diaminobenzidine, 4 µl of 30 % hydrogen peroxide and 20 ml of TBS).

## Diffusion blotting

Since experience revealed that K99 hardly transfers from the gel to nitrocellulose membrane by electroblotting, we applied a method similar to that described by other investigators (Brian Bowen, 1980; Heukeshoven and Dernick, 1995; Olsen and Wiker, 1998; Chen and Chang, 2001).

A Whatman filter paper was wrapped around a plastic box (10 cm × 7 cm × 5 cm). The box was then put inside a container. Then two pieces of filter paper, the gel, nitrocellulose membrane, and the final two layers of filter papers were added to the top of the box. Next, some paper towels were cut to the same size as the nitrocellulose membrane. These were stacked on top of the other layers. Finally, a heavy box (about 1 Kg) was added to



**Figure 2.3** Assembling materials for diffusion blotting

compress the assembly. One litre of PU buffer was added to the container, which was then covered with a plastic sheet to prevent evaporation of the buffer. The assembled diffusion blot was incubated overnight at room temperature. This method is illustrated in Figure 2.3.

## ***Purification of K99 by ion-exchange chromatography***

As experiments showed that a crude fimbrial extract was not sufficiently pure for isolation of antibodies specific for K99, the extract was purified by ion-exchange chromatography.

### **Preparation of sample for chromatography**

After dialysis of the fimbriae extract into PBS, conditions were not appropriate for binding to the ion exchange column. Dialysis into distilled water was found to cause some aggregation of the protein. To solve these problems, fimbriae precipitated with ammonium sulphate was dissolved prior to purification at 1:50 of culture volume in 2M of warm urea solution. It was then exchanged into binding buffer of the same volume using an Amicon Ultra-4 centrifugal filter (Millipore, UK). Finally, the sample was filtered by passing through a 0.45  $\mu\text{m}$  filter before running onto the column.

### **Choice of starting buffer**

Based upon the isoelectric point (pI) of the K99 protein (<http://ca.expasy.org/cgi-bin/protparam1?P18103@noft@>) (Roosendaal *et al.*, 1984), two binding buffers of 50 mM phosphate buffer and 25 mM Tris base pH 7.0 and 7.5 were checked on an SP XL column to assess which resulted in higher binding of K99 to the column matrix. After purification, the initial sample, flow through and eluted fractions were run on SDS PAGE to assess the outcome of the experiment. From these experiments, a binding buffer of 25 mM Tris base pH 7.0 was chosen for subsequent purification.

### **Purification procedure**

Before running the sample on a 5 ml HiTrap SP XL column, the column was washed with 5 column volumes (25 ml) of binding buffer using a Pharmacia Biotech P-1 peristaltic pump (Sweden), at a flow rate of 5ml/min. Then the column was charged by washing with 10 column volumes of binding buffer containing 2M NaCl and finally it was equilibrated with 5-10 column volumes of binding buffer.

Next, 5-10 ml of sample for purification was applied at 5 ml/min and the column was washed with at least 5 column volumes of starting buffer or until no material appeared in the effluent. Contaminating proteins were eluted with 5 column volumes of 25 mM Tris base pH 7.0 containing 20 mM NaCl. K99 was then recovered by pumping through 5 column volumes of the second elution buffer (25 mM Tris base pH 7.0 containing 80mM NaCl).

Fractions containing K99 were identified by SDS PAGE then pooled, concentrated to 1/3 initial volume using an Amicon Ultra-4 centrifugal filter (Millipore, UK) and dialysed overnight against PBS at 4°C.

### ***Protein assay***

Protein concentrations were measured using a BCA Protein Assay Kit (Collins et al.). After preparation of BSA standards of known concentration and the working reagent, 0.1 ml samples of each standard and the fimbrial extract were added to 2.0ml of the working reagent. All tubes were incubated at 37 °C for 30 minutes and then cooled to room temperature. The colorimetric reaction in each tube was measured at 562 nm using Unicam UV/VIS spectrometer. The concentration of the fimbrial extract was determined by comparison against a standard curve.

### ***Biological activity of purified K99***

The biological activity of purified K99 was tested by experiments to compare its properties with those of the starting extract.

#### **Detection by Fimbrex K99 kit**

Two drops of purified K99 antigen was added to a test card and after addition of latex agglutination, the reaction was compared with controls.

#### **Detection by ELISA**

To coat an ELISA plate, 100 µl of purified K99 fimbriae (approximately 30 µg/ml) and 100 µl of 2 % MPBS (as a negative control) were added to wells of an ELISA plate. The plate was incubated at room temperature overnight. The following day the wells were washed three times with PBS and were blocked with 0.1 % BSA/PBS for 2 hrs at 37 °C.

After washing wells as above, they were probed with 100  $\mu$ l of anti-K99 mouse monoclonal antibody (Biogenesis, UK) at 1:1000 dilution for 1 hr at 37 °C. The wells were then washed three times with PBS containing 0.05 % Tween and the bound antibodies were detected by adding 100  $\mu$ l of a rabbit anti-mouse HRP conjugate (Sigma, UK) at a dilution of 1:1000. The reaction was developed with 100  $\mu$ l of developer solution containing OPD substrate. The absorbance was measured after 10 min at 450nm.

## Biotinylation of K99 fimbriae

Since heating the K99 fimbriae appeared to destroy the epitope recognised by the monoclonal anti-K99 antibody, alternative methods were sought to test the specificity of antibody recognition of the bacterial protein. In preparation for immunoprecipitation, purified K99 was biotinylated using an EZ-Link™ Sulfo-NHS-LC-Biotinylation Kit (Collins et al.). In this strategy, biotinylated protein would be detectable using ExtrAvidin-Peroxidase. The biotinylation procedure was based on the method of Hnatowich (Hnatowich *et al.*, 1987) and instructions provided with the kit were followed.

### ***EZ-Link™ Sulfo-NHS-LC-Biotinylation Kit***

Biotin is a small naturally occurring vitamin that binds with high affinity to avidin and streptavidin proteins. As it is so small (244 Da), biotin can be conjugated to many proteins without altering their biological activities. The labelled protein or other molecule may then be detected easily in ELISA, dot blot or Western blot applications using streptavidin or avidin probes. *N*-hydroxysuccinimide (NHS) esters of biotin are a popular type of biotinylation reagent. NHS-activated biotins react efficiently with primary amino groups (-NH<sub>2</sub>) in buffers of pH 7-9 to form stable amide bonds (Figure 2.4). Proteins generally have several primary amines in the side chain of lysine (K) residues and at the N-terminus that are available as targets for labeling with NHS-activated biotin reagents.

The EZ-Link™ Sulfo-NHS-LC-Biotinylation Kit was containing; EZ-Link® Sulfo-NHS-LC-Biotin (25 mg), BupH™ Phosphate Buffered Saline Pack (1 pack, 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2 when reconstituted in 500 ml of ultrapure water), D-Salt™ Dextran Desalting Column (10 ml), HABA (4'-hydroxyazobenzene-2-carboxylic acid) (1 ml) and Affinity Purified Avidin, 10 mg.



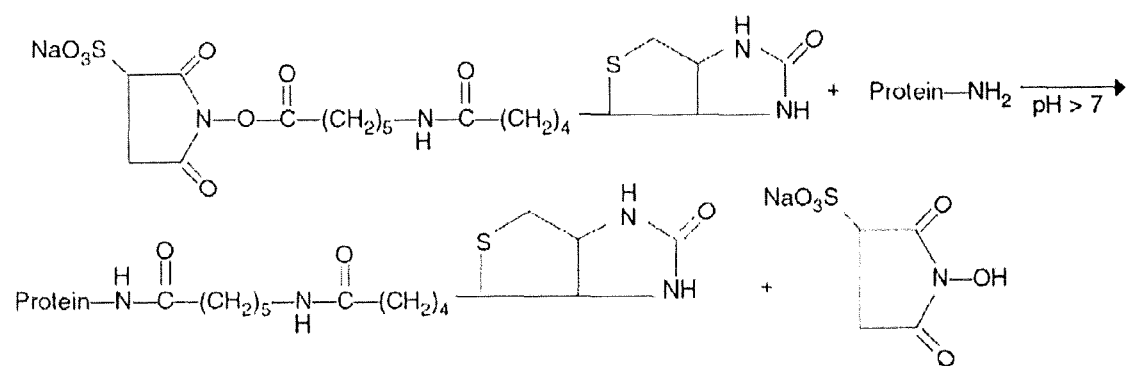


Figure 2.4 The reaction of sulfo-NHS-LC-Biotin with a protein

## **Sample preparation**

Purified K99 was concentrated to approximately 2 mg/ml using an Amicon Ultra-4 centrifugal filter with a 10 KDa cut off. The buffer was exchanged with three volumes of PBS solution from the biotinylation kit (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2) using the same filter. The molar concentration of the K99 was then calculated.

## **Biotinylation procedure**

The Sulfo-NHS-LC-Biotin was warmed to room temperature. Two mg of Sulfo-NHS-LC-Biotin was added to 200  $\mu$ l of ultrapure water and used immediately. A 20-fold (130  $\mu$ l) molar excess of this reagent was added to a 2mg/ml solution of K99. The mixture was incubated on ice for 2 hrs. Next, a 10 ml desalting column supplied with the kit was equilibrated with 30 ml of the PBS buffer. After addition of 1 ml of sample to the column, it was allowed to permeate the gel. Then 12 ml of reconstituted PBS solution was added to the column and the biotinylated protein was recovered in twelve 1 ml fractions. To monitor the presence of protein, 100  $\mu$ l from each fraction was tested at 280 nm using a spectrophotometer. Fractions 5 and 6 were judged from this assay to contain the K99 protein.

## ***HABA assay for measuring biotin incorporation***

To quantitate incorporation of biotin label, a solution containing the biotinylated protein was added to a mixture of HABA and avidin. Since it has a higher affinity for avidin, biotin displaces the HABA from its interaction with avidin and the absorption at 500 nm decreases proportionately. By this method, an unknown amount of biotin present in a solution can be quantitated in a single cuvette by measuring the absorbance of the HABA-avidin solution before and after addition of the biotin-containing sample. The change in absorbance relates to the amount of biotin in the sample.

## **Reagent Preparation**

One mg of avidin and 60  $\mu$ l of 10 mM HABA was added to 1.94 ml of reconstituted PBS. The absorbance of this solution was measured at 500 nm. The absorbance was recorded as 0.966.

## **HABA assay procedure**

Fractions 5 and 6 from the gel filtration column were pooled. One hundred  $\mu\text{l}$  of a 1 in 10 dilution of the mixture was added to 900  $\mu\text{l}$  of the avidin-HABA reagent. After mixing well, the absorbance was measured at 500 nm with spectrophotometer. Then the value moles biotin / mole protein was calculated as protocol instruction.

## ***Detection of biotinylated K99 protein***

### **Dot blotting**

Fractions from gel chromatography judged by spectrophotometry to contain protein were analysed by dot blotting. Three  $\mu\text{l}$  samples from each fraction were spotted three times on a nitrocellulose membrane. After drying the spotted proteins, the membrane was blocked in 3 % MPBS for 1 hr at room temperature with shaking at 100 rpm. The membrane was then washed three times with PBS and incubated in a 1:500 dilution of ExtrAvidin-Peroxidase (Sigma, UK) for 90 minutes at 37 °C with shaking at 200 rpm. Then the membrane was washed three times with PBS containing 0.05 % Tween and was developed using 4-chloro-1-naphthol substrate.

### **SDS-PAGE analysis and Western blotting**

The fractions that had the best reactions in dot blotting were further analysed by SDS-PAGE and Western blotting. Fractions 5 and 6 and also 7 and 8 were mixed and were run on two 12 % SDS acrylamide gels following a standard protocol. A sample of the biotinylated protein prior to desalting and a sample of unbiotinylated K99 were used as positive and negative controls. After running for 1 hr at 200 volts, one gel was stained with Commassie Brilliant Blue solution and the second was used for Western blotting. The proteins were transferred from the gel to a nitrocellulose membrane by diffusion blotting using PU buffer (50 mM phosphate buffer pH 7.2) as described previously. The membrane was then blocked and probed with ExtrAvidin-Peroxidase as described for dot blotting. The membrane was developed using diaminobenzidine substrate.

## ***Immunoprecipitation of biotinylated K99***

To provide further confirmation that the purified protein was K99, the biotinylated K99 was used for immunoprecipitation experiments. Seven  $\mu\text{l}$  of monoclonal anti-K99 antibody

was added to 400  $\mu$ l of biotinylated K99 and was incubated for overnight on a turntable at 4 °C to allow interaction of antigen and antibody. The following day, 100  $\mu$ l of Protein G agarose (Sigma,UK) was washed three times with PBS and was added to the above mixture for rotating incubation for 2 hrs at 4 °C. The mixture was then spun at 10000g for 1 minute to pellet the Protein G agarose. The pellet was washed three times with PBS by centrifugation (10000g, 30 seconds) and resuspension. To release the antibody-antigen complex, 50  $\mu$ l of 3  $\times$  SDS sample buffer was added to the pellet and after mixing it was boiled for 5 minutes and then centrifuged at 10000g for 1 minute. As a control, a sample was also taken from the first supernatant (free antibody) for analysis. A sample of biotinylated K99 was also boiled for analysis.

### **SDS-PAGE analysis and Western blotting**

The test sample and controls were run on two 12 % SDS acrylamide gels following a standard protocol. One gel was stained using a silver staining method; the second was used for diffusion blotting by overnight transfer from the gel to a nitrocellulose membrane overnight using PU buffer (50 mM phosphate buffer pH 7.2) as described previously. The membrane was then washed with PBS, blocked and probed with ExtrAvidin-Peroxidase as described for dot blotting. The membrane was developed using diaminobenzidine substrate.

### ***Haemagglutination assay***

Some erythrocytes (RBCs) such as those from sheep have on their surface receptors to which K99 fimbriae can bind, thereby modelling the interaction of the adhesion factor with enterocytes (Burrows *et al.*, 1976). Haemagglutination (HA) can thus occur with purified fimbriae or with bacteria such as the enterotoxigenic strain of *E.coli* B41 that express K99 (Vazquez *et al.*, 1996). In order to show that purified K99 has the haemagglutination activity, the following experiment was set up.

### **Titration of the haemagglutinating activity**

This was assessed with a test for direct haemagglutinating activity. One colony of *E.coli* B41 grown on sheep blood agar was grown on in 5 ml of Minca medium at 37 °C overnight with shaking at 200 rpm. Following previous investigations (Orskov *et al.*, 1975), another colony was grown as a negative control in the same medium at 18°C since under these conditions, K99 fimbriae are not expressed. 50  $\mu$ l of each culture was added to

the first well of a 96 well microplate (Iwaki, Japan). 25  $\mu$ l aliquots were diluted two fold in PBS containing 1% mannose across the plate. Since the interaction between K99 and its receptor is resistant to mannose, this was intended to inhibit non-specific haemagglutination or the interaction of mannose-sensitive factors with erythrocyte receptors. Bacteria were not added to the last well to provide an additional negative control. To the second row, 50  $\mu$ l of purified K99 at the concentration of about 100  $\mu$ g/ml was added to the first well and diluted across as above. To prepare washed suspensions of sheep red blood cells at a density of about 1.5 % (v/v), 270  $\mu$ l of defibrinated sheep blood (E and O Laboratories, Scotland) was centrifuged at 13000 rpm for 4 min. The pellet was then washed three times with PBS containing 1% mannose (Sigma, UK) by resuspension and spinning for 4 min. Finally the pellet (about 100  $\mu$ l volume) was resuspended in 7.5 ml PBS containing 1% mannose.

25  $\mu$ l of this suspension was added to each well containing bacteria and, after mixing, the plate was incubated at 4 °C overnight. The titre was defined as the highest dilution of bacterial suspension that showed complete haemagglutination.

## Results

### ***Amplification of genes encoding fimbrial subunits of K99 and F41***

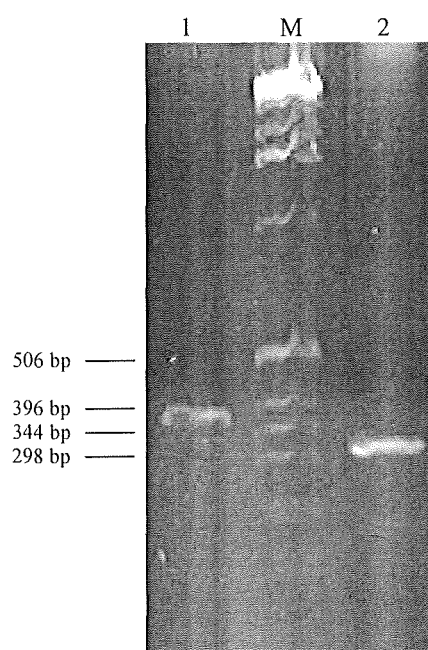
The presence of genes encoding subunits of K99 and F41 fimbriae in *E. coli* B41 was assessed using PCR with specific primers and a bacterial lysate as template. Analysis of the PCR products on 1% agarose gels showed that amplicons of the predicted sizes (314 bp for the K99 product (Roosendaal *et al.*, 1984), 380 bp for the F41 amplicon (Fidock *et al.*, 1989) were present (Figure 2.5).

### ***Fimbrial extraction and analysis***

Fimbriae were extracted from *E. coli* B41 by resuspension in phosphate urea (PU) buffer and heat shock. The fimbriae were then precipitated by ammonium sulphate, resuspended again in PU buffer and dialysed against PBS. Several methods were used to test for the presence of K99 fimbriae in the crude extract.

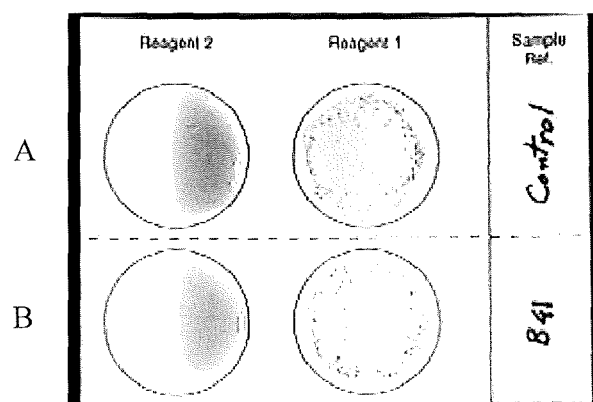
Experiments with the Fimbrex K99 kit revealed the agglutination by latex coated with monoclonal antibody against K99 in the presence of the fimbrial extract. No agglutination was seen in negative controls and the reaction was similar to the positive control antigen provided with the kit (Figure 2.6).

In an ELISA experiment, plates were coated with two-fold serial dilutions of the fimbrial extract and probed with polyclonal and monoclonal anti-K99 antibodies. Both experiments showed recognition of components of the extract by the antibodies. The reaction of the extract with sheep polyclonal anti-K99 antibody is shown in Figure 2.7 and Figure 2.8 shows the reaction with mouse monoclonal anti-K99 antibody. In both cases, the ELISA reaction achieves its strongest response not at the highest coating concentration of extract, but after a degree of dilution. It seems to need further repetition of these ELISA experiments.



**Figure 2.5 Amplification of genes of encoding K99 and F41 subunits by PCR**

A crude lysate of *E. coli* B41 was amplified using primers specific for parts of the F41 (lane 1) and K99 operons (lane 2). The lane marked M shows the migration of a 1 kb DNA ladder (Invitrogen). The migration of markers of 506 bp and 396, 344 and 298 bp is indicated. The predicted size of the K99 product was 314 bp and that for the F41 product was 380 bp.

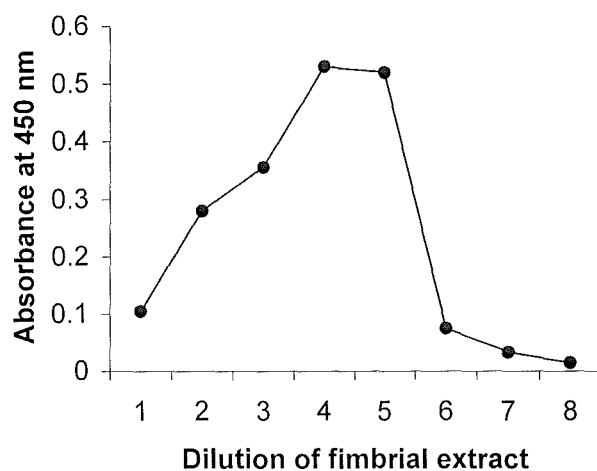


**Figure 2.6** Detection of K99 fimbriae in a crude extract using the Fimbrex K99 kit

A. The antigen provided as a positive control was used with reagent 1 and reagent 2

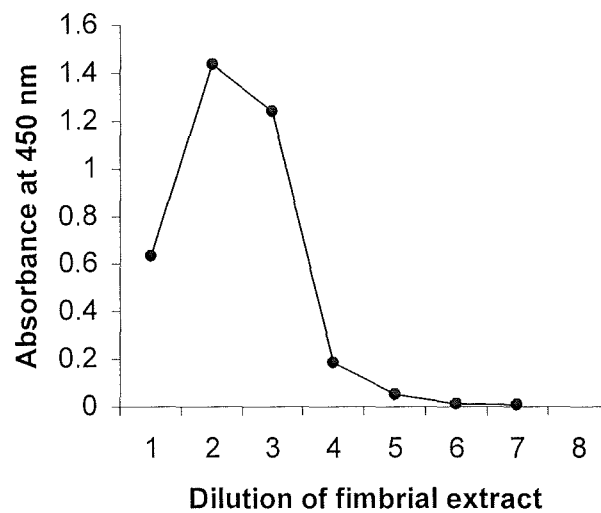
B. The crude fimbrial extract from *E. coli* B41 strain was used. Agglutination of latex coated with monoclonal anti- K99 antibody (reagent 1) was detected whereas unconjugated latex beads (reagent 2) showed no such agglutination.





**Figure 2. 7 Reaction of polyclonal anti-K99 antibody with crude fimbrial extract**

Two-fold serial dilutions of the crude fimbrial extract were coated to wells of an ELISA plate. The samples were probed with polyclonal anti-K99 antibody and anti-sheep HRP conjugate. The reaction was measured at 450 nm.



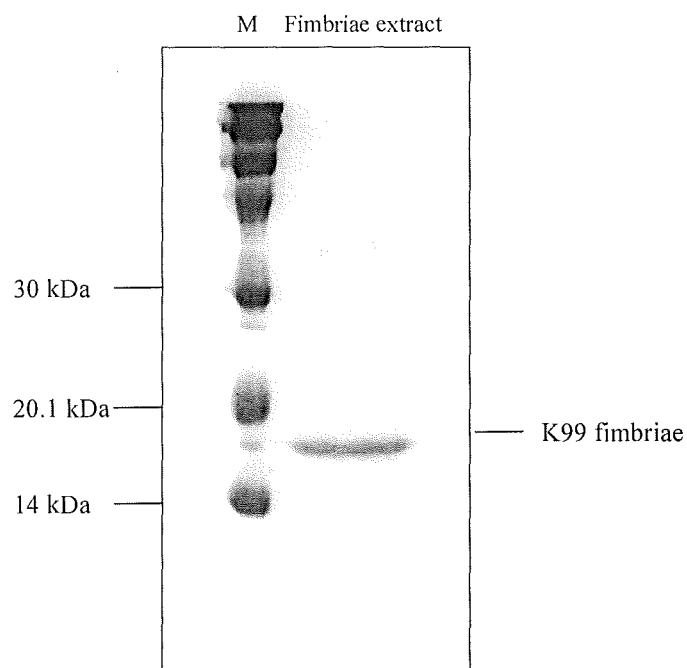
**Figure 2. 8 Reaction of monoclonal anti-K99 antibody with crude fimbrial extract**

Two-fold serial dilutions of the crude fimbrial extract were coated to wells of an ELISA plate. The samples were probed with monoclonal anti-K99 antibody and anti-mouse HRP conjugate. The reaction was measured at 450 nm.

To assess the composition of the crude extract and the presence of subunits of K99 fimbriae, samples were run on a 12 % SDS polyacrylamide gel. The result of this analysis showed that the major constituent in the fimbrial extract was a protein of 16.5 kDa. The size of this protein was similar to that reported previously for the major fimbrial subunit (Vazquez *et al.*, 1996). Other proteins with molecular weights ranging from 30 to more than 100 kDa were also present but at much lower concentrations (Figure 2.9).

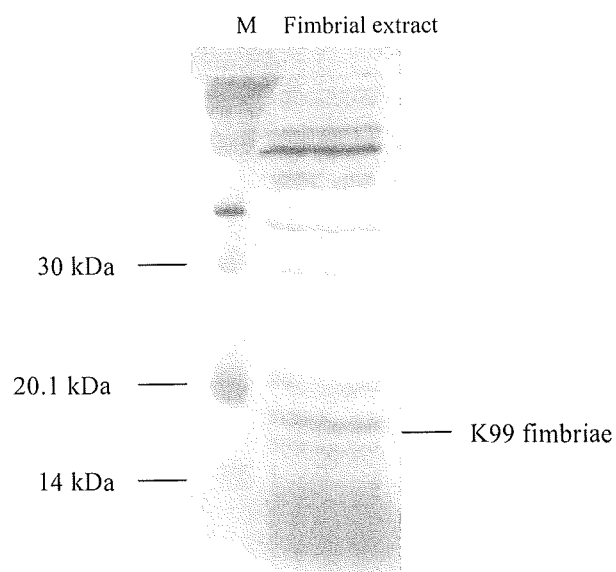
After separating the crude fimbrial extract by SDS-PAGE, proteins were transferred to a nitrocellulose membrane by electroblotting (Western blotting). The membrane was probed with polyclonal anti-K99 antibody and anti-sheep peroxidase conjugate. Figure 2.10 shows the resulting Western blot. A protein of 16.5 kDa, consistent with the size of the major fimbrial subunit reacted with the anti-K99 antibody but extensive cross-reaction with many other proteins was also evident. The reaction of the antibody with proteins on the blot was clearly disproportional to the overall composition of the extract (Figure 2.11). Staining of the membrane with Ponceau red dye immediately after electrotransfer revealed that the 16.5 kDa protein band was transferred poorly to the membrane by this method (data not shown).

Given the poor efficiency of electrotransfer of the 16.5 kDa component from the fimbrial extract, diffusion blotting was attempted. Staining with Ponceau red dye revealed transfer of this protein to nitrocellulose was much-improved. When blots were probed with polyclonal anti-K99 antibody, recognition of the 16.5 kDa protein was much improved and background reaction was significantly reduced (Figure 2.11). The antibody also cross-reacted strongly with two other proteins in the fimbrial extract that in terms of overall composition (Figure 2.11) were minor components. When diffusion blots were probed with a monoclonal anti-K99 antibody, no reaction was detectable.



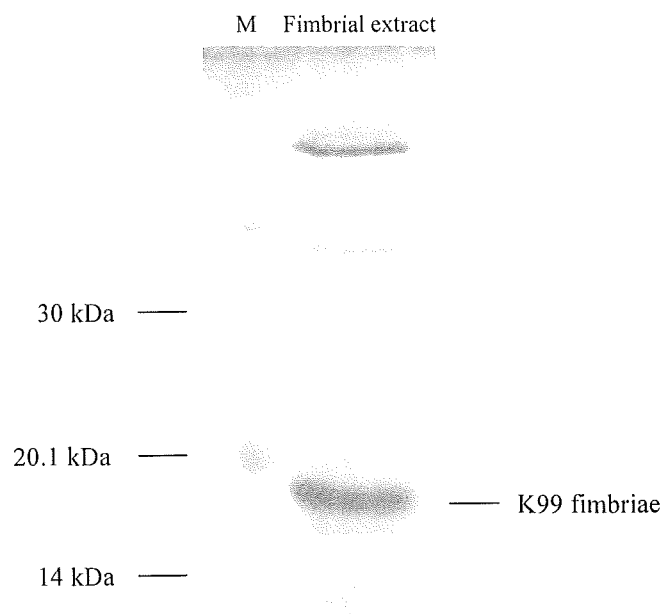
**Figure 2.9 SDS-PAGE analysis of crude fimbrial extract**

A sample of the extract was run on a 12% polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie Blue and photographed. Lane M shows the migration of Rainbow molecular weight markers. The migration of standards of 14, 20.1 and 30 kDa is indicated. The predicted molecular weight of the major K99 subunit is 16.5 kDa.



**Figure 2.10 Western blotting analysis of the fimbrial extract using polyclonal anti-K99 antibody**

The fimbrial extract was separated by SDS-PAGE and transferred to a nitrocellulose membrane by electroblotting. The membrane was probed with sheep polyclonal anti-K99 antibody. Lane M shows the migration of Rainbow molecular weight markers. The migration of standards of 14, 20.1 and 30 kDa is indicated. The predicted molecular weight of the major K99 subunit is 16.5 kDa.



**Figure 2.11** Diffusion blotting analysis of the fimbrial extract using polyclonal anti-K99 antibody

The fimbrial extract was separated by SDS-PAGE and transferred to a nitrocellulose membrane by diffusion blotting. The membrane was probed with sheep polyclonal anti-K99 antibody. Lane M shows the migration of Rainbow molecular weight markers. The migration of standards of 14, 20.1 and 30 kDa is indicated. The predicted molecular weight of the major K99 subunit is 16.5 kDa.

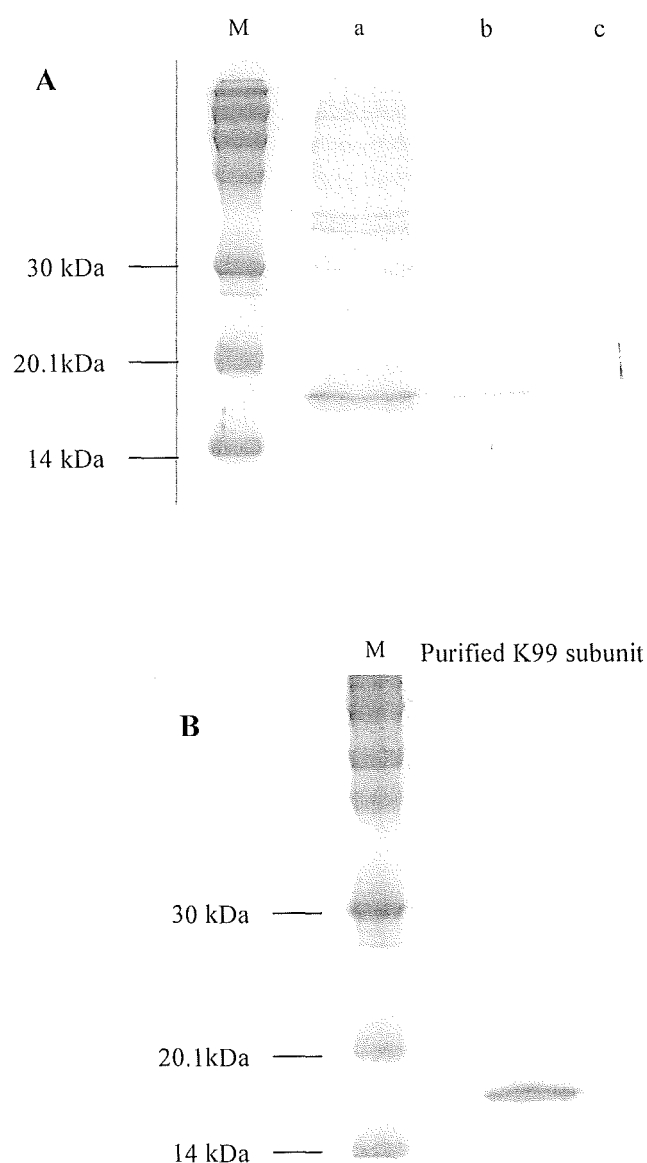
## ***Purification of K99 fimbriae by ion-exchange chromatography***

Isolation of K99 fimbriae by the methods described did not provide material of sufficient purity for the isolation of recombinant antibodies. To overcome this, protein was purified by ion-exchange chromatography. After an initial trial experiment, the best results were achieved using SP XL columns, a binding buffer of 25 mM Tris base pH 7.0 and elution buffers of 25 mM Tris base containing 20 mM and 80mM NaCl. The results are shown in Figure 2.12. After binding protein to the SP XL column, a mixture of proteins could be eluted with binding buffer containing 20 mM NaCl. These conditions released many of the components of the initial extract but there was little evidence of elution of the 16.5 kDa protein, the single most abundant protein in the starting material. By raising the NaCl concentration to 80 mM, the protein of interest (the 16.5 kDa protein), presumed to be the major subunit of K99 was eluted. Figure 2.12B shows the purity of this material after concentration. The preparation appears free of other protein components present in the starting extract (Figure 2.12A).

## ***Biological activity of purified K99***

The purified K99 was tested using the Fimbrex K99 kit as described earlier. The reaction – agglutination of the antibody-coated latex beads – was similar to that of the fimbrial extract and positive the control antigen (Figure 2.6). Given the homogeneity of the purified material (Figure 2.12B) and the specificity of the assay, the result supports presumptive identification of the 16.5 kDa protein as the major subunit of K99 fimbriae,

The protein purified by ion-exchange chromatography was coated to an ELISA plate and assessed using monoclonal anti-K99 antibody. Figure 2.13 shows it was recognised successfully and strongly. This data further supports identification of the protein as the major subunit of K99 fimbriae and shows that so far as recognition by the monoclonal antibody is concerned, it was not denatured during purification.



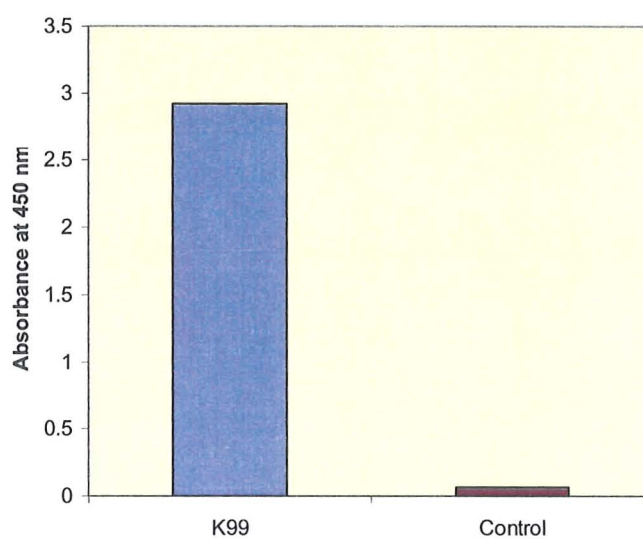
**Figure 2.12 SDS-PAGE analysis of K99 protein purified by ion-exchange chromatography**

Panel A. SDS-PAGE analysis of fractions recovered during the purification of K99. Fimbrial extract was equilibrated with column binding buffer by buffer exchange (lane a). After binding to the column, contaminants were eluted with binding buffer containing 20 mM of NaCl (lane c). By then raising the concentration of NaCl in binding buffer to 80 mM, a single protein of 16.5 kDa could be eluted (lane b).

Panel B. SDS-PAGE analysis of the purified K99 subunit concentrated to approximately 300 µg/ml.

M: Rainbow protein molecular weight markers (Pharmacia). The migration of standards of 14, 20.1 and 30 kDa are indicated.





**Figure 2.13 Reaction of monoclonal anti-K99 with purified 16.5 kDa protein in ELISA**

Purified protein was coated to an ELISA plate and probed with monoclonal anti-K99 and HRP-conjugated antibodies to test for recognition. Negative control wells were coated with 2 % MPBS.

## ***Immunoblotting of purified K99***

### **Effect of preparation conditions on the K99 antigen**

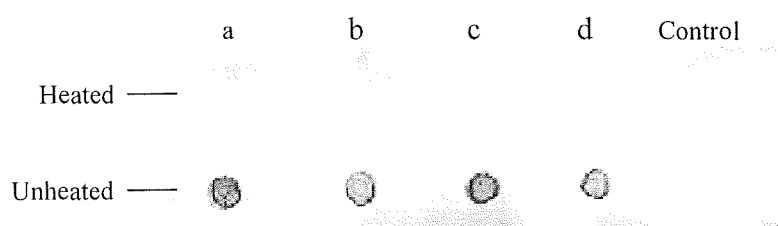
Attempts were made to test the reactivity of the monoclonal anti-K99 antibody against purified fimbrial protein using electroblotting or diffusion blotting. These were unsuccessful. In order to determine the reasons for this, a dot blotting experiment was performed using different conditions for preparation of the sample. Purified K99 was prepared with sample buffers with different additives. Half of these samples were boiled and the remainder were not. After spotting on a strip of nitrocellulose membrane and blocking with 3% BSA/PBS, the samples were probed with monoclonal anti-K99 and anti-mouse peroxidase antibodies using the same reagent concentrations as for ELISA analysis. The result (Figure 2.14) revealed that recognition of K99 antigen with the monoclonal antibody was unaffected by the presence of SDS or  $\beta$ -mercaptoethanol in the sample buffer. In contrast, the epitope recognised by the antibody was highly dependent upon heating of the sample.

### **Effect of heating of K99 to different temperatures**

To find out the temperature at which the K99 epitope was denatured, samples of purified protein were heated in sample buffer to different temperatures. The result of dot blotting (Figure 2.15) showed that recognition of the antigen by monoclonal anti-K99 antibody was retained to a maximum of 50 °C. When samples prepared in this way were analysed by SDS-PAGE (Figure 2.16), it was found that at 40 °C, the 16.5 kDa protein could not be observed, presumably because it was present in the sample in the form of multiple oligomeric forms of insufficient concentration to be visible. When samples were heated to 50 °C or more, migration of the 16.5 kDa monomer was evident. Overall, these results indicate the heat-sensitivity of the K99 protein.

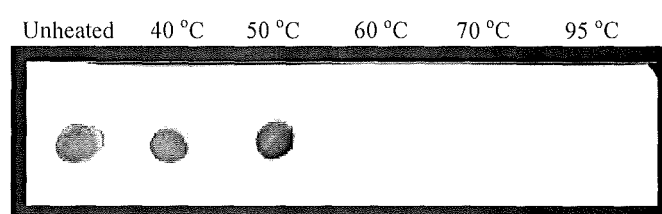
## ***Biotinylation of K99 fimbriae***

To enable its detection in blotting and immunoprecipitation, purified K99 was concentrated and biotinylated. To separate protein from free biotin, the labelling mixture was run through a desalting column and 12 eluted fractions were screened by spectrophotometry for the presence of protein. Samples from fractions with significant absorbance at 280 nm were spotted to nitrocellulose and probed with Extravidin peroxidase



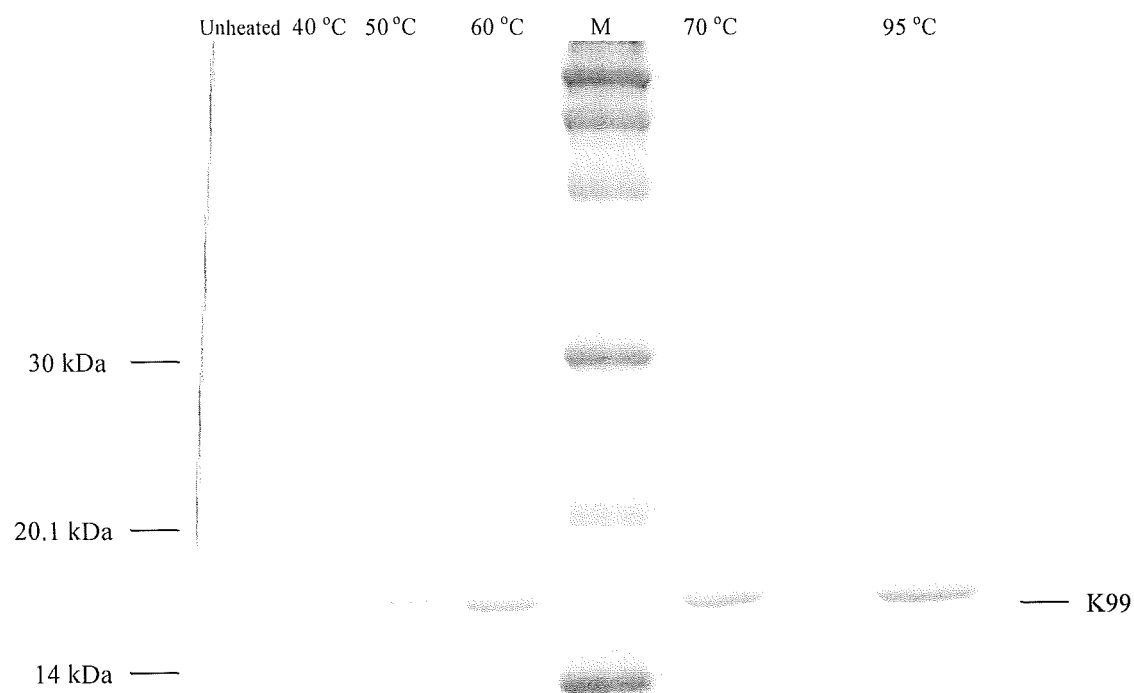
**Figure 2.14 Sample preparation and recognition of purified K99 fimbriae by monoclonal antibody**

Samples of purified protein were prepared in buffers of the following composition and dot blots were probed with anti-K99 monoclonal antibody and anti-mouse HRP conjugate: sample buffer containing both SDS and  $\beta$ -mercaptoethanol (column a); sample buffer containing SDS but not  $\beta$ -mercaptoethanol (column b); sample buffer containing only  $\beta$ -mercaptoethanol (column c); sample buffer without  $\beta$ -mercaptoethanol or SDS (column d). Half of the samples were boiled for 5 minutes (top row) and the remainder were not boiled (bottom row) prior to spotting out. BSA was spotted as a negative control.



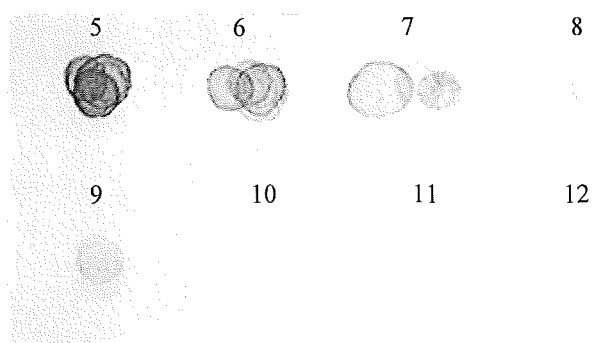
**Figure 2.15 Sample heating and recognition of purified K99 fimbriae by monoclonal antibody**

Purified K99 fimbrial protein was heated in sample buffer to the indicated temperatures before dot blot analysis with monoclonal anti-K99 and HRP-conjugate antibodies.



**Figure 2.16 Migration of K99 in SDS\_PAGE after heating to different temperatures**

Purified K99 was heated in sample buffer to the indicated temperatures. An equal volume of each sample was run on a 12 % polyacrylamide gel and stained with Coomassie Brilliant Blue. M: Rainbow protein molecular weight markers (Pharmacia). The migration of standards of 14, 20.1 and 30 kDa is indicated.



**Figure 2.17 Dot blot analysis of fractions after biotinylation of purified K99**

Fractions from a desalting column were screened by spectrophotometry for the presence of protein. Samples from those with significant absorbance at 280 nm (fractions 5-12 as indicated) were spotted to nitrocellulose and probed with Extravidin peroxidase.

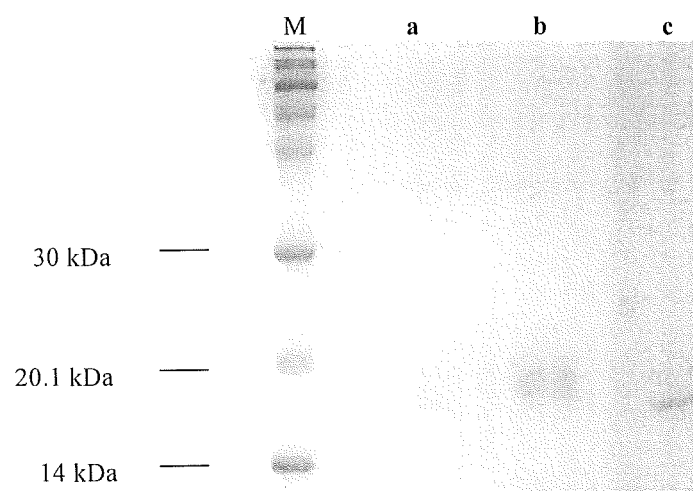
(Figure 2.17). As shown in Figure 2.17, fraction 5 showed strong reaction, the strength of signal decreasing steadily through to fraction 9.

To visualise the biotinylated protein, fractions 5 and 6 were pooled and a sample was separated on SDS-PAGE along with a sample of the biotinylation reaction before desalting and unmodified K99. Figure 2.18 shows that desalting lead to significant dilution of the purified K99 but that a faint band at around 16.5 kDa remained visible. To test if the biotinylated protein was detectable after blotting, samples separated by SDS-PAGE were transferred to a nitrocellulose membrane and probed with Extravidin peroxidase. The result is shown in Figure 2.19. Despite the low concentration of K99 in the desalted fractions, the 16.5 kDa protein was readily and specifically detected with peroxidase conjugate. The analysis revealed that this was the only biotinylated protein present in the desalted preparation. The level of biotin incorporation into the labeled K99 was calculated by HABA assay and found to be 0.133 moles biotin per mole K99 protein. Although this ratio was low, it is clear from Figure 2.19 that the sensitivity of Extravidin peroxidase enabled detection of that fraction of K99 to which biotin was bound.

### ***Immunoprecipitation of biotinylated K99***

To provide further confirmation that the protein purified by ion-exchange chromatography was derived from K99, biotinylated material was used for immunoprecipitation with a monoclonal anti-K99 antibody. After incubation, immune complexes were captured with Protein G agarose and then released by boiling in SDS sample buffer for analysis by SDS-PAGE and Western blotting.

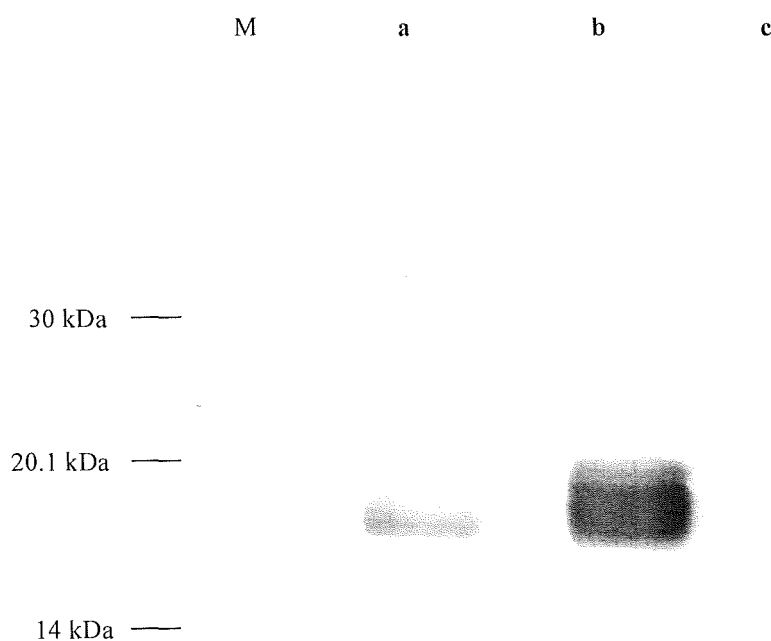
Silver staining was used to reveal proteins separated by SDS-PAGE (Figure 2.20). By comparison with lane c (biotinylated K99), the presence of K99 in the antibody – antigen mixture is evident in lane b and it appears from lane a that the material becomes captured to Protein G, indicating the formation of immune complexes. Bands of higher molecular weight in lane b are likely to be the antibody heavy and light chains although the presence of a protein band close to the 30 kDa marker is higher than might normally be expected for an immunoglobulin light chain (25 kDa). In lane a, proteins of a range of molecular weights appear to have been released from the Protein G agarose by boiling in SDS sample buffer.



