Recombinant antibodies for the study of livestock infection: from basic genetics to single-chain Fvs

Arsalan Hosseini-Nohdani
DVM

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Division of Infection and Immunity
Faculty of Biomedical and Life Sciences
University of Glasgow

This thesis is dedicated to my wife, Mahnaz

and my daughters, Fatemeh and Fahimeh,

always sources of support and inspiration for my future

It is also dedicated to my parents,

who encouraged me throughout my life

Declaration

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

A. Hosseini

Arsalan Hosseini-Nohdani

Summary

Molecular biology has provided new opportunities to understand better the functioning of the immune system and to exploit this information for the construction of specific antibodies against a wide variety of antigens including the pathogens of humans and animals. In spite of the economic importance of cattle, many aspects of the immunology of this animal remain uncharacterised and tools to understand better bovine infections are lacking. This project has addressed aspects of both issues.

The bovine immunoglobulin (Ig) system resembles that of other domesticated mammals in some respects, but other properties (*eg* the length of the third antigen-binding region of the heavy chain) appear unique. The first area for investigation in this project was to characterise the bovine J_H locus and to understand why Ig rearrangement apparently favours a single J_H segment. PCR was used to recover J_H sequence from genomic DNA, either from non-lymphoid tissues or lambda vectors isolated and studied by other investigators. A region of 3200 bp was characterised which included the DQ52 segment, 6 J_H segments and the heavy chain enhancer. The bovine DQ52 sequence is longer than those of other species and differs in sequence from a common consensus. For the most part, the J_H locus is homologous to that of the sheep. The sixth J_H segment identified appears to undergo rearrangement and is expressed in a minority of cattle antibodies. However, none of the segments carried the sequence which is most commonly expressed in bovine Ig. To identify which segment participates in this process, sequence was recovered from the rearranged genomic DNA of isolated bovine B cells using PCR with primers against V_H and J_H regions. This implicated the rearrangement of the fourth J_H segment in the formation of bovine Igs but as the sequence differed between germline and rearranged copies, it appears that a non-conventional process operates in cattle. It is proposed that a gene conversion event or modified rearrangement process introduces sequence to form the fourth framework region of bovine Ig which does not exist at the J_H locus in the germline. The mechanism of this modification requires more investigation.

The second part of this project aimed to construct a library of recombinant bovine Fab antibodies from the Ig repertoire of a calf vaccinated against *Mannheimia haemolytica* (previously named *Pasteurella haemolytica*). Using PCR on lymphoid cDNA, the bovine V_L and V_H repertoires were recovered with ease for direct cloning into the phage display vector pComBov. Complications encountered at the cloning stage led us to an alternative strategy, the cloning of amplicons as blunt fragments into

an intermediate vector, pUC18, in order to increase the efficiency of subsequent restriction enzyme digestion. Intermediate libraries of the light and heavy chain repertoires were constructed successfully and the V_L repertoire was then released for cloning to pComBov by digestion with *Sst*I and *Bst*EII. In contrast, difficulties were encountered in recloning heavy chain *Rsr*II / *Spe*I fragments into the pComBov-V_L library. It was confirmed that the heavy chain fragment could be released from the intermediate heavy chain library and so the obstacle arose at ligation. A thorough investigation of experimental conditions attempted to find a solution but without success. After the failure of indirect cloning of the heavy chain repertoire into a pComBov-V_L library, attention returned to a direct cloning strategy using revised primers, in which the distance between the restriction sites and the termini of heavy chain amplicons was increased. The resulting V_H amplicons could be digested efficiently with *Rsr*II and *Spe*I but attempts to clone the digestion products were unsuccessful again.

In the third part of the project, recombinant antibodies against the outer membrane proteins (OMPs) of *Mannheimia haemolytica* were isolated from the Griffin.1 scFv library. OMPs were prepared using Sarkosyl and their composition assessed by SDS-PAGE. The proteins were coated to plastic and the library screened for binders *via* 3 rounds of selection. Phage antibodies were eluted by either triethylamine or an anti-OMP serum in separate selections. Ninety six clones from each round of selection were screened for the most reactive antibodies through monoclonal phage ELISA. Comparison of eighty clones which reacted best with OMPs showed that the affinity of phage antibodies increased by progressive reduction in the target concentration at the coated surface through the selection process. Reaction was detected between several of the recombinant Igs and an OMP of 40 kDa using Western blotting. By using mass spectrometry, this protein was identified as *Mannheimia haemolytica* PomA.