**Figure 2.18 SDS-PAGE analysis of biotinylated K99 fimbriae**

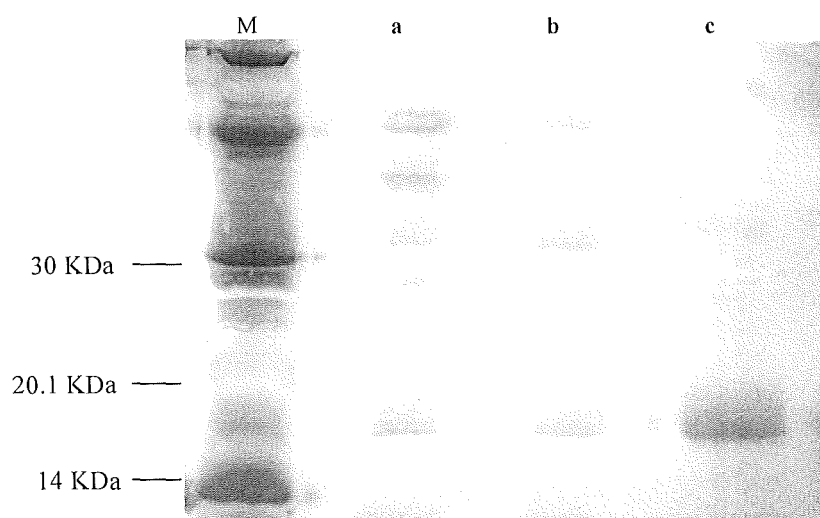
Biotinylated protein, after desalting, was run on a 12 % polyacrylamide gel (lane a) with a sample of the biotinylation reaction before purification (lane b) and purified, unmodified K99 (lane c). The gel was stained with Coomassie Brilliant Blue. M: Rainbow protein molecular weight markers (Pharmacia). The migration of standards of 14, 20.1 and 30 kDa is indicated.





**Figure 2.19 Western blotting of biotinylated K99**

Proteins from a 12 % polyacrylamide gel were transferred to nitrocellulose membrane by Western blotting. The membrane was then probed with Extravidin peroxidase. Lane a shows biotinylated K99 after desalting, lane b shows biotinylated K99 before desalting and lane c shows purified, non-biotinylated K99. M: Rainbow protein molecular weight markers (Pharmacia). The migration of standards of 14, 20.1 and 30 kDa is indicated.



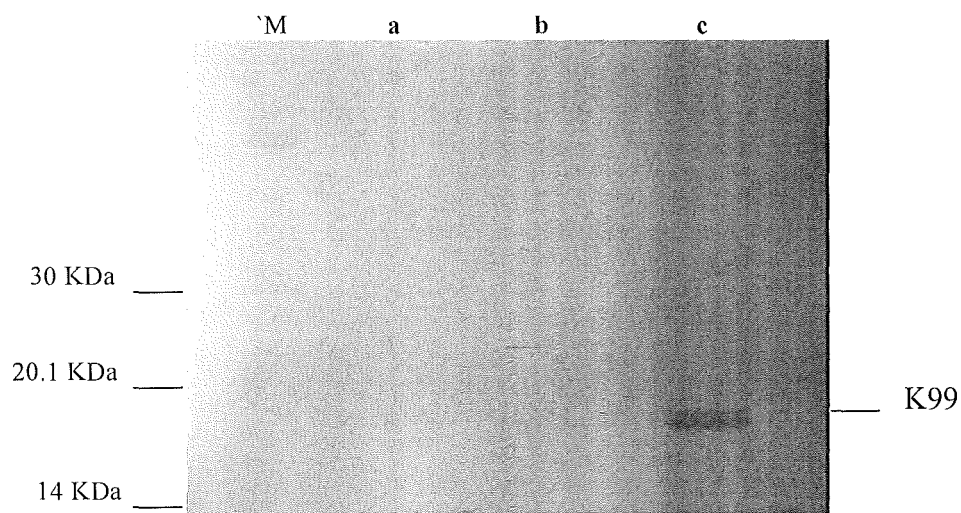
**Figure 2.20 SDS-PAGE analysis of immunoprecipitated K99**

After release of complexes of the antigen (biotinylated K99) and antibody (anti-K99) from Protein G agarose, the mixture was spun and the supernatant was run on an acrylamide gel (lane a). A sample taken before incubation with Protein G agarose (lane b) and a sample of biotinylated K99 before addition of antibody (lane c) were used as controls. After separation, the gel was stained by silver staining. M: Rainbow protein molecular weight markers (Pharmacia). The migration of standards of 14, 20.1 and 30 kDa is indicated.

These tentative findings were strengthened when proteins were transferred from the gel to nitrocellulose and probed with Extavidin peroxidase (Figure 2.21). The efficiency of detection was demonstrated by the strength of signal from a sample of biotinylated K99 alone (lane c). In lane b (mixture of K99 and anti-K99 monoclonal antibody) and lane a (complexes captured to Protein G agarose), the presence of K99 was detectable if faint. Over all, the Figure indicates that the purified protein isolated by ion-exchange chromatography and then biotinylated was successfully recognised by monoclonal anti-K99 antibody.

### ***Haemagglutination assay***

The ability of purified K99 major subunit (Fan C) to haemagglutinate sheep RBC was tested in parallel with B41 bacterial cell. The results showed that the purified K99 can haemagglutinate sheep red blood cells at high dilutions (1 in 32).



**Figure 2.21 Western blotting of immunoprecipitated K99**

SDS-PAGE acrylamide gel to nitrocellulose membrane and probed with Extravidin peroxidase to detect biotinylated material. Lane a: immune complexes release from Protein G agarose by boiling in SDS amples buffer. Lane b: antigen-antibody mixture prior to capture to Protein G agarose. Lane c: sample of biotinylated K99 alone.

M: Rainbow protein molecular weight markers (Pharmacia). The migration of standards of 14, 20.1 and 30 kDa is indicated.

## Discussion

K99 fimbriae are expressed by strains of enterotoxigenic *Escherichia coli* such as B41 strain that cause diarrhoea in calves. The aims of this part of the project were to isolate K99 fimbriae from B41, to purify the major subunit (FanC) and to check the biological activity of the purified protein so that it could be later used as a target for isolation of recombinant antibodies from phage display libraries.

Initial work focussed upon characterisation of *E. coli* B41, specifically to confirm the presence of genes encoding the K99 and F41 fimbriae using PCR. The K99 operon is carried upon a 7.1 kb region of a plasmid of 87.8 kb. Primers were designed using the published sequence of fanC, the forward primer corresponding to nt 21-40 of the coding sequence and the reverse primer annealing to nt 311-334 (Roosendaal *et al.*, 1984). Hence the predicted size of the product was 314 bp. In contrast, the F41 operon is somewhat larger (10.1 kb) and is located on the bacterial chromosome. Again, primers were designed from published sequences corresponding to nt 34-51 (forward) and 390-413 (reverse) of the gene for the fimbrial subunit (Fidock *et al.*, 1989). It was predicted that the amplified product would be 380 bp in size. Successful amplification of products of the expected size from *E. coli* B41 provided preliminary indication of the presence of K99 and F41 genes although this characterisation was not taken on to sequencing to formally confirm this. This approach was however shown to be specific and sensitive and of potential value in the identification of *E. coli* strains capable of causing diarrhoea in calves (Franck *et al.*, 1998).

The bacteria grew well in Minca medium and the expression of K99 fimbriae could be detected using the fimbrex test which is based upon agglutination of latex beads coated with anti-K99 antibody. The test could also be used to demonstrate that thermal shock of bacteria in PU buffer detached the fimbriae. The presence of K99 in the extracts was confirmed with ELISA using polyclonal and monoclonal anti-K99 antibodies. Other authors such as Vasquez (Vasquez *et al.*, 1996) and de Graaf (de Graaf *et al.*, 1981) have also used buffers containing urea for this purpose. In the method described by Vasquez, the final stage of isolation involved dialysis of the fimbrial extract into distilled water. When attempted in this project, overnight dialysing against distilled water at 4 °C led to the formation of dense aggregates. Similar results have been reported previously by Karkhanis and Bhogal (Karkhanis and Bhogal, 1986) and de Graaf (de Graaf *et al.*, 1981). To overcome this problem, the fimbriae could be kept in soluble form by dialysis against PBS. This made possible a thorough characterisation of the isolated K99.

As part of this characterisation, SDS-PAGE was used to reveal the protein composition of fimbrial extracts. Several protein bands could be observed. The strongest band on the gel was of 16.5 kDa, corresponding to the size of the major subunit of K99 (Yoshimatsu *et al.*, 1991; Nagy and Fekete, 1999). A protein band of 30.9 kDa also was seen on the gel. This agrees with the size of the F41 subunit as reported by several different authors (To, 1984; Jacobs, 1985; Raybould *et al.*, 1987; Vazquez *et al.*, 1996). Other bands might have been contaminating components from the bacterial outer membrane given the ability of urea to solubilise mildly hydrophobic proteins.

This preliminary identification of the 16.5 kDa structure was supported by its reaction on blots with a polyclonal antibody raised against K99. However, other protein bands were also detected in these experiments. Since detailed information was unavailable on the preparation of this polyclonal antibody, various explanations are possible. Binding to proteins other than the 16.5 kDa target might be the result of immunological cross-reactivity amongst the proteins on the blot. It is also possible that antigen used to develop the antibody in sheep was not completely pure and hence a proportion of the antibodies present in the serum bound to other proteins isolated from *E. coli* B41. In order to carry out these experiments, technical problems were encountered in transferring the 16.5 kDa protein band from the SDS gel to the nitrocellulose membrane using electroblotting. For this reason, diffusion blotting was more commonly applied: this was found to be more efficient than electroblotting. Although diffusion blotting has been described for a range of applications by other authors (Brian Bowen, 1980; Heukeshoven and Dernick, 1995; Olsen and Wiker, 1998; Chen and Chang, 2001), electrotransfer remains by far the most common method used for the preparation of Western blots. Its failure in this instance might have been attributable to the composition of the transfer buffer. Typically, these are prepared with Tris glycine buffers which have a pH close to the pI of FanC. It has been noted by others that the conditions of electrophoretic transfer can have a significant effect on the transfer of some outer membrane proteins in Western blotting (Davies *et al.*, 1990). Although transfer of the 16.5 kDa protein band to nitrocellulose was much better by diffusion blotting than by electroblotting, some other proteins present on SDS gels did not transfer well by diffusion blotting. This indicates that to some extent, the choice of transfer method and its conditions may depend upon the properties of the protein of interest. This contrasts with the findings of Olsen and Wiker (Olsen and Wiker, 1998) who reported that in diffusion blotting, the same transfer rate could be determined for proteins of different sizes.

Given the presence of other proteins in the bacterial extracts, methods were sought to purify the major subunit of K99 as a preliminary for its use in phage display. Several strategies and methods have been attempted to purify K99 fimbriae. One of the oldest methods was described by Brinton (Brinton, 1965) who devised a series of steps involving mild blending of the bacterial suspension, precipitation at pH 3.9 and aggregation with  $\text{MgCl}_2$ . In contrast, Isaacson (Isaacson, 1977) attempted to purify this protein, after ammonium sulphate precipitation, by an extended series of chromatography steps including ion-exchange and gel filtration chromatography coupled with isoelectric focusing and ultracentrifugation on sucrose density gradients. The method of de Graaf (de Graaf *et al.*, 1981) was less complex. A concentrated preparation of K99 was fractionated on a Sepharose CL-4B column then dialysed against phosphate buffer followed by sodium deoxycholate over a period of more than 5 days. Altmann (Altmann *et al.*, 1983) extracted fimbriae from bacteria using 3 M potassium thiocyanate then fractionated the material by gel filtration and ion-exchange chromatography. All these methods are technically complex and time consuming and were rejected for these reasons when seeking a method to use in this project. One less complex method for the isolation of K99 fimbriae from *E. coli* B44 was described by Karkhanis and Bhogal (Karkhanis and Bhogal, 1986). These authors employed heat shock treatment to extract fimbriae from the bacterial surface. The extracted material was aggregated by incubation at 4 °C for 16 hrs and then centrifuged at 39000g. The pellet was washed two times with phosphate buffer or 4M urea and chromatographed on a Sepharose CL-4B column. Another simple method published by Kuzuya (Kuzuya *et al.*, 1988) purified K99 protein using affinity chromatography. Yoshimatsu (Yoshimatsu *et al.*, 1991) described two purification methods. In both, heat treatment was first used to release fimbriae for purification on a Sepharose 4B gel filtration column. In one method, K99 proteins were separated from F41 fimbrial components by salting-out. In the second method, ultracentrifugation for 4 hrs achieved better objective.

The goal for this project was to find a fast, convenient and inexpensive method for purification of K99 to provide protein of high purity and good biological activity. Attempts were made to purify K99 on a range of ion-exchange chromatography and gel filtration media similar to some of those described in the reports outlined above. These preliminary efforts were not successful. Two problems were encountered: solubility and binding to the columns under test. It was found that if the fimbrial extract was dialysed against distilled water, most proteins aggregated, rendering the material unsuitable for column chromatography. If the fimbrial extract was dialysed against PBS or phosphate buffer, the properties of these buffers inhibited full binding of the K99 major subunit (16.5 kDa) to ion-exchange media. To overcome these problems, the precipitated fimbrial extract was

redissolved in a warm buffer containing 2 M urea immediately before application to the chromatography column. Thus the fimbriae extract was dissolved almost completely. To ensure optimal binding to the column, the composition of this buffer was changed to avoid pH values close to the pI of the major subunit of K99. This achieved high yields of material of good purity. The biological activity of this protein was confirmed by several tests. It had similar reaction in Fimbrex tests as the original fimbrial extract from which it was derived, it had high activity in ELISA using anti-K99 monoclonal antibody, and could be recognised in dot blotting and immunoprecipitation assays. Most importantly, the protein showed a high agglutinating activity with sheep red blood cells.

Comparison of this new purification method with other reported protocols reveals several significant advantages. The K99 major subunit can be purified in a single chromatography step whereas most other published methods involve at least two steps. The aggregation of the protein during extraction and purification can be easily controlled. In the method described by Karkhanis and Bhogal (Karkhanis and Bhogal, 1986) for *E. coli* B44, the protein initially aggregates and then dissolves. The SDS-PAGE analysis of protein reported here showed a single band at the expected molecular weight of 16.5 kDa whereas in some methods, particularly those from the older literature, different molecular weight for the K99 subunit were reported. The materials used during purification by the method described here are cheap and available to many labs, in contrast to the requirements stipulated by Altmann *et al.* (Altmann *et al.*, 1982), Isaacson (Isaacson, 1977) and Kuzuya (Kuzuya *et al.*, 1988). As described in this chapter, the purified K99 protein was exchanged into PBS. It remained soluble and stable in this buffer for several years when stored at  $-20^{\circ}\text{C}$ , but this appeared equally true of the protein when eluted in 25mM Tris base pH 7.0 containing 80 mM NaCl. Significantly, the biological activity of the purified protein was also excellent. In an effort to ensure this, the method reported here excluded the use of strong detergents or denaturants such as SDS, guanidine hydrochloride or high concentrations of urea. Surprisingly, except one investigator (Yoshimatsu *et al.*, 1991), not all authors who have reported K99 purification methods appear to have tested biological activity and in one example, when haemagglutination was tested, it was negative (Altmann *et al.*, 1982). Overall, the average yield of pure K99 antigen from a one-litre culture of *E. coli* B41 was about 3.5 mg using the method reported here, a little higher than that reported by de Graaf (de Graaf *et al.*, 1981). Together, the simplicity, inexpense, yield, purity and stability of K99 purified by this method make it suitable for many purposes including vaccination. As a first step, this could be tested *in vivo* in the infant mouse diarrhoea model of K99 ETEC infection (Duchet-Suchaux, 1988).



Analysis of purified K99 in this study revealed that heating appeared to destroy the epitope that is recognized by a monoclonal anti-K99 antibody. The heat sensitivity of this antigen has not been reported in the wider literature. The observation might therefore simply reflect the specificity of the monoclonal anti-K99 antibody available for this study. It does however raise the opportunity of further investigations to find monoclonal antibodies that recognise linear epitopes of native or recombinant (Jay *et al.*, 2004) K99.

One report in the literature has suggested a possible role for LPS in the formation of K99 fimbriae (Pilipcinec *et al.*, 1994). In this report, isolated K99 fimbriae were subjected to polyacrylamide gel electrophoresis and then tested in Western blotting with LPS antiserum. It was observed that the K99 major subunit reacted specifically in this analysis suggesting covalent association of LPS with the purified protein. In contrast, it has been reported that purified K99 from *E. coli* strains C1443 and B44 strains was not contaminated with LPS after purification (Altmann *et al.*, 1983; Karkhanis and Bhogal, 1986). This may depend to some extent on the purification method employed. No experiments were done in the present study to test for level of LPS associated with the purified K99 but in future, this could be addressed using commercial endotoxin detection reagents based upon the limulus amoebocyte lysis test. Clearly, this would be necessary before any attempts to assess vaccine potential *in vivo*.

The K99 purified in this study might find other uses. It has been reported that the protein can be used in inhibition assays to detect the presence of specific fimbrial receptors on enterocytes (Isaacson *et al.*, 1981; Dean and Isaacson, 1982). FanC has been cloned in combination with other genes of the K99 operon or as a single or truncated unit (van Embden *et al.*, 1980; Ogunniyi *et al.*, 2002). From natural or recombinant sources, it is possible that purified K99 could be used to block the binding of ETEC bacteria to receptors in an *in vitro* model (Jay *et al.*, 2004) and to block bacterial attachment to enterocytes *in vivo* as a disease prevention strategy based upon competitive inhibition.

Overall, the work reported in this chapter fulfills the initial aim to prepare a pure and biologically active antigen for the isolation of recombinant anti-K99 antibodies. In meeting this goal, an improved method for the purification of the major subunit of K99 has been devised that has potential in several areas of application.

## **Chapter 3**

### **Griffin.1 scFv library**

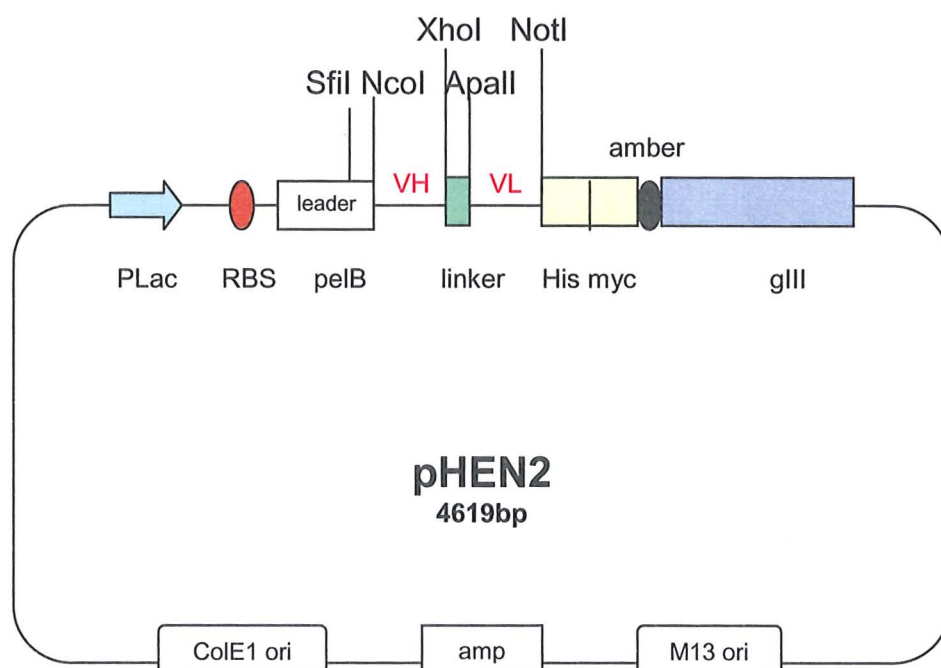
### 3. Griffin.1 scFv library

#### Introduction

The Griffin.1 library is one of the largest synthetic human single chain libraries distributed by the Medical Research Council Centre for Protein Engineering. The library is composed of human scFvs containing highly diverse VH and VL CDR3 sequences, prepared from human synthetic Fab lox library vectors (Griffiths *et al.*, 1994) and then cloned into the phagemid vector pHEN2 (Figure 3.1) (Hoogenboom *et al.*, 1991). The resulting library, distributed in *E. coli* TG1, contains more than  $10^9$  different clones. The kappa and lambda light chain variable regions were PCR amplified from the fdDOG-2loxVk and VL constructs. The PCR fragments were purified and digested with *Apa*II and *Not*I. The gel purified fragments were then ligated into pHEN2. Heavy chain variable regions were PCR amplified from the pUC19-2loxVH vector. The products were purified and digested with *Sfi*I or *Nco*I and *Xho*I. The gel purified fragments were then ligated into Vk-pHEN2 or VL-pHEN2. As cloned, the VH and VL fragments are attached to one another by a flexible Glycine-Serine linker. This vector also contains a c-myc tag that can be used for detection of the expressed antibody fragments. It also provides a HIS6 tag, allowing purification of scFv antibodies using nickel affinity columns (Figure 3.2).

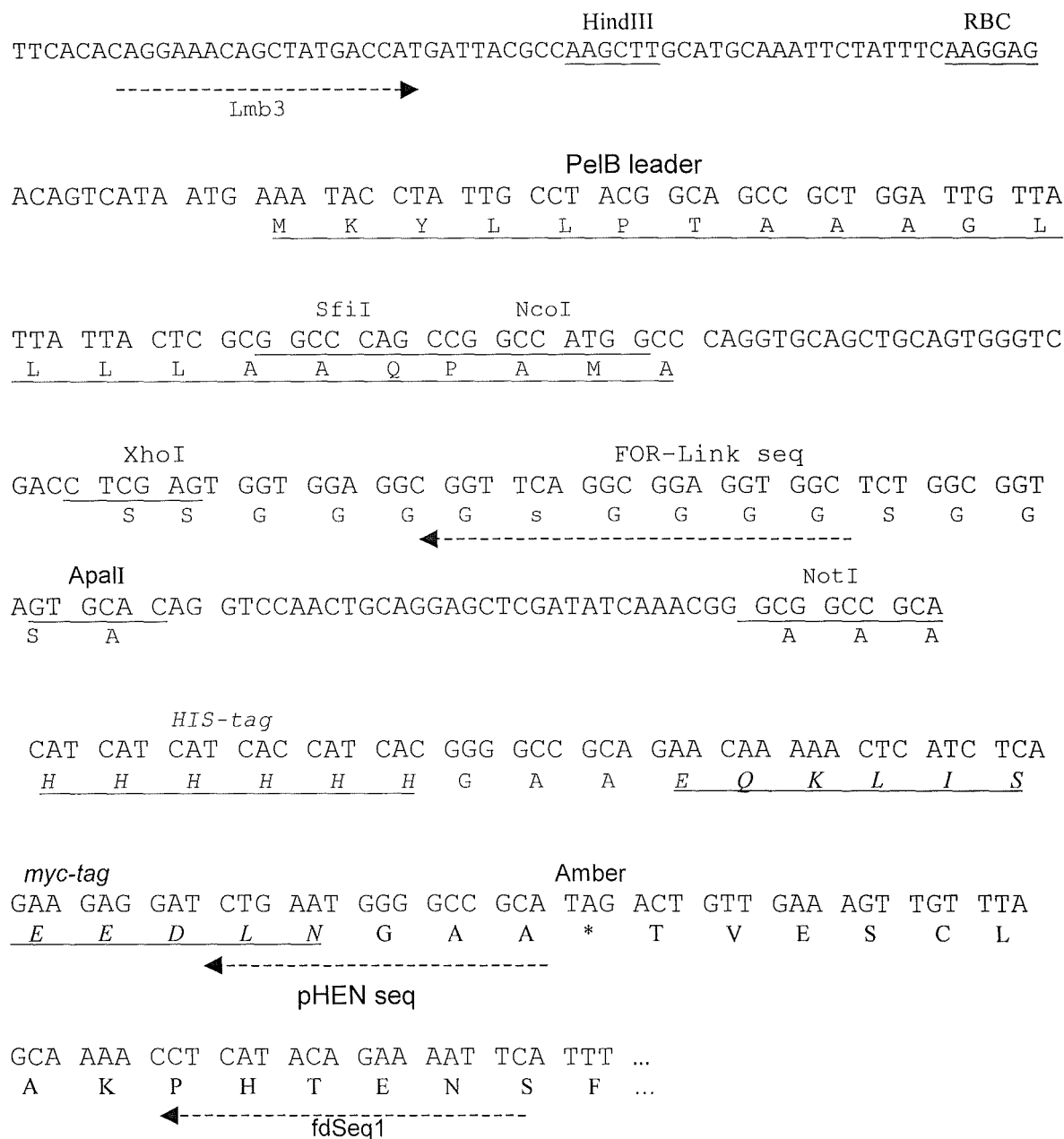
The VH and VL lox libraries form the foundation for the Griffin.1 library. The repertoire of heavy chains ( $>10^8$  different clones) was built from 49 cloned  $V_H$  segments (Tomlinson *et al.*, 1992), with CDR3 loops of 4 to 12 residues of random sequence. The repertoire of  $\kappa$  light chains ( $9 \times 10^4$  clones) was built from 26 cloned  $V_\kappa$  segments (Cox *et al.*, 1994) with CDR3 loops of 8 to 10 residues that included one, two or three residues of random sequence in all cases. The repertoire of  $\lambda$  light chains ( $7.4 \times 10^5$  clones) was built from 21 cloned  $V_\lambda$  segments (Williams and Winter, 1993), with CDR3 loops of 8 to 13 residues that included zero, one, two, three, four or five residues of random sequence.

The Griffin.1 library can be produced to select specific binders to target molecules that are attached to the surface of a tube or biotinylated and captured by streptavidin coated beads. By panning the library for two or three rounds, high affinity and specific phage antibodies can be selected. The monoclonal scFvs can then be screened for binding and used for further analysis of the target molecules.



**Figure 3.1 Structure of the pHEN2phagemid.**

The main features of the vector are indicated. Transcription takes place from a *lac* promoter and in a suppressor strain of *E. coli*, the translated product comprises a PelB leader, the single-chain antibody created by insertion of VH and VL sequences at the sites indicated separated by a linker, peptide tags, and the phage pIII protein.



**Figure 3.2 DNA sequence of pHEN2 vector**

The Figure presents a part of DNA sequence of pHEN2 vector. The restriction enzyme sites are underlined. The whole VH sequence locates between *NcoI* and *XhoI* and the whole VL sequence locates between *ApaI* and *NotI*. Primers are shown by dotted arrows. The amino acid sequences of HIS and myc tags and PelB leader are underlined.

## **VCS M13 helper phage**

In scFv libraries such as Griffin.1, the encoded antibody is genetically fused to the phage coat protein, pIII, in a phagemid vector. These are plasmids containing a phage origin of replication and morphogenetic signal, both required for packaging of the phagemid into phage particles. The pIII protein, present in three to five copies per phage particle, consists of N-terminal N1 and N2 domains involved in phage infectivity and a C-terminal domain essential for phage assembly. Importantly, fusion of scFv to the N-terminus of N1 does not abolish functional activity of the pIII protein. Transformation of a phagemid library into bacteria enables propagation of the DNA but for selection, phage assembly is required. Since the phagemid carries insufficient information to achieve this, bacteria carrying the phagemid need to be infected by helper phage such as VCSM13 that provides all the genes required. When the extruded phage particle is packaged with DNA, it is the scFv-encoding phagemid that enters the capsid with greatest frequency, and viral assembly is completed by capping with a variable proportion of wild-type and scFv-linked pIII molecules translated from helper phage and phagemid copies of the gene respectively. This physical association of the scFv protein at the viral surface and the encoding gene carried on the phagemid within the capsid enables selection of antigen-specific phages from libraries by incubation with the antigen, removal of non-bound phages and elution of bound phages. Phage carrying irrelevant scFv or entirely wild-type copies of pIII are lost in the selection process. Phage that successfully attach to the target are usually recovered by incubation at low or high pH, conditions that do not impair the ability of the N1 and N2 domains of pIII to interact with the bacterial receptor (the F pilus) and thereby mediate infection. Since the phagemid encodes ampicillin resistance, successfully infected bacteria can be isolated on antibiotic-containing media, a step that eliminates any virus carrying helper phage DNA. Culture of bacteria that now harbour phagemids emerging from selection is possible and a further round of superinfection with helper phage prepares stocks for further enrichment.

## **Application of Griffin.1 library**

The Griffin.1 scFv library has the potential to carry high affinity antibodies against any target antigen including foreign antigens, self antigens, non-immunogenic antigens and toxic antigens (Winter *et al.*, 1994). The isolated antibodies can be used for many purposes such as research, diagnostic and therapeutics applications. Some examples follow of the previous use of this library for isolation of scFv antibodies by phage display technology.

## **Antibodies against leukemic cells**

To select phage antibodies with therapeutic potential, the Griffin.1 library was panned on a premyelocytic leukemia cell line (HL60). Phage that bound to targets shared with non-leukemic cells were subtracted by incubating the library with human glioma cells. Phage carrying high affinity scFvs to HL60 cells were enriched by fluorescence-activated cell sorting. The selection procedure was repeated for six rounds to show significant binding to HL60 cells. Also, the inhibitory effect of isolated antibodies on HL60 cell proliferation was tested (Shadidi and Sioud, 2001).

## ***Streptococcus suis***

The Griffin.1 antibody phage display library was used to select recombinant antibodies directed against surface components of a pathogenic strain of *Streptococcus suis* serotype 2 and against extracellular factor (EF), a protein known to be exclusively associated with pathogenic *S. suis* serotype 2 strains. The investigators applied a subtractive selection procedure, to isolate antibody fragments that recognized epitopes specific for a pathogenic *S. suis* serotype 2 strain, compared to a non-pathogenic serotype 2 strain (de Greeff *et al.*, 2000). After 5 and 6 rounds of selection, three phage antibodies from each selection were chosen for further analysis.

## **Anti-hapten antibodies**

The hapten target investigated in this study was microcystin LR (1000 Da), a hepatotoxin produced by certain strains of cyanobacteria (Codd *et al.*, 1997). To isolate antibodies against this hapten, microcystin LR was conjugated to either bovine serum albumin or keyhole limpet haemocyanin. The Griffin.1 library and Tomlinson library I were used for screening. Three rounds of panning were performed on antigen-coated immunotubes. After screening, a selection of scFv antibodies that showed reaction with free microcystin were recloned into pIMS147 vector for expression of soluble scFv fragments. Finally, the activity of the purified antibodies isolated from each library were compared by a competition ELISA test (Strachan *et al.*, 2002).

## **Antibodies against an N-glycosylation site**

To identify antibodies that recognize the Asn-X-Ser/Thr N-glycosylation site that guides oligosaccharyltransferase activity, the Griffin.1 library was used to select antibodies

against synthetic Asn–Cys–Ser/Thr tripeptides conjugated to bovine serum albumin. The selection was performed over several rounds and soluble scFv fragments were expressed in *E. coli* HB2151 for purification on an immobilised nickel column. The interaction between the isolated scFv and Asn–X–Ser/Thr was characterized (Kikuchi *et al.*, 2004).

### **Sickle cell disease**

Sickle red blood cells have enhanced adhesion to the plasma and extracellular matrix protein thrombospondin-1 (TSP) *in vitro*. In this study, the authors aimed to isolate antibodies from the Griffin.1 library that bind TSP. Following three rounds of selection, six unique scFvs that bound to purified TSP were isolated. Using an *in vitro* flow adhesion assay, the isolated scFvs were assessed for their inhibition of the adhesion of sickle red cells to immobilized TSP. Antibodies that had an inhibitory effect were used in epitope mapping studies to identify the binding site for sickle cells (Watkins *et al.*, 2003).

### **Protein disulphide-isomerase (PDI)**

The Griffin.1 phage display library was used in this study to isolate antibodies that cross-react with all proteins in the PDI family through recognition of the motif Cys–X–X–Cys. After selection, the binding of scFvs to synthetic peptides and to mutant members of the PDI family was assessed by surface plasmon resonance. Clones that recognized sequences containing the CGHC or CGHCK motif were identified (Kimura *et al.*, 2004).

### **Objectives**

The main objective of this part of the project was to use human semi-synthetic Griffin.1 library for the isolation of recombinant single-chain antibodies against purified K99 major subunit (Fan C). It was planned to characterise the isolated antibodies and then assess their biological activity to determine whether they have the ability to neutralise K99 function.



## Materials and Methods

### **Griffin1. library**

The Griffin.1 library was obtained from the Centre for Protein Engineering (CPE), Cambridge (<http://www.mrc-cpe.cam.ac.uk/phage/g1mtaf.html>) and used to select phage antibodies. Griffin.1 is a semi-synthetic phage library containing more than  $10^9$  different scFv antibodies and was constructed by re-coloning the  $V_H$  and  $V_L$  variable regions from lox library vectors into the phagemid vector pHEN2 (Tomlinson *et al.*, 1992; Griffiths *et al.*, 1994; Nissim *et al.*, 1994; Winter *et al.*, 1994).

The materials supplied from the CPE comprised a tube of the semi-synthetic library in *E. coli* TG1 cells, a glycerol stock of the positive control (TG1 containing an anti-thyroglobulin clone), a glycerol stock of the negative control (TG1 containing the empty pHEN2 vector), a glycerol stock of T-phage resistant *E. coli* TG1 for propagation of phage (K12  $\Delta(lac-proAB)$  *supE thi hsdD5/F' traD36 proA<sup>+</sup>B lacIq lacZAM15*), and a glycerol stock of *E. coli* HB2151 for expression of antibody fragments (K12 *ara*  $\Delta(lac-proAB)$  *thi/F' proA<sup>+</sup>B lacIq lacZAM15*).

The helper phage VCS-M13 (Stratagene, La Jolla, California) was provided by Dr. P. Ogston, University of Glasgow. The titre of helper phage was  $2.2 \times 10^{14}$  pfu/ml.

### **Growing a secondary stock of library**

To make a secondary stock,  $1 \times 10^{10}$  clones of the Griffin.1 library in *E. coli* TG1 were inoculated into 100 ml of 2xTY (Tryptone 16g, Yeast Extract 10g, NaCl 5g and distilled water up to 1 litre) containing 100  $\mu$ g/ml ampicillin and 1% glucose and grown with shaking at 37 °C until the OD at 600 nm was approximately 0.5. At this OD, 10 ml of the library culture was used for phage rescue. The remaining culture (90 ml) was allowed to continue growing for a further two hours and the cells were then collected by centrifugation at 3300g for 10 minutes. The pellet was resuspended in 4 ml of fresh 2xTY media and after addition of glycerol to a final concentration of 15 %, it was stored at -70 °C.

## ***Phage rescue of the library***

To rescue the library, ten millilitres of library culture at an OD 600 nm of about 0.5, was infected with VCS-M13 helper phage, added in the ratio of bacteria to phage of 1 in 20. Appropriate volumes were calculated from the titre of helper phage and the fact that a bacterial culture of 1 OD unit at 600 nm represents a bacterial density of around  $8 \times 10^8$  bacteria per ml. The mixture of phage and bacteria was incubated for 30 minutes at 37°C in a water bath. The infected cells were then collected by centrifugation (10 min at 3,300 g) and resuspended in 2 ml of 2× TY broth containing 100 µg /ml ampicillin and 25 µg/ml kanamycin (Sigma). Subsequently, the cells were incubated overnight at 30°C and with shaking at 200 rpm. Bacterial cells were removed by centrifugation (30 min at 3,300 g), and phage presented in the supernatant (8 ml) were precipitated with 1.6 ml of polyethylene glycol-NaCl (20% polyethylene glycol 6000, 2.5 M NaCl). The mixture was left for 1 hour at 4 °C and was spun at 3300g for 30 minutes. The pellet was then resuspended in 4 ml of PBS and spun at 11600g for 10 minutes to remove most of the remaining bacterial debris. The phage supernatant was stored at 4 °C and for long term storage, after addition of glycerol (15 % final concentration), at -70 °C.

## ***Titration of phage library***

To titre the phage stock, 1 µl of phage was diluted into 1 ml of PBS and 1 µl of this was used to infect 1 ml of TG1 at an OD 600 nm about 0.5. The culture was incubated at 37 °C water bath for 30 minutes. 50 µl of this, 50 µl of a 1:10<sup>2</sup> dilution and 50 µl of 1:10<sup>4</sup> were plated on TYE (Tryptone 10g, Yeast Extract 5g, NaCl 8g, Agar 15g and distilled water up to 1 litre) plates containing 100 µg/ml ampicillin and 1% glucose. The plates were incubated at 37 °C overnight. The following day, the number of colonies on each plate was counted and titre of phage was determined.

## ***PCR screening for full-length scFv genes***

To estimate the frequency of full-length inserts in the secondary stock of the library, 20 colonies were picked at random from TYE plates used for library titration. Bacterial DNA was obtained by suspending each colony in 50 µl of H<sub>2</sub>O and boiling at 100 °C for 10 minutes. Primers LMB3 and gIII were used to amplify across the inserts in 25-µl PCR reactions containing 0.5 µM concentrations of each primer, 0.25 mM concentrations of each dNTP (Promega), 4 mM MgCl<sub>2</sub>, 1x reaction buffer (containing 50 mM KCL, 10 mM

Tris-HCL pH 9.0 and 0.1% Triton<sup>®</sup>X-100), 2.5 units of Taq DNA polymerase (Promega) and 19 µl of bacterial lysate. Samples were amplified on a Techne thermal cycler under the following conditions: the reaction was heated to 94 °C for 3 min and then 30 cycles of 30 second denaturation at 94 °C, 1 min annealing at 55 °C, 1 min extension at 72 °C. Final extension was for 10 min at 72 °C. As a positive control, an anti-thyroglobulin scFv in the pHEN2 vector was used for amplified since this carries a full-length scFv insert.

PCR products were analysed on a 1% agarose gel for 45 min at 100 V, stained with ethidium bromide, and photographed under UV light.

### ***Restriction analysis of plasmids from secondary stock of library***

To confirm the presence of full-length inserts, plasmid DNA was isolated from 12 clones picked at random and digested with two restriction enzymes. Plasmids were isolated and purified using Qiagen miniprep reagents following the instruction provided with the kit. 20 µl reaction mixtures were set up for each sample comprising 5 µl of plasmid DNA, 1 µl (10U) of *NcoI* (Promega, UK), 1µl (10U) of *NotI* (Promega, UK), 1.8 µl of distilled water, 0.2 µl of BSA and 2 µl of buffer D (60 mM Tris-HCL (pH 7.9), 1.5 M NaCl, 60 mM MgCl<sub>2</sub> and 10 mM DTT). The reaction mixtures were incubated at 37 °C for 1.5 hrs. 5 µl of the digestion products were analysed on a 1% agarose gel for 45 min at 100 V, stained with ethidium bromide, and photographed under UV light.

## **Selection procedure**

### ***Panning against purified K99***

#### **First round of selection**

To select recombinant phage antibodies against purified K99, one immunotube (Nunc, Denmark) was coated overnight at room temperature with 4 ml of purified K99 at 90 mg/ml in PBS. The following day, the immunotube was washed 3 times with PBS by pouring into the tube and pouring out again immediately to remove unbound antigen. The immunotube was then blocked by filling with 4 ml of 2 % MPBS and incubating at 37°C for 2 hours. The immunotube was washed again three times with PBS. Then about  $1 \times 10^{13}$  pfu of phage from the Griffin.1 library was made up to a total volume of 4 ml with 2 % MPBS. To block phage that might be specific for milk constituents, the phage suspension

was incubated for about 30 minutes at room temperature before transfer to the K99-coated immunotube. After addition of the phage library, the tube was incubated on a turntable for 30 min at room temperature, followed by a standing incubation for 90 min at room temperature. The supernatant was discarded and the tube was washed 10 times with PBS containing 0.1 % Tween 20 and 10 times with PBS. After shaking out the excess PBS, phage were eluted by adding 1ml 100 mM triethylamine (700  $\mu$ l of triethylamine (7.18 M) in 50 ml water, diluted on the day of use) and rotating continuously for 20 minutes at room temperature (de Bruin *et al.*, 1999). The eluted phage suspension was neutralized with 0.5 ml of 1 M Tris-HCl (pH 7.4). A further 200  $\mu$ l of Tris buffer was added to the immunotube to neutralise the phage remaining in the tube. Elutes were stored at 4°C to use in the next round of selection and for titration.

### **Infecting TG1 with eluted phage antibodies**

The day before selection, one colony of *E. coli* TG1 was taken from a minimal medium plate and grown in 5 ml of 2xTY overnight at 37 °C with shaking at 200 rpm. On the day of selection, 50  $\mu$ l of this culture was inoculated to 50 ml of 2xTY and incubated at 37 °C with shaking until the culture entered exponential growth and the OD 600nm reached 0.5. At this point, 750  $\mu$ l of the eluted phage was added to 9.25 ml of exponentially growing TG1 cells. Four ml of bacterial culture was also added to the tubes used for selection in order to recover any phage remaining in the tube. Both mixtures were incubated at 37 °C in a water bath for 30 minutes to allow infection to take place. After incubation, the 10 ml and 4 ml cultures were pooled. 100  $\mu$ l of culture was used for titration of output phage. This was done by making 5, 100-fold serial dilutions of 100  $\mu$ l of culture in 2xTY media and plating all 100 $\mu$ l of each dilution on TYE plates containing 100  $\mu$ g/ml of ampicillin and 1 % glucose. The plates were then incubated at 37 °C overnight. The following day, the number of colonies on the plates was counted and from this, the titre of output phage was determined. The plates were then stored at 4 °C for analysis of those phage recovered from the first round of selection.

The remaining culture of infected *E. coli* TG1 was spun at 3300g for 10 minutes. The pellet was resuspended in 2 ml of 2xTY and was plated onto a large Nunc Bio-Assay dish (Gibco-BRL) containing TYE, 100  $\mu$ g/ml of ampicillin and 1 % glucose. The plate was incubated at 30 °C overnight. The following day, bacterial colonies were collected from plate by addition of 5 ml of 2xTY containing 15 % glycerol and cells were loosened into

suspension with a glass spreader. 50  $\mu$ l of the bacterial suspension was used for phage rescue for the second round of selection and the remainder was stored at  $-70^{\circ}\text{C}$ .

### **Rescue of selected phage antibodies**

To rescue the phage antibodies from clones selected at round 1, 50  $\mu$ l of bacteria recovered from the Nunc Bio-Assay dish were inoculated to 100 ml of 2xTY containing 100  $\mu\text{g/ml}$  ampicillin and 1% glucose, and were grown at  $37^{\circ}\text{C}$  with shaking at 200 rpm until OD 600 nm was approximately 0.5. Then 10 ml of bacterial culture was infected with VCS-M13 helper phage at a bacteria : phage ratio of 1 in 20 and the mixture incubated without shaking at  $37^{\circ}\text{C}$  in a water bath for 30 minutes. After that, the infected cells were centrifuged at 3300g for 10 minutes. The pelleted bacteria were then resuspended in 50 ml of 2xTY containing 100  $\mu\text{g/ml}$  ampicillin, 50  $\mu\text{g/ml}$  kanamycin and incubated with shaking at  $30^{\circ}\text{C}$  overnight. The following day, 40 ml of overnight cultures was spun at 3300g for 30 minutes.

To precipitate phage from the culture supernatants, 8 ml of cold PEG/NaCl (20% polyethylene glycol 6000, 2.5 M NaCl) was added to 40 ml of each supernatant. After mixing well, the mixtures were left for 1 hr on ice and then centrifuged at 3300g for 30 minutes. Next, the supernatants were poured away to remove PEG/NaCl and the precipitated phage were resuspended in 2 ml of PBS and spun at 11600g for 10 minutes in a micro centrifuge to remove remaining bacterial debris. 1ml of the supernatant containing the rescued phage was stored at  $4^{\circ}\text{C}$  and remainder was used for the next round of selection.

### **Titration of rescued phage**

To titre phage rescued from the first round of selection and thereby determine the phage input for the second round of selection, 50  $\mu$ l of rescued phage was diluted into 50  $\mu$ l PBS. 1  $\mu$ l of this and 1  $\mu$ l of 5, 100-fold serial dilutions were used to infect 1 ml of TG1 culture previously grown to an OD 600 nm of about 0.5. The cultures were then incubated at  $37^{\circ}\text{C}$  water bath for 30 minutes. 50  $\mu$ l of each dilution was spread onto a TYE plate containing 100  $\mu\text{g/ml}$  ampicillin and 1% glucose and grown overnight at  $30^{\circ}\text{C}$ . The next day, the number of colonies was counted and from this, the titre of phage was determined.

## Second round of selection

In the second round of selection, one immunotube (Nunc, Denmark) was coated overnight at room temperature with a lower concentration of purified K99 (30 µg/ml) to isolate phage antibodies with higher specificity and affinity. The following day the immunotube was washed and blocked as described for the first round of selection.  $3.2 \times 10^{13}$  pfu of PEG precipitated phage in 2% MPBS recovered from the first round of selection was added to the immunotube as input phage. The immunotube was then incubated for 30 minutes with rotation and for 90 minutes standing at room temperature and then was washed 20 times with PBS containing 0.1% Tween 20 and 20 times with PBS. The remaining procedures including elution of selected phage, infection of TG1 cells, superinfection with VCS-M13 helper phage, recovery of phage and titration were done as described for the first round.

## Third round and fourth round of selection

All procedures in the third round and fourth round of selections were done as for previous rounds. The coating of immunotubes with purified K99 and washing steps were carried out as for round two. The titre of input phage antibodies for the third round was  $2.6 \times 10^{14}$  and for the fourth round, it was  $5.3 \times 10^{14}$  pfu in 2% MPBS.

## Screening by monoclonal phage ELISA

The activity of 96 individual isolated clones from each round of selection against purified K99 was assessed by ELISA screening.

## Preparation monoclonal phage antibodies

From plates used to titre phage recovered after selection, 96 individual colonies were picked at random and inoculated into 100 µl of 2xTY containing 100 µg/ml ampicillin and 1% glucose. A separate microtitre plate (Iwaki, Japan) was prepared from each round of selection. The plates were incubated overnight at 37 °C with shaking at 200 rpm. The following day, using a multi-channel pipette, approximately 2 µl of overnight culture was transferred to further 96 well plates containing 200 µl of 2xTY, containing 100 µg/ml ampicillin and 1% glucose per well. Plates were incubated at 37 °C for 1.5 hrs to reach to an OD 600 nm of about 0.4. Then 25 µl of 2xTY containing 100 µg/ml ampicillin, 1% glucose and  $10^9$  pfu helper phage (final concentration) was added to each well for

superinfection. Plates were left to stand at 37 °C for 30 minutes and then shaken at 200 rpm for a further 1 hour at 37 °C. The plates were then centrifuged at 1800g for 10 minutes in a Sigma centrifuge fitted with appropriate carriers and the supernatants were aspirated off. Each pellet was resuspended in 200 µl of 2xTY containing 100 µg/ml ampicillin and 50 µg/ml kanamycin. Cells were grown overnight at 30 °C with shaking at 200 rpm to allow replication and rescue of the phage antibodies. The next day, plates were spun at 1800g for 10 minutes. Each supernatant was then recovered for use as monoclonal phage antibody in ELISA assay.

### **Antigen coating**

Wells of an ELISA plate (Nunc, Denmark) were coated with 100 µl per well of 20 µg/ml of purified K99 in PBS. Plates were left overnight at room temperature to allow the K99 antigen to bind to the plastic wells. Four plates were coated in all so that individual plated were available for screening phage from each round of selection. To screen clones from each round of selection for reaction against dry skimmed milk, wells of another four ELISA plates (one plate for each round) were coated with 100 µl of 2 % MPBS. These plates were also left overnight at room temperature.

### **Blocking**

The next day, unbound antigen was discarded and the wells were washed three times with PBS. The wells were then blocked by addition of 200 µl of 3% BSA/PBS and incubation for 2 hrs at 37 °C.

### **Addition of monoclonal phage antibodies**

After washing the wells three times with PBS, each culture supernatant containing phage antibodies was diluted 1/2 in 3% BSA/PBS. 100 µl of each was added to the wells of a plate coated with K99, and 100 µl was added to the wells of a plate coated with 2 % MPBS. Plates were left for 90 minutes at room temperature to allow phage attachment.

### **Detection of bound phage antibodies**

After washing three times with PBS containing 0.05% Tween 20 to remove unbound phage antibodies, 100 µl of 1:1000 dilution of monoclonal anti-M13-HRP antibody

(Amersham Pharmacia Biotech, UK) in 3% BSA/PBS was added to all wells. Plates were then incubated at room temperature for 90 minutes.

## **Development**

Wells were washed three times with PBS containing 0.05% Tween 20 and three times with PBS to remove unbound antibodies. 100  $\mu$ l of developer solution (composition as described previously) was added to each well. The colorimetric reactions were read at 450 nm in an ELISA reader.

## ***Restriction analysis of clones from round 4***

Thirteen clones showed higher than average activity against K99 antigen in monoclonal phage ELISA. These were taken for further analysis. Each was grown in 5 ml of 2xTY containing 100  $\mu$ g/ml ampicillin, 1% glucose, overnight at 37 °C and 200rpm shaking. The following day, plasmid DNA was isolated and purified using Qiagen miniprep reagents according the manufacturer's instructions. The purified DNA was digested with restriction enzymes of *Nco*I and *Not*I, as described previously for analysis of the secondary stock of the Griffin.1 library. 10  $\mu$ l of the digestion products were electrophoresed on a 1% agarose gel for 45 min at 100 V, stained with ethidium bromide, and photographed under UV light.

## ***Production of soluble scFv fragments***

Four clones from round four of selection that had full-length scFv inserts and one clone that had a smaller insert size, were taken forward to production of soluble scFv antibodies. Selection took place with the phagemids in *E. coli* TG1 cell because of its ability to suppress translational termination at the TAG codon lying between the scFv and gIII sequences. For scFv expression, the chosen phage were infected into the non-suppressing *E. coli* strain HB2151 (K12 *ara*  $\Delta$ (*lac-proAB*) *Thi/F'* *proA*<sup>+</sup>*B* *lacI* *lacZ*  $\Delta$ M15). To achieve this transfer, a colony (TG1) of each sample was picked from TYE plates and grown in 5 ml of 2xTY overnight at 37 °C with shaking at 200 rpm. The following day, 500  $\mu$ l of culture was added to 50 ml of 2xTY media and was grown at 37 °C with shaking until the OD at 600 nm was about 0.5. VCS-M13 helper phage was then added to the cultures at the appropriate ratio (see above). The cultures were then incubated at 30 °C and were then spun at 3300g for 10 minutes. The pellets were resuspended in 50 ml of 2xTY



containing 100 µg/ml ampicillin and 50 µg/ml kanamycin. Cells were grown overnight at 30 °C with shaking at 200 rpm to allow replication and rescue of the phage antibodies. The next day, the cultures were centrifuged at 3300g for 10 minutes. Samples from each supernatant (50 µl) were then used for infection of exponentially growing *E. coli* HB1251 cells (1 ml), prepared to an OD 600 nm of about 0.5. The cultures were incubated at 37 °C water bath for 30 minutes to allow for infection. Then 50 µl, 50 µl of a 1:10<sup>2</sup> dilution, 50 µl of a 1:10<sup>4</sup> dilution and 50 µl of a 1:10<sup>6</sup> dilution of infected bacteria were plated onto TYE plates containing 100 µg/ml ampicillin, 1% glucose. Plates were then incubated overnight at 30 °C. The following day, one colony of each sample was grown in 5 ml of 2xTY overnight at 37 °C with shaking at 200 rpm. 500 µl of overnight cultures were then were grown in 50 ml of 2xTY containing 100 µg/ml ampicillin and 0.1% glucose to an OD approximately 0.9 at 600 nm. At this point, IPTG (Isopropyl-β-D-thiogalactopyranoside) was added to each culture to a final concentration of 1mM of IPTG to induce expression. The cultures were incubated for a further 21 hrs at 30 °C with shaking to allow expression of soluble scFv antibodies.

### **SDS-PAGE analysis**

After growing selected clones for expression of soluble scFv fragments, the cultures were centrifuged at 5000g for 20 minutes. The culture supernatants were then filtered through a 0.45 µm filter and concentrated about 30-fold using an Amicon Ultra-4 centrifugal filter (Millipore, UK). Samples were boiled for 5 minutes in a reducing sample buffer for analysis on 12 % discontinuous SDS- polyacrylamide gels (Laemmli, 1970). After running the gels in a BioRad apparatus at 200 volts for 1 hr, one gel was stained with Coomassie brilliant blue stain and another gel was used for Western blotting.

### **Western blotting**

Proteins were transferred from the gel to nitrocellulose membrane using a standard protocol for blotting (Sambrook *et al.*, 1989). After transfer, the membrane was washed three times with PBS and was blocked with 3% MPBS for 1 hr at room temperature with shaking. The membrane was washed three times with PBS and was incubated in a 1:200 dilution of anti-c-myc polyclonal antibody (Sigma, UK) in 2 % MPBS for 1.5 hr at 37 °C with shaking at 200 rpm. After washing the membrane three times with PBS containing 0.05 % Tween 20 and three times with PBS, a goat anti-rabbit-HRP conjugate (Sigma, UK) was added at 1:1000 dilution in 2 % MPBS and incubated for 1.5 hr at 37 °C with

shaking. The membrane was washed as before and 4-chloro-1-naphthol substrate (Sigma, UK) added.

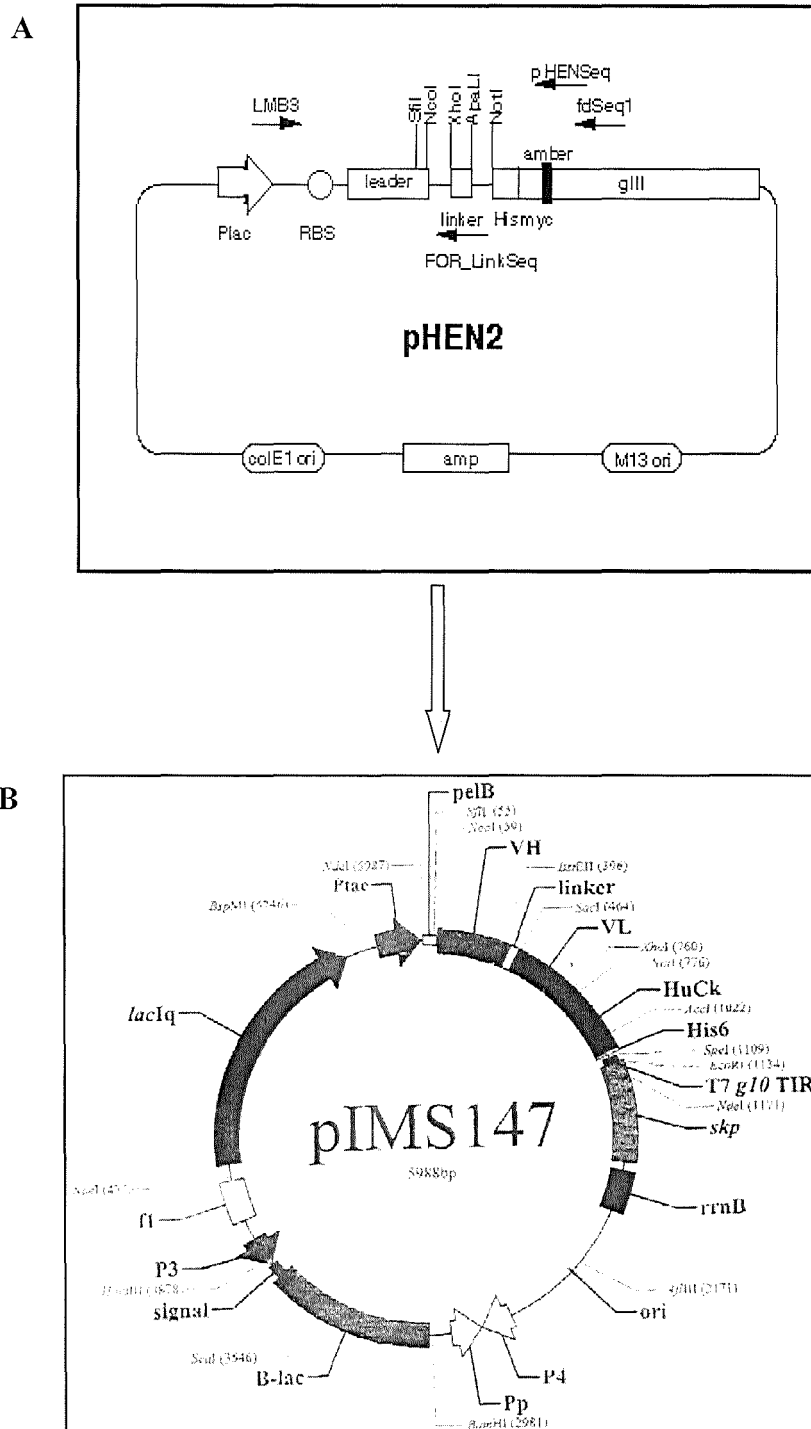
## ***Recloning into pIMS147***

In an attempt to increase the expression level of soluble scFv fragments and provide better detection, the scFv antibody sequences from a number of clones were transferred into pIMS147 (Figure 3.3) (Schafer *et al.*, 1999). This places expression of the construct under the control of the strong, synthetic *tac* promoter, retaining induction with IPTG. It also fuses the scFv gene with sequence encoding a human C  $\kappa$  domain to form a single chain antibody or scAb. This provides a larger target than c-myc for immunochemical detection. A hexahistidine tail lies at the carboxy-terminus of the translation product allowing for purification by nickel chelate affinity chromatography. Finally, the vector provides for co-expression of the Skp periplasmic chaperone to assist folding of the exported product. The antibody expression vector pIMS147 was maintained in *E. coli* XL-1 Blue (Stratagene) (Strachan *et al.*, 2002).

## ***Preparation of the scFv fragments and pIMS147 vector***

### **Digestion of selected clones**

Plasmid DNA was prepared from five clones chosen from round 4 of selection. DNA was isolated and purified using Qiagen miniprep reagents. Purified plasmids were then digested using the restriction enzymes *Nco*I and *Not*I to isolate the scFv inserts. 150  $\mu$ l reaction mixtures were set up for each sample comprising 120  $\mu$ l of plasmid DNA, 4  $\mu$ l (40U) of *Nco*I (Promega, UK), 4  $\mu$ l (40U) of *Not*I (Promega, UK), 55  $\mu$ l of distilled water, 1.5  $\mu$ l of BSA and 15  $\mu$ l of buffer D (60 mM Tris-HCL (pH 7.9), 1.5 M NaCl, 60 mM MgCl<sub>2</sub> and 10 mM DTT). The reaction mixtures were incubated at 37 °C for 1.5 hrs. Each entire reaction was run on a 1 % agarose gel at 100 volts for 45 minutes were stained with ethidium bromide. Then under UV light, the smaller bands were cut from the gel for DNA purification.



**Figure 3.3 pHEN2 and pIMS147 vectors**

A: Map and restriction sites of the pHEN2 phage display vector B: Map and restriction sites of pIMS147

## Digestion of pIMS147 vector

*E. coli* strain XL-1 Blue carrying pIMS147 was grown overnight in 25 ml of LB broth (Tryptone, 10 g; Yeast extract, 5 g; NaCl, 10 g; Distilled water up to 1 litre) containing 100 µg/ml ampicillin and 1% glucose at 37 °C and 200 rpm shaking. The following day, plasmid DNA was extracted and purified using Qiagen miniprep reagents. Purified plasmid DNA was then digested using the restriction enzymes *Nco*I and *Not*I to prepare the vector for ligation. Details of the reaction mixture, incubation time and extraction of vector fragment were as described above.

## Purification of inserts and vector

DNA was isolated from the gel slices using QIAQuick<sup>®</sup> Gel extraction reagents following the instructions provided. To increase the concentrations of DNA, elution from the filter units was with only 15 µl of distilled water. The concentrations of the purified fragments was estimated by running 5 µl samples of each on an agarose gel.

## Cloning of scFv antibody fragments into pIMS147

To ligate the scFv antibody fragments into pIMS147, 20 µl ligation reactions were set up containing 2 µl of vector, 5 µl of insert (this ensured approximately equal molar concentrations of vector and insert), 2 µl of T4 DNA ligase (Promega) at 3 units/µl and 2 µl of 10x buffer (300mM Tris-HCl pH7.8, 100mM MgCl<sub>2</sub>, 100mM DTT and 10mM ATP) and 9 µl of distilled water. Reactions were then incubated at 16 °C for about 18 hrs.

## Transformation of ligation products

*E. coli* XL-1-Blue super competent cells (Stratagene) were used for transformation. After thawing the cells on ice from -80 °C, 0.85 µl of 1.42 M β-mercaptoethanol provided by the manufacturer was added to each 50 µl aliquot of cells giving a final concentration of 25 mM. Cells were kept on ice for 10 minutes before adding 5 µl of each ligation reaction. They were then incubated on ice for a further 30 minutes. Transformation with 0.1 ng of pUC18 plasmid (provided with the cells) was used to estimate transformation efficiency. After 30 minutes incubation on ice, the cells were heat shocked by transferring to a water

bath at 42 °C for 45 second, then returned to ice for 2 minutes before addition of 950 µl of SOC medium (Tryptone 20 g; Yeast extract 5 g; NaCl 0.5 g; Distilled water up to 980 ml; after autoclaving, 10 ml of 1 M MgCl<sub>2</sub> and 10 ml of 1 M MgSO<sub>4</sub> added). The cultures were incubated at 37 °C with shaking for 1 hour and then 100 µl of each was plated on LA (Tryptone, 10 g ;Yeast extract, 5 g; NaCl, 10 g; Agar 6 g, Distilled water up to 1 litre) containing 100 µg/ml ampicillin and 1 % glucose. Plates were incubated at 37 °C overnight and next day were checked for the appearance of colonies.

To confirm that successful ligation had taken place, one colony was picked from each transformation plate and was grown in 5 ml of LB broth containing 100 µg/ml ampicillin and 1 % glucose for overnight at 37 °C and 200 rpm shaking. The following day, plasmid DNA was extracted and purified using Qiagen miniprep reagents. Purified plasmids were then digested using the restriction enzymes *Nco*I and *Not*I to detect the presence of inserts. The composition of reaction mixtures, incubation times and analysis were as described earlier.

To further confirm successful ligation, 5 µl of each recombinant plasmid was digested with *Eco*RI restriction enzyme to check the size of the linearised plasmid. The 10 µl reaction mixture contained 2 µl of *Eco*RI enzyme, 1 µl of 10x buffer, 1 µl of distilled water and 5 µl of plasmid DNA. The entire reaction was then run on 1 % agarose gel at 120 Volts for 45 minutes, stained with ethidium bromide and photographed under UV light.

## ***Characterisation of recloned antibodies***

### **Expression and isolation of soluble secreted scFv fragments**

After confirmation of the presence of scFv inserts, colonies to be characterised were grown in 5 ml of LB broth containing 100 µg/ml ampicillin and 1 % glucose at 37 °C and 200 rpm shaking overnight. 2 ml of each overnight culture was inoculated into 50 ml of LB broth containing 100 µg/ml ampicillin and 1 % glucose and grown on at 37 °C with shaking at 200 rpm until the OD at 600 nm was 0.8-0.9. At this point, IPTG was added to a final concentration of 1mM and growth continued overnight at the lower temperature of 30 °C with shaking at 200 rpm. The cultures were then centrifuged at 5000g for 20 minutes at 4 °C. The supernatants were collected and after filtration, were stored at 4 °C. Periplasmic extracts were prepared from the bacterial pellets as described by Kipriyanov (Kipriyanov, 2002)((O'Brien and Aitken, 2002) by gently resuspending to 5 % of the initial culture

volume in cold 50 mM Tris-HCl, 20 % sucrose, 1mM EDTA, pH 8.0. The cell suspensions were then incubated on ice for 60 minutes with occasional stirring and then were centrifuged in microfuge tubes at 25000g for 30 minutes at 4 °C. The supernatants - the soluble periplasmic extracts - were carefully collected and filtered through filters with a pore size of 0.2 micrometres. Samples were stored at 4 °C for future analysis.

### **Western blotting analysis**

The expression of scFv fragments was assessed in culture supernatants and periplasmic extracts by Western blotting. 20 µl of each culture supernatant and periplasmic extract and also a positive control scFv were run on a 12% SDS-acrylamide gel and transferred to a nitrocellulose membrane as described previously. After blocking with 3 % MPBS and washing three times with PBS, the membrane was incubated in a 1:500 dilution of goat anti-human-ck in 2% MPBS at 37 °C for 60 minutes with 200 rpm shaking. After that, the membrane was washed three times with PBS containing 0.05% Tween 20. The bound antibody was detected using rabbit anti-goat-HRP at a dilution of 1:1000 in 2 % MPBS. Finally, the reaction was developed using 4-chloro-N-naphthol substrate.

### **Screening by ELISA**

Supernatants from induced cultures of *E. coli* were applied to ELISA plates coated with K99 30 µg/ml or a goat anti-human kappa antibody at 1:500 dilutions. Next day after blocking with 3% BSA/PBS, binding of protein was detected by addition of a goat anti-human kappa HRP conjugate and OPD substrate. Colour change was recorded 450 nm.

### ***Soluble scFv-pIII fusion proteins***

Since the expression of soluble scFvs from selected clones in *E. coli* HB2151 was unsuccessful and recloning into pIMS147 vector did not solve the problem, expression of these antibodies as scFv-pIII fusion proteins as described by Mersmann M. *et al.* (Mersmann *et al.*, 1998) was attempted.

In this method, scFv constructs were translated as fusions to pIII by inducing expression in the suppressor strain *E. coli* TG1. This leads to accumulation of the scFv-pIII protein in the cytoplasmic membrane from which it can be released with detergents and detected with anti-pIII antibodies (Tesar *et al.*, 1995).

## ***Expression of scFv-pIII fusion proteins***

Single colonies of the clones for analysis were grown overnight in 5 ml of 2xTY containing 100 µg/ml ampicillin and 1 % glucose at 37 °C and 200 rpm shaking. Next day, 250 µl from each culture was added to 25 ml of fresh 2xTY media containing 100 µg/ml ampicillin and 1 % glucose. The cultures were grown at 37 °C with shaking until OD at 600 nm reached about 0.5. The cultures were then spun at 3300g for 10 minutes. The supernatants were discarded and the pellets were resuspended in 25 ml of fresh 2xTY containing 100 µg/ml ampicillin (without glucose) and 1 mM IPTG (Kipriyanov *et al.*, 1997). Then, cultures were incubated at 30 °C for 20 hours. To extract proteins from the cells, cultures were centrifuged at 5200g for 20 minutes and the pellets were resuspended in 1.6 ml of Bugbuster protein extraction reagent (Novagen, UK) containing 1.5 µl of RNAase. The suspensions were left shaking at 30 °C for 30 minutes and then were spun at 20,000g for 40 minutes at 4 °C. The supernatants predicted to contain the extracted scFv-pIII fusion proteins were collected and concentrated to 1/3 their initial volumes for use in ELISA and Western blotting.

## ***Detection scFv-pIII fusion proteins by Western blotting***

The concentrated Bugbuster extracts were run on 8 % SDS-polyacrylamide gels and transferred to a nitrocellulose membrane as described earlier. The membrane was blocked with 5 % MPBS at room temperature and after washing three times with PBS containing 0.05 % Tween 20, the membrane was incubated in a 1:1000 dilution of monoclonal anti-pIII antibody (Mo Bi Tec, Germany) in blocking buffer at 37 °C for 90 minutes. Next, the membrane was washed and a 1:1000 dilution of an HRP-conjugated anti-mouse antibody was added for a further 90 minutes. After washing, the reaction was developed with 4-chloro-1-naphthol substrate.

## ***ELISA assay of scFv-pIII recognition of K99***

Wells of one row of an ELISA plate were coated overnight at 4 °C with 100 µl of K99 at 100 µg/ml. A second row was coated with 100 µl of 2 % MPBS as a negative control. The coated wells were washed three times with PBS and blocked with 200 µl of 3 % BSA/PBS for 2 hours at 37 °C. After washing, 100 µl of Bugbuster extracts, diluted 2:3 in blocking buffer, were added to designated wells. The plate was then incubated for 60 minutes at 37 °C and then washed three times with PBS containing 0.05 % Tween 20. To

detect bound scFv-pIIIs, 100 µl of a 1:1000 dilution of monoclonal anti-pIII antibody was added and plate was left for 90 minutes at room temperature. Next, the plate was washed and 100 µl of a 1:1000 dilution of HRP anti-mouse antibody was added to each well. After incubating for 90 minutes at room temperature, the plate was washed and developed using OPD substrate. The reaction was measured at 450 nm after 10 minutes.

### ***Sequencing of clones from round 4***

Individual colonies were grown in 5 ml of 2xTY containing 100 µg/ml ampicillin and 1% glucose overnight at 37 °C with shaking. Plasmid DNA was isolated from 3 ml of culture using Qiagen miniprep reagents. The primers pelB and gIII were chosen for sequencing. Each was diluted to 3.2 µM. Sequencing was done at the MBSU, University of Glasgow using Big Dye (Applied Biosystems) and ET-Dye Terminator (Amersham Bioscience) chemistries. Samples were analysed on a MegaBACE1000 (96 capillary) sequencing machine. The reaction chemistry is based on the dideoxy method developed by Sanger (Sanger *et al.*, 1977). The sequence data was analysed using DNA Star software. Sequences were aligned to V BASE using DNAPLOT database at the Centre for Protein Engineering, MRC, Cambridge, UK ( [www.mrc-cpe.cam.ac.uk/vbase-ok.php?menu=901](http://www.mrc-cpe.cam.ac.uk/vbase-ok.php?menu=901) ). The confirmed nucleic acid sequences were translated and aligned using MEGALIGN software. Since clone E4 had an unexpected sequence, matches on the GenBank database were sought by BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>).

### ***Screening of additional clones from rounds 3 and 4 of selection***

The outcome of analysis forced the screening of additional clones from round 4 of selection. Purified DNA was prepared from overnight cultures as previously described and full-length inserts sought by PCR with primers LMB3 and gIII. An anti-thyroglobulin scFv construct was used as a positive control. For the expression of soluble scFv proteins, purified DNA was introduced into *E. coli* HB2151 made competent for transformation (Chung and Miller, 1988). Briefly, 500 µl of a overnight culture of HB2151 was added to 50 ml of 2xTY at 37 °C and grown with shaking at 200 rpm until the OD at 600 nm reached 0.3-0.4. The culture was centrifuged at 1000g for 10 minutes at 4 °C. The pellet was then resuspended in 5 ml of an ice-cold solution containing 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, 10 % PEG and 5 % DMSO (Sigma) and was incubated on ice for 10 minutes. After that 1 µl of a 1 in 10 dilution of each plasmid was added to 100 µl of competent cells. The plasmids were transferred to cells during fifteen minutes incubation on ice. The



bacteria were grown in 900  $\mu$ l of LB broth for 1 hour at 37 °C and 200 rpm shaking before plating 30  $\mu$ l of each culture onto LB agar plates containing 100  $\mu$ g/ml ampicillin. Plates were incubated overnight at 37 °C.

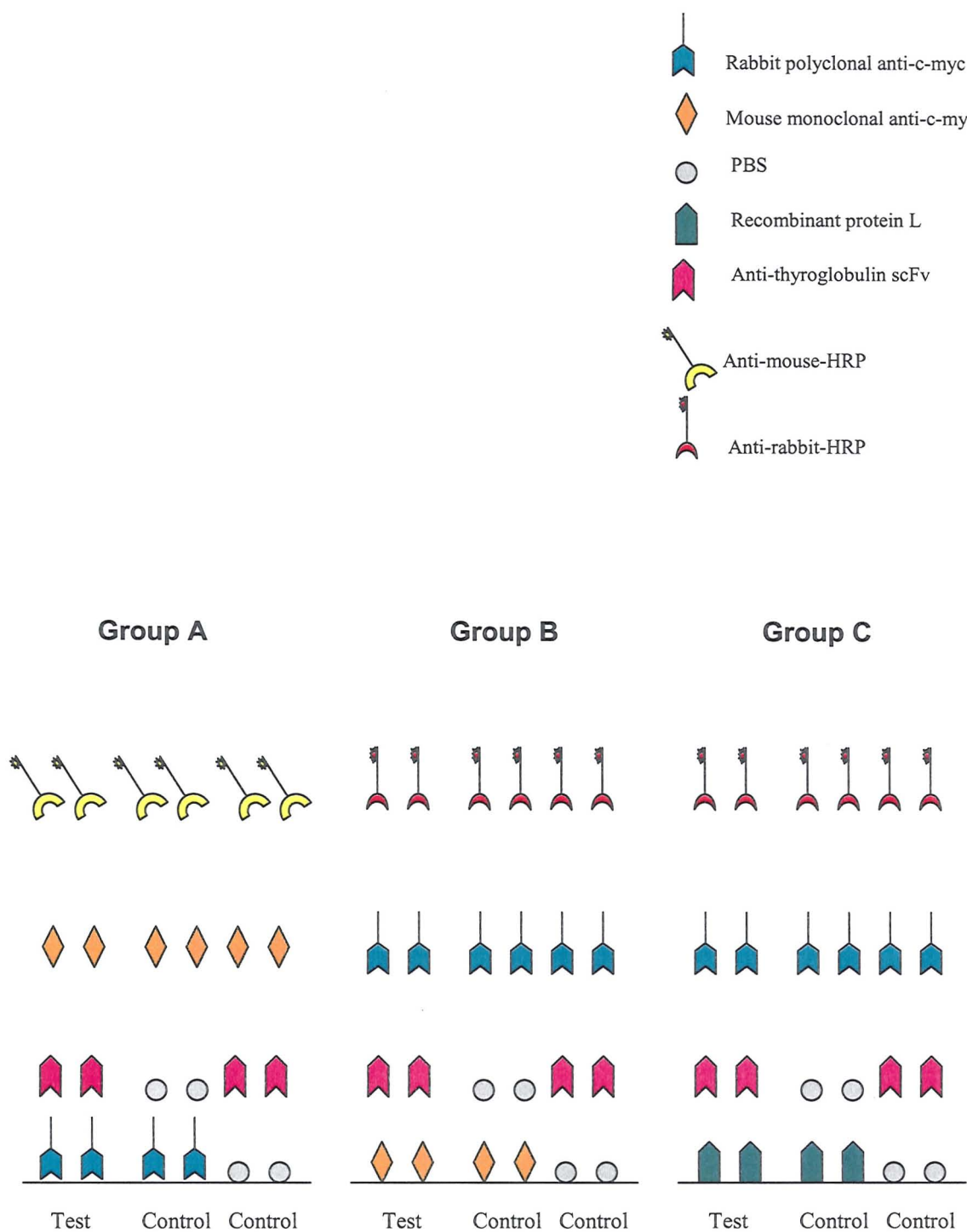
Single transformants were then grown and expression of soluble scFv induced with IPTG as described earlier. Concentrated culture supernatants were analysed by ELISA and Western blotting for recognition of K99 and the presence of scFv protein. These analyses were carried out with anti-c-myc reagents. Sequencing of scFv inserts was carried out as described earlier.

### ***Large scale screening for scFv expression***

Three different capture protocols were tested with a culture supernatant known to contain an anti-thyroglobulin scFv. In group A, wells of an ELISA plate were coated with a rabbit polyclonal anti-c-myc antibody at a dilution of 1:200 dilutions in PBS. For group B, the coating was a 1:500 dilution of a mouse monoclonal anti-c-myc in PBS. For group C, wells were coated with recombinant protein L (rProtein L™ from *Peptostreptococcus magnus* binds immunoglobulins (Ig) primarily through the  $\kappa$  light chain without interfering with the antigen binding site. Specifically it has been shown to bind strongly to human  $\kappa$  light chain subclasses I, III, and IV and to some  $\kappa$  chains from other species such as rat and mouse) at a concentration of 1  $\mu$ g/ml. As negative controls, for each group, two wells were coated only with PBS. Coating was done by incubation overnight at 4 °C. After the wells had been washed three times with PBS, the plastic was blocked by addition of 200  $\mu$ l of 3% BSA/PBS and incubation for 2 hours at 37 °C. After that, the wells were washed three times with PBS and 100  $\mu$ l of unconcentrated culture supernatant containing the anti-thyroglobulin scFv was added to half of the prepared wells; to the other half, 100  $\mu$ l of PBS was added to serve as a negative control, testing for cross-reactions between capture and detection layers in the assay. The ELISA plate was then incubated for 90 minutes at 37 °C. After washing three times with PBS containing 0.05 % Tween 20, bound scFvs were detected as follows. To wells in group A, a 1:1000 dilution of mouse monoclonal anti-c-myc antibody was added. Groups B and C received a 1:500 dilution of rabbit polyclonal anti-c-myc. The plate was incubated for further 90 minutes at 37 °C. Finally, wells were washed and were incubated for 1 hour at 37 °C with a 1:1000 dilution of anti-mouse-HRP (group A) or goat anti-rabbit-HRP (groups B and C). The wells, after washing, were developed using OPD substrate and the reaction was measured after 10 minutes at 450 nm. The format of this ELISA experiment is illustrated in Figure 3.4.

To assess the expression of scFv fragments, the clones to be tested (typically 94 picked from the original Griffin.1 library or a round of selection, along with an anti-thyroglobulin positive control) were grown in microtitre plates to aid the analysis of large numbers. For secondary stocks of the Griffin.1 library, phage antibodies were prepared from *E. coli* TG1 stocks by superinfection as described earlier. These were used to infect *E. coli* HB2151 and clones transduced to ampicillin resistance were picked into microtitre plates for analysis (see below). Phage eluted at rounds 3 and 4 of selection against K99 were infected into *E. coli* TG1 to determine phage recoveries. These clones were carried forward individually to produce phage for infection of *E. coli* HB2151 and then analysis for scFv expression. This was carried out as follows. *E. coli* TG1 carrying the phagemids were picked and grown at 37 °C in the wells of microtitre plates containing 100 µl of 2xTY, 100µg/ml ampicillin and 1 % glucose. Plates were shaken at 200 rpm. The following day, 2 µl of each overnight culture was transferred to a fresh plate with wells containing 200 µl of 2xTY containing 100µg/ml ampicillin and 1 % glucose. The plates were shaken at 37 °C for 1.5 hrs to reach an OD 600 nm of about 0.4. Then 25 µl of 2xTY containing 100 µg/ml ampicillin, 1% glucose and  $10^9$  pfu helper phage (final concentration) was added to each well to allow superinfection. Plates were left to stand at 37 °C for 30 minutes and then shaken at 200 rpm for a further 1 hour at 37 °C. The plates were centrifuged in a Sigma centrifuge fitted with plate carriers at 1800g for 10 minutes and the supernatants were aspirated off. Each pellet was resuspended in 200 µl of 2xTY containing 100 µg/ml ampicillin and 50 µg/ml kanamycin. Cells were grown overnight at 30 °C with shaking at 200 rpm to allow replication and rescue of the phage antibodies. The next day, plates were spun at 1800g for 10 minutes. The culture supernatants were used as monoclonal phage antibodies.

To express soluble scFv antibodies in HB2151 cells, 0.5 ml of an overnight culture of HB2151 cell was grown in 50 ml of 2xTY until OD at 600nm reached about 0.5. 200 µl aliquots were dispensed to the wells of a microtitre plate. Then 10 µl of each phage antibody was added and held for 30 minutes at 37 °C without shaking to allow infection to take place. The infected bacteria were then allowed to grow overnight at the same temperature with 200 rpm shaking. Next day, using a multi-channel pipette, about 2 µl samples from these wells were transferred to a further microtitre plate containing 200 µl of 2xTY, 100 µg/ml ampicillin and 0.1% glucose per well. The cultures were grown at 37 °C, shaking until OD at 600 nm was approximately 0.9. 25 µl of 2xTY containing 100 µg/ml ampicillin and 9 mM IPTG was added and growth continued at 30 °C for a further 20 hours. At the same day, ELISA plates were prepared to the optimal capture format as



**Figure 3.4 Three formats for a capture ELISA for detection of scFv**

Group A: wells of an ELISA plate were coated with polyclonal anti-c-myc

Group B: wells of an ELISA plate were coated with monoclonal anti-c-myc

Group C: wells of an ELISA plate were coated with recombinant protein L

determined by the experiments described earlier. The plates containing bacterial cultures were centrifuged at 1800g for 10 minutes at 4 °C and 100 µl samples of the supernatants were used for ELISA. As a negative control, a culture supernatant from *E. coli* HB2151 without the pHEN2 plasmid was added.

### **Western blotting**

For independent confirmation of the expression of soluble scFv fragments, *E. coli* HB2151 clones and an anti-thyroglobulin positive control were grown and induced by addition of IPTG. Culture supernatants were concentrated about 20 times and after running on SDS-PAGE, transferred to nitrocellulose membranes. The membranes were then blocked and probed with anti-c-myc reagents and a diaminobenzidine substrate (Harlow and Lane, 1988) comprising 6 mg of diaminobenzidine (Sigma) in 10 ml of 50 mM Tris (pH 7.6). Before application, 10 µl of 30 % H<sub>2</sub>O<sub>2</sub> was added. The membranes were incubated in this solution at room temperature for about 20 minutes.

To enhance the sensitivity of this reaction, membranes were washed with PBS three times and then developed with a chemiluminescent substrate (Dunbar, 1994). Briefly, two detection reagents were prepared. ECL reagents A contained 1 ml of luminol solution (0.8858g 5-amino-2,3-dihydro-1,4 phthalazine in 20 ml of DMSO) 0.44 ml of a p-coumeric acid solution (0.2956g coumeric acid in 20 ml of DMSO) and 10 ml of 1M Tris, pH 8.5. ECL reagents B comprised 10 ml of 1 M Tris pH 8.5 and 64 µl of 30 % H<sub>2</sub>O<sub>2</sub>. Equal volumes of A and B reagents were mixed to give sufficient working solution (125 µl for each cm<sup>2</sup> of membrane). Membranes were allowed to drain briefly and were placed between two plastic sheets. The mixture of solutions A and B was added to membranes just prior to development. After 1 minute incubation, blots were placed into an X-ray cassette against a sheet of X-ray film, the protein-coated side facing the film, was put onto two blots and left to expose for 30 second. Next, the x-ray film was removed and was developed in an automatic film processor.

### **ELISA**

ELISA was used to compare the activity of polyclonal phage isolated from the secondary stock of library with scFv isolated from the same source. The assay was conducted against purified K99 fimbriae and 2 % MPBS using variations to ELISA methods described earlier.

Wells of an ELISA plate were coated with 100  $\mu$ l of purified K99 at 90  $\mu$ g/ml and 2 % MPBS. To capture phage antibodies or soluble scFv fragments, wells were also coated with a 1:1000 dilution of monoclonal anti-c-myc. The plate was incubated overnight at 4 °C then washed three times with PBS and blocked with 200  $\mu$ l of 3 % BSA/PBS for two hours at 37 °C.

After washing wells three times with PBS, 100  $\mu$ l of a 1/5 dilution of phage rescued from the secondary stock of library was added to half the coated wells. To the remaining coated wells, 100  $\mu$ l of supernatant was added, taken from an induced culture of HB2151 infected with phage rescued from secondary stock of library. The plate was incubated for 90 minutes at 37 °C.

All wells were washed three times with PBST (0.05 %) and three times with PBS. To detect bound phage, 100  $\mu$ l of a 1:1000 dilution of monoclonal anti-M13-HRP was dispensed to those wells to which the phage antibodies had been added. To wells previously incubated with scFv, 100  $\mu$ l of a 1:500 dilution of polyclonal anti-c-myc was added for 60 minutes at 37 °C, followed by 100  $\mu$ l of a 1:1000 dilution of anti-rabbit-HRP. Reactions were developed with OPD substrate as described earlier.

## Results

### ***PCR screening of the Griffin.1 library for full-length scFv genes***

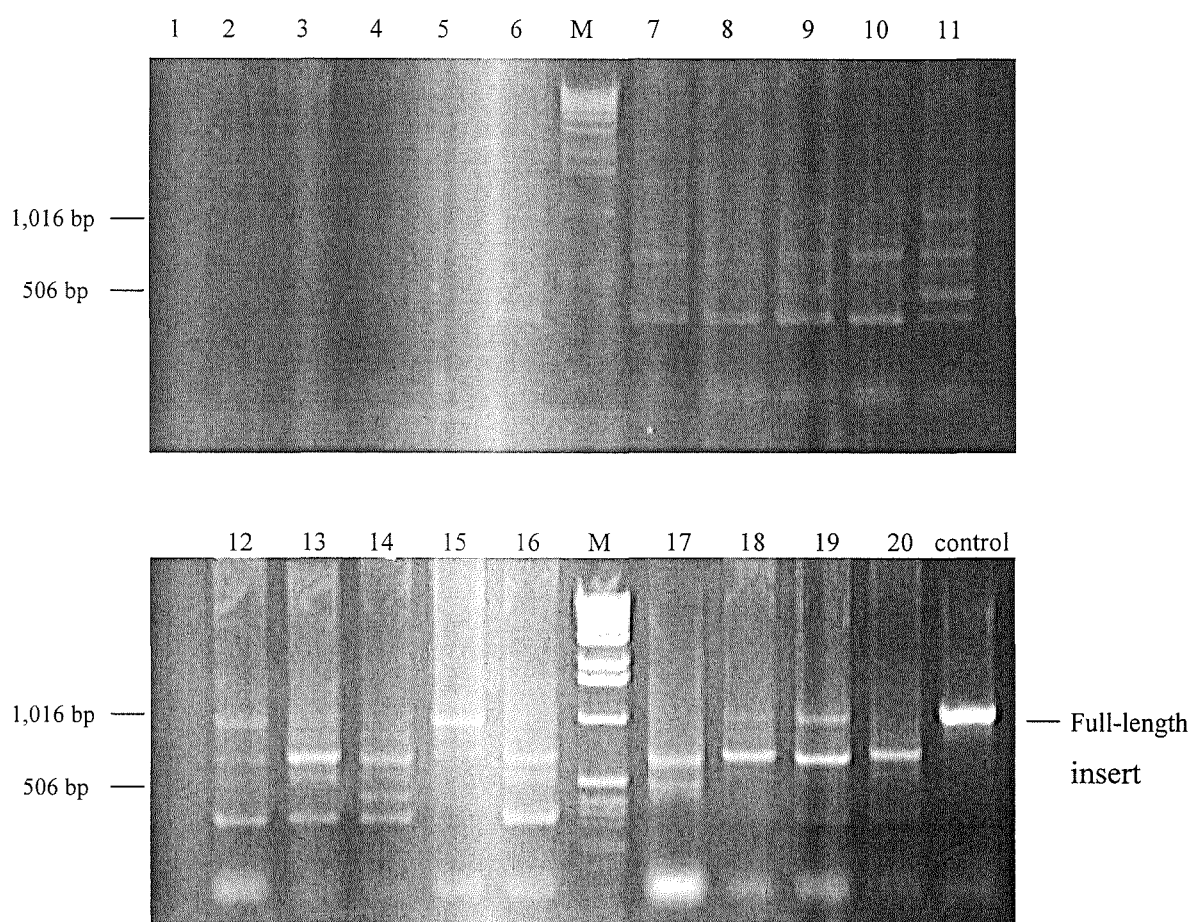
Twenty colonies were picked at random from the secondary stock of the Griffin.1 library. Crude lysates were prepared and screened by PCR using primers LMB3 and gIII for the presence of full-length scFv inserts. The products were analysed by agarose gel electrophoresis. This showed that less than 1/3 of the colonies had full-length inserts comprising V<sub>H</sub>, linker and V<sub>L</sub> sequences (Figure 3.5). No products were recovered from three clones, and for others, PCR indicated that only the V<sub>H</sub> or the V<sub>L</sub> fragments were present. Overall, the results indicate the possibility that phage might be amplified from the library carrying only V<sub>H</sub> or V<sub>L</sub> components during selection for anti-K99 antibodies.

### ***Restriction analysis of plasmids from the Griffin.1 library***

Plasmid DNA was isolated from 12 colonies picked at random from a secondary stock of the Griffin.1 library. Samples were digested with the restriction enzymes *Nco*I and *Not*I and analysed on an agarose gel. Since sites for these enzymes lie at the 5' terminus of the V<sub>H</sub> (*Nco*I) and the 3' terminus of the V<sub>L</sub> (*Not*I) sequences, the size of the released fragment can be used to confirm the presence of full-length insert. The results showed 1/3 of plasmids contained full-length inserts of approximately 720 bp. Other plasmids were not cut by both enzymes suggesting the absence of one or other of the scFv components (data not shown).

### ***Phage library preparation and titration***

The Griffin.1 library was supplied in *E. coli* TG1. A stock was grown and infected with VCS-M13 helper phage, thereby amplifying and releasing to the culture supernatant phage displaying scFvs. These phage antibodies were isolated by precipitation with



**Figure 3.5 PCR screening of random clones from the Griffin.1 library**

Crude lysates were prepared from 20 colonies picked from the library. Amplicons generated with primers LMB3 and gIII were analysed by agarose gel electrophoresis. Clone numbers are indicated at the top of each photograph. PCR of lysate from an anti-tyroglobulin scFv (control) confirmed the size of a full-length scFv insert (indicated). M indicates the migration of a 1 kb DNA ladder (Invitrogen, UK). The migration of markers of 1.016 bp and 506 bp are indicated.

PEG/NaCl and resuspended in PBS. Phage numbers were assessed by preparing serial dilutions of the phage stock, infecting *E. coli* TG1 and plating to TYE containing ampicillin. The resulting titration showed the phage titre to be  $2.6 \times 10^{13}$  pfu/ml, sufficient for panning the library for the selection of anti-K99 scFvs.

### ***Panning the Griffin.1 library against purified K99***

Data supplied with the Griffin.1 library indicated that it contained about  $1 \times 10^9$  unique scFvs. Phage antibodies against K99 fimbriae were isolated through four rounds of conventional panning using immunotubes coated with the purified K99 target at 90 µg/ml in round 1 and 30 µg/ml in rounds 2, 3 and 4. The number of phage added to the immunotube and the number recovered at elution was determined for each round of selection by infecting TG1 cells with serial dilutions. Table 3.1 shows this input and output data and also the percentage of phage recovered after each round of selection. The results show that the percentage of phage recovered increased slightly in the second round of selection over that recorded for round one. A sharp rise was noted from round two to round three. This was taken to indicate that phage carrying antibodies specific for K99 were isolated and enriched by panning. In the fourth round, the titre of output phage decreased.

### ***Screening phage antibodies by monoclonal phage ELISA***

Ninety-six colonies were picked from TYE titration plates after each round of selection. After superinfection with helper phage, the clones were analysed by monoclonal phage ELISA to determine the frequency with which they reacted with purified K99. The phage antibodies were also screened on ELISA plates that were coated with 2% MPBS, the blocking buffer used in selection, to check for the specificity of any reaction with K99. The results of analysis with K99 coated plates is shown in Figure 3.6. As the Figure shows, the number of clones that recognised K99 increased through rounds of selection and at round 4, all clones showed a marked recognition of the fimbriae. The results of screening against a coating of skimmed milk indicated that some clones that reacted with K99 fimbriae also bound to milk proteins. This was observed most commonly amongst clones isolated from rounds 2 and round 3 but not from round 4 (data not shown).

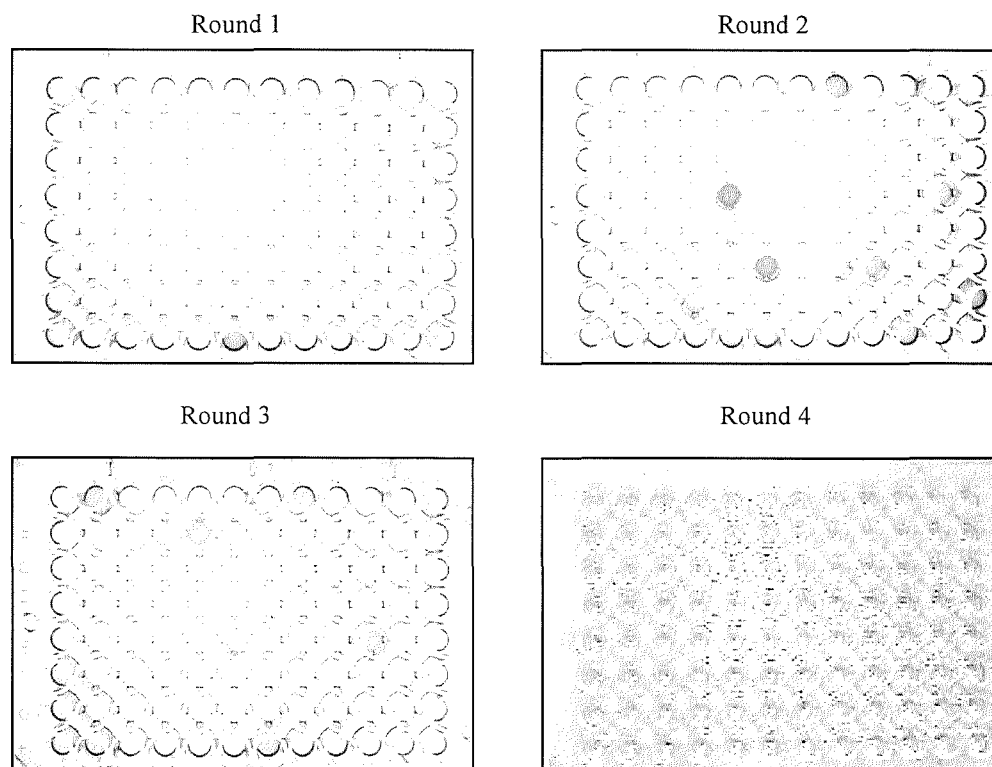


**Table 3.1** Phage titres obtained during selection of anti-K99 antibodies from the Griffin.1 library

Selection Round	Phage titre (CFU/ml)		
	Input <sup>a</sup>	Output <sup>a</sup>	% recovery <sup>b</sup>
1	$1 \times 10^{13}$	$3.9 \times 10^4$	$3.9 \times 10^{-7}$
2	$3.2 \times 10^{13}$	$8.4 \times 10^4$	$2.6 \times 10^{-7}$
3	$2.4 \times 10^{14}$	$4.7 \times 10^9$	$1.9 \times 10^{-3}$
4	$5.3 \times 10^{14}$	$5.6 \times 10^5$	$1.0 \times 10^{-7}$

<sup>a</sup> Input and output phage titres were determined by transduction of *E.coli* to ampicillin resistance using serial dilutions of sample

<sup>b</sup> Percentage recovery was determined by division of the output phage titre by the input titre, and multiplication by 100



**Figure 3.6 Monoclonal phage ELISA against K99**

Ninety six clones picked at random after each round of selection (as indicated) were superinfected with helper phage to generate monoclonal phage antibodies. The phage were then applied to ELISA plates coated with K99 and binding detected with anti-M13 reagents and OPD substrate. Final ODs were measured at 450 nm (data not shown) and images of plates were captured.

## **Plasmid analysis of clones from round 4**

Given the apparent high frequency of recognition of the K99 target amongst clones recovered from round 4, plasmid DNA was isolated from 13 clones for further analysis. Clones were chosen because they showed the strongest signals in ELISA against K99. To test for the presence of full-length insert, plasmid DNA was digested with the restriction enzymes *Nco*I and *Not*I. Recognition sites for these enzymes are located at the 5' and 3' termini of the scFv sequence in the pHEN2 vector in which the Griffin.1 library was constructed (Figure 3.1).

The digestion products were analysed by agarose gel electrophoresis (Figure 3.7), it being predicted that release of a DNA fragment of about 720 bp would indicate the presence of full-length scFv insert. The results showed that only 4 clones of the 13 chosen were likely to carry a full-length insert. Based upon this result, clones E1, E7, C8, G6 were taken forward to further analysis with clone E4 was chosen because release of a small DNA fragment suggested the presence of a  $V_H$  or  $V_L$  component.