Overall, the project identified aspects of the immunology of cows that appear to be entirely novel, and applied recombinant antibody technology to understand better an important bacterial pathogen for this livestock species.

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Abbreviations

BSA: bovine serum albumin

βME: Beta mercaptoethanol

C_H: constant region of heavy chain

C_L: variable region of light chain

cDNA: complementary DNA

CDR: complementarity determining region

D gene: diversity gene

DTT: dithiothreitol

ECL: Enhanced chemiluminescence

FR: framework region

GST: glutathione S-transferase

H chain: heavy chain

Ig: immunoglobulin

IPTG: Isopropyl-β-D- thiogalactopyranoside

J: joining region

L chain: light chain

LA: Luria agar

LB: Luria broth

LMP agrose: low melting point agarose

MABs: Monoclonal antibodies

MPBS: milk in phosphate buffered saline

PBS: phosphate buffered saline

PCR: polymerase chain reaction

RSS: recombination signal sequence

scFv: single chain antibody fragment

TE: Tris-EDTA buffer

TEA: triethylamine

TCL: temperature-cycle ligation

U: unit

V: variable region

V_H: variable region of heavy chain

V_L: variable region of light chain

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Chapter 1

General Introduction

1 General Introduction

1.1 Antibodies as passive therapeutics

The survival of animals in a hostile environment teeming with pathogens has necessitated the evolution of complex systems of immune defence. These systems contain molecules, primarily antibodies, and T-cell receptors, with exquisite specificities which have naturally attracted attention as potential therapeutic agents. Antibodies are clearly more straightforward to use in this role since they are soluble protein molecules whereas T-cell receptors are of course cell associated and require MHC compatibility for recognition.

Indeed over 100 years ago, Emil von Behring demonstrated the efficacy of hyperimmune horse serum containing high titres of antibody to diphtheria in combating an outbreak of disease in humans. However, despite this early success, the use of passive immunisation through the 20th Century has been very limited. This probably reflects the great success of antibiotics in combating bacterial diseases, of vaccines in the prevention of viral diseases, and the difficulties of obtaining antibodies useful for passive immunisation. With regard to the latter issue, the stimulation of high titre sera in animals is a relatively trivial matter but their use as passive therapeutics in human patients inevitably elicits a response leading to elimination of the foreign immunoglobulin from the circulation. This severely constrains heterologous passive transfer as a therapeutic strategy. The risks inherent in human to human transfer are sufficiently formidable to effectively rule out this option as an alternative and hydridoma techniques for the production of monoclonal antibody (Kohler and Milstein, 1975) have never fulfilled their potential as a means to produce of human antibody *in vitro*. However, the emergence of AIDS in the 1980s and of a spectrum of infections in a large pool of immunocompromised patients has renewed interest in passive immunisation for the treatment of infectious disease. This has coincided with a period of rapid progress in the generation and design of antibodies for medicine using recombinant DNA techniques.

1.2 Antibody engineering

1.2.1 Hybridomas

Antibody engineering became possible with the development of hybridoma technology by Kohler and Milstein in 1975. In common with the generation of polyclonal antibodies, it requires the initial immunisation of a laboratory animal (usually mouse or rat) to raise a high titre of circulating antibodies against the immunising

antigen. B lymphocytes are then isolated from the spleen. Through rearrangement of immunoglobulin genes, each B lymphocyte produces antibodies with affinity for a single antigenic unit or epitope. Monospecific antibodies with this same specificity cannot be obtained directly from isolated B lymphocytes as they rapidly die in cell culture. To overcome this obstacle, 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 a monoclonal antibody as coded 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. A disadvantage of this method, however, is the labour-intensive procedure needed for the selection of the desired antibody-producing clone from thousands of hybridomas that manufacture irrelevant antibodies.

In principle unlimited quantities of a monospecific antibody could be produced from the immortalised cells, but the technology is consistently more successful for production of rodent monoclonal antbodies than for human (James and Bell, 1987). Clinical testing of murine monoclonal antibodies in human immunotherapy also has generally yielded disappointing results; murine immunoglobulins invariably evoke a human anti-mouse immune response; they are ineffective as cytotoxic agents and in their ability to recruit effector cells or molecules of the complement system. This situation has stimulated endeavours aimed at the construction of genetically engineered antibodies with reduced immunogenicity and improved effector functions (de Kruif *et al.*, 1996)

1.2.2 Recombinant antibodies

In recombinant antibody technology, synthesis of the protein is removed from a laboratory animal and takes place in bacteria or in eukaryotic cell culture, *in vitro*. Expression of the full-length protein complete with antigen-binding and effector domains is just one option. Genetic manipulation of the coding sequence can result in expression of Fab fragments or entirely artificial products such as single chain antibodies (scFvs), isolated heavy chain domains (Fvs) or constructs with dual antigenic specificities (diabodies).

Why recombinant antibodies?

Recombinant antibodies can be obtained in vitro

In contrast to all other methods mentioned, recombinant antibodies can be expressed in a bacterial host that is completely independent of a vertebrate. This eliminates the chance of possible contamination with contagious pathogens, a risk always presents when human or animal products are used. These recombinant antibodies are, of course, also monoclonal because they originate from a single (bacterial) cell. Moreover, handling bacterial clones is usually simpler and cheaper than for hybridoma cells in term of their culture, analysis and storage. More important however is the fact that the power of bacterial genetics can be applied to the analysis and modification of the encoded immunoglobulin. Consequently, sequencing and countless modifications are simplified.

Recombinant antibodies can be produced from many different host species

Currently, recombinant antibodies can be produced without the need for prior immunisation, by using immunoglobulin libraries carried in bacteriophage (see below), thereby reducing laboratory animal experimentation. Recombinant antibodies can be produced against native sequence from a wide range of species. For many of these animals, hybridoma technology is not available; where heterohybridomas have been used, their stability is frequently poor. To date, the recombinant approach has been most popular for the production of human and rodent immunoglobulins, but it has also proved successful with rabbits, a range of domesticated livestock and primates (O'Brien and Aitken 2002). As noted above, antibodies of native sequence are specially favoured over those from other animals when used *in vivo* for diagnosis or therapy. They could be applied as passive therapeutics against pathogens or toxins and are of proven value in the fight against cancer, due to their specificity, while avoiding the immune response which would be triggered by antibodies from a different species (Breitling and Dubel, 1999).

Chimeric antibodies

The first generation of recombinant monoclonal antibodies consisted of rodent-derived variable (V) regions fused to human constant (C) regions. It is thought that the most immunogenic regions of antibodies are the conserved C domains (Khazaeli *et al.*, 1994). Because the antigen binding site of the antibody is localised within the V regions, the chimeric molecules retain their binding affinity for the antigen and acquire the function of substituted C regions. For examples, mouse::human IgG1 chimeric

antibodies have been shown to mediate the lysis of tumour cells in the presence of human complement (Liu *et al.,* 1987).

The chimeric antibodies are easily produced but retain the mouse variable domains with possible consequences for immunogenicity. Clinical experience with rodent monoclonal and mouse::human chimeric antibodies indicates that for some antibodies a strong anti-idiotypic reaction is elicited (Jaffers *et al.,* 1986). Therefore, to reduce the murine content even further, procedures have been developed for humanising the Fv regions (Kipriyanov and Little, 1999).