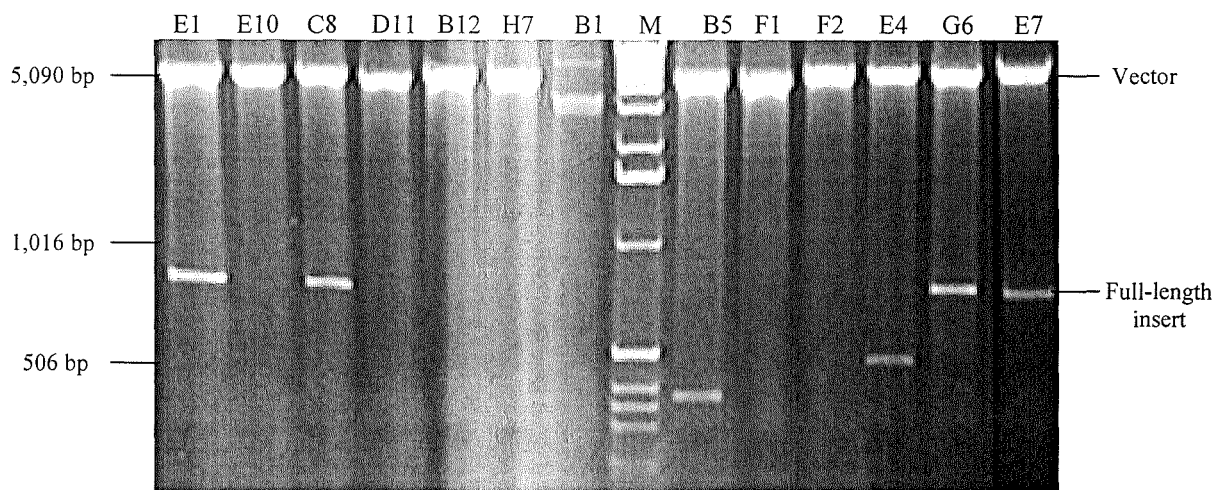
## **Production of soluble scFv proteins**

After choosing five clones from round 4 of selection, *E. coli* HB2151 cells were infected with phage to allow expression of soluble scFv fragments. The infected cells were grown on TYE plates and one colony from each plate was transferred to liquid culture and induced with IPTG. The culture supernatants were then isolated and tested for the presence of scFv fragments by SDS-PAGE analysis and Western blotting using polyclonal anti-c-myc antibody. SDS-PAGE analysis of the concentrated culture supernatants (Figure 3.8) showed some evidence of proteins of the size predicted for a scFv (30 kDa; clones G6 and C8). Induction of clone E1 with IPTG consistently resulted in the release of a large number of proteins to the culture supernatant suggesting that partial lysis of the bacteria was triggered. It was hoped that Western blotting would provide confirmation of the presence of scFv products but no signal could be detected around 30 kDa (Figure 3.9). For clone E1, a component of the culture supernatant appeared to react with the anti-c-myc reagents, but its size was higher than expected. Despite extensive investigation of culture and induction conditions (temperature, IPTG concentration, addition of sucrose (Kipriyanov, 2002),) the overall conclusion was that soluble anti-K99 scFvs could not be expressed from the clones chosen for investigation.

## **Recloning into pIMS147**

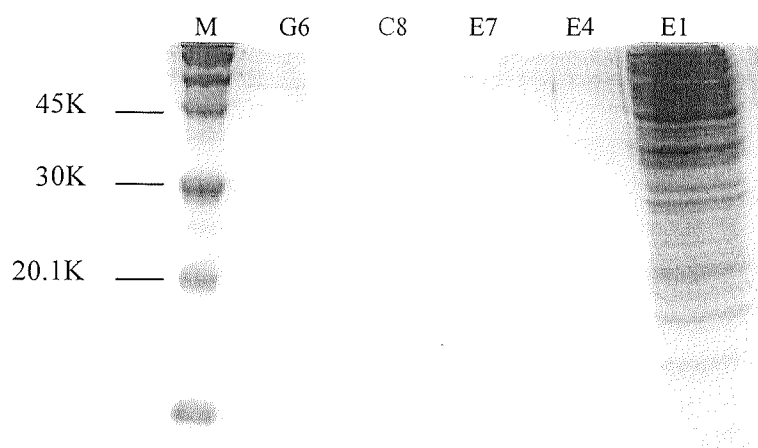
Some investigators have reported that expression of scFvs as fusion proteins with an attached human kappa constant domain can stabilise the products and enable easy and sensitive detection with anti-kappa reagents. The vector pIMS147 has been designed to create these fusions with scFv sequences recovered from phage display vectors (Strachan *et al.*, 2002). The scFv sequences were recovered as *NcoI* / *NotI* fragments from clones picked from round 4 of selection and ligated into pIMS147 prepared with the same restriction enzymes. Ligation products were transformed into *E. coli*. Without a scFv insert, the predicted size of the pIMS147 vector is 5988 bp. Hence the migration of linearised plasmid DNA at around 7 kb showed that the scFv sequences had been successfully ligated into the new vector (Figure 3.10A). Since pIMS147 and the pHEN2 phage display vector differ significantly in size (Figure 3.10B), the data exclude the possibility that religation of the original constructs had taken place.

The recloned scFvs were transformed into *E. coli* XL1 Blue competent cells and after growing, expression was induced with IPTG. Although the presence of recombinant protein could be confirmed by capture *via* the human kappa domain and detection of the same component, culture supernatants showed no significant activity against K99 (Figure 3.11).



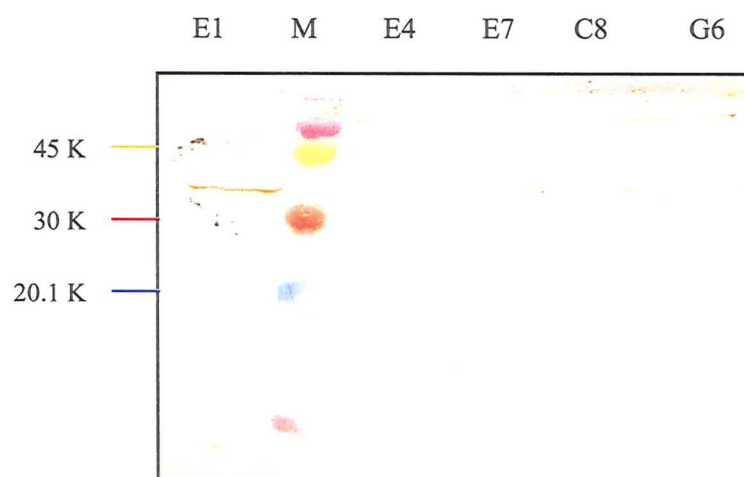
**Figure 3.7 Restriction analysis of plasmid DNA from 13 anti-K99 scFv clones**

Clones were taken from the fourth round of selection against K99. They were chosen for their high apparent reaction against K99 in monoclonal phage ELISA. Plasmid DNA was isolated from each clone, digested by the restriction enzymes *Nco*I and *Not*I and analysed by agarose gel electrophoresis. The expected size of fragment released from a full-length scFv insert (about 720 bp) is indicated on the right. The migration of the vector fragment is also shown. Clone designations are shown at the top of the figure, M loading of a 1 kb DNA ladder (Invitrogen). The migration of markers of 506 bp, 1,016 bp and 5,090 bp are indicated on the left of the figure.



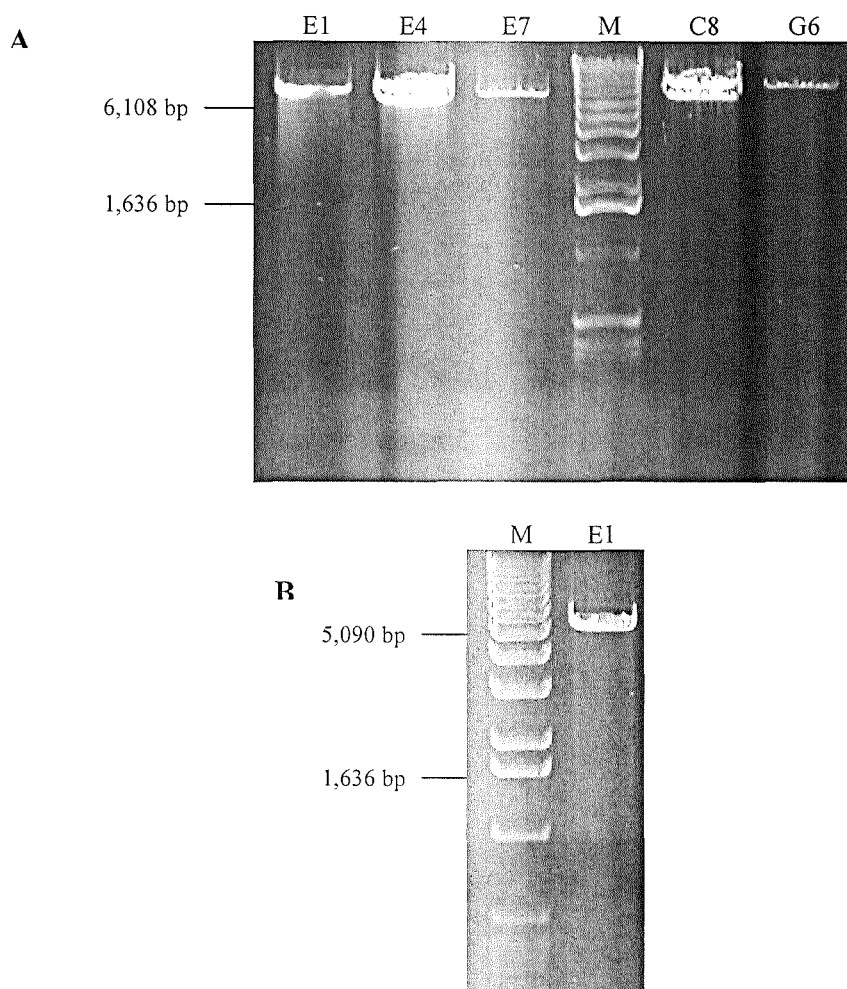
**Figure 3.8 SDS-PAGE analysis for the presence of scFvs in concentrated culture supernatants**

Supernatants from induced cultures of *E. coli* HB2151 were concentrated about 30-fold and analysed on SDS polyacryamide gels. The image from a Coomassie Blue stained gel is shown. M indicates the loading of Rainbow protein molecular weight marker (Pharmacia). The migration of standards of 45, 30 and 20.1 kDa is indicated. Clone designations are shown at the top of the figure. The expected size for a soluble scFv is about 30 kDa.



**Figure 3.9 Western blotting of concentrated culture supernatants of selected clones after attempting to express soluble scFv fragments with HB2151 *E. coli* strain**

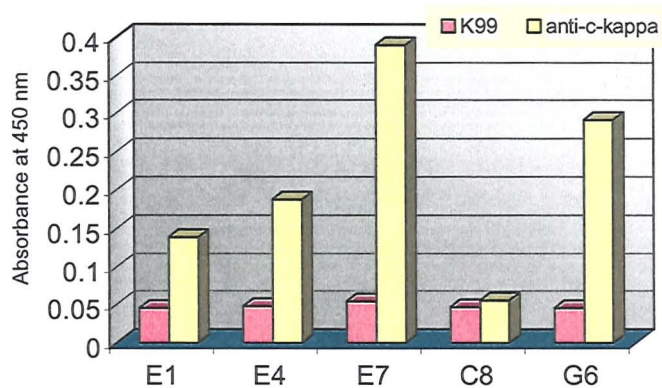
Supernatants from induced cultures of *E. coli* HB2151 were concentrated about 30-fold and analysed on SDS polyacrylamide gels. Proteins were transferred to nitrocellulose and probed with anti-c-myc reagents and diaminobenzidine substrate before image capture. M indicates the loading of Rainbow protein molecular weight marker (Pharmacia). The migration of standards of 45, 30 and 20.1 kDa is indicated. Clone designations are shown at the top of the figure. The expected size for a soluble scFv is about 30 kDa.



**Figure 3.10 Restriction analysis of recombinant pIMS147 containing anti-K99 scFvs**

Plasmid DNA was isolated from transformed *E. coli*, linearised by digestion with *EcoRI* and analysed on agarose gels. Panel A: recloning of scFv sequences from clones E1, E4, E7, C8 and G6 into pIMS147. Clone designations are shown at the top, M indicating loading of a 1 kb ladder to the gel. The migration of markers of 1,636 bp and 6,108 bp is indicated. Panel B: analysis of clone E1 in the original pHEN2 phage display vector. The migration of markers of 1,636 and 5,090 bp is indicated.





**Figure 3.11 Expression of scFv fusion proteins in pIMS147**

Supernatants from induced cultures of *E. coli* were applied to ELISA plates coated with K99 (pink) or a goat anti-human kappa antibody (yellow). Binding of protein was detected by addition of a goat anti-human kappa HRP conjugate and OPD substrate. Colour change was recorded at 450 nm.

## ***Expression scFv-pIII fusion proteins***

Given the failure to confirm the anti-K99 specificity of clones from round 4 by expression of soluble scFv fragments or kappa fusion proteins, the expression of scFv-pIII fusion proteins was attempted as an alternative strategy.

In the pHEN2 vector, an amber stop codon lies between the scFv coding sequence and the phage gIII gene. Conventionally, suppressor strains of *E. coli* such as TG1 are used for the propagation of phage that bear phagemid-encoded scFvs fused to the viral minor coat protein, pIII. The constructs are transferred to non-suppressor strains such as HB2151 for expression of the scFv in its soluble form. Detection of the recombinant antibody then depends upon use of peptide tags at the carboxy-terminus of the scFv (e.g. histidine repeats or the c-myc epitope). Expression of phagemids in suppressor strains triggers synthesis of the scFv-pIII fusion which in the absence of superinfecting helper phage, accumulates in the bacterial membrane. If liberated from this location with detergent, the attached pIII sequence provides a tag which is readily detectable with anti-pIII reagents (Mersmann *et al.*, 1998). The expression of candidate anti-K99 scFv-pIII proteins was assessed by Western blotting and the activity of these proteins against K99 antigen was tested by ELISA.

In preliminary experiments, the expression of scFv-pIII fusion proteins was tested for clones E1 and G6 and their detection with an anti-pIII monoclonal antibody was confirmed (data not shown). The clones chosen from round four of selection (see above) grown in *E. coli* TG1, induced with IPTG and the cells collected by centrifugation. The cell pellets were lysed by resuspension in Bugbuster reagent and extracts were run on an 8 % SDS-polyacrylamide gel. The proteins were then transferred to a nitrocellulose membrane and the presence of scFv-pIII fusion proteins was detected using a mouse monoclonal anti-pIII antibody and anti-mouse-HRP reagents. The Western blotting result (Figure 3.12) demonstrated that a scFv-pIII fusion protein of about 80 kDa size was expressed in all clones. A second protein present in the samples and reactive with the anti-pIII antibodies was of a size (approximately 60 kDa) consistent with that of the wild type pIII. This result is as reported by others (Mersmann *et al.*, 1998). Since the TG1 clones carry only the recombinant phagemid, the appearance of wild type pIII most likely arises through the proteolytic cleavage of the scFv from the pIII fusion.

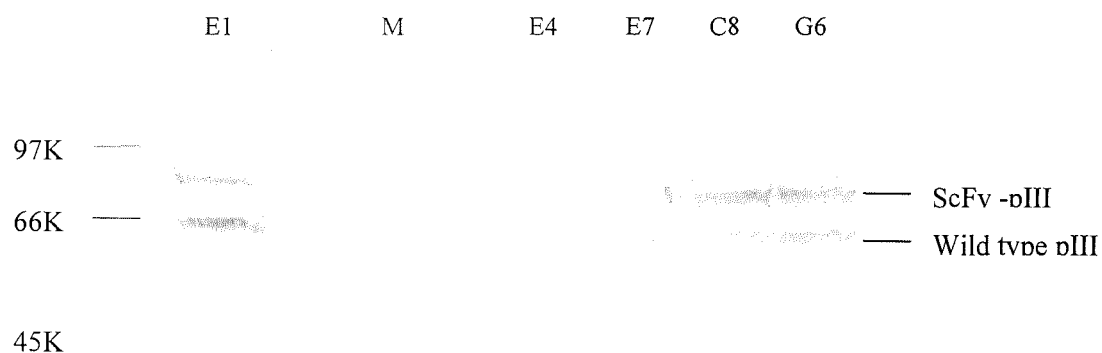
For ELISA experiment, the wells of a microtitre plate were first coated with purified K99 or 2 % MPBS. The bacterial lysates prepared with Bugbuster reagent and confirmed to contain pIII fusion proteins, were added and the binding of scFv- pIII proteins to either target was detected with monoclonal anti-pIII and anti-mouse-HRP reagents. The experiment showed that scFv-pIII fusion proteins from each of the clones bound to milk proteins to a low but detectable level. However, binding to K99 fimbriae generated signal that was at least 5-fold higher (Figure 3.13).

Clones chosen from round 4 of selection were sequenced. DNA and amino acid sequence results of 5 selected clones from round four were analysed by DNASTAR software and VBASE database. The analysis of heavy chain sequences showed clones E1, E7 and C8 were related to VH1 family and clone G6 to the VH3 family (Figure 3.14). Clones E1 and C8 had very similar sequence in their CDRs and there was a stop codon in the same position in both CRD3. CDR3 sequence of Clone E7 was completely different from the others. For clone E4 no V<sub>H</sub> component was recognized by comparison with VBASE database.

The V<sub>L</sub> sequences revealed that except for clone E4, other clones had consensus sequence of V<sub>κ</sub> subgroup and VL1 family with high diversity. The clone E4 had a truncated VL domain (Figure 3.15). The VBASE database analysis did not show consensus sequence of CDR1 and CDR2 of this clone.

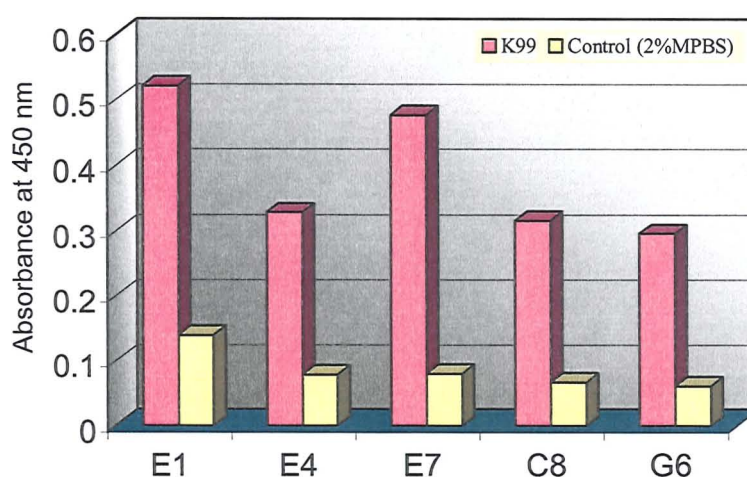
### ***Further screening of clones from round 4 of selection***

Another 10 clones from the fourth round of panning against K99 were chosen on the basis of their high reaction with K99 antigen in monoclonal phage ELISA. These were taken forward to PCR analysis for the presence of full-length scFv inserts and for expression and analysis of soluble scFv antibodies.



**Figure 3.12 Western blot analysis of expression of scFv-pIII fusion proteins**

Detergent extracts were prepared from induced cells of *E. coli* TG1 and separated by SDS-PAGE. Proteins were transferred to nitrocellulose membrane and probed with an anti-pIII monoclonal antibody and anti-mouse-HRP reagents. The blot was developed with chloronaphthol substrate and the image captured. M indicates the loading of Rainbow protein molecular weight markers (Pharmacia). The migration of standards of 45, 66 and 97 kDa is indicated at the left. Clones are as shown at the top of the image. The predicted migration of scFv-pIII fusions (80 kDa) and wild-type pIII (60 kDa) is shown at the right of the image.



**Figure 3.13 Reaction scFv-pIII fusion proteins with K99 and milk proteins**

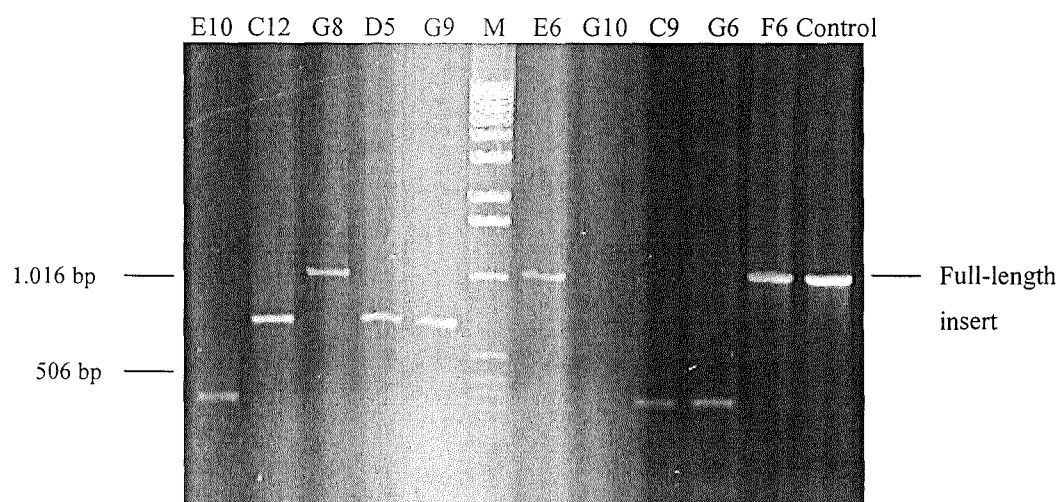
Wells of an ELISA plate were coated with K99 (pink) or skimmed milk (yellow). Detergent extracts were prepared from induced cells of *E. coli* TG1 and applied to the coated surfaces. Binding of the scFv-pIII fusion proteins was detected with an anti-pIII monoclonal antibody and anti-mouse-HRP reagents. The assay was developed with OPD substrate and absorbance values at 450 nm measured in a microplate reader. Clones are indicated along the x axis of the histogram.

Clone	Subgroup	CDR1	CDR2	CDR3
E1	VH1 family	GYVMH	WINPTSGGTNYAQKFQG	CVRNVV.LSE
E7	VH1 family	NNFMH	WINAGNGNTTYAQKFQG	HCARHTWNR
C8	VH1 family	GYVMH	WINPNSGGTNYAQKFQG	CVRNVV.LSE
G6	VH3 family	GFTFDDYAMH	GISWNSGSIGYADSVKG	CERHTQ
E4	.....	.....	.....	.....

**Figure 3.14 Heavy chain amino acid sequence of five best clones from round 4 of selection**

Clone	Subgroup	CDR1	CDR2	CDR3
E1	VL2 family	TGTSSDVGDYDHVF	NVNTRPS	SLYSSSYCSLYSSS
E7	VKI subgroup	RASQGISSYLAWY	AASTLQS	QQLNSYPACQQLNSYPAA
C8	VKI subgroup	RASQGISSWLA	AASSLQS	QQANRFPPCQQANRFPPF
G6	VL1 family	SGSSSNIGNNAVN	YDDLPS	AAWDDSLCAAWDDSL
E4	VKvI subgroup	.....	.....	HQSSSLPR

**Figure 3.15 Light chain amino acid sequence of five best clones of round 4 of selection**



**Figure 3.16 PCR screening of 10 clones from round four of panning on K99.**

Plasmid DNA was prepared from 10 colonies picked from the fourth round of panning against K99 and used as template in PCR with primers LMB3 and gIII. Amplicons were analysed by agarose gel electrophoresis. Clone numbers are indicated at the top the photograph, "Control" indicating sample from a construct confirmed to carry a full-length scFv. M indicates the migration of a 1 kb DNA ladder (Invitrogen, UK). The migration of markers of 1.016 bp and 506 bp are indicated.



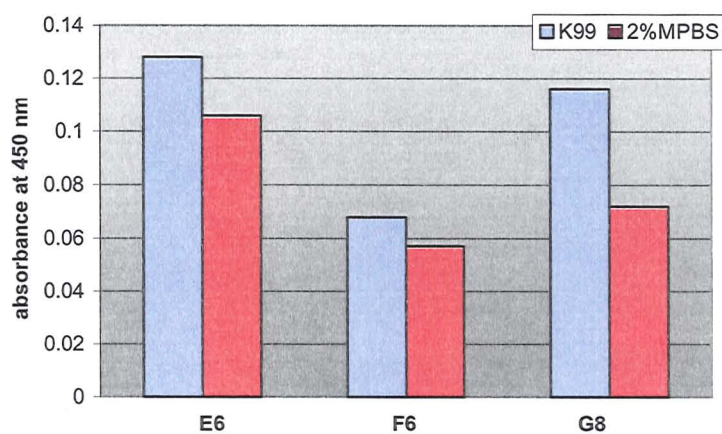
Plasmid DNA was purified from the 10 clones and was PCR carried out using the primers LMB3 and gIII. Figure 3.16 shows the results of this screening. The Figure illustrates that of the 10 samples analysed only three PCR products were of a size (about 1 kb) similar to the positive control and therefore consistent with the presence of a full-length scFv insert.

The three clones identified by PCR as carrying full-length scFv inserts were carried forward to expression and analysis of soluble scFvs. Plasmid DNA was transformed into competent HB2151 cells. After growth in liquid culture and induction with IPTG, culture supernatants were collected by centrifugation and added to an ELISA plate coated with K99 and 2 % MPBS. The binding of scFvs to these target proteins was detected using a monoclonal anti-c-myc antibody and anti-mouse-HRP reagents. The resulting immunoassay data (Figure 3.17) showed that scFvs from all three clones reacted very weakly with K99 fimbriae. The final absorbance values in wells coated with K99 were similar to those in wells coated with skimmed milk.

The culture supernatants used in ELISA were also tested in Western blotting to assess if scFvs were present. The results (data not shown) revealed that none of the selected clones appeared to express scFv since bands of the predicted molecular weight (about 30 kDa) were absent. Overall, the analysis suggested that these three clones were unable to express scFv antibodies.

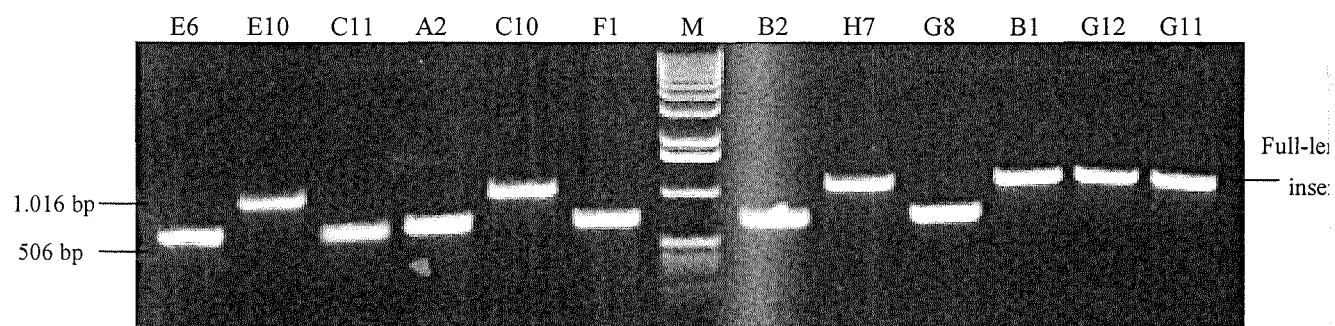
### ***Screening of clones from round 3 of selection***

From the 96 clones originating from round 3 of selection and previously tested in monoclonal phage ELISA, 12 were chosen for further characterisation. Of these, 6 showed activity against K99 and 6 showed activity against skimmed milk. The selected clones were checked for the presence of full-length scFv inserts, the expression of soluble scFv antibodies and they were sequenced.



**Figure 3.17 ELISA analysis of culture supernatants from 3 clones chosen from round 4.**

Supernatants from induced cultures were applied to wells coated with purified K99 (blue) or skimmed milk (pink) and the binding of scFv detected with anti-c-myc antibodies, anti-mouse HRP conjugate and OPD substrate. The absorbance at 450 nm was recorded (y axis). Clone designations are shown on the x-axis.



**Figure 3.18 PCR screening of clones from round 3 of selection**

Purified plasmid DNA from clones E6, E10, A2, H7, C10 and F1 (candidate anti-K99 antibodies), and C11, B2, G8, B1, G12 and G11 (candidate anti-milk antibodies) was used in PCR with primers LMB3 and gIII. Amplicons were analysed by agarose gel electrophoresis and images captured. M shows the loading of a 1 kb DNA ladder (Invitrogen, UK). The migration of markers of 506 bp and 1,016 bp is indicated (left) with the predicted size of a full-length scFv amplicon (right). Clone designations are shown at the top of the photograph.

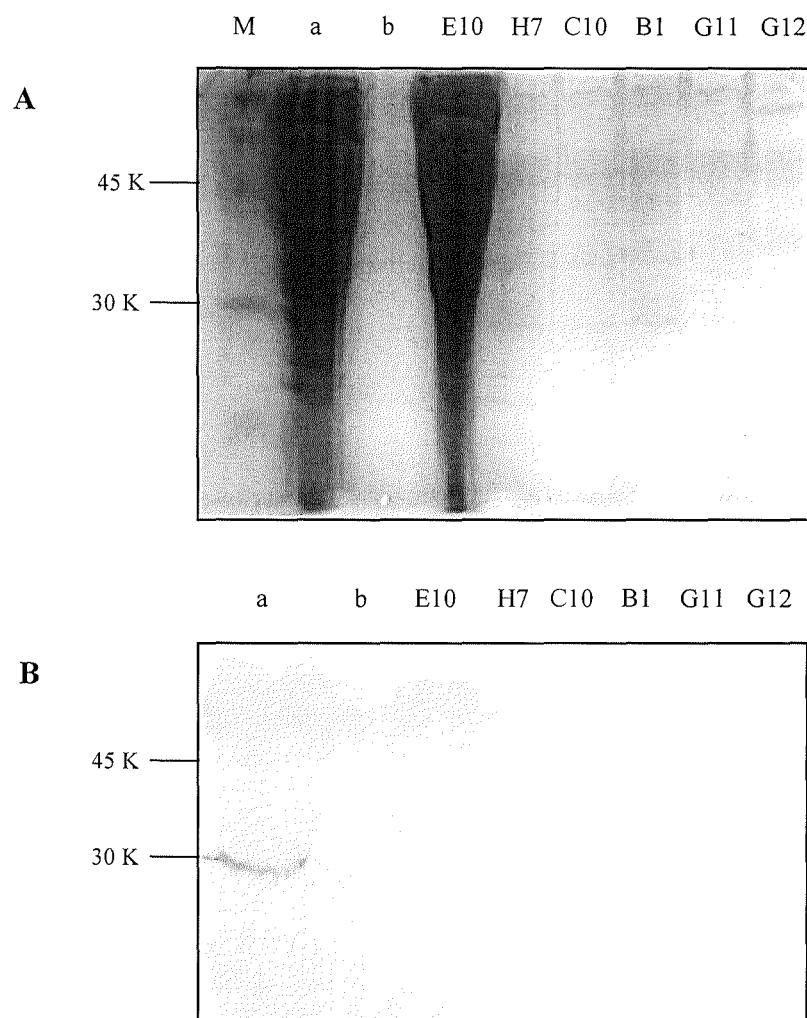
Plasmid DNA was purified from the 12 selected clones and used as template in PCR with primers LMB3 and gIII. Figure 3.18 shows that half of the clones chosen appeared contain full-length inserts. Interestingly, these were evenly divided between clones chosen for their activity against K99 and against skimmed milk.

All six clones carrying full-length inserts were transformed into competent *E. coli* HB2151. The transformants were grown in liquid culture and induced by addition of IPTG in an attempt to express soluble scFv protein. As a positive control, a clone carrying an anti-thyroglobulin scFv was grown in parallel. Untransformed *E. coli* HB2151 lacking a plasmid was grown as a negative control. The culture supernatants were concentrated about 50 times and were used for further analysis.

SDS-PAGE analysis (Figure 3.19A) showed the presence of many proteins in culture supernatants from clones E10 (a candidate anti-K99 construct) and the anti-thyroglobulin positive control. Supernatants from other clones had very few proteins despite extensive concentration from the original cultures. The proteins were transferred to nitrocellulose membrane by Western blotting and probed with anti-c-myc and anti-mouse-HRP antibodies (Figure 3.19B). The results showed that scFv protein was clearly detectable from the positive control and that other clones failed to express this recombinant protein.

## **ELISA**

Culture supernatants prepared from clones chosen at round 3 were tested in ELISA against purified K99. This analysis was carried out as previously described for clones chosen from round 4. None of the culture supernatants showed significant activity against K99 (results not shown).



**Figure 3.19 SDS-PAGE and Western blotting of full-length scFv clones from round 3**

Concentrated supernatants from induced cultures were separated by SDS-PAGE, stained with Coomassie Blue and photographed (panel A). M shows the loading of a set of molecular weight markers with the migration of standards of 45 and 30 kDa indicated. Clone designations are shown at the top with “a” and “b” showing the loading of positive and negative control supernatants respectively. Blots (panel B) were probed with anti-c-myc reagents and DAB substrate before image capture.

**Table 3.2 Summary of the properties of 12 clones chosen from round 3 of selection**

Clone name	Dominant reactivity <sup>a</sup>	Insert	Anti-K99 activity <sup>b</sup>	scFv expression <sup>c</sup>	Sequencing
E6	K99	V <sub>H</sub> or V <sub>L</sub>	.....	.....	.....
E10	K99	V <sub>H</sub> -V <sub>L</sub>	Negative	Negative	Stop codon
C11	Milk	V <sub>H</sub> or V <sub>L</sub>	.....	.....	.....
A2	K99	V <sub>H</sub> or V <sub>L</sub>	.....	.....	.....
C10	K99	V <sub>H</sub> -V <sub>L</sub>	Negative	Negative	Intact sequence
B2	Milk	V <sub>H</sub> or V <sub>L</sub>	.....	.....	.....
F1	K99	V <sub>H</sub> or V <sub>L</sub>	.....	.....	.....
G12	Milk	V <sub>H</sub> -V <sub>L</sub>	Negative	Negative	Stop codon
G8	Milk	V <sub>H</sub> or V <sub>L</sub>	.....	.....	.....
B1	Milk	V <sub>H</sub> -V <sub>L</sub>	Negative	Negative	Stop codon
H7	K99	V <sub>H</sub> -V <sub>L</sub>	Negative	Negative	Intact sequence
G11	Milk	V <sub>H</sub> -V <sub>L</sub>	Negative	Negative	Stop codon

<sup>a</sup> Reactivity as assessed by monoclonal phage ELISA against K99 or 2% MPBS

<sup>b</sup> Reactivity towards K99 as assessed by ELISA using supernatant from induced HB2151 cultures

<sup>c</sup> Expression of soluble scFv protein as assessed by Western analysis of supernatant from induced HB2151 cultures

The six plasmids that contained full-length scFv inserts were prepared for sequencing with primers PelB and gIII. Analysis of the sequence data revealed that only two clones (H7 and C10, both putative anti-K99 constructs) had scFv sequences free of stop codons in either framework or CDR regions. Table 3.2 summarises the data gathered on the properties of all 12 clones chosen from round 3 of panning.

### ***High-throughput methods to assess expression of soluble scFv***

Since screening of clones from rounds 3 and 4 failed to identify any able to express soluble scFv antibodies, methods were sought that could be applied in large scale screening to meet this goal. ELISAs were tested using the anti-thyroglobulin clone known to express a scFv protein.

Briefly, wells of an ELISA plate were coated with either recombinant protein L, a mouse monoclonal against c-myc or polyclonal anti-c-myc antibodies. After blocking the wells, supernatant from an induced culture of HB2151 carrying the anti-thyroglobulin construct was added. Binding of the scFv to protein L was then detected using polyclonal anti-c-myc and goat anti-rabbit-HRP antibodies. In wells coated with monoclonal anti-c-myc, binding of the scFv was assessed with the same reagents. Finally, binding of the scFv in wells coated with polyclonal anti-c-myc was detected with the monoclonal anti-c-myc reagent and anti-mouse-HRP antibodies. Figure 3.20 shows the results of these three tests. Signal strength was highest when monoclonal anti-c-myc was used as the capturing layer, implying that this assay format had the highest sensitivity. This method also showed the highest specificity when compared with controls which either lacked the capture layer, or the scFv ligand (Figure 3.20).

### **Screening clones from rounds 3 and 4 for scFv expression**

Ninety four clones from round 3, and ninety four from round 4 were picked at random and grown to produce phage that were then infected into exponentially growing *E. coli* HB2151 cells. As a positive control, a clone carrying an anti-thyroglobulin scFv was also prepared, with HB2151 lacking plasmid serving as a negative control for the experiment. The cells were grown in the wells of microtitre plates and induced by addition of IPTG. The culture supernatants were then used in a capture ELISA set up to the format described above.

The ELISA results showed that all clones from round 4 were unable to produce a soluble scFv product and reactions in the assay were similar to the negative control. Three clones from round three showed a very weak reaction in the assay but further experiments with these clones was unable to confirm the production of soluble scFv fragments (data not shown). Results from the anti-thyroglobulin control were strongly positive as in preliminary experiments (Figure 3.20).

### ***Screening secondary stock of Griffin.1 library for expression of soluble scFv fragments***

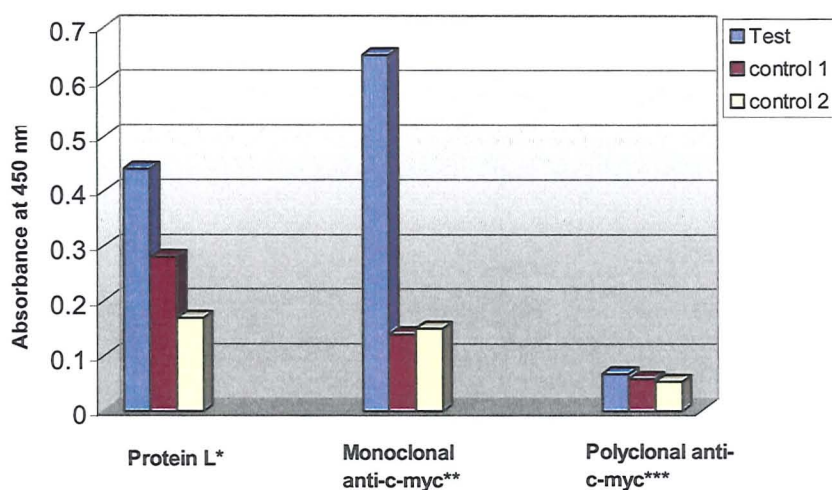
Since large scale screening of clones from rounds 3 and 4 of selection against K99 revealed an inability to express soluble fragments, the assay was repeated with clones picked at random from the original Griffin.1. This attempted to establish if selection for binding to K99 had in some way driven the isolation of clones unable to express a soluble scFv product, or if this was a feature of the original library.

Library clones in TG1 cells were picked at random, grown and phage antibodies produced by infection with VCS-M13 helper phage. The phage were then precipitated by PEG/NaCl and resuspended in PBS. Exponentially growing *E. coli* HB2151 cells were infected with diluted aliquots of the phage stock and then used for screening.

Ninety four individual HB2151 clones were grown in the wells of a microtitre plate and soluble scFv expression was induced by the addition of IPTG. After collection, the culture supernatants were tested by ELISA using the capture format described earlier. The assay showed some clones were able to produce soluble scFv. Fourteen clones that showed the strongest reaction in the assay were chosen for further analysis.

Crude lysates of all 14 clones was used as template in PCR with the primers LMB3 and gIII. Although the crude lysates gave results that were not as clear-cut as in reactions using purified plasmid DNA (e.g. Figure 3.18), the experiment indicated that most of the clones probably carried full-length scFv inserts (Figure 13.21). Only three clones (A3, H3, H5) showed sharp products that were too small for full-length scFv sequences. For the remainder, there was some evidence of amplicons of the size predicted for a full-length insert and this conclusion was beyond doubt for clones A5, B5 and F6.





**Figure 3.20 Capture ELISAs for the detection of scFv**

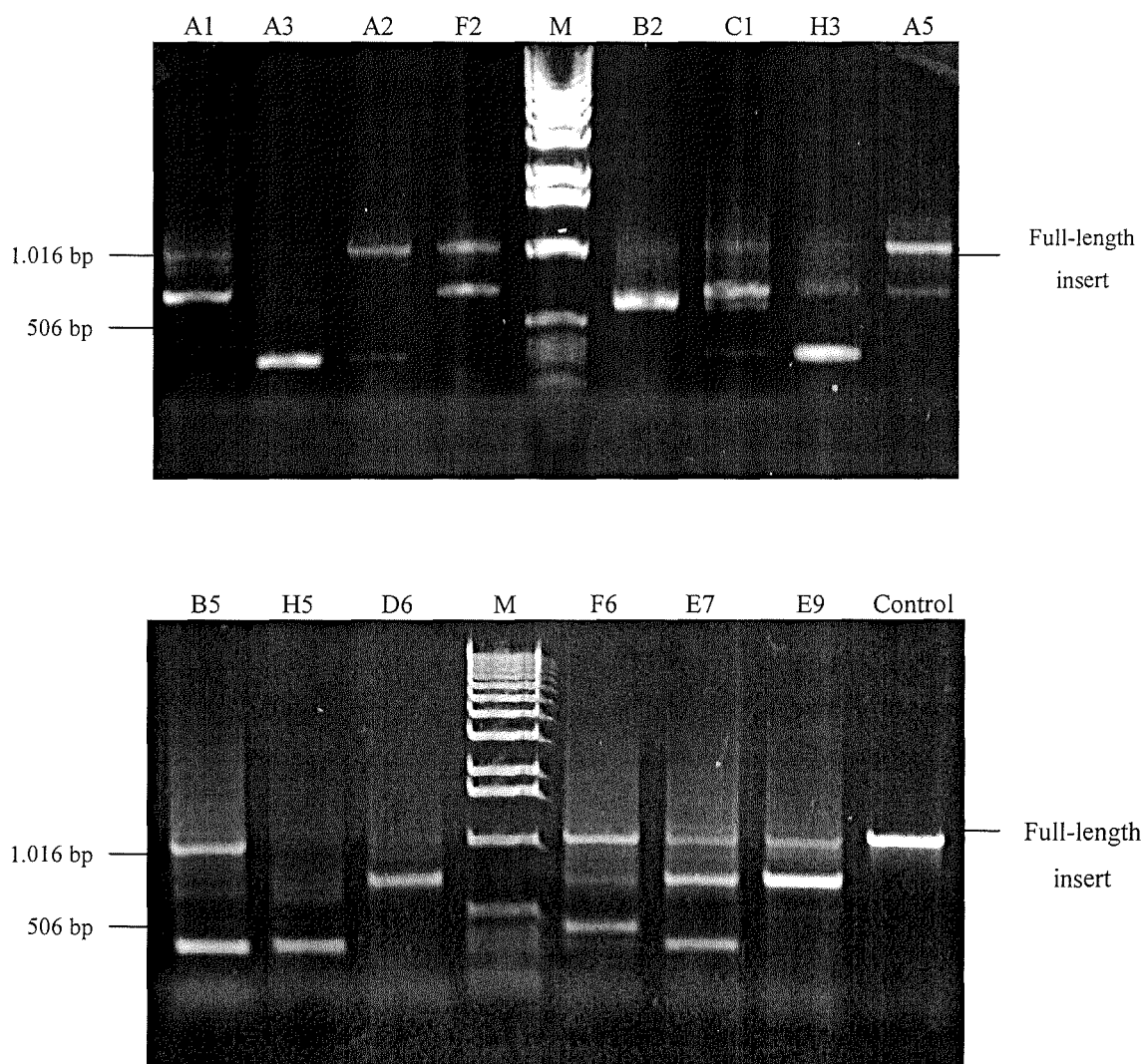
Wells of an ELISA plate were coated with the reagents indicated on the x-axis of the histogram. Culture supernatant containing soluble scFv was added and scFv binding was detected as follows: Protein L capture layer, rabbit polyclonal anti-c-myc reagents; mouse monoclonal anti-c-myc capture, rabbit polyclonal anti-c-myc reagents; rabbit polyclonal anti-c-myc capture, mouse monoclonal anti-c-myc reagents. After addition of OPD substrate, absorbance at 450 nm was recorded (y-axis). Test reactions are shown in blue. Controls lacked either the scFv ligand (purple) or the capture reagent (yellow).

Supernatants from induced cultures of the 14 clones chosen by capture ELISA and from an anti-thyroglobulin positive control were analysed by Western blotting. The samples were concentrated about 20-fold and after separation by SDS-PAGE, they were transferred to nitrocellulose membranes and probed with anti-c-myc reagents and diaminobenzidine substrate. Only clone F6 and the positive control showed reaction with a protein of about 30 kDa (data not shown).

To increase the sensitivity of detection, Western blotting was repeated and the reactions developed with a chemiluminescent substrate. As shown in Figure 3.22, there was convincing reaction of the anti-c-myc antibody with proteins of 30 kDa expressed by clones E9 and F6. The Figure also shows some evidence of low level production of scFv by clones A5, B2, B5, and C1. Overall, the results of ELISA and Western blotting suggested that most clones from the original library expressed scFv antibodies poorly.

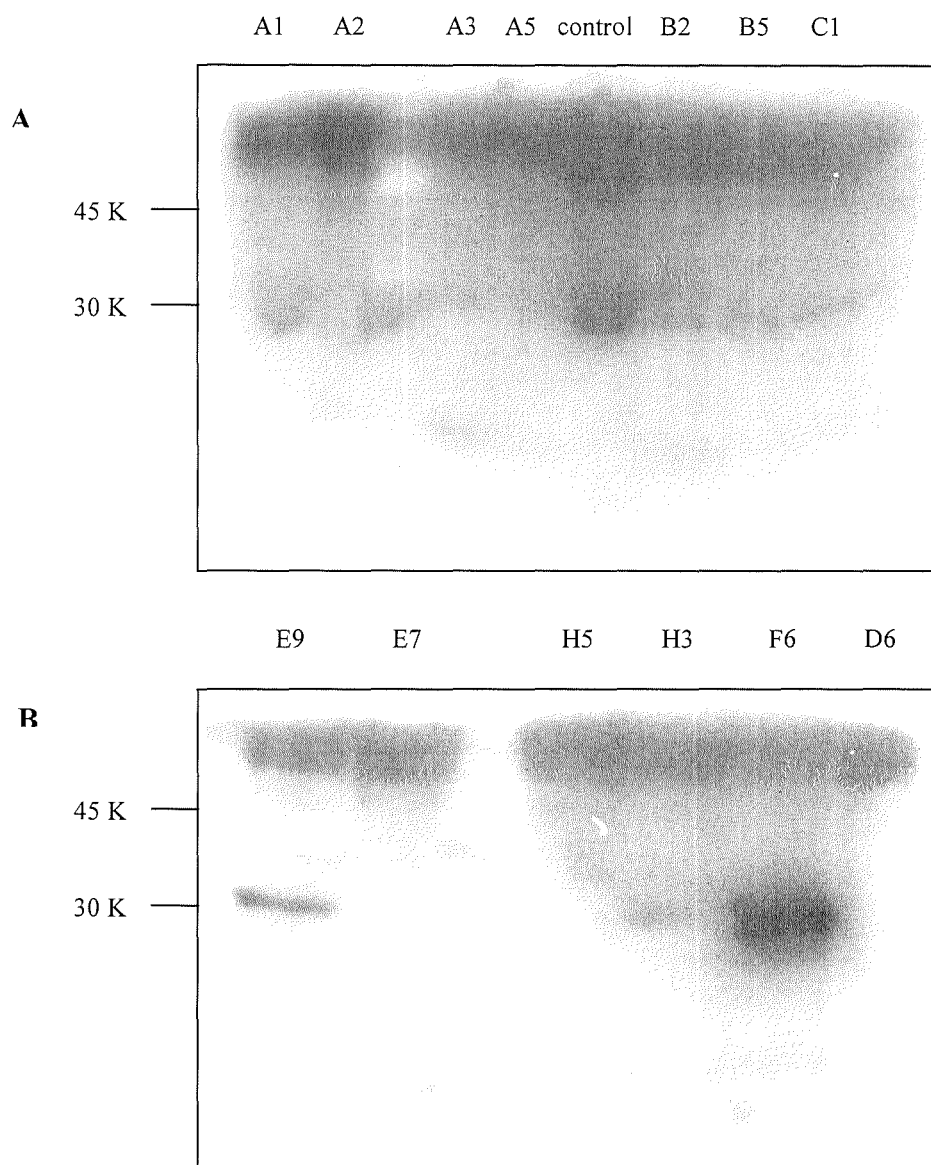
### **Final ELISA analysis**

To conclude experiments with the Griffin.1 library, a comparison was made between the reactivity of polyclonal, unselected phage antibodies prepared from the library on wells coated with K99 and skimmed milk. The assay was also conducted with scFv made from the library stock. The binding of phage antibodies was detected with a monoclonal anti-M13-HRP conjugate and the binding of scFv antibodies was detected with anti-c-myc reagents. The Figure 3.23 shows the results of this comparison. The assay showed low levels of reactivity with milk protein when phage or scFv were applied. Whilst the scFv reaction with K99 was somewhat lower than with milk, the phage ELISA generated a signal on K99 that was twice that on milk.