Humanised antibodies

A large decrease in the immunogenicity of an antibody can be achieved by variable domain replacement (Boulianne *et al.,* 1984; Morrison *et al.,* 1984; Neuberger *et al.,* 1985) or by grafting the complementary-determining regions (CDRs) of foreign antibodies onto human framework (FR) and C regions (Jones *et al.,* 1986; Verhoeyen *et al.,* 1988). This can generate antibodies suitable for use *in vivo* but humanised antibodies are tedious to construct and often yield molecules with reduced affinity for the target antigen. Indeed, it is frequently found that some FR residues from the original antibody need to be preserved in the humanised molecule if significant antigen-binding affinity is to be retained (Queen *et al.,* 1989; Ohtomo *et al.,* 1995). The following approaches have been used to produce effective antibodies by CDR-grafting (Co and Queen, 1991):

a- In one approach, human V regions showing the greatest sequence homology to murine V regions are chosen from a database in order to provide the human FR. The selection of human FRs can be either from human consensus sequences or from individual human antibodies (Kolbinger *et al.,* 1993).

b- In an alternative approach, murine CDRs are grafted onto a given human V region. If the humanised antibody has reduced or no affinity for the target antigen, new constructs are made that incorporate one to three additional mouse residue near the CDRs, and so on until binding is restored.

c- In a third approach, the surface of the xenogenic antibody is humanised. A statistical analysis of unique human and murine immunoglobulin heavy and light chain V regions revealed that the precise patterns of exposed residues are different in human and murine antibodies, and most surface locations have a strong preference for a small number of different residues (Padlan, 1991; Pedersen *et al.,* 1993). Therefore, it may be possible to reduce the immunogenicity of a non-human Fv, while preserving its antigen-

binding properties, by replacing exposed FR residues that differ from those usually found in human antibodies (Padlan, 1991). This would humanise the surface of the xenogenic antibody while retaining the interior and contact residues that influence antigen-binding and inter-domain contacts. Since protein antigenicity can be correlated with surface accessibility, replacement of the surface residues may be sufficient to render the mouse variable region " invisible" to the human immune system. This procedure of humanisation is referred to as "veneering" since only the outer surface of the antibody is altered and the supporting residues remain unaltered. Another approach to minimise the immune response to foreign antibodies is to alter the surface epitopes which may be reactive with host T cells (de-immunisation). In this process, computer modelling is used to identify potential T cell epitopes on the antibody fragment. These amino acids are then substituted with less reactive residues without altering the conformation of the antibody (discussed by Adair, 2000).

Combinatorial libraries

Hybridoma methodologies allow for a limited sampling of the immune repertoire. Cloning the entire repertoire or a significant proportion of it in *E. coli* would allow a much more extensive survey of the immune response and might be essential for screening or selection for rare specificities. This approach became feasible through two developments. First, it was demonstrated in 1988 that Fvs and Fab constructs could be expressed and functionally assembled in *E. coli* (Better *et al.,* 1988; Skerra and Plukthun, 1988). In these experiments, antibody fragments were secreted from the cytoplasm to the oxidising environment of the periplasm of *E. coli* guided by bacterial leader sequences. The oxidising environment is necessary for disulfide bond formation and proper folding of antibody domains. Second, the development of the polymerase chain reaction (PCR) allowed for the rapid cloning of antibody genes from hybridomas (Chiang *et al.,* 1989; Larrick *et al.,* 1989; Orlandi *et al.,* 1989) and large collections of variable genes (Huse *et al.,* 1989; Sastry *et al.*, 1989 Ward *et al.,* 1989).

The first combinatorial library experiment reported in 1989 (Huse *et al.,* 1989) involved plaque-screening antibody Fab fragments with labelled antigen. In this case the libraries were expressed in lambda phage, a lytic phage which upon lysis of infected bacteria, releases the periplasmically sequestered Fab. In these experiments a mouse was immunised with a hapten designed to elicit catalytic antibodies and RNA was prepared from the spleen. After reverse transcription, cDNAs encoding antibody heavy chains (Fd

part of IgG1) and light chains were amplified by the PCR reaction and ligated into modified lambda phage vectors to give libraries of heavy and light chains. The two libraries were then combined by digestion of opposite arms of the vectors and religation to generate a random combinatorial library containing the genetic information for the production of Fab fragments. The library was screened by transferring Fabs from lambda plaques on to nitrocellulose filters. The filters were then probed with an ^{125}I-labeled hapten conjugate. This revealed a high frequency of positives in the library (about 1 in 5000) which allowed 200 monoclonal Fab fragments to be identified following an examination of 10^6 Fabs. The screening of this library of a million clones required 20 filter lifts, a relatively modest task.

1.3 Phage display of antibody fragments

The screening procedure limits the size of the library that may be examined in lambda phage system. For example, the screening of a library of $5x10^8$ antibodies would require an examination of a minimum of 10000 filters lifts at 50000 plaques per plate. Furthermore, the screening procedure places restrictions on the antigens which are being examined in that the antigen must be available in significant quantities in purified form for labelling with ^{125}I or enzymes, and should not stick significantly to filters in the absence of antibody. This is very restrictive if the interest is in isolating antibodies against proteins which have yet to be identified or characterised, found for example on the surface of a cell or in a crude protein extract such as a viral lysate.

Selection is a more efficient method for driving the isolation of positive clones from a library than screening since virtually no effort is wasted examining the majority of negative clones. Selection is practised very effectively by the immune system through the expression of immunoglobulin on the surface of B cells. The selected clones are then amplified by the linkage of this recognition device to the replication of the genetic information within the B cell, the process of clonal expansion. It was therefore logical to seek a selection system in which recognition and replication could be linked. George Smith (1985) had shown that peptides could be expressed on the surface of filamentous phage (Ff phage) indicating how this linkage might be achieved. The concept of selectable phage display libraries had been established in 1990 with peptide libraries (Cwirla *et al.*, 1990; Devlin *et al.*, 1990; Scott and Smith, 1990). Fragments of beta-galactosidase had previously been displayed on phage (Parmley and Smith, 1988). The concept needed only be extended to libraries of proteins. Numerous kind of proteins

have been displayed on phage including enzymes such alkaline phosphatase (Meola *et al.*, 1995) and β-lactamase (Soumillion *et al.*, 1994); hormones such angiotensin (McConnell *et al.*, 1994) and endothelin (McConnel and Hoess, 1995); cytokines such IL-3 (Gram *et al.*, 1993); toxins such ricin B chain (Swimmer *et al.*, 1992) and *Aspergillus fumigatus* ribotoxin (Crameri and Suter, 1993). This would allow selection of specific antibodies based on their ability to bind to immobilised antigen largely circumventing the problems associated with screening. In 1990 McCafferty showed that antibody fragments could be displayed on the surface of filamentous phage particles by fusion of the antibody variable genes to one of the phage coat proteins. Antigen-specific phage antibodies could subsequently be enriched by multiple round of affinity selection, because the phage particle carried the gene encoding the displayed antibody. This was originally reported for scFvs (McCafferty *et al.*, 1990), and later for Fab fragments (Chang *et al.*, 1991; Garrard *et al.*, 1991; Hoogenboom *et al.*, 1991) and other antibody derivatives such as diabodies (McGuinness *et al.*, 1996) and has been extended to other display systems.

1.4 Filamentous (Ff) phage

Ff phage, most notably F1, fd and M13 (the three are almost identical) infect Gram-negative bacteria by virtue of a specific interaction between one end of the phage and the F pilus. Ff phage are long and thin (ca. 900 x 6-10nm); essentially they are tubes of protein encapsulating a single-stranded closed circular DNA genome. The mature Ff phage comprises five proteins and a covalently closed genome of approximately 6.4 kb. Approximately 2700 copies of the protein encoded by gene 8 (g8p or cpVIII) exist. Minor coat proteins (g3p or cpIII, g6p, g7p, g9p) are present in about five copies each. The proteins are arranged so that g3p and g6p are expressed at the tail of the phage, g7p and g9p at the head. g3p is the phage protein that makes specific contact with the bacterial receptor. Rough genetic mapping has demonstrated that g3p has two domains, an amino-terminal domain important for phage infectivity and a carboxy-terminal domain important for closing or capping the phage tube. An important regulatory element, the intergenic region (IR), is present in the genome aside from reading frames for structural and non-structural proteins. The IR contains a DNA origin of replication and a DNA packaging signal. A plasmid with an Ff IR, most commonly F1, is known as a phagemid. The IR is sufficient to allow helper phage-mediated replication and packaging of the phagemid into phage particles as single-stranded closed circular DNA.