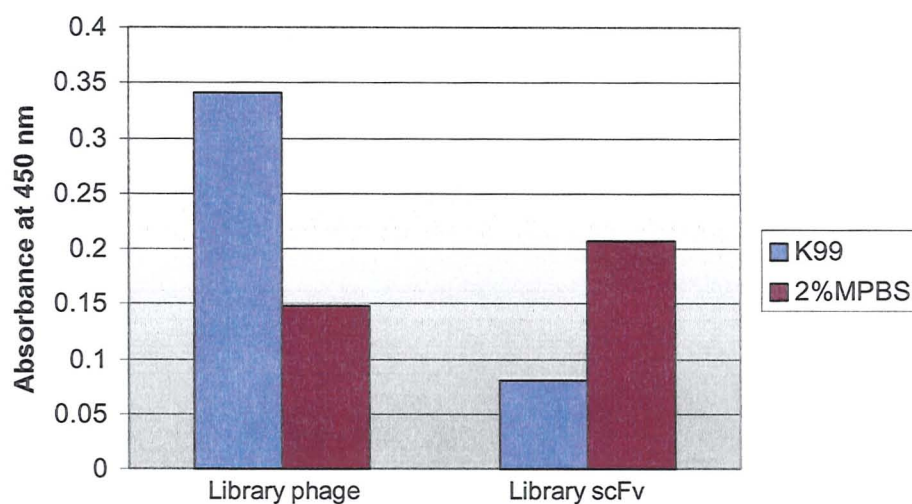
**Figure 3.21 PCR screening of 14 clones positive in a scFv capture ELISA**

Fourteen clones were chosen from screening the unselected Griffin.1 library in scFv capture ELISA. Crude bacterial lysates were prepared and used as templates in PCR with the primers LMB3 and gIII along with a full-length anti-thyroglobulin scFv as a positive control. Products were analysed by agarose gel electrophoresis and photographed. Clone numbers and products from the positive control are indicated, "M" showing loading of a 1 kb DNA ladder. The migration of markers of 506 bp and 1,016 bp is indicated, with the predicted size (1 kb) of a full-length scFv insert.



**Figure 3.22 Western blotting of 14 clones positive in a scFv capture ELISA**

Fourteen clones were chosen from screening the unselected Griffin.1 library in scFv capture ELISA. Phagemids were transfected to *E. coli* HB2151 and supernatants collected from cultures induced with IPTG. After SDS-PAGE separation, samples were transferred to nitrocellulose and probed with anti-c-myc reagents and chemiluminescent substrate. The image was captured to x-ray film. Clone designations are indicated with "Control" indicating loading of an anti-thyroglobulin scFv. The migration of marker proteins of 45 and 30 kDa is indicated to the left of each panel.



**Figure 3.23 Polyclonal phage and scFv ELISA with the Griffin.1 library**

Phage antibodies and scFvs were prepared from aliquots of the unselected Griffin.1 library. The antibodies were then applied to wells of ELISA plates coated with K99 (blue) and skimmed milk (purple). Binding of phage was detected with a monoclonal anti-M13-HRP conjugate; the binding of scFv antibodies was detected with anti-c-myc reagents. After development with OPD substrate, absorbance values were measured at 450 nm using a microplate reader.

## Discussion

Progress in antibody engineering has been directed toward the expression of antibody fragments in bacterial and phage display systems, leading to increase in application of these antibodies in research, biology, clinical diagnosis and therapy. The power of the phage display system to express antibody fragments offers several advantages over hybridoma technology. Making these antibodies is quick and cheap, the affinity can be increased and it is easy to manipulate them.

Many investigations have been done to study the applications of recombinant antibody technology for human or animal diseases. Most of these are focused on the study of tumors, viral and parasites diseases but less on bacterial diseases. Our aim was to choose a virulence factor from a well-known bacterial agent and to isolate small antibody fragments (scFvs) from a large synthetic single chain antibody library for further characterisation. Griffin.1 is a very large library of about  $10^9$  clones that was chosen for the study.

Initial attempts used a fimbriae extract containing K99 antigen for selection. After four rounds of selection, many phage antibodies were found to have higher activity with contaminant proteins rather than the fimbrial major subunit. Thus, this protein was purified by ion-exchange chromatography and its activity was checked by several tests. Before using the secondary stock of the library for selection, the frequency of full-length inserts was estimated by PCR and restriction enzyme analysis of clones chosen at random. The results indicated only about 1/3 selected clones had full-length insert. Similar findings with the Griffin.1 library have been reported (de Bruin *et al.*, 1999; de Greeff *et al.*, 2000) and have been discussed (<http://www.mrc-cpe.cam.ac.uk/~plw/phageqa.html>) by several investigators previously. De Bruin *et al.* (de Bruin *et al.*, 1999) showed that the percentage of phage with full-size inserts decreased at every cycle of enrichment. They also reported PCR and sequencing of clones from the final round of panning revealed that the only one from 100 phage antibodies carried a complete scFv insert, the majority lacking the V<sub>H</sub> component.

During the screening of clones isolated throughout four rounds of selection, we observed the number of positive clones rose from one round to the next but many positive clones had reaction with either K99 or with 2 % MPBS. Also the plasmid digestion of thirteen positive clones from round 4 with restriction enzymes, revealed only 4 clones were

contained full-length inserts, two clones had truncated inserts and the rest did not show any inserts. Similar results were observed for clones isolated from round 3 of selection that appeared to react with K99. Moreover when we repeated monoclonal phage ELISA, levels of different background reaction were seen each time. These results could be due to the quality of the library and using VCS-M13 helper phage. A well-known problem in phage display is the inefficient incorporation into the phage coat of phagemid-encoded scFv-pIII, compared with native pIII encoded by the helper phage genome. As a result the majority of phage do not contain scFv in their coat. In addition, phagemid libraries often contain many clones that do not express a scFv-pIII fusion, due to stop codons and frame shifts introduced during library construction. Unwanted enrichment of such aberrant phagemids may result during amplification steps, caused by enhanced growth rates of bacteria not expressing scFv-pIII. As a consequence, rescue of phages using conventional helper phage like VCSM13 generally results in a high frequency (up to 99.5%) of phages not displaying the protein of interest (Azzazy, 2002). This could explain why the enrichment rate of specific antibodies appeared to decrease from round 3 to round 4. To overcome this problem with VCS-M13 helper phage, several strategies have been attempted by investigators such as making KM13 helper phage (Kristensen and Winter, 1998), which has a proteolytic cleavage site, and construction of CT helper phage (Kramer *et al.*, 2003). Rescue of a library with these novel helper phage yields virus that are only infectious when they contain a phagemid-encoded pIII-fusion protein (Kramer *et al.*, 2003) using VCS-M13 and CT helper phage in three experiments showed 28% of the clones infected with VCSM13-rescued phages produced scFv, whereas 84% of CT-rescued phages produced scFv. Also ELISA analysis of scFv incorporation in rescued phages showed about 8-fold higher absorbance when CT helper phage was used instead of VCSM13.

After choosing clones from round 4 that had higher reaction than most in monoclonal phage ELISA, attempts were made to express soluble scFv fragments in the non-suppressor *E. coli* strain HB2151. This was not successful. Working on the idea that this was due to low level of expression and inefficient detection, inserts from the chosen clones were transferred into pIMS147 expression vector and transformed into *E. coli* XL-1 blue (Strachan *et al.*, 2002). This strategy was also unsuccessful. Moreover, changing expression conditions (Kipriyanov *et al.*, 1997; Kipriyanov, 2002) like temperature, the concentration of IPTG and addition of sucrose did not affect the expression level. In contrast, the expression of these clones as scFv-pIII fusion proteins (Mersmann *et al.*, 1998) in TG1 cells, showed activity with K99 in ELISA and expression was confirmed by Western blotting. To find the reason for the failure to express soluble scFvs, the DNA sequences of these clones and others from round 4 were determined. The results revealed

all of these clones had at least one stop codon. These stop codons were located either in CDRs or frameworks. Since the Griffin.1 library was constructed from random residues in CDRs, it was natural to find some clones containing TAG stop codons in these positions. These clones can express phage antibodies in suppressor *E. coli* strains like TG1 but could not express scFv antibodies in non-suppressor strains. Unexpectedly, we found some clones had stop codons in their frameworks. This was observed particularly in one position of framework two of heavy chain in several clones. We can explain that the above problem could happen during construction of the library when the frameworks were isolated and amplified by PCR.

By comparing the sequences of clones from the secondary stock of library, from round three and from round four of selection, it was observed that the number of clones containing stop codons rose. This indicates that these clones were amplified during selection.

Following screening of many clones from round 4 and round 3 that had higher reaction with K99 in monoclonal phage ELISA, but failed to express soluble scFv fragments, a sensitive and specific method to detect the expression of soluble scFvs was devised for large scale screening. Using a positive control clone, an ELISA method was established that was sensitive for estimation of expression of scFv antibodies but also is probably sensitive for estimation of expression of any c-myc tag recombinant protein. Using this technique, 94 clones from round 4 and 94 clones from round 3 were screened but no reaction was seen. When 94 clones from the secondary stock of library were tested to assess the ability of the library to express soluble fragments, different levels of expression were discovered in ELISA. Further analysis by PCR and western blotting of fourteen clones with higher signals in above ELISA revealed that most of these clones contained full-length inserts but except for two clones, the isolates were unable to express soluble scFv antibodies at high levels. This result supports the sensitivity of the ELISA method for estimation of expression of soluble scFvs and also indicates the expression of these recombinant proteins depends upon other factors. It is known that the expression of recombinant antibodies can vary widely on a case by case basis. For example, some antibody frameworks (like VH<sub>3</sub> family) have better expression levels than the majority. Proper folding of antibody within the bacterial periplasm has effect on levels of active antibody (Knappik *et al.*, 1993; Bothmann and Pluckthun, 1998). Some recombinant antibodies are toxic to their host cells (Krebber *et al.*, 1996). Growth temperature (Somerville *et al.*, 1994; Kipriyanov *et al.*, 1997), addition of osmotic stress reagents (Kipriyanov *et al.*, 1997), strength of promoter (Clark *et al.*, 1997) and the concentration of



IPTG and the time of growth culture (Kipriyanov *et al.*, 1997) may have an effect on the expression of recombinant proteins. Moreover, O'Brien *et al.* showed the *E. coli* strain can also affect the level of expression of soluble Fab fragments (O'Brien *et al.*, 2002). Therefore the variation of expression level of clones of library could be the result of some of the above factors. Since these clones were chosen from the library before selection, the effect of each factor upon expression of scFv was not investigated.

The overall activity of unselected phage from the library and scFv product was tested with K99 and BSA (as comparison antigen) in an ELISA experiment. This experiment clearly revealed that although unselected phage antibodies in the library have higher activity with K99 than 2% MPBS, the reaction of population of soluble scFvs with K99 is much lower than the reaction with 2% MPBS. Thus, this result indicated the chance of finding high affinity soluble scFv antibodies against K99 antigen with this library was very low. Also, the experience of using this library with two other antigens in our lab had similar results (unpublished data).

Despite these results and the problems that were encountered with the library during these experiments, it may be still possible to use the library for the isolation of single chain antibodies against some antigens as a few previous reports have shown. It is possible that helper phage like KM13 will assist the isolation of phage antibodies with complete inserts at each round of selection. The success of Strachan *et al.* (Strachan *et al.*, 2002) for isolating anti-hapten scFvs from the Griffin.1 library shows that this should be possible. Moreover, the polyclonal phage ELISA and polyclonal scFv ELISA will help quick review of the selection procedure. One important lesson learned from use of the Griffin.1 library was the value of screening the ability of any antibody display library to produce soluble scFv before starting selection.

## **Chapter 4**

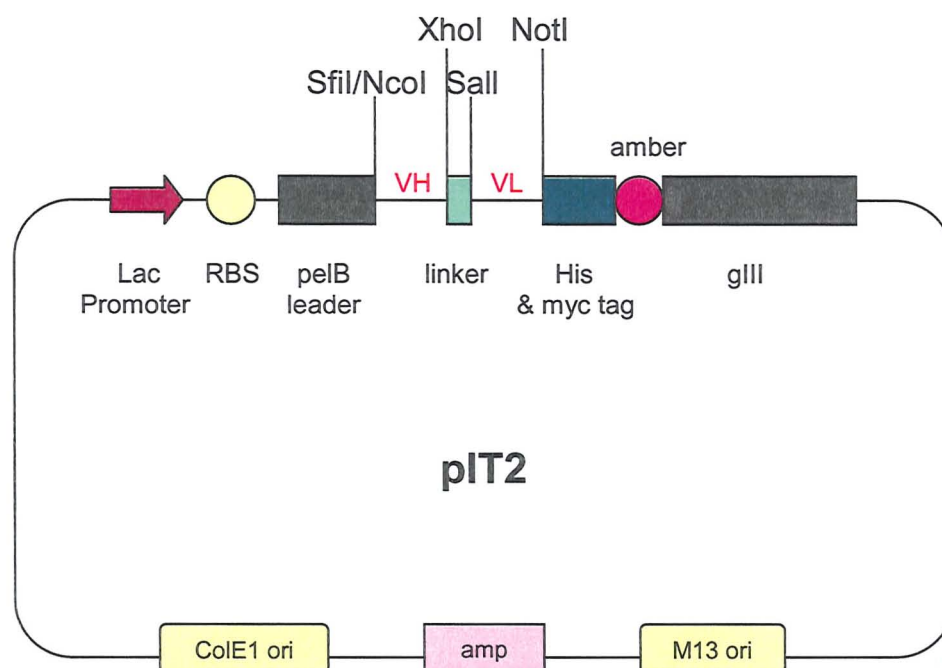
### **Tomlinson scFv libraries**

## 4. Tomlinson scFv libraries

### Introduction

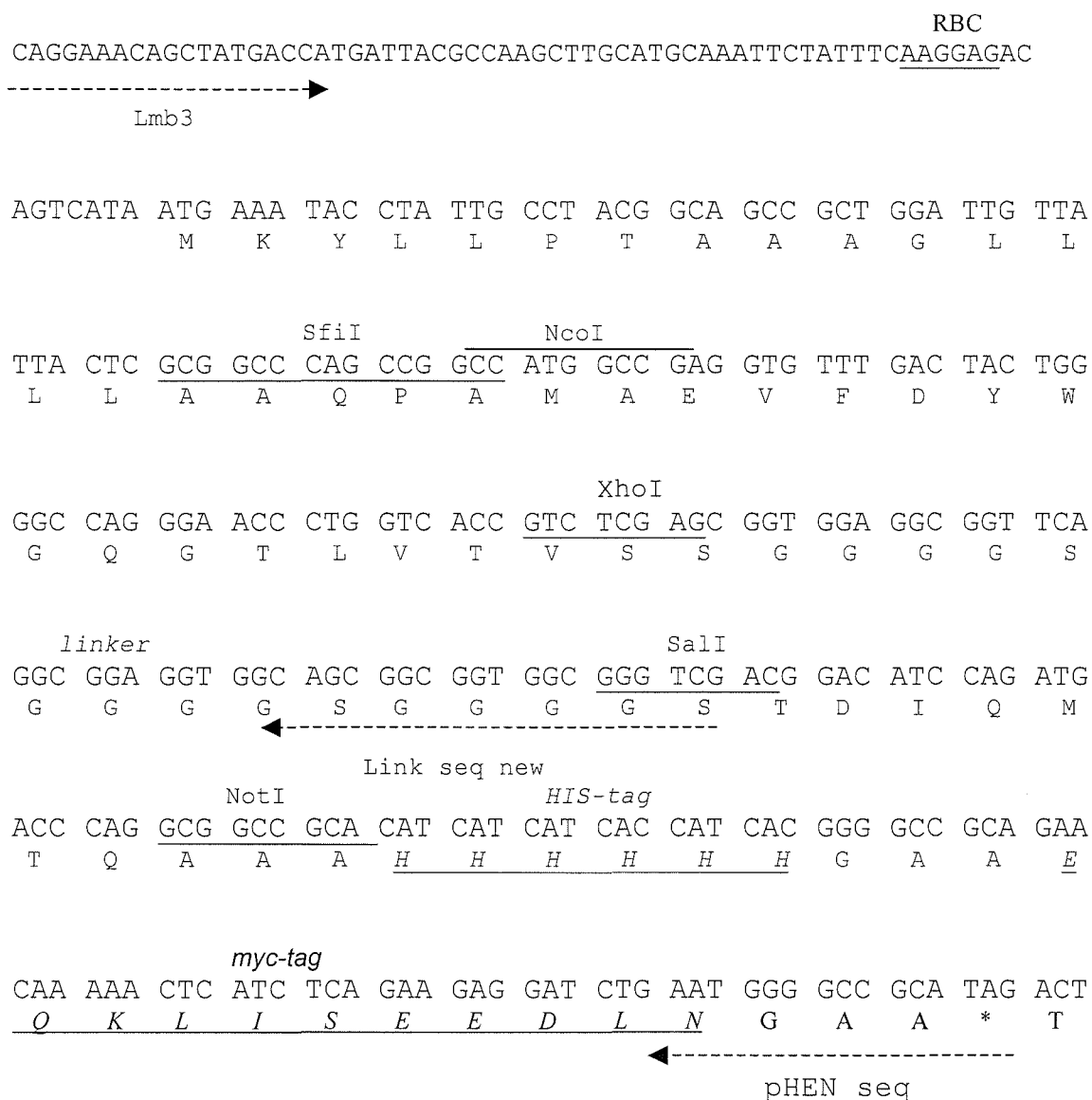
The Tomlinson I and J libraries are the latest semi-synthetic libraries to be distributed by the MRC. Each library comprises over 100 million different scFv fragments cloned in an ampicillin resistant phagemid vector, pIT2 (Figures 4.1, 4.2) which is derived from pHEN1 (Hoogenboom *et al.*, 1991). The libraries are distributed in *E. coli* TG1. The scFv is conventional, consisting of a single polypeptide with the V<sub>H</sub> and V<sub>L</sub> domains attached to one another by a flexible Glycine-Serine linker. Using these libraries, specific binders to target molecules can be selected by normal panning methods. Typically two or three rounds of selection are required to ensure that more than half of the scFvs in selected population bind to the target molecule. The monoclonal scFvs can then be screened for binding and then used for further analysis of the target molecule. All the functional scFvs in the Tomlinson I and J libraries can bind Protein A (Protein A from *Staphylococcus aureus* has five domains that mediate interaction with the Fc region of most mammalian IgGs. Protein A also binds to the Fab region of a subset of Ig with heavy chains belonging to the V<sub>H</sub>III family) (Harboe and Folling, 1974) through the V<sub>H</sub> domain (Sasso *et al.*, 1989; Sasso *et al.*, 1991) and Protein L through the V<sub>κ</sub> domain (Bjorck, 1988). This allows either of these secondary reagents to be used for detection, purification or immobilisation. Alternatively, secondary reagents that bind the attached myc or histidine tags can be used.

Both libraries are constructed on single human frameworks for the V<sub>H</sub> (V3-23/DP-47 and JH4b) and V<sub>κ</sub> domains (O12/O2/DPK9 and Jκ1). The canonical structure (V<sub>H</sub>: 1-3, V<sub>κ</sub>: 2-1-1) encoded by the chosen frameworks are by far the most common in the human antibody repertoire (de Wildt *et al.*, 1999). Diversity is incorporated at positions that make contacts to antigen in known structures and are known to be highly diverse in the mature antibody repertoire. This comprises a total of 18 residues – H50, H52, H52a, H53, H55, H56, H58, H95, H96, H97, H98, L50, L53, L91, L92, L93, L94 and L96. The CDR3 of the heavy chain was designed to be as short as possible yet still able to form an antigen binding surface. In library I, diversity is created through incorporation of DVT (D: A, G or T; V: A, C or G) codons at these positions. In library J, NNK (N: any base; K G or T) codons is used instead. Both libraries are constructed in the vector pIT2 which provides HIS and myc tags (Figures 4.1 and 4.2). After selection, scFvs can be matured by incorporating additional diversity based on somatic mutation. Both libraries contain about



**Figure 4.1 Structure of phagemid pIT2.**

The main features of the vector are indicated. Transcription takes place from a *lac* promoter and in a suppressor strain of *E. coli*, the translated product comprises a PelB leader, the single-chain antibody created by insertion of VH and VL sequences at the sites indicated separated by a linker, peptide tags, and the phage pIII protein.



**Figure 4.2 DNA sequence of pIT2 vector**

The Figure presents part of the DNA sequence of the pIT2 vector. The restriction enzyme sites are underlined. The VH sequence is located between *NcoI* and *XhoI* sites and the VL sequence is located between *SalI* and *NotI* sites. Primers are shown by dotted arrows. The amber stop codon is presented as a star. The amino acid sequences of HIS and myc tags are underlined.

$1.4 \times 10^8$  clones and it is estimated that 96% of clones in library I contain an insert whereas the value is somewhat lower (88%) for library J.

### ***KM13 helper phage***

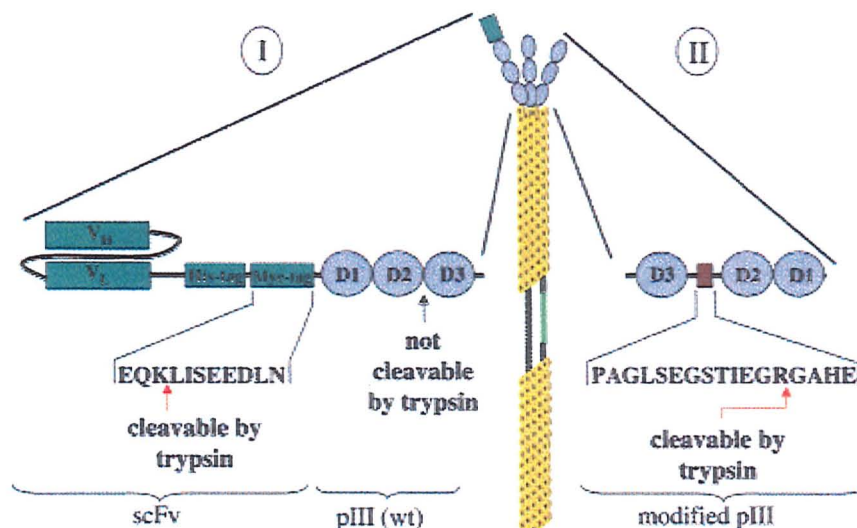
The KM13 helper phage was created by Kristensen and Winter (Kristensen and Winter, 1998) to overcome problems inherent to use of other helper systems. In the modified helper phage, a trypsin cleavage site was introduced into the pIII gene between the coding regions for the second and third domains of the protein (indicated as II in Figure 4.3). While it is possible to attach sequence to the amino terminus of pIII or even to insert peptides between domain boundaries without abolishing infectivity (Smith, 1985; Endemann et al., 1992; Krebber et al., 1997b), all three domains of pIII are essential for the successful infection of bacteria (Riechmann and Holliger, 1997). Hence, trypsin treatment of phage destroys the participation of helper phage-derived pIII in bacterial infection. In contrast, the phagemid-encoded gene for pIII to which the scFv for display is attached, is wild-type and is insensitive to trypsin (indicated as I in Figure 4.3). Protease treatment therefore leaves intact the ability of this protein to mediate infection of bacteria. By placing a trypsin-sensitive site between the scFv and pIII, Kristensen and Winter broadened the utility of protease treatment to include an efficient elution method. Since the method was published, proteolytic selection has been employed for other kinds of selection (Demartis *et al.*, 1999; de Wildt *et al.*, 2000; Riechmann and Winter, 2000; Goletz *et al.*, 2002).

### ***Application of Tomlinson I and J libraries***

These scFv libraries can be used to derive binders to almost any target molecule using phage display and selection. These binders can be used for all the same applications as conventional monoclonal antibodies like ELISA, Western blotting, FACS, immunochemistry *etc.* The sections that follow illustrate some of the investigations carried out using these libraries.

#### **Human fibrin clots**

A scFv antibody was developed against human fibrin clots by using the Tomlinson libraries. One purified soluble scFv was verified by Western blot analysis. It specifically recognized human fibrin clots but showed no binding to human fibrinogen in ELISA



**Figure 4.3 The proteolytic selection principle.**

The Figure shows an scFv-phage from a phagemid library prepared using KM13 as helper phage. During packaging and rescue of the library, the pIII from the helper phage (II) competes with the scFv-pIII fusion protein (I) for incorporation into phage particles. The helper phage KM13 encodes a modified pIII with an additional trypsin-sensitive site between domains D2 and D3 (II). All three domains of pIII are essential for phage infectivity hence trypsin treatment inactivates the function of these proteins. The trypsin cleavage site in the myc tag of the scFv-pIII (wt) fusion protein allows the proteolytic elution of an infective phage from its antigen but in the absence of other trypsin-sensitive sites, the infective function of this form of pIII is not lost after protease treatment. The Figure has taken from (Kristensen and Winter, 1998).

analysis. The potential of this scFv for the diagnosis and therapy of thrombus-correlated diseases is being explored (Jun Peng Yan, 2004).

### **Antiidiotypic antibodies as immunogens**

Antiidiotypic antibodies that mimic carbohydrate or conformational epitopes (Ab2 $\beta$ ) are of considerable interest as surrogate immunogens for cancer vaccination. To investigate this area, scFvs from the Tomlinson libraries were isolated against an idiotypic antibody (Goletz *et al.*, 2002). Elution of binders was accomplished with the original antigen followed by trypsin treatment of the recovered phages before rescue with the protease sensitive helper phage KM13. These methods were compared with various conventional selection and elution methods and the diversity of the scFv clones was determined.

### **Antibody arrays**

The Tomlinson I and J libraries have also been used to develop a novel technique for high-throughput screening of recombinant antibodies, based on the creation of antibody arrays. Using robotic picking and high-density gridding of bacteria containing antibody genes followed by filter-based ELISA screening, clones were identified that expressed scFvs of interest. The investigators applied this technique in several different applications, including isolating antibodies against impure proteins and complex antigens, where several rounds of phage display often fail (de Wildt *et al.*, 2000).

### **Protein arrays**

To identify highly specific antibody–antigen interactions by protein array screening, 12 well-expressed antibody fragments were chosen from the Tomlinson I and J libraries. After capturing the scFv antibodies, their interaction with 27 648 human foetal brain proteins arrayed on a PVDF membrane was tested. The authors suggested that this ‘naive’ screening approach could be applied to the high throughput isolation of specific antibodies against many different targets in the human proteome (Holt *et al.*, 2000).

### **Bovine complex I**

The Tomlinson I and J libraries have also been used to isolate an antibody that binds to a specific 51kDa subunit of complex I, a macromolecular assembly. Antibodies that bound



to the intact complex were selected and screened with a high-density Western blot to identify a subunit-specific binder. Conventional Western blotting and competition ELISA were then used to confirm the identity of the target subunit and that the antibody bound to the native protein complex and not to an epitope that is only revealed when the antibody is immobilized for phage selection (Rubinstein *et al.*, 2003).

## **Objectives**

Since the results of previous chapter did not fulfil our aims, the Tomlinson libraries of I and J were chosen. It was planned to use these two semi-synthetic libraries for isolation of recombinant single-chain antibodies against purified K99 major subunit. Then we wanted to characterise the isolated antibodies and assess the biological activities of them and then find out whether these antibodies have the ability to neutralise the attachment of B41 *E. coli* strain to villi *in vitro*.

## Materials and methods

### ***Tomlinson library I and library J***

The human synthetic scFv libraries of Tomlinson I + J (MRC, Centre for Protein Engineering, Cambridge, UK.) were used to select antibodies against purified K99 fimbriae. The materials supplied comprised 500 µl of library I and 500 µl of library J in TG1 *E. coli* cells, glycerol stocks of positive control scFvs against BSA and ubiquitin in bacterial strain TG1, a glycerol stock of T-phage resistant *E. coli* TG1 for propagation of phage (K12  $\Delta(lac-proAB)$  *supE thi hsdD5/F' traD36 proA<sup>+</sup>B lacIq lacZ $\Delta$ M15*), a glycerol stock of *E. coli* HB2151 for expression of antibody fragments (K12 *ara*  $\Delta(lac-proAB)$  *thi/F' proA<sup>+</sup>B lacIq lacZ $\Delta$ M15*) and 100 µl of phage KM13 at 10<sup>7</sup> pfu/ml. All materials were stored frozen. Before screening, some initial preparations were carried out.

### ***Production of large quantities of KM13 helper phage***

To make a stock of KM13 helper phage, one colony of TG1 from a plate of minimal medium was grown overnight in 5ml of 2xTY containing 100 µg/ml ampicillin and 1% glucose at 37°C with shaking at 200 rpm. 500 µl of this culture was inoculated into 50ml of 2xTY and was grown until OD at 600 nm was 0.3-0.4. Then 200 µl aliquots of the culture were infected with 10 µl of 10x serial dilutions of KM13 helper phage and incubated at 37 °C in a water bath for 30 min. 3ml of H-top agar (Tryptone 10g, NaCl 8g, Agar 6g and distilled water up to 1 litre) warmed at 42 °C was added to each tube and after mixing, poured onto warm TYE plates. The plates were allowed to set and then incubated overnight at 37 °C. Next day a single small plaque was picked into 5 ml of fresh TG1 at an OD 600 nm of 0.4, grown for 2hr with shaking at 37 °C and then added to 500ml of 2xTY in a 2 litre flask to grow with shaking at 37 °C for 1hr. After that kanamycin was added to a final concentration of 50 µg/ml and the culture was grown overnight with shaking at 30 °C. The overnight culture was centrifuged at 10800g for 15 minutes and 100ml PEG/NaCl (20 % polyethylene glycol 6000, 2.5 M NaCl) was added to 400 ml of supernatant. After 1 hr incubation on ice, the mixture was spun at 10800g for 30 min. The pellet of precipitated phage was resuspended in 8 ml of PBS and 2ml of PEG/NaCl. After mixing and incubation for 20 minutes on ice, the mixture was spun at 11600g for 10 min in a micro centrifuge to remove any remaining bacterial debris. The supernatant containing prepared phage was then stored at -20 °C.

### ***Titration of KM13 helper phage stock***

To titre the helper phage, 5  $\mu$ l of trypsin stock solution was added to 45  $\mu$ l of phage and incubated for 30 min at 37 °C. 1  $\mu$ l of trypsin treated phage was diluted into 1ml of PBS and five 100 fold serial dilutions prepared in 1ml aliquots of PBS. Then 50  $\mu$ l from each dilution was added to separate tubes containing 1 ml of TG1 at an OD 600 nm of 0.4. After mixing, 3 ml of molten H-Top agar was added to each tube and poured evenly onto TYE plates. Dilutions were also prepared using 1  $\mu$ l of non-trypsin treated phage. These were processed as described for trypsin treated phage. The difference between titres of trypsin treated and trypsin non-treated phage was calculated.

### ***Growing the libraries***

Library stocks of Tomlinson I and Tomlinson J (500  $\mu$ l) were added to 200 ml of pre-warmed 2xTY containing 100  $\mu$ g/ml ampicillin and 1% glucose and grown with shaking at 37 °C until the OD at 600 nm was 0.4. 150 ml of these cultures were used for making secondary stocks and the remaining cultures were infected with  $2 \times 10^{11}$  (1.88  $\mu$ l) pfu of KM13 helper phage. Cultures were incubated without shaking in a 37 °C water bath for 30 minutes, spun at 3300g for 10 minutes and the bacterial pellets resuspended in 100 ml of 2xTY containing 100  $\mu$ g/ml ampicillin, 50  $\mu$ g/ml kanamycin and 0.1% glucose. The suspensions were incubated overnight with shaking at 30 °C. The next day, after spinning at 3300g for 30 minutes, 20 ml PEG/NaCl was added to 80 ml of each supernatant. The mixtures were incubated for an hour on ice, spun at 3300g for 30 minutes and the pellets resuspended in 4ml PBS. After a further spin at 11600g for 10 minutes, the supernatants containing prepared phage libraries were stored at 4 °C.

### ***Titration of phage libraries***

To titre the library phage stocks, 1  $\mu$ l of each phage library was diluted into 100  $\mu$ l PBS. 1  $\mu$ l of was withdrawn and diluted in a further 100  $\mu$ l PBS and so on until there were 6 dilutions in total. 900  $\mu$ l of TG1 at an OD 600 nm of 0.4 was added to each tube and incubated at 37 °C in a water bath for 30 minutes. 10  $\mu$ l of each dilution was spread onto a TYE plate containing 100  $\mu$ g/ml ampicillin and 1% glucose and grown overnight at 37 °C.

## ***Growing TG1 and HB2151 cells***

To generate secondary stocks of TG1 and HB2151, about 10 µl of each strain was withdrawn from the glycerol suspensions and streaked onto minimal agar plates for overnight growth at 37 °C. One colony from each plate was grown on in 5 ml of 2xTY overnight with shaking at 37 °C. The following day, the cultures were centrifuged at 3300g for 10 minutes and pellets resuspended in 1 ml 2xTY containing 15 % glycerol. Secondary stocks were then stored at -80 °C. Also minimal media agar (di-Sodium Hydrogen orthophosphate 6g, Potassium dihydrogen orthophosphate 3g, NaCl 0.5g, Ammonium Chloride 1g, Agar 15g and distilled water up to 1 litre) plates of these cells were stored at 4 °C for future use.

## ***Initial experiments before selection***

Before using the libraries for selection of phage antibodies, some preliminary experiments were performed.

## ***Activity of purified K99 at low concentration***

In order to check that purified K99 would retain integrity at the low concentrations to be used for selection in immunotubes (Nunc, Denmark), duplicate wells of an ELISA plate (Nunc, Denmark) were coated overnight at room temperature with K99 diluted to 20, 10, 5, 2.5, 1.25, 0.625, 0.312 µg/ml. As a negative control, two wells were coated with PBS. The wells were blocked with 3% BSA/PBS for 2 hr at 37 °C and then probed with 100µl of 1:1000 dilution of anti- K99 mouse monoclonal antibody (Biogenesis, UK) in blocking buffer for 90 minutes at 37 °C. After probing with an anti-mouse peroxidase conjugate at 1:1000 dilutions, the reaction was developed with 100 µl of substrate solution (1 mg/ml OPD in 0.1 M citrate buffer pH 4.5 and 0.012% (v/v) H<sub>2</sub>O<sub>2</sub>) and stopped after 10 minutes with 50 µl of 1M sulphuric acid. The absorbance was measured at 492 nm.

## ***Growing positive controls***

To generate positive control scFvs for future experiments, TG1 cells carrying anti-BSA and anti-ubiquitin antibodies were grown on TYE plates containing 100 µg/ml ampicillin and 1% glucose overnight at 37 °C in an incubator. A single colony from each plate was grown in 5 ml of 2xTY containing 100 µg/ml ampicillin and 1% glucose

overnight at 37 °C with 200 rpm shaking. Plasmid DNA was isolated from 3ml of these cultures using a Qiagen miniprep kit. Plasmid purification was done as recommended by the manufacturer. 2 µl of each purified plasmid was transformed into 100 µl of competent HB2151 (K12 *ara*  $\Delta(lac-proAB)$  *Thi/F'* *proA<sup>+</sup>B* *lacIq* *lacZ* $\Delta$ M15) using standard protocols (Sambrook *et al.*, 1989). Transformants were plated onto TYE plates containing 100 µg/ml ampicillin and 1% glucose and grown overnight at 37 °C.

### ***Expression soluble positive control scFvs***

For expression of soluble anti-BSA and anti-ubiquitin scFvs, single colonies of HB2151 cells carrying the respective plasmids were grown in 5 ml of 2xTY containing 100 µg/ml ampicillin and 1% glucose overnight at 37 °C with shaking (200 rpm). 1ml of these cultures were added to 100 ml of fresh 2xTY containing 100 µg/ml ampicillin and 0.1% glucose. The cultures were grown with shaking (200 rpm) at 37 °C until the OD 600nm was approximately 0.8-0.9. IPTG (Roche, Germany) was added to a final concentration of 1mM and incubation continued with shaking (200 rpm) at 30 °C for about 20 hr. The cultures were centrifuged at 5200g for 20 min and the supernatants concentrated approximately 20 times, with simultaneous exchanged of buffer to PBS using Amicon Ultra-4 centrifugal filters with a 10 kDa cut-off (Millipore, UK). The filtrates were then stored at -20 °C for future use.

### ***Activity of anti-BSA and anti-ubiquitin scFvs with Protein L-HRP, LA-HRP and anti-c-myc antibody***

In order to optimise a high sensitivity detection method with for checking expression of scFvs, three equally concentrated culture supernatants of anti-BSA and anti-ubiquitin scFvs were run on an SDS-PAGE gel and transferred to a nitrocellulose membrane (Amersham, UK) at 100v for 1hr. After blocking the membrane with 3% MPBS for 1hr at room temperature, the membrane was cut into three parts. To the first, recombinant Protein LA-HRP which is a combined L and A protein with the binding characteristics of both, conjugated to HRP (Actigen, Norway), was added at 1:1000 dilutions. The second strip was probed with recombinant Protein L-HRP (Actigen, Norway); the third part was incubated with anti-c-myc mouse monoclonal antibody (Sigma, UK) at 1:3000 dilution in 2% MPBS for 90 min at 37 °C with 200 rpm shaking. A secondary rabbit anti-mouse (Sigma, UK) at 1:1000 dilutions in 2%MPBS was added to the third strip only to detect binding of the mouse monoclonal antibody; this was incubated at 37 °C with shaking for an

hour. Finally, all complexes were developed with 4-chloro-1-naphthol substrate (Sigma, UK).

### ***PCR screening for full-length scFv genes***

It is common that recombinant scFv antibody libraries contain some clones containing only the  $V_H$  or the  $V_L$  components or totally lacking inserts. Any truncated scFvs able to react with a target antigen can then be isolated by selection and may come to dominate the population of recovered phage if they have marginally faster replication times or other selective advantages. To assess the presence of truncated inserts in the Tomlinson library I and Tomlinson library J, 21 colonies were picked at random from TYE plates used to titre each library. A colony carrying the anti-ubiquitin scFv was also taken as a positive control. Bacterial DNA was obtained by suspending each colony in 50  $\mu$ l of  $H_2O$  and boiling at 100 °C for 10 minutes. Primers LMB3 and pHEN seq (Tables 4.1 and 4.2) were used to amplify across the inserts in 25- $\mu$ l PCR reactions containing 0.5  $\mu$ M concentrations of each primer, 0.25 mM concentrations of each dNTP (Promega), 4mM  $MgCl_2$ , 1x reaction buffer (containing 50 mM KCL, 10 mM Tris-HCL pH 9.0 and 0.1% Triton®X-100), 2.5 units of Taq DNA polymerase (Promega) and 2  $\mu$ l of bacterial lysate. Samples were amplified on a Techne thermal cycler under the following conditions: the reaction was heated to 94 °C for 3 min and then 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 55 °C, 2 min extension at 72 °C. Final extension was for 10 min at 72 °C.

PCR products were analysed on a 1% agarose gel for 45 min at 100 V, stained with ethidium bromide, and photographed under UV light.

**Table 4.1 List of primers used for characterisation of scFv genes in pIT2 vector**

Primers	Sequences from 5' to 3'
LMB 3:	CAGGAAACAGCTATGAC
Link seq new:	CGACCCGCCACCGCCCGCTG
DPK9 FR1 seq:	CATCTGTAGGAGACAGAGTC
pHEN seq:	CTATGCGGCCCCATTCA
g III:	CCCTCATAGTTAGCGTAACG
pelB:	ATGAAATACCTATTGCCTACGGCAGC

**Table 4.2 Expected sizes of PCR products using different primers**

<b>Primers</b>	<b>Isolation of fragment(s)</b>	<b>Expected size (bp)</b>
LMB3—link seq new	V <sub>H</sub> only	with insert = 527 without insert = 227
DPK9 FR1 seq—pHEN seq	V <sub>K</sub> only	with insert = 368 Without insert = no band
LMB3—pHEN seq	V <sub>H</sub> and V <sub>K</sub>	with insert = 935 without insert = 329
pelB—pHEN seq	V <sub>H</sub> and V <sub>K</sub>	with insert = 885 without insert = 279
gIII—LMB3	V <sub>H</sub> and V <sub>K</sub>	with insert = 1035 without insert = 429
gIII—pelB	V <sub>H</sub> and V <sub>K</sub>	with insert = 985 without insert = 379
pelB—link seq new	V <sub>H</sub> only	with insert = 477 without insert = 177
DPK9 FR1 seq—gIII	V <sub>K</sub> only	with insert = 468 without insert = no band



## ***Selection procedure***

### ***Panning Tomlinson library I and library J against purified K99***

#### **First round of selection**

Two immunotubes (Nunc, Denmark) were coated overnight at room temperature with 4 ml of purified K99 at 20 mg/ml in PBS. The following day, the immunotubes were washed 3 times with PBS by pouring into the tubes and pouring out again immediately to remove unbound antigen. The immunotubes were blocked by filling with 2% MPBS and incubating at room temperature for 2 hrs. Immunotubes were washed again 3 times with PBS. After that,  $1.28 \times 10^{13}$  phage of library J were added in 4 ml of 2% MPBS to one immunotube and approximately  $1.28 \times 10^{13}$  phage of library I in 4 ml of 2% MPBS were added to a second. To block phage that might be specific for milk constituents, the phage suspensions were incubated for about 30 minutes at room temperature before addition to the K99-coated immunotubes. The tubes were incubated for 60 minutes at room temperature with continuous rotation, and then allowed to stand for a further 60 minutes at room temperature. The supernatants were discarded and the tubes were washed 10 times with PBS containing 0.1 % Tween 20. After shaking out the excess PBS, phage were eluted by adding 500  $\mu$ l of trypsin-PBS solution and rotating for 10 minutes at room temperature. The trypsin solution was prepared by sampling 50  $\mu$ l of a stock solution prepared at 10mg/ml (Fluka, UK) in 50mM Tris-HCl pH 7.4, 1mM  $\text{CaCl}_2$  and adding this to 450  $\mu$ l PBS.

#### **Infecting TG1 cell with eluted phage antibodies**

The day before selection, one colony of *E. coli* TG1 was taken from a minimal medium plate and grown in 5 ml of 2xTY overnight at 37 °C with shaking at 200 rpm. On the day of selection, 50 $\mu$ l of this culture was inoculated to 50 ml of 2xTY and incubated at 37 °C with shaking until the culture entered exponential growth and the OD 600nm was 0.45. Then 250  $\mu$ l of the eluted phage from each immunotube was added to 1.75 ml of exponentially growing TG1 cells and incubated at 37 °C in a water bath for 30 minutes. 10 $\mu$ l of infected TG1 cells, 10  $\mu$ l of a 1 in  $10^2$  dilution and 10  $\mu$ l of a 1 in  $10^4$  dilution were spotted on TYE plates containing 100  $\mu$ g/ml ampicillin and 1% glucose and grown overnight at 30°C to titre the phage. The remaining 250  $\mu$ l of phage were stored at 4 °C for later use. The following day, after calculating the output of phage from the first round of

selection from the number of colonies on titration plates and first round titre, these plates were stored at 4 °C for future analysis of first round phage antibodies

The remaining cultures of infected TG1 were spun in a microcentrifuge at 11600 g for 5 minutes. The pelleted bacteria were resuspended in 50 µl of 2xTY and plated on two TYE plates containing 100 µg/ml ampicillin and 1% glucose and grown overnight at 30 °C. Colonies were then scraped from the plates with a glass spreader and inoculated into 2 ml of 2xTY containing 100 µg/ml ampicillin and 1% glucose. Care was taken to keep the I and J libraries separate throughout. One ml of these bacteria were stored at -80 °C in 15% glycerol and 50 µl of the remaining bacteria were used in phage rescue for the next round of selection.

### **Rescue of selected phage antibodies**

50 µl of bacteria from each library from the first round of selection were inoculated to 50 ml of 2xTY containing 100 µg/ml ampicillin and 1% glucose and grown at 37 °C with shaking at 200 rpm until OD 600 nm was approximately 0.4. At this OD,  $5 \times 10^{10}$  pfu of KM13 helper phage were added to 10 ml of culture and incubated without shaking at 37 °C in a water bath for 30 minutes. The cultures were then centrifuged at 3000g for 10 minutes. The pelleted bacteria were resuspended in 50 ml of 2xTY containing 100 µg/ml ampicillin, 50 µg/ml kanamycin and 0.1% glucose and incubated shaking at 30 °C overnight. The following day, overnight cultures were spun at 3300g for 25 minutes.

To precipitate phage from the culture supernatants, 10 ml of cold PEG/NaCl (20% polyethylene glycol 6000, 2.5 M NaCl) was added to 40 ml of each supernatant. After mixing well, the mixtures were left for 1 hr on ice and then centrifuged at 3300g for 30 minutes. Supernatants were poured away to remove PEG/NaCl and the precipitated phage were resuspended in 2 ml of PBS and spun at 11600g for 10 minutes in a micro centrifuge to remove remaining bacterial debris. 1ml of each rescued phage library (supernatant) was stored at 4 °C and remaining 1 ml was used for next round of selection.

### **Titration of rescued phage**

To assay the titre of phage rescued from the first round of selection and thereby determine the phage input for the second round of selection, 1 µl of rescued phage from each library was diluted into 100 µl PBS and the dilution repeated to generate six serial

100-fold dilutions in total. 900  $\mu$ l of TG1 at an OD 600 nm of about 0.4 was added to each tube and incubated at 37 °C for 30 minutes. 10  $\mu$ l of each dilution was spread onto a TYE plate containing 100  $\mu$ g/ml ampicillin and 1% glucose and grown overnight at 30 °C.

### **Second round of selection**

In the second round of selection, two immunotubes (Nunc, Denmark) were coated overnight at room temperature with a lower concentration of purified K99 (10  $\mu$ g/ml) to isolate phage antibodies with higher specificity and affinity as were done previously by some other investigators (Shadidi and Sioud, 2001).

The next day, immunotubes were washed and blocked as for the first round of selection.  $1.4 \times 10^{12}$  PEG precipitated phage in 2% MPBS from the first round of selection of library I were added to one immunotube and  $1.3 \times 10^{12}$  PEG precipitated phage in 2% MPBS from the first round of selection of library J were added to a second tube as input phage. Immunotubes were incubated for 1hr with rotation and for 1hr standing at room temperature and were washed 20 times with PBS containing 0.1% Tween 20. The remaining procedures including elution of selected phage, infection of TG1 cells, superinfection with KM13, recovery of phage and titration were done as described for the first round.

### **Third round of selection**

All procedures in the third round of selection of phage library I and library J were done as for previous rounds. The coating of immunotubes with purified K99 and washing steps were carried out as for round two. The titre of input phage antibodies for library I was  $1.4 \times 10^{12}$  and for library J, it was  $3 \times 10^{10}$  pfu in 2% MPBS.

### ***Screening phage antibodies by ELISA***

After three rounds of selection, phage antibodies from each round were screened for reaction against purified K99. Screening was done with the entire population of phage eluted at each round of selection; this is termed “polyclonal phage ELISA”. It was also carried out with individual phage clones generated from single colonies of each round of selection; this is termed “monoclonal phage ELISA”.

## ***Polyclonal phage ELISA***

### **Antigen coating**

Two rows of an ELISA plate (Nunc, Denmark) were coated with 100  $\mu$ l of purified K99 at 20  $\mu$ g/ml in PBS. As 2% MPBS was used during phage antibody selection, another two rows of the plate were coated with 100  $\mu$ l of 2% MPBS to check the specificity of the selected phage antibodies (Table 4.3). The plate was incubated at room temperature overnight to allow antigen binding.

### **Blocking**

Next day, unbound antigen was discarded by inverting the plate, shaking it, wells were washed three times with PBS. The wells were then blocked by adding 200  $\mu$ l of 2% MPBS and incubating for 2 hrs at 37 °C.

### **Preparation and addition of phage antibodies**

40  $\mu$ l of PEG precipitated phage from the original libraries prior to selection, and from each round of selection was diluted in 400  $\mu$ l of 2% MPBS. As a negative control for this experiment, 10  $\mu$ l of KM13 helper phage was diluted in 100  $\mu$ l of 2% MPBS.

The blocked wells of the ELISA plate were washed three times with PBS and 100  $\mu$ l of each diluted phage stock was added to wells coated with K99 and milk as shown in Table 4.3. The plate was incubated for 1hr at room temperature.

### **Detection of bound phage antibodies**

After washing three times wells with PBS containing 0.1% Tween 20 to remove unbound phage, 100  $\mu$ l of a 1:2500 dilution of HRP-anti-M13 monoclonal antibody (Amersham Pharmacia Biotech, UK) in 2% MPBS was added to all wells. By binding to pVIII protein on the phage capsid, this antibody detected phage captured to the coated plastic. The plate was incubated at room temperature for 1 hr.

## Development

Wells were washed three times with PBS containing 0.1% Tween 20 to remove unbound antibodies. 100  $\mu$ l of developer solution was added to each well. The composition of this solution was 0.1 M citric buffer pH 4.5, 0.4 mg/ml o-phenylenediamine (Sigma, UK) and 0.4  $\mu$ l/ml of 30% hydrogen peroxide. The colorimetric reaction was read after 5 min at 450 nm on an ELISA reader.

## ***Monoclonal Phage ELISA***

### **Preparation of monoclonal phage antibodies**

20 individual colonies picked at random from the original libraries, the first, and the second rounds of selection and 36 individual colonies from the third round of selection were inoculated into 100  $\mu$ l of 2xTY containing 100  $\mu$ g/ml ampicillin and 1% glucose in 96 well plates. Separate plates were used for library I and for library J. Colonies were taken from plates used to titrate phage stocks. The plates were incubated overnight at 37 °C with shaking at 200 rpm.

The following day, approximately 2  $\mu$ l of overnight cultures were transferred to further 96 well plates containing 200  $\mu$ l of 2xTY, 100  $\mu$ g/ml ampicillin and 1% glucose per well using a multi-channel pipette. Plates were incubated at 37 °C for 2 hrs to reach to an OD 600 nm of about 0.4. Then 25  $\mu$ l of 2xTY containing 100  $\mu$ g/ml ampicillin, 1% glucose and  $10^9$  helper phage was added to each well to initiate superinfection. Plates were incubated again at 37 °C with shaking at 200 rpm for 1 hr to allow infection of the TG1 cells with helper phage. The plates were then centrifuged at 1800g for 10 minutes and the supernatants were aspirated off. Each pellet was resuspended in 200  $\mu$ l of 2xTY containing 100  $\mu$ g/ml ampicillin and 50  $\mu$ g/ml kanamycin. Cells were grown overnight at 30 °C with shaking at 200 rpm to allow replication and rescue of the phage antibodies.

The next day, plates were spun at 1800g for 10 minutes. Each supernatant was then recovered for use as monoclonal phage antibody in ELISA assay.

Table 4.3 Adding phage antibodies to ELISA plate

Coated with K99	Lib I	Lib I	R1 I	R1 I	R2 I	R2 I	R3 I	R3 I		
	Lib J	Lib J	R1 J	R1 J	R2 J	R2 J	R3 J	R3 J	KM13	KM13
Coated with 2%MPBS	Lib I	Lib I	R1 I	R1 I	R2 I	R2 I	R3 I	R3 I		
	Lib J	Lib J	R1 J	R1 J	R2 J	R2 J	R3 J	R3 J	KM13	KM13

### **Antigen coating**

Two ELISA plates (Nunc, Denmark) were coated with 100  $\mu$ l per well of 10  $\mu$ g/ml of purified K99 in PBS. Plates were left overnight at room temperature to allow the K99 antigen to bind to the plastic wells.

### **Blocking**

The next day, unbound antigen was discarded and the wells were washed three times with PBS. The wells were blocked by addition of 200  $\mu$ l of 2% MPBS and incubation for 2 hrs at 37 °C.

### **Addition monoclonal phage antibodies**

After washing the wells three times with PBS, each culture supernatant containing phage antibodies was diluted 1/2 in 2% MPBS and 100  $\mu$ l was added to each well to check their reaction against K99. Plates were left for 1 hr at room temperature for binding.

### **Detection of bound phage antibodies**

After washing three times wells with PBS containing 0.1% Tween 20 to remove unbound phage antibodies, 100  $\mu$ l of 1:2500 dilution of HRP-anti-M13 monoclonal antibody (Amersham Pharmacia Biotech, UK) in 2% MPBS was added to all wells. Plates were then incubated at room temperature for 1 hr.

### **Development**

Wells were washed three times with PBS containing 0.1% Tween 20 to remove unbound antibodies. 100  $\mu$ l of developer solution (composition as described previously) was added to each well. The colorimetric reactions were stopped after 10 minutes by adding 50  $\mu$ l of 1 M sulphuric acid and read at 490 nm in an ELISA reader.

## ***Production soluble scFv fragments***

Expression of soluble scFv antibodies can be achieved by induction of individual colonies of TG1 cells picked from the various rounds of selection. Since TG1 cell is able to suppress termination at the TAG codon by introduction of a glutamate residue, this will ensure the expression of all clones including those in which the TAG codon lies in the scFv coding sequence. Since this stop codon also lies between the scFv and gIII sequences, expression of a scFv-pIII fusion occurs which tends to lower the overall levels of antibody. To overcome this problem, the selected phage can be used to infect HB2151 (K12 *ara*  $\Delta(lac-proAB)$  *Thi/F'* *proA<sup>+</sup>B* *lacIq* *lacZ* $\Delta$ M15) which is a non-suppressor strain and can be induced to give soluble scFv fragments. Expression in this strain produces higher levels of soluble scFv than in TG1 but scFvs with TAG stop codons in the reading frame are not expressed.

## ***Transfection of E.coli HB2151 cell by phage antibodies***

To infect *E. coli* HB2151, a single colony was picked from a minimal medium plate and was grown in 5 ml of 2xTY overnight at 37 °C with shaking at 200 rpm. The following day, 300  $\mu$ l of culture was added to 30 ml of 2xTY media and was grown at 37 °C with shaking until the OD at 600 nm was about 0.4. At this point, 10  $\mu$ l of phage antibodies taken from the original I and J libraries or eluted from each round of selection were added to 200  $\mu$ l aliquots of culture in microfuge tubes. The tubes were incubated at 37 °C in a water bath for 30 minutes to allow infection of the exponentially growing HB2151 bacteria to take place. Then 50  $\mu$ l, 50  $\mu$ l of a 1:10<sup>2</sup> dilution, 50  $\mu$ l of a 1:10<sup>4</sup> dilution and 50  $\mu$ l of a 1:10<sup>6</sup> dilution of infected bacteria were plated onto TYE plates containing 100  $\mu$ g/ml ampicillin, 1% glucose. Plates were incubated overnight at 30 °C in an incubator.

## ***Growing selected colonies***

From the TYE plates, 20 colonies from the original libraries, from round one and from two, and 36 colonies from round three were picked into 100  $\mu$ l aliquots of 2xTY containing 100  $\mu$ g/ml ampicillin, 1% glucose set out in two 96 well plates. Separate plates were used for each library. The samples were grown overnight at 37 °C with shaking at 200 rpm .



The next day, small inocula (about 2  $\mu$ l) were transferred to two fresh 96 well plates containing 200  $\mu$ l per well 2xTY containing 100  $\mu$ g/ml ampicillin and 0.1% glucose. Cultures were grown on at 37 °C with shaking until the OD 600 nm was approximately 0.9 (about 3 hrs). Then, 25  $\mu$ l 2xTY containing 100  $\mu$ g/ml ampicillin and 9 mM IPTG was added to each well to initiate expression. Plates were incubated for a further 20 hrs at 30 °C with shaking to allow expression of soluble scFv antibodies.

### ***Isolation and preparation of scFv antibodies for ELISA***

Since scFvs expressed in HB2151 cells are released into the culture supernatant, plates were centrifuged at 1800g for 10 minutes to pellet the bacteria. 50  $\mu$ l of each supernatant was diluted with 50  $\mu$ l of BSA/PBS to a final concentration of 3% for use in ELISA screening.

### ***Screening monoclonal soluble scFvs by ELISA***

#### **Antigen coating**

Two ELISA plates (Nunc, Denmark) were coated with 100  $\mu$ l per well of 20  $\mu$ g/ml purified K99 in PBS. Plates were left overnight at room temperature to allow binding of K99 to the plastic.

#### **Blocking**

The next day, wells were washed three times with PBS and then blocked by adding 200  $\mu$ l of 3% BSA/PBS for incubation at 37 °C for 2 hrs.

#### **Addition of scFv antibodies**

After washing wells three times with PBS, 100  $\mu$ l of the diluted culture supernatants were added to wells of the ELISA plates to check their reaction against K99. Plates were left for 1.5 hr at room temperature to allow binding of scFvs to antigen.

#### **Detection bound scFv antibodies**

All wells were washed three times with PBS containing 0.1% Tween 20 to remove unbound scFv antibodies. 100  $\mu$ l of a 1:3000 dilution of recombinant Protein L-HRP

(Actigen, Norway) in 3% BSA/PBS was added to all wells. Protein L-HRP recognises to scFv antibodies from the Tomlinson I and J libraries via the kappa light chain but this interaction does not effect the binding of the antibody to its antigen. Plates were then incubated at room temperature for 1 hr.

## **Development**

Wells were washed three times with PBS containing 0.1% Tween 20 to remove unbound Protein L-HRP. 100  $\mu$ l of developer solution (composition as described previously) was added to each well. The colorimetric reactions were stopped after 10 minutes by adding 50  $\mu$ l of 1 M sulphuric acid and read at 490 nm on an ELISA reader.

## ***PCR characterisation of selected scFvs***

To assess the presence of scFv genes in 10 selected clones from the Tomlinson library I and 5 from library J, the following PCR was performed. The clones were chosen because of the strength of their recognition of purified K99 in ELISA.

Single colonies were picked from TYE plates into 5 ml of 2xTY containing 100  $\mu$ g/ml ampicillin and 1% glucose and grown overnight at 37°C. Plasmid DNA was isolated and purified from 3 ml of culture by using a miniprep kit (Qiagen, UK). As a positive control, plasmid DNA was prepared from a clone carrying the sequence of an anti-ubiquitin scFv. Primers pelB and pHEN seq were chosen to amplify across the full insert in each clone. The 25- $\mu$ l PCR mixture contained each primer at 0.5  $\mu$ M of, dNTPs 0.25 mM (Promega), 4mM MgCl<sub>2</sub>, 1x reaction buffer (containing 50 mM KCL, 10 mM Tris-HCL pH 9.0 and 0.1% Triton<sup>®</sup>X-100), 2.5 units of Taq DNA polymerase (Promega) and 1  $\mu$ l of plasmid DNA as template. The samples were amplified in a Techne thermal cycler under the following conditions: the reaction was heated to 94 °C for 10 min and then 35 cycles of amplification were performed beginning with a 1 min denaturation at 94 °C, primer annealing at 55 °C for 1 min, and extension at 72 °C for 3 min. Final extension took place for 10 min at 72 °C.

10  $\mu$ l samples of the PCR products were analysed by electrophoresis on 1% agarose gels for 45 min at 100 V. Gels were stained with ethidium bromide, and photographed under UV light.

## ***Plasmid digestion with restriction enzymes***

Purified plasmids of clones chosen for detailed analysis (10 from library I and 5 from library J isolated from rounds 2 and 3) were digested with restriction enzymes to confirm the size of the inserts. 20 µl reaction mixtures were set up for each sample comprising 6 µl of plasmid DNA, 1 µl (10U) of *NcoI* (Promega, UK), 1 µl (10U) of *NotI* (Promega, UK), 10 µl of distilled water, and 2 µl of buffer D (60 mM Tris-HCL (pH 7.9), 1.5 M NaCl, 60 mM MgCl<sub>2</sub> and 10 mM DTT). The reaction mixtures were incubated at 37 °C for 2 hrs.

4 µl of the digestion products was electrophoresed on a 1% agarose gel for 45 min at 100 V, stained with ethidium bromide, and photographed under UV light.

## ***Western blotting of selected clones from library I and library J***

Western blotting experiments were performed to characterise the scFv antibodies from libraries I and J.

### **Sample preparation**

One colony of each selected clone in HB2151 was picked from TYE plates and grown in 5 ml of 2xTY containing 100 µg/ml ampicillin and 1% glucose overnight at 37°C. The following day, 500 µl of overnight culture was added to 50 ml of 2xTY containing 100 µg/ml ampicillin and 0.1% glucose and incubated at 37 °C with 200 rpm shaking until the OD at 600 nm was about 0.900. IPTG was then added to a final concentration of 1 mM to induce expression of scFv antibodies. Cultures were grown on at 30 °C for 22 hrs with shaking at 200 rpm. The cultures were spun at 5000g for 20 minutes, the supernatants were recovered and after filtration, concentrated using Amicon Ultra-4 centrifugal filters (Millipore, UK) to approximately 1/25 initial volume. All concentrated culture supernatants were exchanged into PBS to assist clear resolution on SDS gels and Western blots. 20 µl of each sample was mixed with 10 µl of 3x SDS boiling buffer and heated for 5 min in boiling water. 15 µl of each sample was then loaded to a 12.5 % SDS-polyacrylamide gel. Gels were run for 1 hr at 100 volts in a BioRad electrophoresis apparatus.

## Blotting

For blotting, nitrocellulose membrane (Amersham Pharmacia Biotech, UK) and four pieces of Watman filter paper were cut to the same size as the gel and soaked with the gel in transfer buffer for 20 min. For transfer, a fibre pad was overlaid with two pieces of filter paper, the gel, nitrocellulose membrane, and the final two layers of filter papers. After addition of a second fibre pad, the sandwich was assembled into a blotting cassette and loaded into a BioRad electrophoresis tank. Transfer buffer was added along with a frozen insert to avoid overheating during transfer. Care was taken to ensure that the gel lay to the anode (-) side of the assembly and the membrane lay to the cathode (+) side. Proteins were transferred from the gel to the membrane for 1 hr at 100 volts. After transfer, the membrane was carefully separated, washed three times with PBS and stained with Ponceau red dye (Sigma, USA) by shaking for 5 min at room temperature to confirm that proteins had been transferred from the gel to the membrane. The membrane was then washed with distilled water to remove the dye. The membrane was blocked with 3% MPBS for 1 hr at room temperature with shaking. The membrane was then washed three times with PBS and was incubated in a 1:3000 dilution of anti-c-myc monoclonal antibody (Sigma, UK) in 2 % MPBS for 1 hr at 37 °C with shaking at 200 rpm. After washing the membrane three times with PBS containing 0.05 % Tween 20, an anti-mouse HRP conjugate (Sigma, UK) was added at 1:1000 dilution in 2 % MPBS and incubated for 1 hr at 37 °C with shaking. The membrane was washed as before and developed with 4-chloro-1-naphthol substrate (Sigma, UK).

### ***Dot blotting of selected clones from libraries I and J***

Since previous experiments had shown that K99 loses its reactivity with anti-K99 mouse monoclonal antibodies after heating, dot blot experiments were performed to assess the binding of selected soluble scFvs to purified K99 on nitrocellulose.

Sixteen small strips of nitrocellulose (Amersham, UK) were each spotted three times with two 2 µl aliquots of purified K99. The strips were allowed to dry between spotting to increase the concentration of antigen at the point of application. To test whether the anti-K99 reactivity of selected scFvs was retained after heating the antigen, an extra spot of K99 that had been heated to boiling for 5 minutes in SDS boiling buffer was spotted out on 11 of the strips. The nitrocellulose strips were then blocked with 3 % MPBS for 1 hr at room temperature with shaking. Strips were washed three times with PBS and then were incubated for 1 hr at 37 °C with shaking with 200 µl of concentrated culture supernatants

containing the scFvs under analysis (1/2 diluted in 2 % MPBS). Next, the membrane strips were washed three times with PBS containing 0.05 % Tween 20, once with PBS and incubated with 1:3000 anti-c-myc monoclonal antibody (Sigma, UK) in 2 % MPBS for 1 hr at 37 °C with shaking at 200 rpm. After washing membrane three times with PBS containing 0.05 % Tween 20, a 1:1000 dilution of anti-mouse HRP conjugate in 2 % MPBS was added to all strips and incubated at 37 °C for another 1 hr. Membrane strips were washed as previous step and developed with 4-chloro-1-naphthol substrate.

### ***Sequencing of selected clones***

To determine the diversity of the selected scFv genes (10 from library I and 5 from library J), their DNA and predicted amino acid sequences were determined.

After growing individual colonies in 5 ml of 2xTY containing 100 µg/ml ampicillin and 0.1% glucose overnight at 37 °C with shaking, plasmid DNA was isolated from 3 ml of culture using Qiagen miniprep reagents. The primers forward pelB and reverse gIII (Table 4.1) were chosen for sequencing and diluted to 3.2 µM. Sequencing was done at the MBSU, University of Glasgow using Big Dye (Applied Biosystems) and ET-Dye Terminator (Amersham Bioscience) chemistries. Samples were analysed on a MegaBACE1000 (96 capillary) sequencing machine. The reaction chemistry is based on the dideoxy method developed by Sanger et al.(1977). The sequence data was analysed using DNA Star software. Sequences were aligned to V BASE using DNAPLOT database at the Centre for Protein Engineering, MRC, Cambridge, UK ( [www.mrc-cpe.cam.ac.uk/vbase-ok.php?menu=901](http://www.mrc-cpe.cam.ac.uk/vbase-ok.php?menu=901) ). The confirmed nucleic acid sequences were translated and aligned using MEGALIN software.

### ***Final selection of scFv antibodies***

Four scFvs from the Tomlinson library I and two scFvs from library J, which had diverse sequences, high levels of expression as soluble protein and high activity in dot blotting experiments with purified K99 were chosen for purification and further investigation.

## ***High level production and purification of soluble single chain antibodies***

### **Growth conditions**

In order to make large amounts of soluble scFv, one colony of each clone was grown overnight in 5 ml of 2xTY containing 100 µg/ml ampicillin and 1% glucose at 37 °C with shaking at 200 rpm. The 5ml overnight culture was added to 1 litre of 2xTY containing 100 µg/ml ampicillin and 0.1% glucose in a 2 litre shake flask and grown on at 37 with shaking at 200 rpm. When cultures reached an OD at 600 nm of approximately 0.9, IPTG was added to 1mM and incubation continued with shaking at 200 rpm at 30 °C for 20-22 hr.

### **Concentration of culture supernatants**

As the expressed soluble scFvs are released into the culture supernatants, bacterial cells were removed by centrifugation for 20 minutes at 5000g at 4 °C. Then the culture supernatants were concentrated using a tangential flow filter (Vivaflow 200, Vivascience, Germany) containing a polyethersulfone membrane using a peristaltic pump. Briefly, the membrane was initially washed by pumping 400 ml of deionised water through the system and recirculating at 90 ml/min to remove residual chemicals and any air pockets. Then, the culture supernatant was pumped through the system at the same rate. As the last few millilitres of sample entered the system, the input was switched to 100 ml of 20mM phosphate buffer containing 0.5M NaCl, pH 7.2, to adjust the buffer composition of the concentrated culture supernatant to those used for binding in subsequent affinity purification methods. As the desired volume of concentrate approached (20-25 ml), the recirculation rate was reduced to 30-40ml/min for 1-2 min to increase sample recovery. For recovery of concentrated protein, the feed line was removed from the sample and the residual volume present in the system was pumped back into a container. To increase efficiency of recovery, 25ml of 20mM phosphate, 0.5M NaCl, pH7.2 was rinsed through the system, and recovered as before.

## **Purification of soluble scFvs**

### **Buffer composition**

For preparation of binding buffer (starting buffer), 20mM sodium phosphate buffer pH 7.6 was made first and then NaCl was added to a final concentration of 0.5 M. The final pH was about 7.3. Three elution buffers were prepared in binding buffer containing 20mM, 80mM and 200mM imidazole (Sigma, USA). All buffers were sterilised by passing them through a 0.2  $\mu$ M filter before use.

### **Column preparation and equilibration**

To purify soluble antibodies, a 1ml HiTrap Chelating HP column (Amersham Pharmacia Biotech, UK) was chosen, exploiting the histidine repeats present at the carboxy-terminus of scFvs from the Tomlinson libraries. To prepare this column, it was washed with 5 ml of distilled water at 1 ml/min by using a Pharmacia Biotech pump P-1 (Amersham Pharmacia Biotech AB, Sweden). The column was then charged with 0.5 ml 0.1 M  $\text{NiSO}_4$  and washed again with 5 ml of distilled water. After that, a blank run was performed to elute non-specifically bound metal ions that might otherwise be eluted during the desorption. To do this, 5 ml of binding buffer was pumped through the column, followed by 5 ml of elution buffer (200mM imidazole). The column re-equilibrated with 5-10 ml of binding buffer before application of the sample.

### **Sample preparation**

The concentrated sample that had been exchanged into binding buffer during concentration of culture supernatant was filtered through a 0.2  $\mu$ M filter immediately before applying to the column.

### **Purification procedure**

20 ml of sample was applied to an equilibrated HiTrap column at 1 ml/min. The column was then washed with 5 ml of binding buffer and elution buffer containing 20 mM and 80 mM imidazole (Sigma, USA) to elute non-specifically bound proteins and thereby increase the purity of the final eluate. Finally, 5 ml of elution buffer containing 200 mM imidazole was pumped through to elute the attached scFv antibody. The eluate was collected in fractions and concentrated to 1/5 their initial volume using an Amicon Ultra-4

centrifugal filter (Millipore, UK) and exchanged into PBS buffer at 4°C by. The purified scFv was stored at -20 °C for future use.

### ***Measurement of purified scFv concentration***

Before testing the biological activity of the 6 purified scFvs, their concentration and that of an anti-BSA scFv purified by the same methods were measured by BCA Protein Assay Kit (Collins et al.). The method has been described previously.

## **Biological activity of selected scFvs**

### ***Comparison of activity of purified scFvs by ELISA***

The purified scFvs were adjusted to the same protein concentration and their reactivity towards K99 was tested in an ELISA experiment.

#### **Antigen coating**

An ELISA plate (Nunc, Denmark) was coated with 100 µl per well of 20 µg/ml of purified K99 in PBS. The plate was left overnight at 4 °C to allow binding of the K99 antigen to the plastic.

#### **Blocking**

The next day, the wells were washed three times with PBS. Then 200 µl of 3% BSA/PBS was added to all wells except two wells that were to be probed with the anti-BSA scFv negative control. These wells were blocked by adding 2% MPBS. The plate was then incubated for 2 hrs at 37 °C.

#### **Addition of scFv antibodies**

After washing the wells three times with PBS, 100 µl of the purified scFv samples were added, diluted to an scFv concentration of 8 µg/ml in blocking buffer. For a positive control, a sheep anti-K99 polyclonal antibody was used; for negative controls, the anti-BSA scFv and a PBS blank were added to K99-coated wells. Each antibody was added in duplicate. Plates were left for 1.5 hr at room temperature to allow binding of the antibodies to antigen.



## Detection bound scFv antibodies

All wells were washed three times with PBS containing 0.1% Tween 20 to remove unbound antibodies. 100 µl of 1:3000 dilution of recombinant Protein L-HRP (Actigen, Norway) were added in blocking buffer to all wells containing scFv antibodies. 100 µl of anti-sheep HRP (Sigma, UK) at a dilution of 1:1000 was added to positive control wells. The plate was then incubated at room temperature for 1 hr.

## Development

Wells were washed three times with PBS containing 0.1% Tween 20 to remove unbound HRP conjugates. 100 µl of developer solution (composition described previously) was added to each well. The colorimetric reactions were measured after 10 minutes at 450 nm on an ELISA reader.

## *Haemagglutination inhibition assay*

Some erythrocytes (RBCs) such as those from sheep have on their surface receptors to which K99 fimbriae can bind, thereby modelling the interaction of the adhesion factor with enterocytes (Burrows *et al.*, 1976). Haemagglutination (HA) can thus occur with purified fimbriae or with bacteria such as the enterotoxigenic strain of *E. coli* B41 that express K99 (Vazquez *et al.*, 1996). In some cases, this haemagglutination activity can be inhibited using anti-fimbrial antibody. Also some investigators have reported that antibodies with haemagglutination inhibiting activity can have the potential to block attachment of bacteria to epithelial cells (Burrows *et al.*, 1976). The following experiments were performed to assess if the selected scFvs possessed these biological activities.

## Titration of the haemagglutinating activity of *E. coli* B41

This was assessed with a test for direct haemagglutinating activity.

## Preparation of bacteria

One colony of *E. coli* B41 grown on sheep blood agar was grown in 5 ml of Minca medium at 37 °C overnight with shaking at 200 rpm. Following previous investigations (Orskov *et al.*, 1975), another colony was grown as a negative control in the same medium at 18°C since under these conditions, K99 fimbriae are not expressed. 50 µl of each culture

was added to the first well of a 96 well microplate (Iwaki, Japan). 25  $\mu$ l aliquots were diluted two fold in PBS containing 1% mannose across the plate. Since the interaction between K99 and its receptor is resistant to mannose, this was intended to inhibit non-specific haemagglutination or the interaction of mannose-sensitive factors with erythrocyte receptors. Bacteria were not added to the last well to provide an additional negative control.

### **Preparation of sheep RBC and haemagglutination procedure**

To prepare washed suspensions of sheep red blood cells at a density of about 1.5 % (v/v), 270  $\mu$ l of defibrinated sheep blood (E and O Laboratories, Scotland) was centrifuged at 13000 rpm for 4 min. The pellet was then washed three times with PBS containing 1% mannose (Sigma, UK) by resuspension and spinning for 4 min. Finally the pellet (about 100  $\mu$ l volume) was resuspended in 7.5 ml PBS containing 1% mannose.

25  $\mu$ l of this suspension was added to each well containing bacteria and, after mixing, the plate was incubated at 4 °C overnight. The titre was defined as the highest dilution of bacterial suspension that showed complete haemagglutination.

### **Haemagglutination Inhibition**

The haemagglutination inhibition test was performed by setting up serial dilutions of the selected anti-K99 scFv antibodies, a polyclonal anti-K99 antibody as a positive control and an anti-ubiquitin scFv antibody as negative control. 50  $\mu$ l of each of each purified antibody, adjusted to similar protein concentrations, was added to the first wells of a 96 well U bottom microplate (Iwaki, Japan). 25  $\mu$ l of each antibody was then diluted two-fold across the plate in 25  $\mu$ l of PBS containing 1% mannose. The last well in each row was left blank and contained only 25  $\mu$ l of dilution buffer as an additional negative control. Then 25  $\mu$ l of 4 haemagglutinating units of bacterial suspension in PBS containing 1% mannose were added to all wells. After mixing, plate was incubated at 37°C for two hrs. After that the plate was cooled and 25  $\mu$ l of a 1.5 % suspension of sheep red blood cells was added to all wells. Haemagglutination inhibition (HI) titres were determined by the highest dilutions of antibodies that inhibited haemagglutination after 1 hr at 4 °C.

## **Indirect Fluorescent Antibody Test (IFA)**

Since K99 fimbriae are located at the surface of the *E. coli* strain B41, the binding of antibody against K99 should be visible under the immunofluorescent microscope using fluorescently labelled reagents. To detect whether the isolated scFv antibodies recognised K99 fimbriae on bacteria, they were tested by an indirect fluorescence antibody assay.

### **Slide preparation**

Nine clean glass microscope slides (LDH, UK) were printed with a circle with a diameter of about 10 mm by using nail varnish. 5 µl of an overnight culture of B41 grown in Minca medium at 37 °C was added to eight slides and as a negative control, 5 µl of the same strain grown at 18 °C was added to the last slide. The bacteria were spread inside the circles and incubated at room temperature until dry. The bacteria were then fixed by adding 50 µl of 4 % paraformaldehyde/1 % glutaraldehyde in PBS and incubating for 15 minutes at room temperature.

### **Blocking**

After fixation, the slides were washed three times with PBS to remove remaining paraformaldehyde, glutaraldehyde and unfixed bacteria. Then the isolated areas on the slides were blocked by adding 50 µl of 3 % BSA/PBS. Slides were incubated for 30 minutes at room temperature inside a plastic container.

### **Addition of scFv antibodies**

The excess blocking buffer was removed by washing three times with PBS. 50 µl of the purified scFvs, adjusted to similar protein concentrations, was added to each slide. As a negative control, an anti-BSA scFv antibody was added to one slide carrying fixed B41 bacteria, grown at 37 °C. Also, a commercial monoclonal anti-K99 antibody was added to one slide carrying B41 grown at 37 °C and one slide with B41 grown at 18 °C as positive and negative controls respectively. The monoclonal was diluted to 1:500. Slides were then incubated at 37 °C for 1 hr.

## **Addition of secondary and fluorescent antibodies**

All slides were washed three times with PBS, and 50 µl of a mouse monoclonal antibody against c-myc at was added at a dilution of 1:200 in 3 % BSA/PBS to all slides carrying scFvs. These slides were incubated for 1 hr at 37 °C.

To label all samples with fluorescent antibody, 50 µl of a 1:200 dilution of a fluorescein linked anti-mouse Ig antibody (Amersham Life Science) was added to all. The incubation time for labelling was 30 minutes at 37 °C.

To observe the binding of antibodies to the K99 fimbriae, all slides were washed with PBS and after adding cover slips, they were observed under normal and UV illumination on a Zeiss microscope under 1000× magnification. Images were captured to a computer running OpenLab software.

## ***Electron Microscopy and Immunolabelling***

### **Staining of fimbriae for EM observation**

In order to find an effective and convenient staining method for observation of K99 fimbriae by electron microscopy, two different protocols were tested.

### **Growing of bacteria**

A single colony of *E. coli* B41 was picked from a sheep blood agar plate and grown overnight with shaking in 5 ml of Minca medium at 37 °C. As a negative control, a second colony of bacteria was grown at 18 °C. At this temperature, the bacteria do not express K99 fimbriae (Orskov *et al.*, 1975).

### **Preparation of grids**

Nickel 300 mesh grids coated with formvar and carbon were prepared in the Integrated Microscopy Facility, IBLS. Before using the grids for samples, they were pre-cooled on ice for 10 minutes.

Droplets of 40 µl of each bacterial culture were put onto a clean surface of Parafilm. Grids were then put on top of these droplets and incubated for 30 seconds. The excess fluid

from grids was removed by touching the edge to filter paper, held between the tips of a pair of forceps.

### **Fixation**

Bacteria were fixed onto the grids by incubation on top of droplets of fixative buffer for 30 minutes, in a fume hood. The composition of the fixative buffer was 2 % paraformaldehyde in PBS containing 0.1 % glutaraldehyde. After that, the fixative buffer was removed from grids by touching the edge to filter paper and washing three times for 2 minutes on droplets of PBS and distilled buffer under a plastic cover.

### **Negative staining**

This staining was the method chosen since the K99 fimbriae are located around the bacteria. This staining is well-established, extremely simple and quick to execute. The grids were simply incubated on droplets of Nanovan stain (Nanoprobes, USA) by holding on forceps. The stain was then removed by touching the grids onto the edge of filter paper. Grids were rinsed by transferring grids six times (3 minutes each) to droplets of distilled water. After drying at room temperature, the bacteria were observed by electron microscopy.

### **Negative staining by methylcellulose method**

This staining was done by incubating the grids on 40 µl droplets of 2 % uranyl acetate, 0.15 M oxalic acid, pH 7.2 for 10 minutes. Grids were then washed four times for 30 seconds aqueous on droplets of distilled water and briefly on droplets of containing 2 % methylcellulose and 0.1 % uranyl acetate. Excess stain was removed by touching the edge of filter paper to grids mounted on wire loops. Grids were then left overnight at room temperature to dry.

### **Immunolabelling of K99 fimbriae**

To detect the binding of selected scFv antibodies to K99 fimbriae, all six scFvs were analysed by immunogold electron microscopy. B41 bacteria were grown in Minca medium at 37 °C or 18 °C and were added to Nickel 300 mesh grids coated with formvar and carbon as described above. They were fixed as described for staining.

## Washing and blocking

In order to removed fixative, the grids were washed first four times for 2 minutes each wash on droplets of PBS and then three times for 5 minutes each on droplets of 0.1M glycine in PBS. To prevent non-specific binding and suppress background in immunostaining, the grids were then blocked by incubation for five minutes for three times on droplets of 0.2% /PBS/acetylated BSA at room temperature.

## Addition of selected scFv antibodies

For immunolabelling of the bacteria, 40  $\mu$ l droplets of concentrated scFv antibodies were put on to a clean surface of Parafilm inside a petri dish. As positive controls, a drop of a 1:500 dilution of mouse monoclonal anti-K99 antibody and a drop of a 1:500 dilution of sheep polyclonal anti-K99 were also set up. For a negative control, a drop of anti-ubiquitin scFv antibody used. Grids carrying fixed B41 grown at 37 °C were placed on top of each droplet. Another negative control was prepared by adding a grid carrying fixed of B41 grown at 18 °C to the top of a droplet of the mouse monoclonal antibody. The petri dish containing all samples and moist filter paper was incubated at 37 °C for 90 minutes.

After incubation, the grids were washed six times for 5 minutes, each wash on droplets of PBS/acetylated BSA (Aurion, Netherland) to remove unbound antibodies.

## Double labelling with protein A-gold

Protein A from *Staphylococcus aureus* has five domains that mediate interaction with the Fc region of most mammalian IgGs. Protein A also binds to the Fabs or scFvs carrying heavy chains belonging to the V<sub>H</sub>III family. Since all scFv antibodies in the Tomlinson libraries are derived from the V3-23/DP-47 and J<sub>H</sub>4b family segments, labelling with Protein A is possible.

To label the scFv and control antibodies, 50  $\mu$ l of 5nm Protein A – gold colloidal particles –5nm (EY Laboratories, CA) was diluted in 450  $\mu$ l of 1% BSA in PBS, pH 7.4. Then the grids were incubated on droplets of this diluted protein for 30 minutes at room temperature and washed six times for 5 minutes per wash on droplets of PBS/acetylated BSA three times. They were then further washed, six times for 3 minutes per wash on droplets of PBS and and six times on droplets of distilled water. Finally the grids were

stained with negative staining using methylcellulose uranylacetate as described above to stain the bacteria and fimbriae.

### **Imaging colloidal gold by electron microscopy**

Images of the bacterial surfaces were obtained with a Zeiss 902 transmission electron microscope at 30,000 or 50,000 magnification. Images were then captured to computer and edited by SIS Esi Vision software.

### ***In vitro villus attachment assay***

Attachment of K99 fimbriae to the villi of the small intestine villi in calves is essential for colonisation by enteropathogenic *Escherichia coli* and the stimulation of diarrhoea (Orskov *et al.*, 1975; Guinee *et al.*, 1976; Gaastra and de Graaf, 1982). This attachment can be modelled *in vitro* using isolated epithelial cells (Wilson and Hohmann, 1974), or preparations of the intestinal villi and brush border (Van den Broeck *et al.*, 1999). In the experiments that follow, *in vitro* assessment of bacterial colonisation was based on the method described by Girardeau previously (Girardeau, 1980).

### **Preparation of bacteria**

*E. coli* strain B41 was grown on sheep blood agar overnight at 37 °C. Next day, a single colony was picked from the plate and grown overnight in 5 ml of Minca medium at 37 °C with shaking to trigger expression of K99 fimbriae. As a negative control, a second colony was grown overnight in the same medium at 18 °C with shaking. At this temperature, K99 is not expressed (de Graaf *et al.*, 1980b).

### **Preparation of villi**

The villi were isolated from a healthy calf, approximately 2 days old. At this stage, the villi are long and easily isolated. Slaughter and tissue isolation was carried out at the Moredun Research Institute, Edinburgh, by a qualified veterinary pathologist using authorised procedures. After slaughter, about 40 cm of jejunum was removed and opened longitudinally. The tissue washed immediately with ice-cold sterile Krebs buffer pH 7.2 and transferred to a plastic bag containing cold Krebs buffer for transport back to Glasgow. There, a glass slide was used to gently detach the villi by scraping. The material was suspended in cold Krebs buffer and allowed to settle. The villi were rinsed with the

same buffer several times until a clear supernatant was obtained. To store the villi and preserve the cells, the villi were taken up in Hank's-DMSO medium. The composition of the medium was 20 % calf serum containing ultra low levels of immunoglobulin (Gibco, UK), 10 % DMSO, 10 % glycerol, 15 % lactalbumin buffer (40mg/ml) and 45 % Hank's medium (Gibco, UK). The suspension was then divided between 1.5 ml cryotubes, placed at  $-20^{\circ}\text{C}$  for several hours and then transferred for storage at  $-80^{\circ}\text{C}$ .

### **Thawing and fixation of villi**

Cryotubes containing the villi suspension were thawed at room temperature for about 10 minutes. The villi were placed into a Kerbs buffer with 2 % formaldehyde (Sigma, USA) for 1 hour in order to stop all enzyme activity which could potentially lyse the epithelial cells. Next, the villi were rinsed twice in 5 ml of Krebs buffer pH 7.2 to remove residual formaldehyde. The villi were then put in Krebs buffer pH 7.2 for at least 18 hours at  $4^{\circ}\text{C}$  to stabilize. The stabilized villi were used within one week of fixation.

### **Bacterial attachment**

To verify attachment of bacteria to villi via K99 fimbriae, villi were transferred with an automatic pipette into 1.5 ml of Krebs buffer pH 6.8. Overnight bacterial cultures were spun at 13000 rpm and resuspended in Krebs buffer pH 6.8 containing 1 % mannose to a final concentration of  $4.8 \times 10^8$  bacteria. The density of bacteria was established by measuring OD at 600 nm and plate counts. The villi were transferred to the bacterial suspension, set up inside a sterile petri dish and the mixture was gently agitated at room temperature for 20 minutes or 1 hour.

### **Observations**

After incubation, villi were removed with an automatic pipette and placed gently on a glass slide and covered with a cover slip. Observations were made on a Zeiss phase contrast microscope at 600 to 1000-fold magnification. The edge of villi, and in particular the brush-borders could be clearly distinguished. Bacterial attachment was confirmed by the presence of many cells on the villi surface or brush border after co-incubation for 20 or 60 minutes.



## ***Bacterial adherence inhibition assay***

The six selected, purified scFv antibodies were tested for their ability to inhibit the binding of *E. coli* B41 to isolated villi.

### **Preparation of antibodies**

All six purified antibodies were diluted in PBS and adjusted to the same final concentration of 30 µg/ml. As a negative control, a purified anti-BSA scFv was diluted to the same concentration. A sheep polyclonal anti-K99 antibody at a dilution of 1:1000 in PBS was used as a positive control.

### **Preparation of bacteria**

B41 were added to 1.5 ml Eppendorff tubes and adjusted to a density of about  $5 \times 10^8$  bacteria/ml. Bacteria grown at 18 °C served as negative controls. All tubes were spun at 13000 rpm for 1 min. The supernatants were discarded. The pellet was used for testing.

### ***In vitro* inhibition assay**

The bacterial pellets were resuspended in each antibody to a final volume of 50 µl and incubated at 37 °C for 45 min with agitation at 100 rpm. Then 500 µl of Krebs buffer pH 6.8 / 1% mannose containing about 50 stabilized villi were mixed with the bacterial suspension in sterile petri dishes. Next, the petri dishes were incubated at room temperature for 1 hour with gentle shaking. After incubation, the villi were examined by phase-contrast microscopy at a magnification of 600 or 1000×. The adhesion of bacteria to villi was observed along the length of the villi brush border at several random locations.

## Results

### ***Production and titration of KM13 helper phage***

*E. coli* TG1 cells were infected with serial dilutions of KM13 and plaques allowed to form in TYE H-top agar. A single plaque was isolated and used to infect a fresh exponential culture of TG1 cells. Phage were precipitated from the culture supernatant with PEG/NaCl and resuspended in PBS. The titre of this helper phage stock was measured by infecting TG1 cells with serial dilutions of trypsin treated phage, plating to TYE plates containing H-top agar, counting the resulting plaques and adjusting for dilution. As a control, the titre of non-trypsinised phage was also measured. As can be seen from the data in Table 4.4A, the difference in titre between treated and untreated phage was around 9 orders of magnitude. The KM13 helper phage produced were then used for the preparation of the scFv antibody libraries.

### ***Preparation and titration of phage libraries***

TG1 bacteria comprising the libraries were grown in 2xTY medium and infected with KM13 helper phage. Phage displaying the scFvs were then isolated from culture supernatants by precipitation with PEG/NaCl and resuspended in PBS. The size of the Tomlinson I and J libraries were then determined by infection of *E. coli* TG1 cells with serial dilutions of phage and counting the number of ampicillin-resistant colonies that then grew on TYE plates. Table 4.4B shows the titre of each phage library as calculated in this way.

### ***Immunoreactivity of purified K99 at low concentrations***

To check that the K99 target would bind to plastic at low concentrations and remain detectable by specific antibody, ELISA was set up with serial dilutions of purified K99. The results are shown in Figure 4.4; it was clear that coating concentrations as low as 2.5 µg/ml could still be efficiently detected with an anti-K99 monoclonal antibody. Phage antibodies are typically selected in immunotubes coated with target in the range 10-100 µg/ml. It was clear from this preliminary experiment that purified K99 retains integrity when coated to plastic at low concentrations in this range.

### ***SDS-PAGE and Western analysis of control scFvs***

Conditions for the detection of expressed scFvs were validated with anti-ubiquitin and anti-BSA control antibodies. Culture supernatants from *E. coli* HB2151 expressing each scFv were concentrated to about 1/20 initial volume, exchanged into PBS and analysed by SDS-PAGE. Many proteins were detected on the gel (Figure 4.5) but bands of around 29 kDa, the estimated molecular weight of a soluble scFv, could be seen in each sample.

In principle, scFvs from the Tomlinson I and J libraries should be recognised by Protein L and the hybrid immunoglobulin-binding protein, Protein LA through their interaction with light and heavy chain frameworks. The scFvs from each library should also carry a c-myc tag at the carboxy-terminus, detectable with immunochemical reagents against this sequence. A Western blotting experiment was performed to compare detection with these three reagents. Concentrated supernatants from HB2151 expressing anti-BSA and anti-ubiquitin scFvs were loaded in triplicate to an SDS-PAGE gel, separated and then blotted onto nitrocellulose membrane. The membrane was then cut into three parts and each was probed with rProtein L-HRP, rProtein LA-HRP or anti-c-myc reagents. Figure 4.6 shows both control scFvs were detectable by all methods with bands appearing at the expected molecular weight at different strengths. Detection through recognition of the c-myc tag showed the greatest signal strength, probably because it is done with primary and secondary (HRP) antibodies. This method was therefore considered most appropriate when the expected concentration of a scFv is low.

Table 4.4 A Titre of KM13 phage after production in large quantity (pfu/ml)

Titre of trypsin treated phage	Titre of non-trypsin treated phage	Difference <sup>a</sup>
$1.66 \times 10^{14}$	$1.2 \times 10^5$	$0.46 \times 10^9$

<sup>a</sup> Difference was calculated by the titre of trypsin treated phage minus non-trypsin treated phage

Table 4.4B Titre of library phage (pfu/ml)

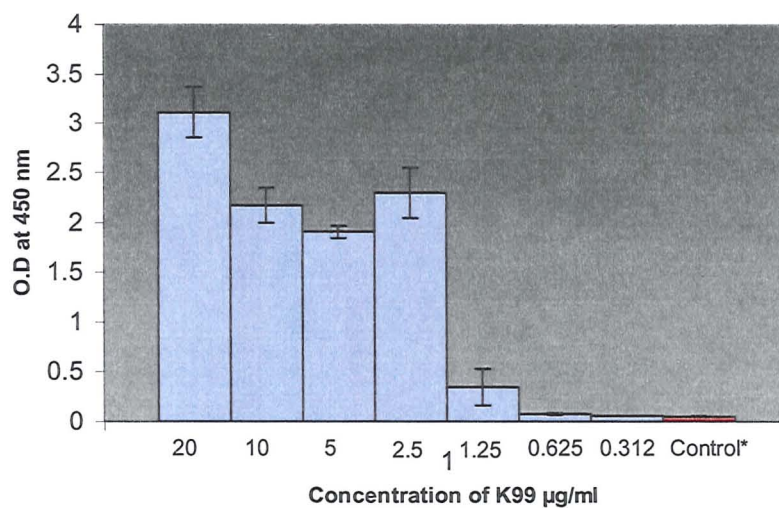
Tomlinson library I	Tomlinson Library J
$2.08 \times 10^{13}$	$1.28 \times 10^{12}$

## ***PCR screening for full-length scFv genes***

To determine the frequency of full-length scFv genes in the Tomlinson I and J libraries, 21 colonies were picked at random from initial titration experiments for PCR screening. Bacteria carrying genes for an anti-ubiquitin scFv served as a positive control. PCR was performed on crude lysates using LMB3 and pHEN seq primers to amplify across the light chain, linker and heavy chain sequences. PCR products were then analysed on 1% agarose gels. Figure 4.4 shows that all clones from the Tomlinson library I had full length inserts according to the predicted sizes (935 bp) and comparison with the positive control although in several cases (lanes 16, 18 and 21) the amplification products were faint. Given that the lysates were very crude, the presence of non-specific products was unsurprising and they were also apparent in the positive control reaction. Only one clone from Tomlinson library J did not show insert (Figure 4.7, lane 6). The absence of non-specific products in this reaction suggests either a fault with the reaction or low template concentration rather than absence of an scFv insert. These results indicated that both libraries had a very high percentage of inserts of full length, in contrast to the Griffin library used in earlier experiments.

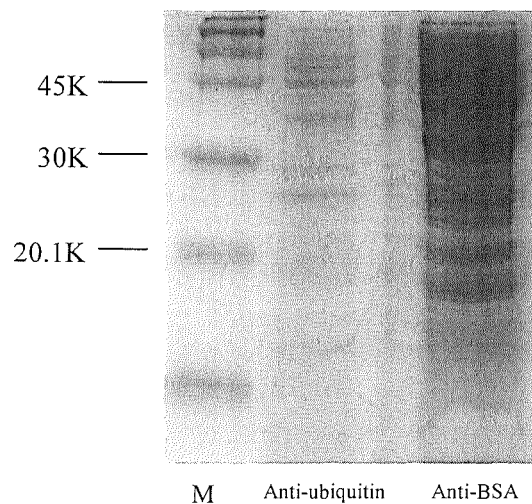
## ***Panning Tomlinson library I and Tomlinson library J against purified K99***

The libraries used for this section of the project were each predicted to carry in excess of  $10^8$  unique specificities (Tomlinson library I:  $1.47 \times 10^8$ ; Tomlinson library J:  $1.37 \times 10^8$ ). Phage antibodies against purified K99 were isolated in immunotubes coated with purified K99 at 20 µg/ml in the first round of selection and 10 µg/ml in the second and third rounds. The target antigen was coated at low concentrations in order to select antibodies of high specificity. Before and after each round of selection, the number of phage was determined by infecting TG1 cells. The data in Table 4.5 shows the titre of input and output phage antibodies and the percentage recovery after each round of selection. Initial recovery of phage from library I was low but detectable. Thereafter, percentage recoveries rose by about 100-fold from round one to two and from two to three. Initial recovery of phage from Tomlinson library J was almost 10-fold higher than from library I (Table 4.5). Progression to round two of selection saw a rise in recovery that was lower than from library J, but phage output in round three was nearly 1000-fold higher in percentage terms than from round two. These data and the enrichment rates (Figure 4.9) suggested that in both selections, specific phage antibodies were recovered and amplified.



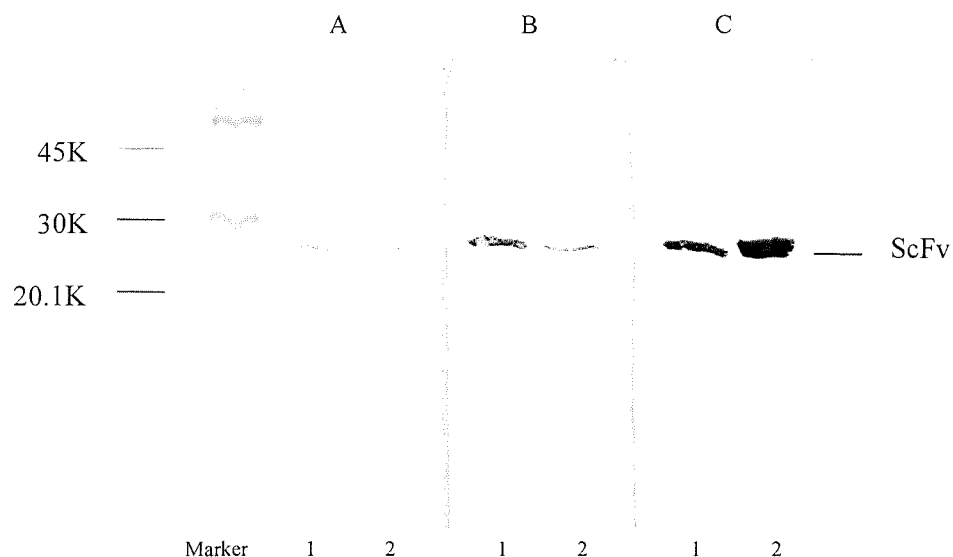
**Figure 4.4 Recognition of purified K99 at different dilutions using an anti-K99 monoclonal antibody**

Purified K99 was coated to plastic at the concentrations indicated and then detected with a mouse monoclonal and HRP-conjugated antibodies. Coating was carried out in duplicate; the mean value is presented, the error bars indicating the standard deviation of the two values. 2% MPBS was coated as a negative control (\*)



**Figure 4.5 SDS-PAGE analysis of concentrated culture supernatants from bacteria expressing anti-ubiquitin and anti-BSA scFvs**

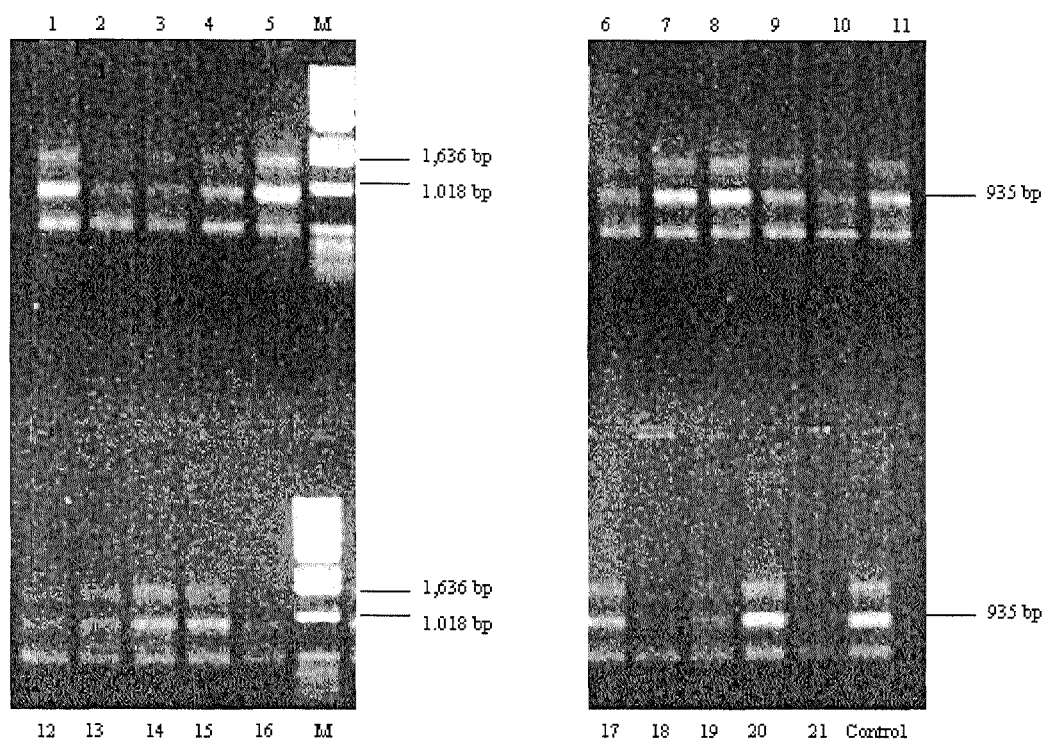
After electrophoresis, the gels was stained with Coomassie Blue and photographed. Lane M shows the migration of Rainbow molecular weight markers. The migration of standards of 45, 30 and 20.1 kDa is indicated



**Figure 4.6 Western blotting analysis of concentrated culture supernatants from bacteria expressing anti-ubiquitin and anti-BSA scFvs**

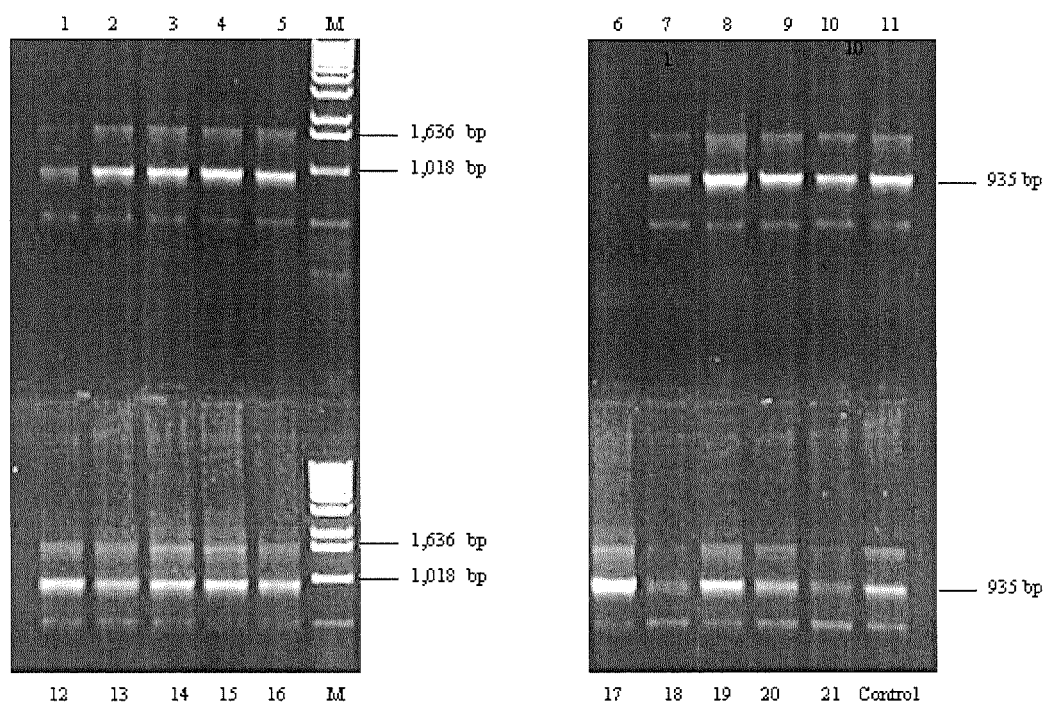
Culture supernatants from bacteria expressing an anti-ubiquitin (lane 1) and anti-BSA (lane 2) scFvs were separated by SDS-PAGE and blotted to nitrocellulose. Membrane strips were probed with rProtein LA-HRP (panel A), rProtein L-HRP (panel B) and anti-c-myc reagents (panel C). Lane M shows the migration of Rainbow molecular weight markers. The migration of standards of 45, 30 and 20.1 kDa is indicated. The predicted molecular weight of a soluble scFv is approximately 29 kDa.





**Figure 4.7 PCR screening for scFv genes in Tomlinson library I**

Crude lysates from 21 clones (lanes 1 to 21) picked at random were amplified with primers flanking the scFv insert. The products were separated on a 12% agarose gel containing ethidium bromide and photographed under ultraviolet illumination. Lane M shows the migration of a 1 kb DNA ladder. The migration of markers of 1,018 bp and 1,636 bp is indicated. In the lane marked "Control", products from bacteria carrying genes for an anti-ubiquitin scFv are shown.



**Figure 4.8 PCR screening for scFv genes in Tomlinson library J**

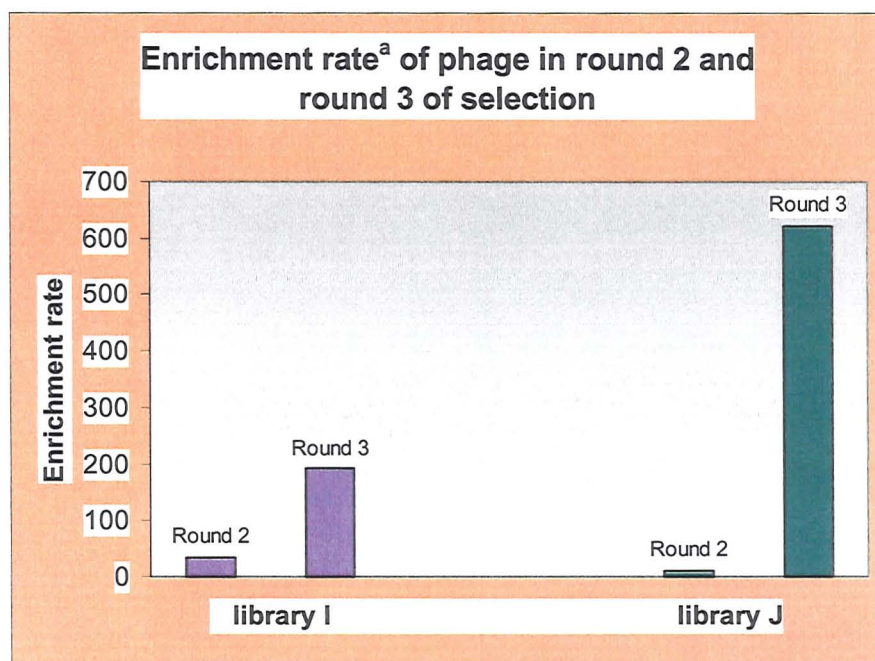
Crude lysates from 21 clones (lanes 1 to 21) picked at random were amplified with primers flanking the scFv insert. The products were separated on a 12 % agarose gel containing ethidium bromide and photographed under ultraviolet illumination. Lane M shows the migration of a 1 kb DNA ladder. The migration of markers of 1,018 bp and 1,636 bp is indicated. In the lane marked "Control", products from bacteria carrying genes for an anti-ubiquitin scFv are shown.

**Table 4.5 Progress of selection for anti-K99 scFv by panning**

Library	Tomlinson I			Tomlinson J		
Selection	Input <sup>a</sup>	Output <sup>a</sup>	% of Recovery <sup>b</sup>	Input	Output	% of Recovery
Round 1	$1.28 \times 10^{13}$	$4.38 \times 10^5$	$3.42 \times 10^{-8}$	$1.28 \times 10^{13}$	$3.00 \times 10^6$	$2.34 \times 10^{-7}$
Round 2	$1.40 \times 10^{12}$	$1.60 \times 10^6$	$1.14 \times 10^{-6}$	$1.30 \times 10^{12}$	$3.04 \times 10^6$	$2.33 \times 10^{-6}$
Round 3	$1.40 \times 10^{12}$	$3.08 \times 10^8$	$2.20 \times 10^{-4}$	$3.00 \times 10^{11}$	$4.36 \times 10^8$	$1.45 \times 10^{-3}$

<sup>a</sup> Input and output phage titres were determined by transduction of *E. coli* to ampicillin resistance using serial dilutions of sample

<sup>b</sup> Percent recovery determined by division of output phage titre by input titre and multiplication by 100



**Figure 4.9 Enrichment rates during panning of the Tomlinson libraries I and J against K99**

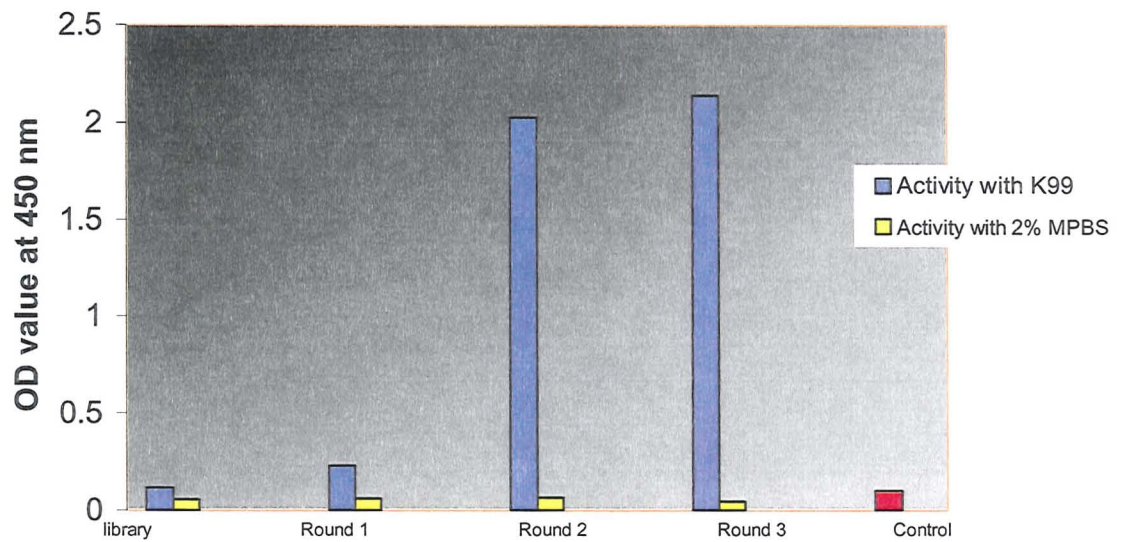
<sup>a</sup> Enrichment rate was calculated by dividing the percentage recovery in the given round of selection by the percentage recovery achieved in the previous round

## ***Polyclonal phage ELISA***

The populations of phage recovered after each round of selection from each library, were tested against purified K99 in ELISA. This screening was designed to test whether the elevated recoveries seen in Table 4.5 could be attributed to the selection of scFvs at the phage surface directed against target antigen or non-specific effects. Wells of an ELISA plate were coated with either 100  $\mu$ l of purified K99 at the concentration of 20  $\mu$ g/ml or 2% MPBS that was used as blocker during panning of the libraries. Samples of phage were taken from the original libraries prior to selection and after each round of panning. The phage were incubated in the coated wells, washed, and binding detected with virus-specific antibodies. Figures 4.10 and Figure 4.11 show the results of these assays for Tomlinson libraries I and J respectively. In each case, recognition of K99 or milk protein is very low in samples from the unselected libraries and comparable to the reaction of KM13 helper phage with K99. Only a slight bias towards K99 is evident after one round of panning. However, reactivity towards the K99 antigen rose sharply after round two and strong reaction against this target was evident after round three. Interestingly, phage antibodies isolated from library J after round two had a stronger reaction with 2% MPBS than at other stages of the screening or at any point from Tomlinson library J. This could be taken as evidence that in selection from the Tomlinson library I, about one third of phage isolated in rounds one and two had the potential to bind to milk protein but bias in favour of K99 was restored in round three. Overall, the results of polyclonal phage ELISA indicated that phage antibodies reactive with K99 fimbriae were successfully isolated and enriched from both Tomlinson libraries I and J.

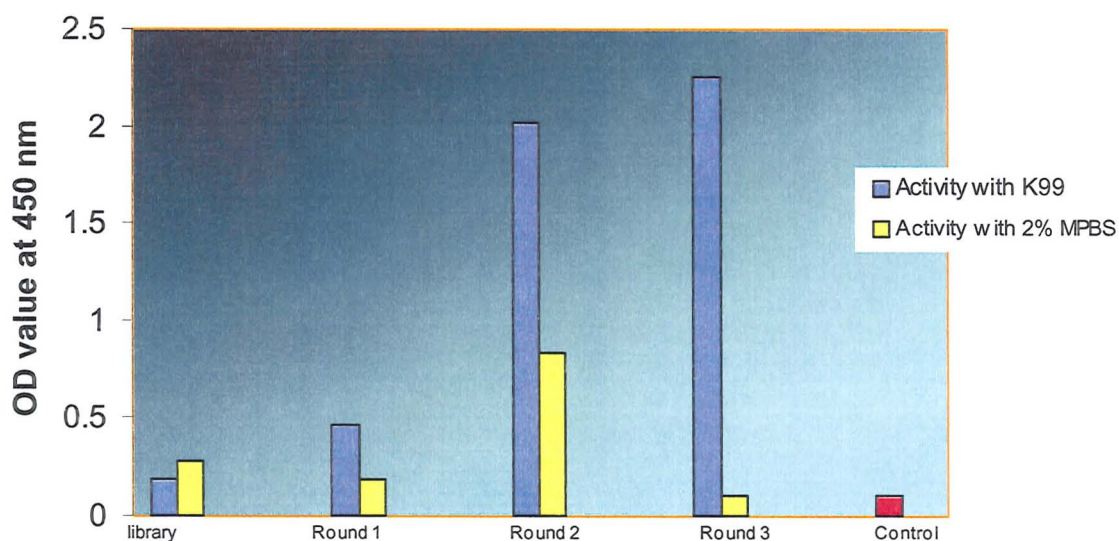
## ***Monoclonal phage ELISA***

A total of 96 bacterial colonies were picked at random from each library during selection against K99. Twenty colonies were chosen from each original library prior to panning, and after rounds one and two. A further 36 were picked after round three. These individual colonies were grown, infected with helper phage, culture supernatants were then used in monoclonal phage ELISA. The phage samples were added to wells of ELISA plates that had been coated with purified K99 at 20  $\mu$ g/ml and blocked with 3% BSA/PBS. Binding was tested as before with phage-specific antibodies. The results are shown in



**Figure 4.10 Polyclonal phage ELISA of Tomlinson library I during panning against K99**

Phage from the original library and each round of selection by were tested in ELISA against purified K99 and 2% MPBS using an anti-M13-HRP monoclonal conjugate. The control shows the reaction of KM13 helper phage against the K99 antigen. Phage samples were tested against each antigen in duplicate and the mean value is presented.



**Figure 4.11 Polyclonal phage ELISA of Tomlinson library J during panning against K99**

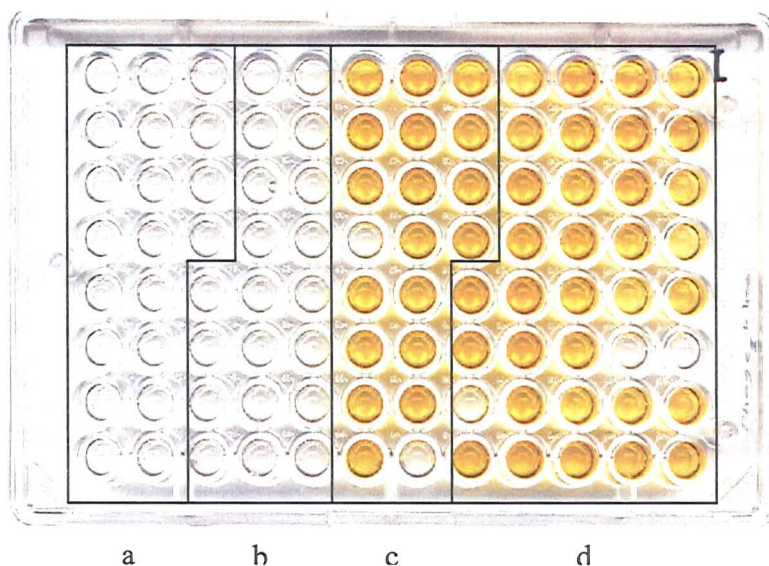
Phage from the original library and each round of selection by were tested in ELISA against purified K99 and 2% MPBS using an anti-M13-HRP monoclonal conjugate. The control shows the reaction of KM13 helper phage against the K99 antigen. Phage samples were tested against each antigen in duplicate and the mean value is presented.

Figures 4.12 (library I) and 4.13 (library J). The Figures show that none of the randomly chosen phage antibodies prepared from the original libraries recognised K99 fimbriae to a detectable extent. After a single round of selection, only one phage antibody selected from the Tomlinson library J had activity against K99 antigen and all clones isolated from library I at this point were unreactive. In contrast most of the phage antibodies isolated from each library after two and three rounds of panning showed strong reaction with the K99 target. These results indicate that selection for recombinant phage antibodies against K99 by phage display was successful.

### ***Screening monoclonal soluble scFv antibodies by ELISA***

In work with the Griffin library, a discrepancy was noted between the results of phage ELISA and the reactivity towards K99 of soluble svFvs produced from promising clones. It was therefore of considerable importance to assess carefully whether clones from the Tomlinson libraries behaved similarly. This was tackled by ELISA against purified K99. Soluble scFv antibodies were prepared from a number of phage clones picked at random and infected into *E. coli* HB2151. The infected cells were grown on TYE plates under ampicillin selection. Twenty clones were picked from the original libraries I and J before panning on K99 along with 20 from rounds 1 and 2, and 36 colonies from round 3. After infection to HB2151, bacteria were grown and expression of soluble scFvs induced by addition of IPTG. Culture supernatants containing each monoclonal, soluble scFv were used in immunoassay. ELISA plates were coated with purified K99 fimbriae, and were blocked with 2% MPBS. After adding the culture supernatants, bound scFvs were detected using rProtein L-HRP. Table 4.6 shows the number and percentage of scFvs that were judged positive in the assay for each library. The results from library I show that clones able to express soluble scFv with anti-K99 activity first become detectable after round 2 of selection and that they come to dominate the recovered population after round 3. This provides clear evidence that panning the library against K99 successfully selected and enriched antibodies against the fimbriae. It is interesting however to contrast the modest number of anti-K99 clones detected in this assay after round 2 with their apparent frequency in monoclonal phage ELISA (Figure 4.13). A different picture emerged from analysis of clones from Tomlinson library J. Here, the numbers of colonies expressing anti





**Figure 4.12 Monoclonal phage ELISA of Tomlinson library I during panning against K99**

Individual phage clones from the original library and each round of selection by were tested in ELISA against purified K99 using an anti-M13-HRP monoclonal conjugate. Phage were applied as follows:

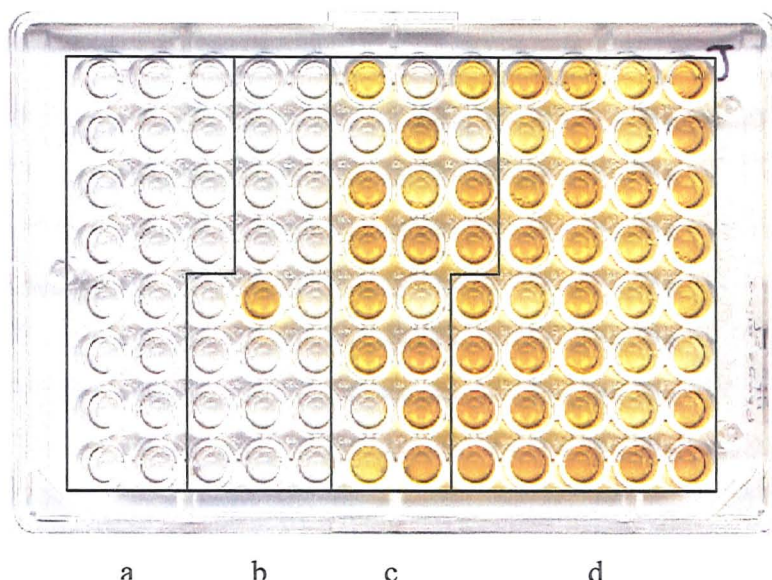
section a: 20 clones picked at random from the Tomlinson library I before selection

section b: 20 clones picked at random after round 1 of selection

section c: 20 clones picked at random after round 2 of selection

section d: 36 clones picked at random after round 3 of selection

The O.D at 492 nm was measured after 10 minutes.



**Figure 4.13 Monoclonal phage ELISA of Tomlinson library J during panning against K99**

Individual phage clones from the original library and each round of selection by were tested in ELISA against purified K99 using an anti-M13-HRP monoclonal conjugate. Phage were applied as follows:

section a: 20 clones picked at random from the Tomlinson library J before selection

section b: 20 clones picked at random after round 1 of selection

section c: 20 clones picked at random after round 2 of selection

section d: 20 clones picked at random after round 3 of selection

The O.D was measured at 492 nm after 10 minutes.

K99 scFvs after round 2 is substantially high after round 3. Since the Tomlinson library J was diversified with NNK codons, this could be due to enrichment of clones containing stop codons in round 3 of selection.

### ***PCR analysis of genes encoding anti-K99 scFvs***

Ten clones from Tomlinson library I were chosen from rounds 2 and round 3 on the basis of their reaction against K99 in ELISA screening of soluble scFv antibodies. Their coding sequences were initially analysed by PCR using primers pelB and pHEN seq to screen for the presence of full-length inserts. The results are shown in Figure 4.14. All 10 reactions yielded products that were identical in size to that from the positive control, an anti-ubiquitin scFv. Comparison of the sizes of these amplicons was consistent with that predicted for a full-length scFv insert (935 bp).

### ***Restriction analysis of genes encoding anti-K99 scFvs***

Plasmid DNA was isolated from 10 clones chosen from screening of Tomlinson library I and 5 from library J. Clones were chosen because they showed higher reaction against K99 in ELISA assays of soluble scFv antibodies than the majority. DNA samples were digested with the restriction enzymes *NcoI* and *NotI* to assess if all clones carried full-length scFv inserts. Figures 4.15 and 4.16 reveal that all the clones selected for detailed analysis contained full-length scFv genes.

### ***Western blotting of clones from libraries I and J***

The expression of scFv antibodies from the 15 clones selected for detailed analysis was confirmed by Western blotting. HB2151 clones were grown in similar conditions and induced by addition of IPTG. Culture supernatants were concentrated to approximately 1/25 initial volume and similar volumes were run on SDS-PAGE gels. Proteins were transferred to nitrocellulose membrane and after blocking with 3% MPBS, single chain Fv fragments were detected using mouse monoclonal anti-c-Myc and anti-mouse-HRP antibodies. Figure 4.17 shows Western blotting analysis of the 10 clones chosen from Tomlinson library I. The expected size of a scFv is approximately 29 kDa. The results confirm that scFv antibodies were expressed by all selected clones but it is apparent that

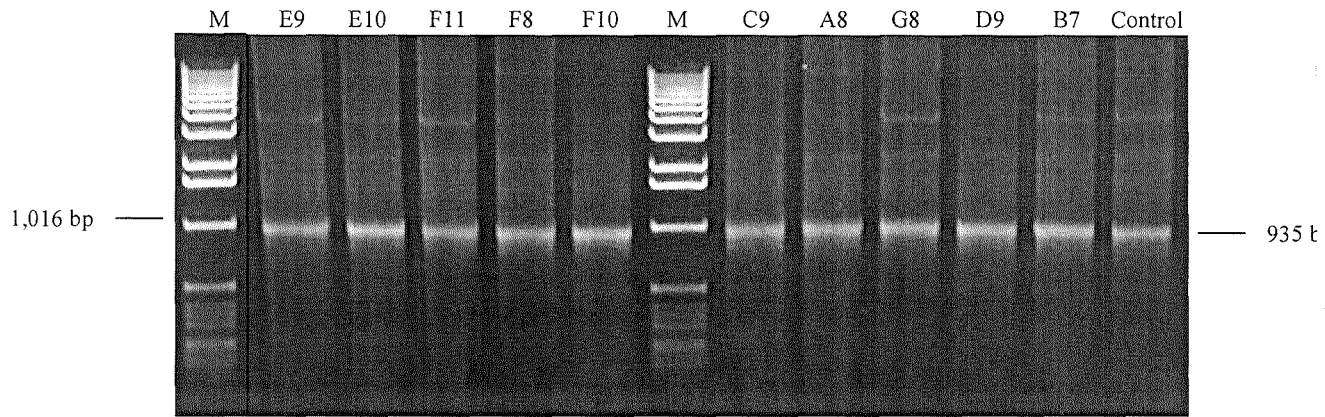
**Table 4.6 ELISA screening of soluble scFv antibodies against K99 fimbriae**

	Tomlinson library I			Tomlinson library J	
	Number of positives scFvs <sup>a</sup>	Percentage of positive scFvs <sup>b</sup>		Number of positive scFvs <sup>a</sup>	Percentage positive of scFvs <sup>b</sup>
Original library	0	0 %	Original library	0	0 %
Round 1	0	0 %	Round 1	1	5 %
Round 2	1	5 %	Round 2	11	55 %
Round 3	31	86 %	Round 3	3	8 %

Supernatants from induced HB2151 cultures were applied to K99-coated ELISA plates and the binding of svFvs detected with rProtein L-HRP. In all, 20 clones were tested from the original libraries prior to selection, and the same number from rounds 1 and 2 of panning. Thirty-six clones were tested from round 3. All clones were picked at random.

a: scFv antibodies that showed an OD at 492 nm of more than 0.1 after 10 minutes of development were considered positive

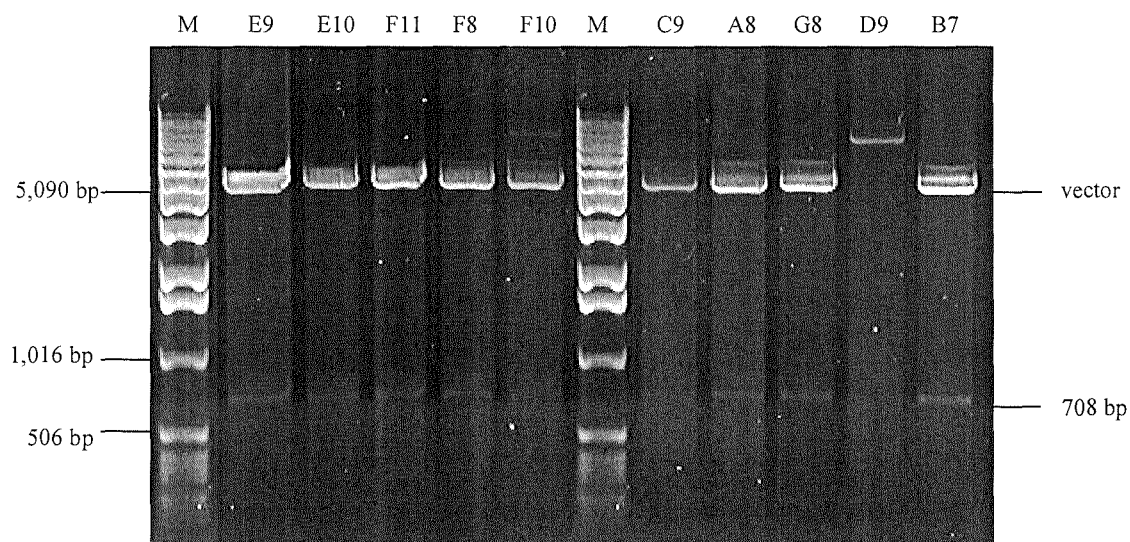
b: The percentage of positive scFvs was calculated by dividing the number of positive scFvs by the total number of clones tested for each stage of the experiment and multiplication by 100



**Figure 4.14** PCR screening of 10 anti-K99 clones from Tomlinson library I

Ten clones were chosen after screening of the Tomlinson library I on the basis of their strong reaction to K99 in ELISA of soluble scFv. The scFv inserts were amplified using primers of LMB3 and pHEN seq. For a full-length scFv insert, the expected size of the amplicon is about 935 bp (indicated).

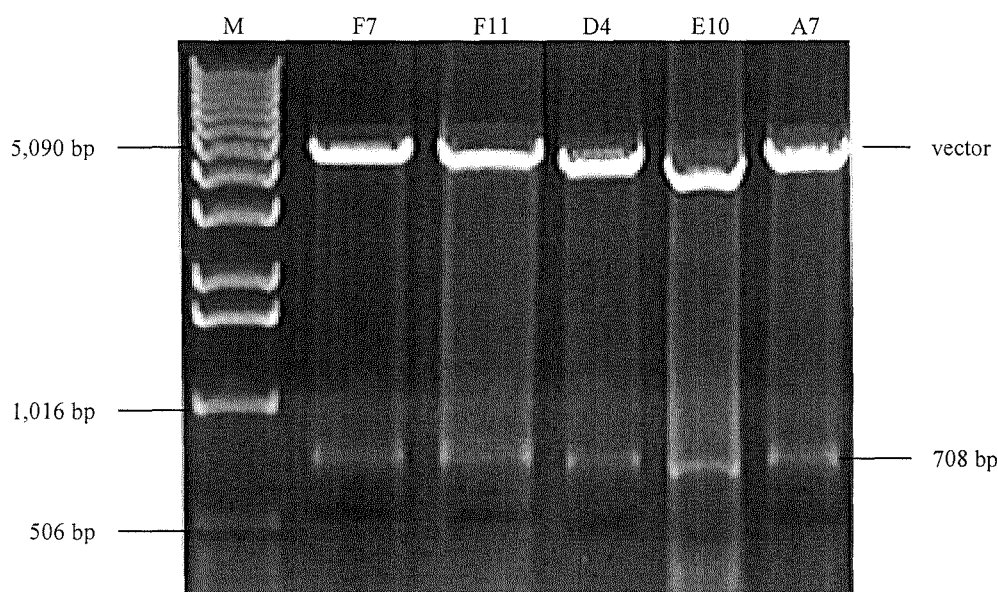
M: 1 kb DNA Ladder (Invitrogen, UK). The migration of a marker of 1,016 bp is indicated  
Control: Positive control PCR amplified from an anti-ubiquitin scFv.



**Figure 4.15** Restriction analysis of 10 anti-K99 clones from Tomlinson library I

Ten clones were chosen after screening of the Tomlinson library I on the basis of their strong reaction to K99 in ELISA of soluble scFv. Plasmid DNA from each clone was digested with *Nco*I and *Not*I. The expected size of the small fragment released is 708 bp (indicated).

M: 1 kb DNA Ladder (Invitrogen, UK). The migration of markers of 506 bp, 1,016 bp and 5,090 bp are indicated



**Figure 4.16** Restriction digestion of plasmids of selected colonies of Tomlinson library J

Five clones were chosen after screening of the Tomlinson library J on the basis of their strong reaction to K99 in ELISA of soluble scFv. Plasmid DNA from each clone was digested with *NcoI* and *NotI*. The expected size of the small fragment released is 708 bp (indicated). Plasmid DNA from each clone was digested with *NcoI* and *NotI*. The expected size of the small fragment released is 708 bp (indicated).

M: 1 kb DNA Ladder (Invitrogen, UK). The migration of markers of 506 bp, 1,016 bp and 5,090 bp is indicated

the proteins are expressed to quite widely differing levels and none of the signals match that from the anti-BSA control used in the experiment.

Figure 4.18 shows the equivalent analysis of the 5 clones from library J. As we can see from the Figure, 4 clones showed good levels of expression of scFv antibody, only clone D4 had very low level of expression. In contrast to Figure 4.17, several clones appeared to produce recombinant protein to a higher level than that achieved by the anti-BSA positive control.

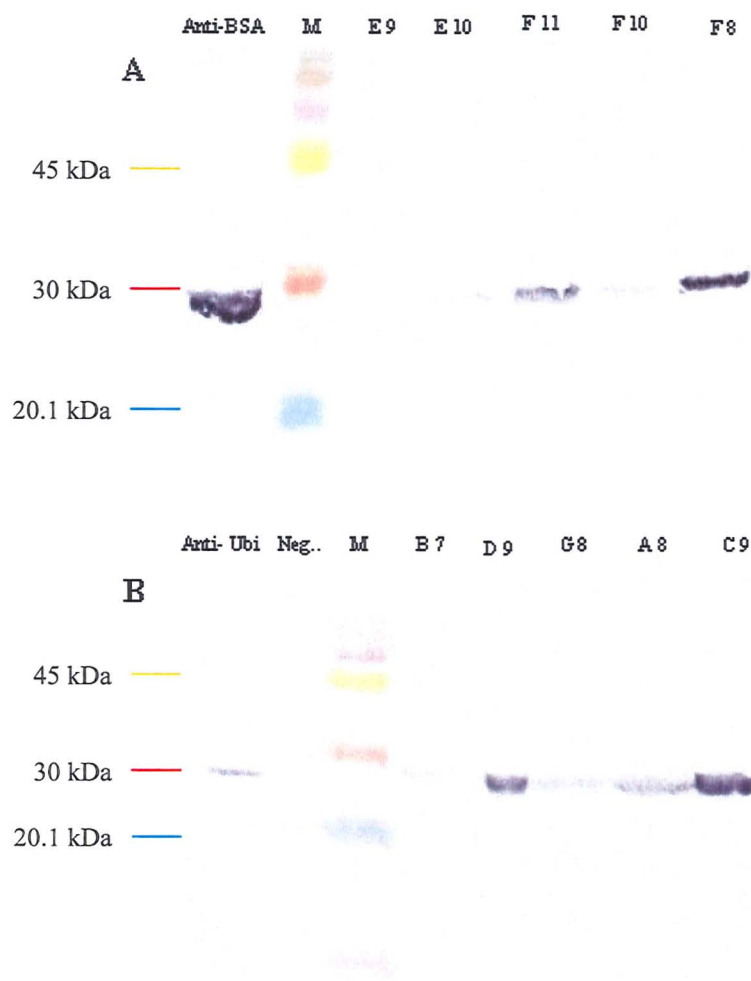
### ***Dot blotting of selected clones of library I and library J***

Attempts were made to test the reactivity of the anti-K99 scFvs against purified fimbriae using Western blotting. These were unsuccessful. In order to determine the reasons for this and to confirm that the recombinant antibodies would bind K99 after attachment to a substrate other than an ELISA plate, the following dot blot experiments were conducted. Purified K99 was boiled in SDS sample buffer and then spotted to strips of nitrocellulose along with unheated samples of the antigen. Concentrated supernatants prepared from induced cultures of the 10 clones selected from library I were added. The binding of scFvs was then detected with anti-c-myc reagents and with 4-chloro-1-naphthol substrate. The results (Figure 4.19) clearly showed that some of the chosen scFv antibodies recognised strongly the purified K99 but that heat treatment abolished this binding. The same effect was observed when a mouse monoclonal antibody against K99 was used (Figure 4.19). In view of this, the experiment was repeated with the 5 clones chosen from analysis of library J, using only unheated samples of K99 (Figure 4.20). Overall, the strongest signals were obtained from clones C9 and F10 from library I and A7 and F7 from library J. Comparison with previous Figure shows that this pattern does not simply reflect the levels of scFv expressed by these clones. The negative control scFv, an anti-BSA scFv, had no reaction with either heated or unheated K99 fimbriae.

### ***Sequencing of anti-K99 scFvs from Tomlinson libraries I and J***

DNA sequences of the 10 selected clones from library I and 5 from library J were analysed to confirm their reading frames were intact, to predict their amino acid sequences and thereby establish their diversity. The sequencing results were initially checked for the

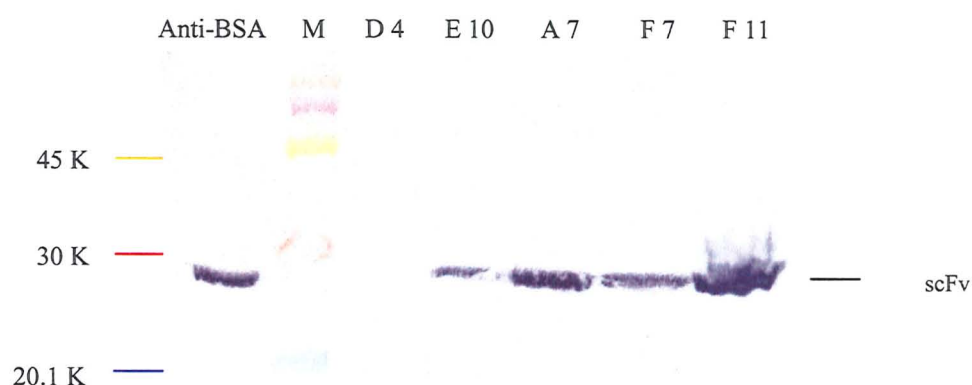




**Figure 4.17 Western blotting of 10 clones selected from Tomlinson library I**

Concentrated supernatants from induced cultures were separated by SDS-PAGE, transferred to membrane and probed with anti-c-myc reagents. The predicted molecular weight of a scFv is approximately 29 kDa (indicated). In panel A, an anti-BSA clone served as a positive control; in panel B, an anti-ubiquitin scFv was used. In panel B, culture supernatant from an induced culture of *E. coli* HB2151 cell carrying plasmid but lacking a scFv insert was used as negative control (Neg).

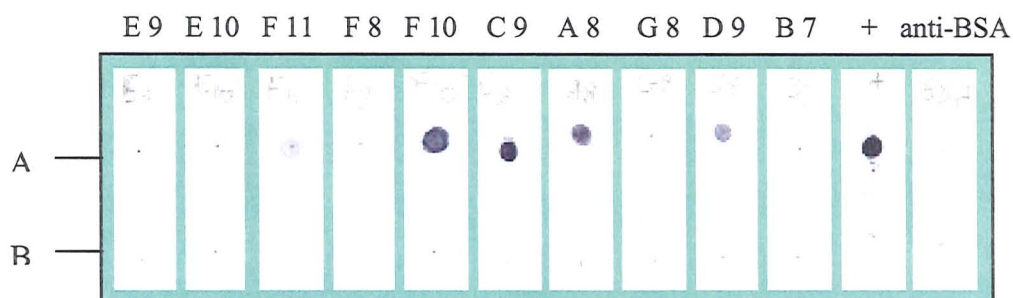
M: Rainbow protein molecular weight markers (Pharmacia). The migration of standards of 45, 30 and 20.1 kDa is indicated.



**Figure 4.18 Western blotting of 5 selected colonies from Tomlinson library J**

Concentrated supernatants from induced cultures were separated by SDS-PAGE, transferred to membrane and probed with anti-c-myc reagents. The predicted molecular weight of a scFv is approximately 29 kDa (indicated). An anti-BSA clone served as a positive control.

M: Rainbow protein molecular weight markers (Pharmacia). The migration of standards of 45, 30 and 20.1 kDa is indicated.

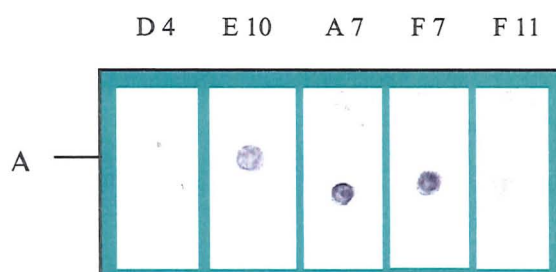


**Figure 4.19** Dot blot analysis of 10 anti-K99 scFvs from Tomlinson library I

Unheated (row A) and heated samples of K99 (row B) were spotted to nitrocellulose and the binding of anti-K99 scFvs detected with anti-c-myc reagents.

+: Positive control, A mouse anti-K99 monoclonal antibody detected with anti-mouse reagents serving as positive control

The anti-BSA scFv (indicated) was used as a negative control



**Figure 4.20 Dot blot analysis of 5 anti-K99 scFvs from Tomlinson library J**

Unheated (row A) samples of K99 were spotted to nitrocellulose and the binding of anti-K99 scFvs detected with anti-c-myc reagents.

presence of a complete  $V_H$ -linker- $V_L$  sequence. This is illustrated for clone C9 (library I) in Figure 4.21. The DNA and predicted amino acid sequences are presented in Tables 4.7 and 4.8. Since both libraries were based on single human  $V_H$  and  $V_L$  frameworks, diversity was confined to the heavy chain complementarity determining regions CDR2 (residues H50, H52, H52a, H53, H55, H56, H58), and CDR3 (H95, H96, H97, H98), and CDRs2 (L50, L53) and 3 (L91, L92, L93, L94, L96) for the light chain component. The data indicate some clones are identical in their amino acid sequences through these regions. For example from library I, B7 and F10 are identical, as are A8, C9 and D9. For others, differences are limited to conservative substitutions. In this regard, the  $V_H$  component of E9 (library I) only differs from the B7/F10 pair at 2 positions (H58: N replaces D; H96: T replaces S). In some cases, identical sequences emerge from different libraries. This is illustrated by comparison of the  $V_L$  components of G8 (library I) and F11/A7 (library J). Whilst the sequences may be unique to a greater or lesser extent, the overall impression is that the clones carry broadly similar sequences.

### ***Purification of soluble scFvs***

Four scFvs from Tomlinson library I and two scFvs from library J were chosen for purification and analysis of biological function. On the basis of the sequence data and activity in dot blots, these were C9, F10, F8 and F11 from library I and F7 and A7 from library J. Supernatants from large scale cultures were concentrated in a tangential flow apparatus and purified by nickel chelating chromatography, exploiting the histidine repeats carried at the carboxyl termini of the scFvs. Fractions eluted from the affinity column were analysed by SDS-PAGE and Western blotting. To illustrate this, data is presented in Figures 4.22 and 4.23 from the purification of scFv C8. Analysis by SDS-PAGE (Figure 4.22) confirmed the presence of a protein of the size expected for a scFv in concentrated culture supernatant (lane a). Some protein of this molecular weight could be observed in flow through and wash fractions (lanes b, c and d) along with material of a range of sizes. A single protein was successfully eluted by raising the imidazole concentration to 200 mM. While this was of the size expected for a scFv, its identity was confirmed by Western

Table 4.7 Amino acid sequence diversity of 10 selected clones from library I

Clone Name	Heavy chain											Light chain						
	CDR2				CDR3							CDR2			CDR3			
	H50	H52	H52a	H53	H55	H56	H58	H95	H96	H97	H98	L50	L53	L91	L92	L93	L94	L96
A8	Y	S	D	S	T	D	S	K	T	Y	A	N	S	Y	S	Y	G	S
B7	S	T	D	N	N	T	D	K	S	Y	A	N	T	Y	N	A	G	T
C9	Y	S	D	S	T	D	S	K	T	Y	A	N	S	Y	S	Y	G	S
D9	Y	S	D	S	T	D	S	K	T	Y	A	N	S	Y	S	Y	G	S
E10	G	A	T	A	D	T	Y	R	A	Y	G	G	A	Y	Y	Y	S	S
E9	S	T	D	N	N	T	N	K	T	Y	A	G	D	Y	D	D	S	D
F10	S	T	D	N	N	T	D	K	S	Y	A	N	T	Y	N	A	G	T
F8	T	S	D	D	G	S	T	K	T	Y	G	N	D	Y	N	S	Y	S
G8	T	E	A	W	N	Y	V	K	K	S	A	A	S	S	Y	S	T	N
F1	Y	T	D	Y	N	D	Y	S	Deletion			N	Y	Y	Y	D	Deletion	

Table 4.8 Amino acid sequence diversity of 5 selected clones from library J

Clone Name	Heavy chain											Light chain						
	CDR2						CDR3					CDR2			CDR3			
	H50	H52	H52a	H53	H55	H56	H58	H95	H96	H97	H98	L50	L53	L91	L92	L93	L94	L96
F11	T	E	A	W	N	Y	V	K	S	A	G	A	S	S	Y	S	T	N
F7	S	V	A	A	D	L	S	N	L	P	R	S	G	G	S	R	L	S
E10	G	A	T	A	D	T	Y	A	Y	G	G	A	S	S	Y	S	T	N
D'4	Y	V	A	S	N	T	S	C	A	K	N	S	S	G	Y	S	S	Y
A7	S	V	A	A	D	L	S	N	L	P	R	A	S	S	Y	S	T	N

Figure 4.21 Complete nucleic acid sequence of clone C9 from library I

Heavy chain

```

FR1
1  2  3  4  5  6  7  8  9 10 11 12 13 14 15
GAG GTG CAG CTG TTG GAG TCT GGG GGA GGC TTG GTA CAG CCT GGG

16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
GGG TCC CTG AGA CTC TCC TGT GCA GCC TCT GGA TTC ACC TTT AGC

CDR1 FR2
31 31a 31b 32 33 34 35 36 37 38 39 40 41 42 43
AGC ... .. TAT GCC ATG AGC TGG GTC CGC CAG GCT CCA GGG AAG

44 45 46 47 48 49 50* 51 52* 52a* 52b 52c 53* 54 55*
GGG CTG GAG TGG GTC TCA TAT ATT AGT GAT ... .. TCT GGT ACT

CDR2 FR3
56* 57 58* 59 60 61 62 63 64 65 66 67 68 69 70
GAT ACA TCT TAC GCA GAC TCC GTG AAG GGC CGG TTC ACC ATC TCC

71 72 73 74 75 76 77 78 79 80 81 82 82a 82b 82c
AGA GAC AAT TCC AAG AAC ACG CTG TAT CTG CAA ATG AAC AGC CTG

83 84 85 86 87 88 89 90 91 92 93 94 95* 96*
AGA GCC GAG GAC ACG GCC GTA TAT TAC TGT GCG AAA TGT GCG

CDR3 FR4
97* 98* 99 100 101 102 103 104 105 106 107 108 109 110 111
AAA ACT TAT GCT GGT TTT GAC TAC TGG GGC CAG GGA ACC CTG GTC

112 113
ACC GTC

```

Linker

GGT GGA GGC GGT TCA GGC GGA GGT GGC AGC GGC GGT GGC GGG



Figure 4.21 (continued)

Light chain

												FR1									
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17					
GAC	ATC	CAG	ATG	ACC	CAG	TCT	CCA	TCC	TCC	CTG	TCT	GCA	TCT	GTA	GGA	GAC					
												CDR1									
18	19	20	21	22	23	24	25	26	27	28	29	30	31	31a	31b	31c	31d				
AGA	GTC	ACC	ATC	ACT	TGC	CGG	GCA	AGT	CAG	AGC	ATT	AGC	AGC	...	...	...	...				
												FR2									
31e	31f	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46					
...	...	TAT	TTA	AAT	TGG	TAT	CAG	CAG	AAA	CCA	GGG	AAA	GCC	CCT	AAG	CTC					
												CDR2									
47	48	49	50*	51	52	53*	54	55	56	57	58	59	60	61	62						
CTG	ATC	TAT	AAT	GCA	TCC	TCT	TTG	CAA	AGT	GGG	GTC	CCA	TCA	AGG	TTC						
												FR3									
63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79					
AGT	GGC	AGT	GGA	TCT	GGG	ACA	GAT	TTC	ACT	CTC	ACC	ATC	AGC	AGT	CTG	CAA					
												CDR3									
80	81	82	83	84	85	86	87	88	89	90	91*	92*	93*	94*	95	96*					
CCT	GAA	GAT	TTT	GCA	ACT	TAC	TAC	TGT	CAA	CAG	TAT	TGT	CAA	CAG	TAT	TCT					
												FR4									
97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112						
TAT	GGT	CCT	AGT	ACG	TTC	GGC	CAA	GGG	ACC	AAG	GTG	GAA	ATC	AAA	CGG						

Codons are numbered according to the Kabat convention with framework and complementarity determining regions indicated over the sequence. Positions that are diverse in the primary repertoire are indicated with an asterisk.

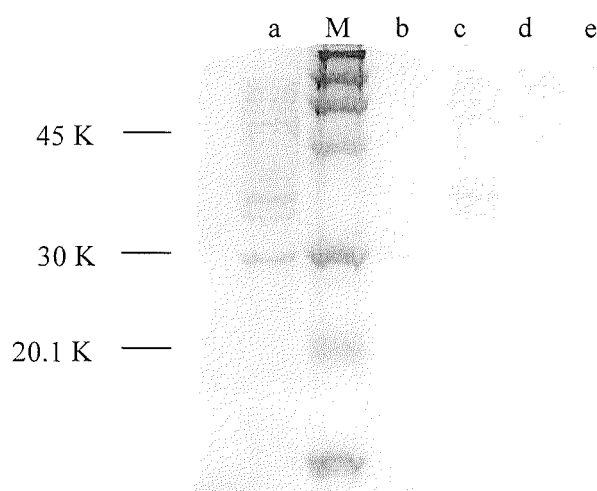
blotting by using monoclonal antibody against the c-myc tag carried by the recombinant antibody (Figure 4.23). The scFv and a cross-reacting contaminant could be detected in the concentrated culture supernatant (lane a). The high molecular weight contaminant failed to bind to the affinity column to a significant extent (lane b) and residual traces were successfully removed during initial washing (lane c). Protein of a size similar to a scFv noted from SDS-PAGE analysis to be present in the column flow through and washes failed to react with the anti c-myc reagents (lanes b-d). In contrast, the 29 kDa protein eluted with 200 mM imidazole reacted strongly in the analysis (lane e) confirming that it was indeed the scFv.

### ***Comparison of activity of purified scFvs by ELISA***

Purification of the scFvs and adjustment to equal protein concentrations allowed assessment of their relative reactivities with purified K99 fimbriae. K99 was coated to an ELISA plate, the scFvs added their binding detected using rProtein L-HRP. Results of this experiment are shown in Figure 4.24 and reveal that the purified scFv antibodies all recognised K99 fimbriae but there was a range of approximately 8-fold in the signals obtained from the assay from the lowest (F8) to the highest (F7). Interestingly, the strongest signals were obtained from the 2 clones chosen from library J. Signals from an anti-BSA scFv negative control were very low.

### ***Haemagglutination inhibition assay***

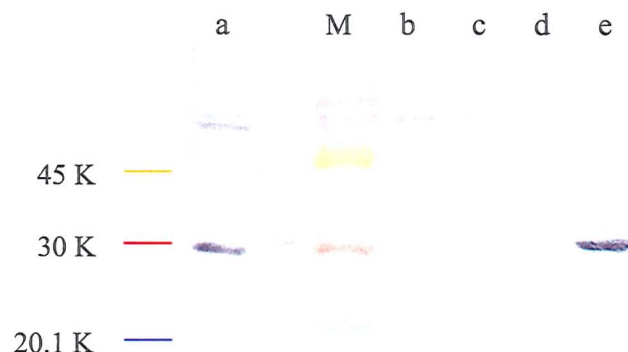
Suspensions of *E. coli* B 41 grown at 37 °C caused haemagglutination of sheep red-blood cells through expression of K99 fimbriae. By testing serial dilutions of the bacterial suspension, the haemagglutination activity could be titred to 1/64. Haemagglutination of sheep red-blood cells was not inhibited by the presence of 0.5 % D-mannose. Also, haemagglutination was not evident when the bacteria were grown at 18 °C or when bacteria were not added to the erythrocytes (Figure 24.26).



**Figure 4.22 SDS-PAGE analysis of fractions recovered during the purification of scFv C 9**

Concentrated culture supernatant was equilibrated with column binding buffer by buffer exchange (lane a). The material was applied to an affinity column charged with nickel and the flow through collected (lane b). The column was washed first with 20 mM imidazole in binding buffer (lane c), then 50mM imidazole (lane d), before eluting bound proteins with 200 mM imidazole. The recovered scFv was concentrated about 5 fold before loading to the gel (lane e).

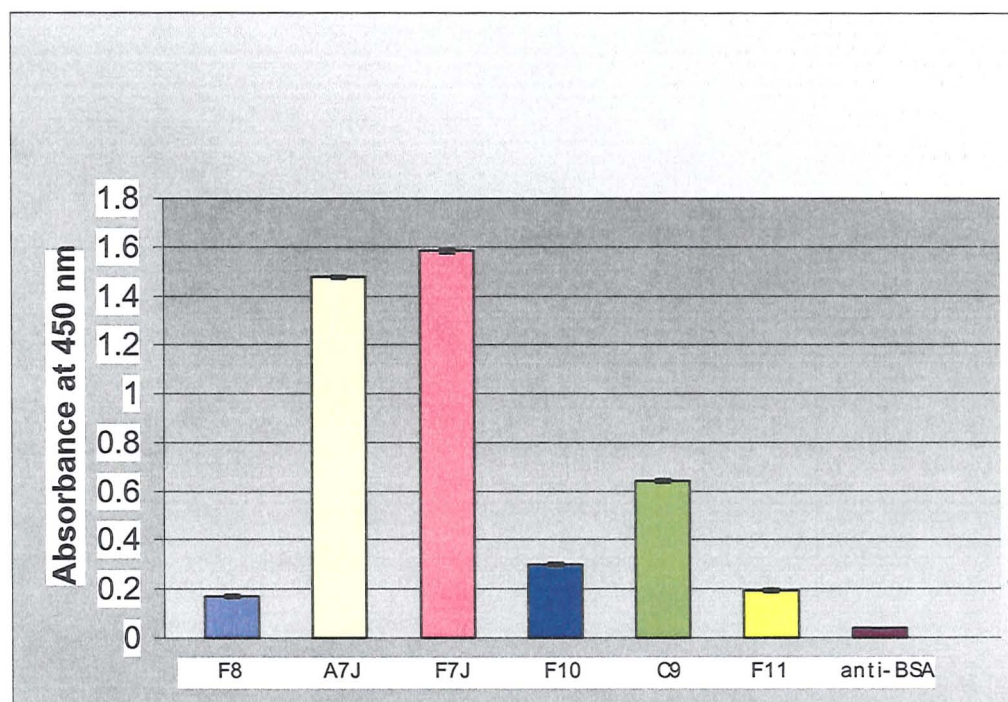
M: Rainbow protein molecular weight markers (Pharmacia). The migration of standards of 45, 30 and 20.1 kDa is indicated.



**Figure 4.23** Western blotting analysis of fractions recovered during the purification of scFv C 9

Concentrated culture supernatant was equilibrated with column binding buffer by buffer exchange (lane a). The material was applied to an affinity column charged with nickel and the flow through collected (lane b). The column was washed first with 20 mM imidazole in binding buffer (lane c), then 50mM imidazole (lane d), before eluting bound proteins with 200 mM imidazole. The recovered scFv was concentrated about 5 fold before loading to the gel (lane e). Samples were run first on an SDS-PAGE gel, then transferred to nitrocellulose and probed with anti c-myc reagents.

M: Rainbow protein molecular weight markers (Pharmacia). The migration of standards of 45, 30 and 20.1 kDa are indicated.



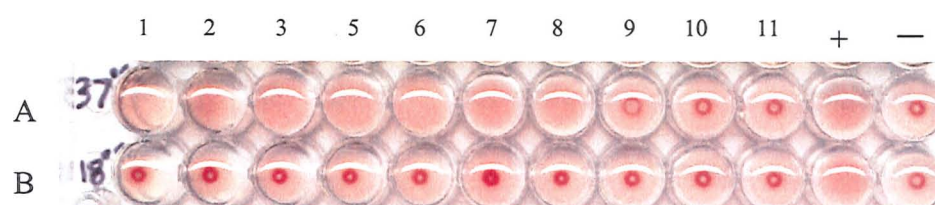
**Figure 4.24 Activity of purified scFvs in ELISA**

The purified scFvs were adjusted to equal total protein concentrations and applied in duplicate to wells coated with K99. Binding was detected with rProtein L-HRP. An anti-BSA scFv was used as a negative control. Error bars show the standard deviation of the duplicated samples.

Bacterial haemagglutination of sheep erythrocytes provided a convenient *in vitro* model in which the biological activity of anti-K99 scFvs could be tested. In these assays, a suspension of B41 bacteria was diluted close to its haemagglutinating titre and then incubated for 2 hrs at 37 °C with dilutions of the isolated scFv antibodies. By then adding sheep erythrocytes and testing for haemagglutination, the inhibitory activity of the antibody fragments could be assessed (Table 4.9). The results show that haemagglutination was inhibited by the binding of the scFvs to K99 fimbriae on the surface of bacteria. A purified scFv against ubiquitin had no effect on the haemagglutinating properties of the bacteria. In contrast, addition of an anti-K99 polyclonal antibody to B41 bacterial suspension grown at 37 °C successfully inhibited haemagglutination to high dilutions. Bacteria grown at 18 °C were unable to agglutinate erythrocytes. The monovalent binding of scFvs to their target in this assay effectively excluded the possibility that bacteria were themselves agglutinated and thereby prevented from interaction with the erythrocytes. It was not possible to rule this out as an explanation of the high HI titre of the polyclonal sheep antibody (Table 4.9).

### ***Indirect fluorescent antibody test (IFA)***

Indirect immunofluorescent staining was used to confirm that all six anti-K99 scFvs were able to bind to fimbriae on the surface of *E.coli* cells. Bacteria were grown at 37 °C, attached to glass slides and fixed. The recombinant antibodies were then added and their binding detected using an anti-c-myc monoclonal and an FITC-conjugated anti-mouse antibody. Fluorescence microscopy confirmed that all anti-K99 scFvs successfully adhered to the surface of bacteria grown at 37 °C though images were difficult to capture (data not shown). A positive control mouse monoclonal antibody against K99 produced identical patterns of greater intensity (Figure 4.26) and confirmed that antibody attachment was not detectable when bacteria were first grown at 18 °C. No binding could be observed when a control scFv against BSA was used (data not shown).



**Figure 4.25** Haemagglutination activity of *E. coli* B41 cells

Bacteria were grown overnight at 37 °C (row A) or at 18 °C (row B), then diluted two folds in PBS (wells 2 to 10) and mixed with sheep erythrocytes. Haemagglutination became evident after 2hrs incubation on ice. Undiluted suspensions of B41 were used as positive controls (+). As negative controls, sheep erythrocytes were incubated in the absence of bacteria (-). Titre was defined as the final dilution of overnight culture in which haemagglutination was evident (1/64; well 7)

Table 4.9 The inhibition of haemagglutination activity of by anti-K99 scFvs

Antibody	HI titre <sup>a</sup>
F11	1/8
C9	1/16
F8	1/16
F10	1/8
A7 J	ND
F7 J	ND
Polyclonal antibody (+) <sup>b</sup>	1/1024
Anti-ubiquitin (-) <sup>c</sup>	1/2

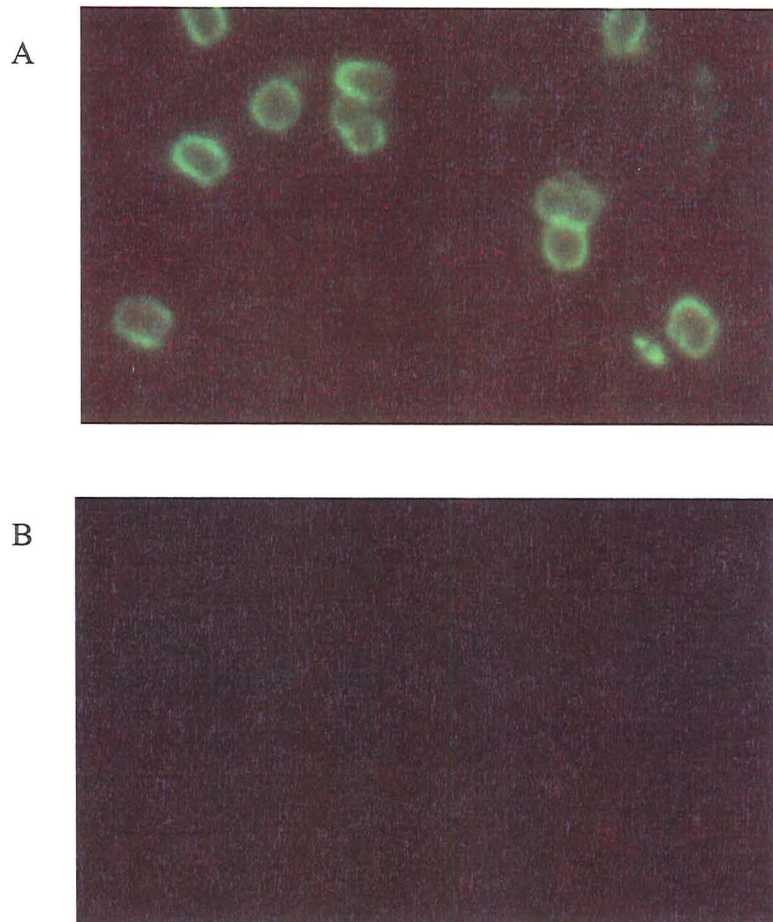
<sup>a</sup> HI titres were defined as the highest dilutions of antibody that successfully inhibited 4 haemagglutinating units of B41 bacteria

<sup>b</sup> A polyclonal sheep anti-K99 antibody was used as a positive control

<sup>c</sup> A scFv against ubiquitin was used as a negative control

ND: Not determined





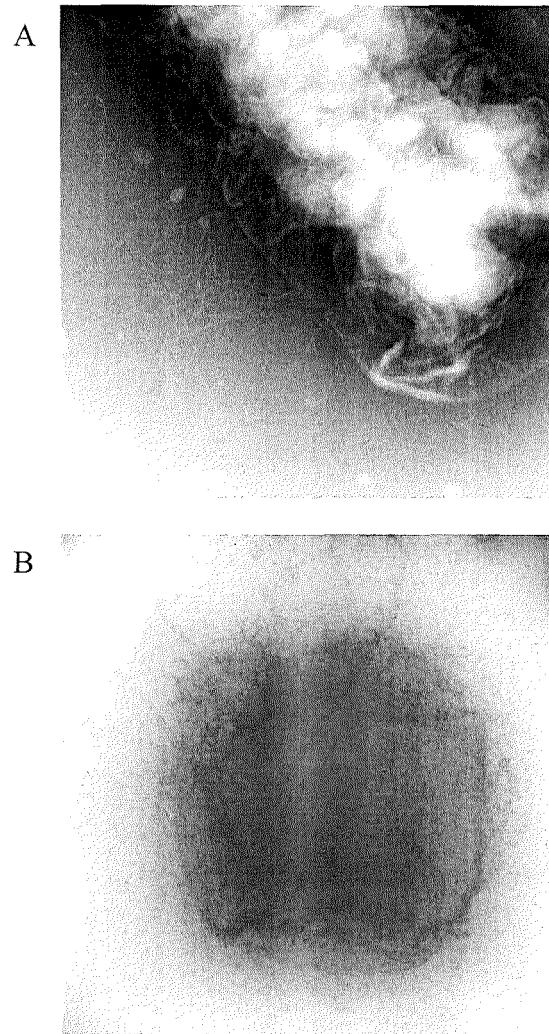
**Figure 4.26 Immunofluorescent staining of B41 bacteria using a monoclonal anti-K99 antibody**

*E. coli* B41 were grown at 37 °C (panel A) or 18 °C (panel B) and imaged by indirect immunofluorescence microscopy with a mouse monoclonal antibody against K99 and an FITC conjugate.

## ***Electron microscopy and immunolabelling***

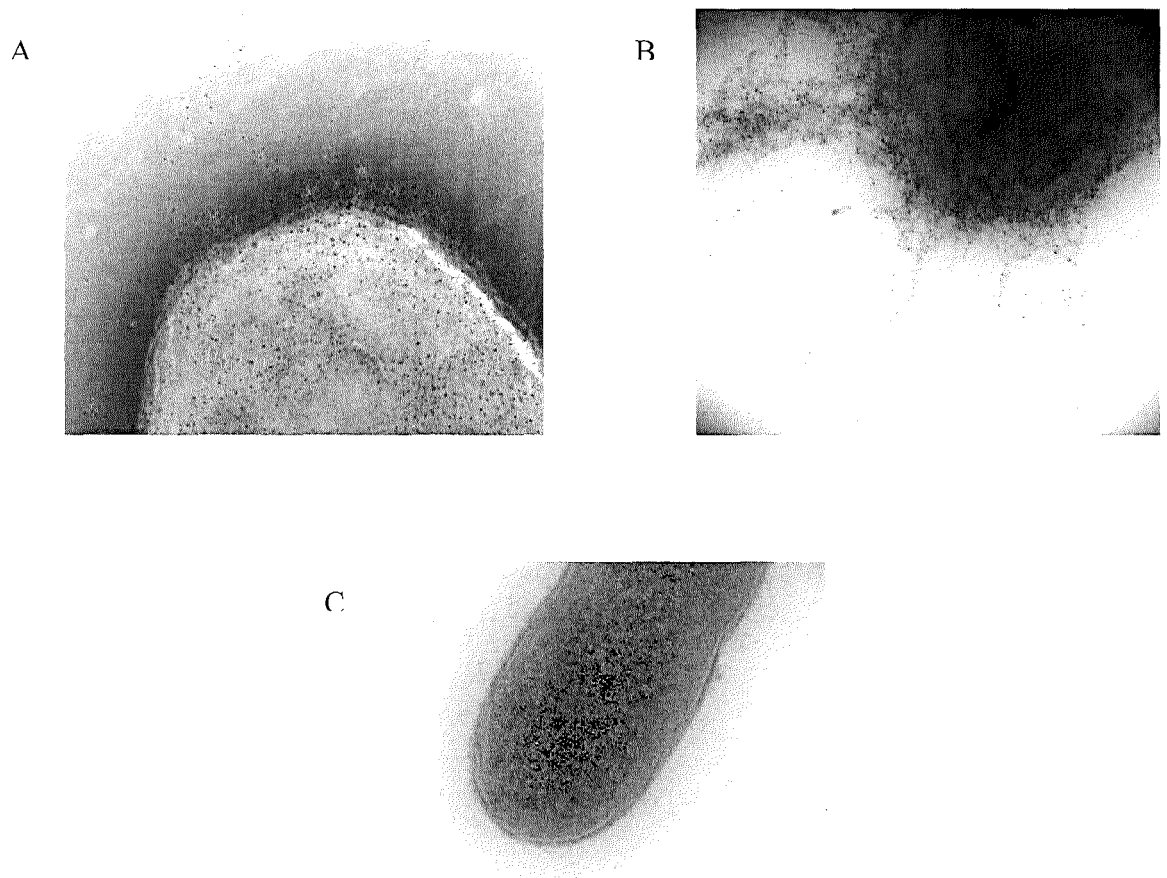
To evaluate staining methods that would reveal K99 fimbriae for transmission electron microscopy, *E. coli* B41 were fixed to grids and treated by negative staining and Negative syaining by methylcellulose. Observation in the EM revealed that fimbriae could be visualised by both staining methods but that the K99 structure was more visible when stained with the negative staining by methylcellulose. Also levels of background staining with this method were lower (Figure 4.27). K99 fimbriae could not be observed when bacteria were grown at 18 °C.

K99 fimbriae on the surface of *E.coli* B41 were labelled with the scFv antibodies selected from the Tomlinson I and J libraries for detailed analysis. To reveal the presence of the scFvs, samples were then incubated with a suspension of 5nm colloidal gold particles conjugated to Protein A and examined in the EM. Polyclonal and monoclonal anti-K99 antibodies served as positive controls while bacteria grown at 18 °C before labelling provided a negative control (Figure 4.28). Control experiments clearly showed patches of immunogold staining with gold particles running along the fimbriae when polyclonal or monoclonal antibodies were applied. Bacteria prepared at 18 °C were free of gold particles (Figure 4.28). Broadly similar patterns of staining were observed when scFvs from Tomlinson library I (Figure 4.29) or library J were used (Figure 4.30). Importantly, these results indicate that in all cases, the scFvs recognise epitopes present on fimbrial subunits present in multiple copies in the structure of the adhesin. To ensure that labelling with the recombinant antibodies was specific, bacteria grown at 37 °C were labelled with an anti-ubiquitin scFv and Protein A-colloidal gold. No particles could be observed at the bacterial surface (Figure 4.31). Similar results were obtained when anti-K99 scFvs were applied to *E. coli* B41 grown at 18 °C.



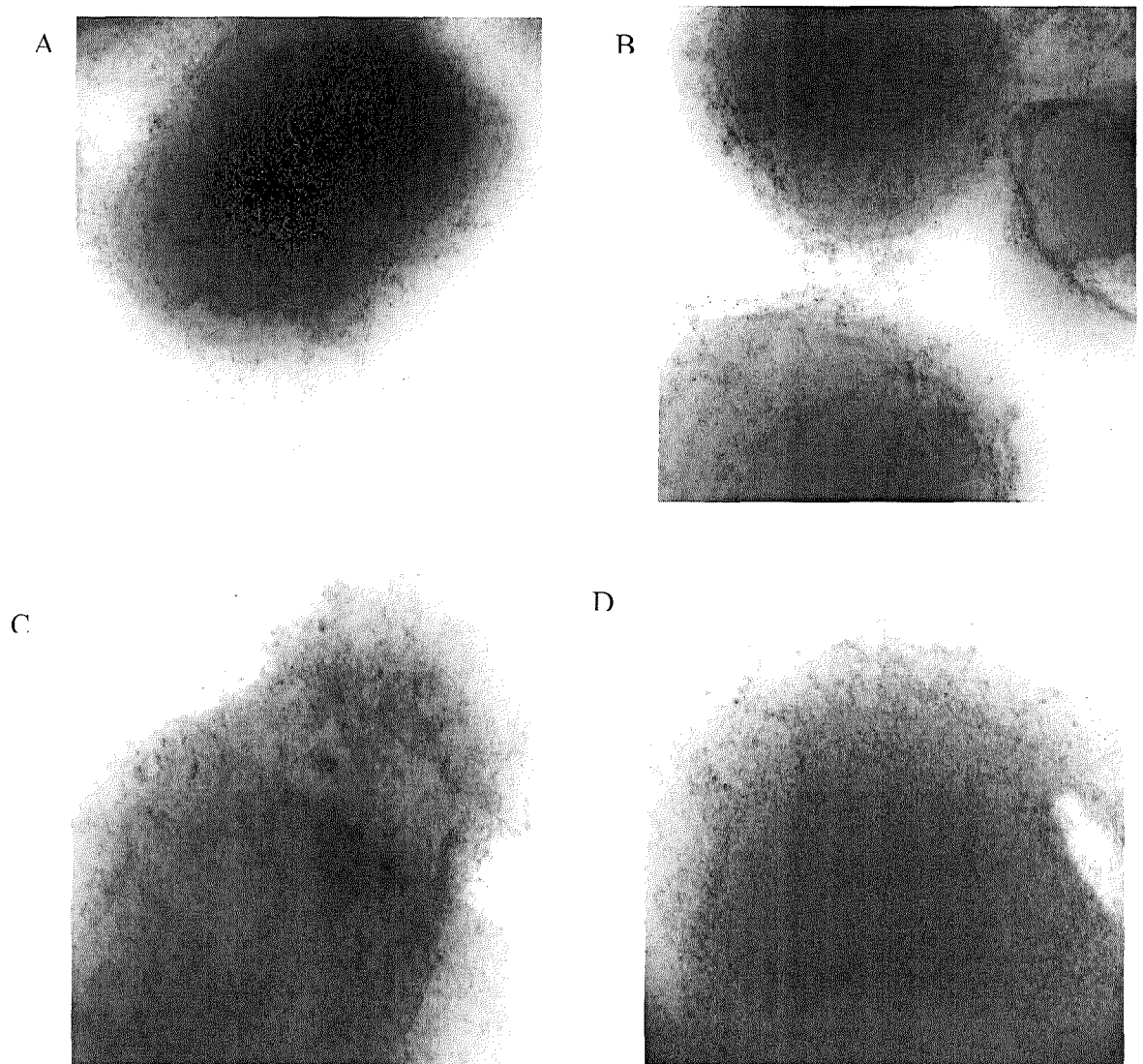
**Figure 4.27 Staining of *E. coli* B41 for electron microscopy**

*E. coli* B41 were grown overnight at 37 °C, fixed to grids and prepared by negative staining (A) or negative staining by methylcellulose (B) for examination by transmission electron microscopy at magnifications of 50 k (A) or 30 k (B).



**Figure 4.28 Immunogold labelling of K99 fimbriae with monoclonal and polyclonal antibodies**

*E. coli* B41 were grown at 37 °C (A and B) or 18 °C (C) and prepared for electron microscopy. Grids were incubated with monoclonal (A) or polyclonal anti-K99 antibodies (B and C) and Protein A-colloidal gold reagents before examination in the electron microscope at 50 (A), 30 (B) or 7 k magnification (C).



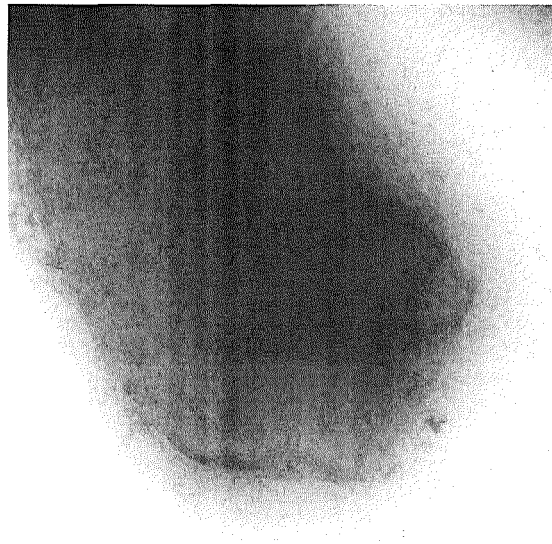
**Figure 4.29** Immunogold labelling of K99 fimbriae with scFv antibodies from Tomlinson library I

*E. coli* B41 were grown at 37 °C and prepared for electron microscopy. Grids were incubated with scFvs F8 (A), F10 (B), F11 (C) or C9 (D) and Protein A-colloidal gold reagents before examination in the electron microscope at 30 k magnification.



**Figure 4.30 Immunogold labelling of K99 fimbriae with scFv antibodies from Tomlinson library J**

*E. coli* B41 were grown at 37 °C and prepared for electron microscopy. Grids were incubated with scFvs A7 (A), or F7 (B) and Protein A-colloidal gold reagents before examination in the electron microscope at 30 k magnification.



**Figure 4.31 Immunogold labelling of K99 fimbriae with an anti-ubiquitin scFv antibody**

*E. coli* B41 were grown at 37 °C and prepared for electron microscopy. Grids were incubated with an anti-ubiquitin scFv and Protein A-colloidal gold reagents before examination in the electron microscope at 30 k magnification.

### ***In vitro villus attachment assay***

Isolated calf villi were stored at  $-80^{\circ}\text{C}$  in aliquots. For initial experiments to determine optimal fixation conditions, aliquots were thawed at room temperature before treatment with a range of reagents. It was established that 1 % formaldehyde was insufficient to stabilise the villi; they disintegrated within a few minutes of incubation at room temperature. Fixation with glutaraldehyde was unsuitable for different reasons. In this case, bacterial attachment to the villi was markedly reduced after treatment. Overall, 2 % formaldehyde proved most suitable against both of these criteria: the isolated villi were sufficiently stable to withstand incubation with bacteria and subsequent washing steps and as shown in Figure 4.32, *E. coli* B41 expressing K99 fimbriae rapidly became attached at the brush border of the tissue fragments. The attachment of bacteria became greater with longer incubation at room temperature. Bacterial attachment could not be observed when *E. coli* B41 were grown at  $18^{\circ}\text{C}$ .

### ***Bacterial adherence inhibition assay***

The ability of six purified scFv antibodies to inhibit the attachment of B41 bacteria to villi were assessed. ScFv antibodies at similar concentration ( $30\text{ }\mu\text{g/ml}$ ) were added to *E. coli* B41 strain and were incubated at  $37^{\circ}\text{C}$  with agitation for 45 min. Then about 50 washed villi (with Krebs buffer pH 6.8 and 1% mannose) were added to the mixture and the villi materials were incubated at room temperature for 1 hr. The observation results revealed that, except for the positive control, none of the purified scFv antibodies could show a significant inhibition of attachment at above concentration.





**Figure 4.32** The villi after fixation with 2 % formaldehyde

## Discussion

Attempts to isolate recombinant soluble scFv antibodies against the major subunit of K99 using the Griffin.1 library encountered several problems. Although antibodies expressed at the surface of phage appeared able to recognise the target, it was not possible to express soluble scFv fragments. The Tomlinson I and J semi-synthetic libraries were chosen as alternative resources from which the aims for the project might be met. To check the quality of these two libraries, they were initially analysed for the presence of full-length inserts by screening clones picked at random. In contrast to the Griffin.1 library in which only about 1/3 of clones chosen in this way carried full-length inserts, these experiments revealed that almost all clones from the Tomlinson libraries were intact. On this basis alone, the quality of the Tomlinson libraries appeared better than Griffin.1. This was reinforced by the expression of soluble scFv by a very high proportion of clones picked at random from the libraries. Positive control clones were then grown and characterised to ensure that methods were working effectively. The activity of the purified K99 antigen was also tested at a range of different concentrations using a monoclonal antibody before panning the libraries.

Selection revealed a pattern of phage recovery that was consistent with the progressive enrichment of target-specific clones and again, contrasted with data from screening the Griffin.1 library. In percentage terms, recoveries rose steadily from round 1 to round 3 and were consistently higher than observed during from the Griffin library. This may be due to multiple factors: the greater proportion of phage in the Tomlinson libraries with full-length inserts and hence a more diverse starting resource; the use of the KM13 helper phage and trypsin-driven elution ensuring that only phage with intact reading frames progress from elution to rescue and amplification (Kristensen and Winter, 1998). Since recovery data only provide a preliminary indication of the progress of selection, monoclonal phage ELISA was a critical benchmark for these experiments. This clearly revealed that most clones in rounds 2 and 3 showed high reaction with K99 and there were far fewer false positive results than obtained with the Griffin.1 library, a problem that others have commented upon (Azzazy, 2002). Clones isolated from Tomlinson I and J libraries were also shown to be highly specific for K99 in polyclonal phage ELISA and, in contrast with Griffin.1 library, they showed almost no reaction with skimmed milk. Expression of scFv was further checked by picking at random a number of clones eluted after each round of

selection and infected into *E. coli* HB2151. Supernatants from induced cultures were screened by ELISA. In contrast to the analysis of samples from the Griffin.1 library where scFvs were only rarely expressed, samples from the Tomlinson libraries expressed successfully. This indicated that stop codons occurred rarely and inserts were usually full-length.

In screening for anti-K99 antibodies, the Tomlinson I and J libraries were run in parallel. These resources only differ in the nature of their diversification. Library I is diversified by possessing DVT (D: A, G or T; V: A, C or G) codons at selected positions in the scFv insert. This only carries 9 possible nucleotide combinations but no stop codons arise. Library J is diversified by the NNK (N: any base; K G or T) motif, each encoding 32 possible nucleotide sequences of which one (TAG) is an amber stop codon. Overall, similar results were obtained from these libraries but the percentage recovery rate was lower for J and a lower proportion of clones expressed soluble scFv at a detectable level, perhaps due to the occurrence of the amber codon.

This said, there were ample clones able to express specific anti-K99 scFvs from which to choose several with high ELISA activity. The biological activity of these scFv antibodies was then assessed. Initial experiments attempted to confirm the specificity of the scFvs for FanC by Western blotting. No signal could be detected on the nitrocellulose membrane but as a similar problem was encountered with monoclonal anti-K99, the dot blotting was used to test for reaction with K99. The results showed that some scFv antibodies had stronger reaction in this assay than others, indicating either that the clones expressed the scFvs to different levels or that the scFvs were able to bind the target with different efficiencies. Although DNA sequencing of these clones revealed some similarity between them, this provided insufficient basis to discriminate between these possibilities. From this point, four clones from Tomlinson library I and two clones from Tomlinson library J were chosen, purified and taken forward to more detailed tests of their activities.

It is known that the major subunit of K99 can adhere to erythrocytes from a variety of animal species due to recognition of a specific receptor on the surface of these cells (Burrows *et al.*, 1976; Gaastra and de Graaf, 1982; Jacobs *et al.*, 1987). This was demonstrated by mixing *E. coli* B41 of purified K99 with sheep erythrocytes and observing haemagglutination. This reaction was dependent upon culture of bacteria at 37°C. Haemagglutination could be inhibited by pre-incubation of bacteria with high dilutions of

anti-K99 antibodies (Burrows *et al.*, 1976; Morris *et al.*, 1980; Vazquez *et al.*, 1996), thereby providing a positive control for an *in vitro* assay for K99-neutralising activity. Anti-K99 scFvs were purified and tested in this assay, revealing that some possessed inhibitory activity. For those scFvs that showed promise, anti-haemagglutinating activity did not persist to high dilutions as was apparent for the polyclonal anti-K99 positive control. This observation needs further repetition and the assay could be expanded to a wider range of scFvs. However, an important difference between the properties of a scFv and a native immunoglobulin molecule should be noted. The scFv interacts with its target on a monomeric basis whereas the native immunoglobulin has the capacity for a dimeric interaction, assuming it to be IgG. On a simple molar basis, one would therefore predict that twice as much scFv compared with IgG would be required to achieve the same biological activity, ignoring the ability of the IgG to make contact with epitopes that might be close together. Perhaps more significant however is the ability of a dimeric molecule like IgG to physically agglutinate the bacteria or K99 in this assay, inhibiting attachment to the red blood cell target. It is also significant that some aggregation of some scFvs could be observed after purification that would probably lead to some reduction in the starting concentration in the assay. Therefore, this assay clearly shows that some anti-K99 scFvs possessed interesting biological properties but the significance of the outcome could be better assessed by comparing the data with Fab fragments generated from the polyclonal anti-K99 antibody that served as positive control and standardising the proteins under test to a common starting concentration. Further work on the stability of the purified scFvs, affinity maturation, or re-engineering so that they were expressed as dimeric proteins ("diabodies") might also raise their neutralisation activities. Finally, it did not prove possible to measure the affinity of the scFv antibodies for FliC; this would also be a valuable objective in future studies.

Use of the purified scFvs in indirect immunofluorescent staining revealed their ability to bind to fimbriae on the surface of *E. coli* cells. This data might serve as the starting point for the use of the isolated scFv antibodies as diagnostic reagents. Since some investigators in recent studies have successfully shown that green fluorescent protein (GFP) or a red-shifted mutant of green fluorescent protein (EGFP) can be fused to scFvs (Casey *et al.*, 2000; Hink *et al.*, 2000), genetic conjugation of an anti-K99 to a reporter molecule that requires no substrate addition could make for faster and cheaper diagnosis of bovine ETEC infection.

The interaction between scFv antibodies and K99 fimbriae was also studied by electron microscopy using Protein A-gold colloidal particles. Initial efforts used simple negative staining (Duchet-Suchaux *et al.*, 1988; Hernandez *et al.*, 1989) to show the presence of fimbriae. Although this method was adequate for the intended purpose, it did create high background staining on the grids. This could be reduced using a methylcellulose method. Immunogold labelling of fimbriae using scFvs from Tomlinson libraries I or J generated similar patterns in the electron microscope compared with polyclonal or monoclonal anti-K99 and binding was noted at numerous positions along the fimbrial structure. For P (Lindberg *et al.*, 1987) or I fimbriae (Buhler *et al.*, 1991), the receptor-binding site of the major subunit only is exposed at the tip of the structure. For K99, multivalent interaction of the major subunit (Fan C) with its ganglioside receptor (Willemsen and de Graaf, 1993) is possible and it has been estimated that receptor can be bound at intervals of about 20 nm (Willemsen and de Graaf, 1993). For all the scFvs tested in electron microscopy, the staining pattern is therefore consistent with recognition of an exposed epitope present on the FanC subunit. This does not necessarily equate with recognition of epitopes in or close to the receptor binding site and hence the capacity to neutralise K99 activity, though it is clear from the earlier discussion that some scFvs possessed this property. However, engineering of scFvs with this pattern of staining to divalent diabodies would likely create proteins with K99-specific agglutinating activity. This strategy for developing recombinant antibodies with agglutinating properties for diagnosis or therapy could be broadened for other microbial pathogens.

Haemagglutination provided a useful model to identify scFvs with biological activities of potential value but it has its limitations as a model. Attempts were made to use the attachment of *E. coli* B41 to fixed intestinal villi to better assess the whether scFvs isolated from Tomlinson libraries I or J could inhibit bacterial attachment *in vitro*. The method of Girardeau (Girardeau, 1980) was sufficient for the isolation and storage of villi and the technique has been used by other in order to set up *in vitro* attachment assays (Van den Broeck *et al.*, 1999; Mercado *et al.*, 2003). Experience showed that thawing the villi from storage and fixation in 1 % formaldehyde was not sufficient to stabilise them. By raising the concentration of formaldehyde to 2 %, disintegration was prevented and after washing with Krebs buffer, they could be maintained for several days at 4 °C. K99-specific attachment to villi was confirmed by using B41 bacteria grown at 18 °C *versus* 37 °C and by inhibition of the attachment process using an anti-K99 monoclonal antibody. It proved difficult to demonstrate inhibition of attachment with purified scFvs. When the inhibitory

effect of monoclonal anti-K99 was checked by light microscopy, it revealed the aggregation of bacteria was taking place. This finding supports the idea proposed above that dimeric interaction of native immunoglobulin with K99 might prevent normal receptor binding at least in part because of agglutination of the bacteria under test. Again, the monomeric interaction of scFvs with their targets dictates that attachment can only be prevented by blocking the K99 receptor-binding site. The use of the anti-K99 scFvs in this model of attachment therefore needs further development, perhaps using scFv antibodies at higher concentration, or increasing the incubation time of the bacteria with the scFvs under test before addition to villi. An alternative model worth consideration might also be the erythrocyte-binding assay (Willemsen and de Graaf, 1993; Jay *et al.*, 2004) in which the attachment of bacteria to red cells (as opposed to the agglutination of these target cells) is studied. Also the binding of isolated fimbriae or bacteria expressing K99 to the receptor purified from solubilized pig brush border (Sun *et al.*, 2000) or calf ileal enterocytes (Isaacson *et al.*, 1978; Jay *et al.*, 2004) could be studied on artificial substrates such as plastic. It was noticeable with villus attachment that bacterial distribution was often irregular, perhaps reflecting the partial integrity of the brush border. The use of erythrocytes or the purified ganglioside receptor might enable some of these variables to be eliminated. Finally, for those recombinant antibodies with best promise a mouse model for the study of the virulence of bovine ETEC strains has been developed (Duchet-Suchaux, 1988; Duchet-Suchaux *et al.*, 1992). It is only in an intact animal that the therapeutic potential of anti-K99 scFvs could be assessed.

Overall, the use of the results Tomlinson I and J libraries enabled the goals of the project to be met: to use phage display for isolation of recombinant antibodies against K99, to express soluble scFv fragments, purify them and assess their biological activity. This phase of the project clearly showed that recombinant antibodies against a bacterial colonisation factor could be isolated with simple selection methods. There is every reason to expect that the strategy could be applied with equal success to human or animal pathogens that colonise the intestine or other mucosal surfaces.

## **Chapter 5**

### **Conclusions**

## 5. Conclusions

The focus of this study has been to isolate recombinant antibodies against the K99 fimbrial structure, a colonisation factor of major importance in the pathogenesis of *E. coli* infection in newborn calves. The goals of the project were to use K99 as a model virulence factor and thereby explore the utility of phage display and recombinant antibody technology as tools in the study of bacterial pathogenesis, specifically to see whether small antibody fragments with the capacity to neutralize bacterial products can be isolated from phage display libraries.

The first objective was to isolate K99 fimbriae from a clinical isolate of *E. coli* and experience demonstrated that the purity of the major fimbrial subunit (Fan C) was a very important factor in the isolation of specific recombinant antibodies. Unless specifically guided, phage display is essentially blind to the nature of the target used in panning and with a large antibody library with millions of potential binding specificities, it seemed relatively easy for selection to drift away from the target of interest if the coated surface was heterogeneous. It was reasoned that if the recombinant antibodies were to possess the ability to neutralise the action of K99, the target protein must not only be pure but also in a correctly folded and fully active state. In the event, methods were devised that successfully purified the major subunit of K99 with ease, at low cost and yielded protein with high biological activity. This method for the purification of K99 may find use in research but given the considerable body of data showing the value of the antigen in vaccination, it may also find commercial application. Future work might explore if simple ion exchange chromatography as used here could be also be applied to the isolation of other colonisation factors of importance in bovine ETEC infection (*eg* F41 and F17). The K99 protein purified by this method showed all the characteristics of the native structure (immunological reactivity, haemagglutination) but formal demonstration of its ability to protect against ETEC infection was beyond the scope of this project. This might be addressed by work in a mouse model of ETEC infection.

The results that were obtained from panning the Griffin.1 library against purified K99 showed that the quality of the recombinant antibody library is the single most important factor in work in this area. The crucial issues to emerge from this phase of the project were that the proportion of clones in the library with full-length inserts should be maximised and



the percentage of clones containing stop codons should be minimised, if at all possible. In an attempt to achieve this, some investigators have tried to make recombinant antibody libraries that do not contain stop codons (Knappik *et al.*, 2000). It is also likely that the properties of helper phage have a role in carrying phage antibodies containing full-length antibodies forward from one round of selection to the next and eliminating those with incomplete inserts. Attempts to isolate recombinant antibodies against K99 from the Griffin.1 library that could be expressed as soluble scFvs were time-consuming and ultimately unsuccessful but valuable experience was gained. It was clear from this phase of the project that at the outset, a recombinant antibody library should be tested before use. Specifically, the percentage of full-length inserts should be assessed, the ability of clones picked at random to express soluble fragments should be tested, clones should be screened for the presence of stop codons, and good controls to confirm the progress of selection and expression levels are important. Experience with the Griffin.1 library has shown that over-amplification can sometimes lead to a drastic reduction in the diversity of the library, probably through the faster growth of clones containing incomplete inserts. After panning the library, polyclonal phage ELISA and enrichment rates appear to be the quickest way to evaluate the results of selection. It is now clear that if these tests fail to show substantial enrichment for clones reactive with the target, it is better to repeat the selection rather than pursuing further screening. Regarding the identification of clones with high activity against the target antigen, direct infection of HB2151 and assay of soluble antibody fragments seems inclined to fewer artefacts than monoclonal phage ELISA. Early progress to sequencing is also recommended as a means to explain low-level expression of antibodies that apparently react with the target.

On the positive side, the Tomlinson libraries proved excellent resources from which to isolate anti-K99 scFvs. The libraries met the criteria of high frequency presence of full-length inserts and the ease of expression of scFvs. It proved relatively straight-forward to isolate numerous anti-K99 scFvs, from which 6 were chosen for detailed analysis. The properties of these met in full the goals of the project: they reacted specifically with K99, they could be used to determine the cellular location of the colonisation factor and the ability of some to block K99-mediated haemagglutination was of particular interest, showing the ability to inhibit the mode of action of the virulence factor. In future, these properties could be further explored with an *in vitro* model of *E. coli* colonisation using isolated intestinal villi from young calves or an *in vivo* mouse model (Duchet-Suchaux, 1988).

Overall, the project has shown that phage display has a useful role as a tool to understand better molecules of importance in bacterial pathogenesis. Is it possible that recombinant antibodies isolated in this way could be used passively for the therapeutic treatment of livestock animals? Given the current economics of livestock farming, it would seem unlikely that purified antibodies against microbial pathogens could be administered for the treatment of disease except in species (*eg* horses) or individuals (*eg* breeding animals) of high commercial value. It may be possible that unit costs could be reduced by alternative delivery methods. For example, considerable effort has been invested in DNA vaccines as alternatives to protein immunogens for the vaccination of livestock animals. Recent work has shown that the expression of recombinant antibodies can be induced in animals *via* DNA immunisation with the encoding sequences and that this can protect against pathogen challenge (Lorenzen, 2000). It has also been shown that attenuated bacterial species able to colonise the host can express recombinant antibodies as either anti-idiotypic immunogens or protective proteins in their own right (Beninati, 2000).

It is difficult to predict if these technologies will eventually impact on livestock animal healthcare but from the studies reported here, it would seem that antibody phage display is likely to find increasing use as a basic tool for the study of the pathogens of livestock animals.

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