Packaged phagemids can infect bacteria and are known as transducing particles. Unlike lambda phage in which the first repertoire cloning experiments were performed, the Ff phage are not assembled in the cytoplasm and are not released by cell lysis. They are instead extruded through the membrane leaving the cell unharmed. Assembly begins with the coating of genomic (or phagemid) single-stranded DNA by g5p. This nucleoprotein complex then migrates to the inner membrane. Coat proteins then replace g5p in a vectorial fashion, g7p and g9p are first, 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 features make them ideal targets for fusion with antibody domains. Secretion into the periplasmic space is a prerequisite for proper folding and assembly of antibodies in all but specifically engineered *E. coli*. Phage which display antibody fragments can then be screened by a process known as panning to isolate those that encode the desired specificity and highest affinity as described below.

1.5 Phage display vector systems

The phage genome itself may serve as a cloning vehicle, i.e. genes can be directly cloned into the phage genome. Genomic g3p fusion systems allow the polyvalent display of foreign peptides. In these systems every copy of the three to five copies of g3p is fused with the displayed protein or peptide. With multivalent display of antibodies on the phage surface, avidity effects minimise the selective discrimination of clones bearing antibodies which differ by two orders of magnitude in affinity for a target antigen. Genomic g3p fusions have been highly successful in the area of peptide libraries but have been superseded for antibody display by phagemid systems.

Phagemid systems offer an attractive alternative to cloning directly in to the phage genome. Phagemids are simply plasmids which bear the IR region of an Ff phage. When propagated in cells superinfected with a helper phage, phagemids will be packaged as phage particles in a fashion identical to the phage itself. Phagemid systems have two distinct advantages over cloning into the phage genome. First, double-stranded DNA is easily obtained making library construction less tedious and, second, the valency of the displayed protein may be controlled. Phagemid systems has been described using both g8p and g3p fusions. During superinfection, native and fused variants of the coat protein are expressed, with native copies derived either from helper phage or from wild type

sequences present on the phagemid. As phage assembly takes place, there is a competition between these proteins for incorporation into the virion.

The g8p system like the genomic system offers multivalent display. The display of Fab fragments as g8p fusions was described initially by Kang *et al.*, (1991) and later by Chang *et al.*, (1991). Sorting of antibodies of different specificities was demonstrated in one case with a 1000-fold enrichment in a single panning step. Analysis of the phage revealed the presence of a variable number of Fab fragments ranging from 1 to 24. Further examination of this system demonstrated that the avidity effect of multiple copies of Fab on the phage surface does not allow for the selection of antibodies of the highest affinity. In panning experiments with human anti-tetanus toxoid antibodies, which differed by 100-fold in affinity, only a 5-fold enrichment could be demonstrated in a single selection (Barbas *et al.*, 1991).

In contrast to g8p fusions, monovalent display is possible by fusion to g3p. In the virion, there are normally 3-5 copies of g3p. The assembly of virus in bacteria carrying a phagemid-encoded g3p fusion after superinfection with helper phage leads to incorporation of recombinant and the native forms of g3p into the virion. The expression of native g3p from sequences carried on the helper phage is necessary for infection of the resulting viral particles. The fusion, generally one copy per phage particle, is displayed in functional form on the surface of the phage and is therefore available for antigen selection. Fusion with g3p needs to retain the two functions carried out by this 406-residue protein, infectivity and normal (nonpolyphage) morphogenesis. These properties map roughly to the first and second halves of the gene, respectively. The N-terminal domain of g3p binds to the F' pilus, allowing for infection of *E. coli*, whereas the membrane-bound C-terminal domain (residues P^{198}-S^{406}) serves the morphogenic role of capping the trailing end of the filament as it is extruded through the bacterial membrane. Another important biological feature of the N-terminal domain of g3p is its role in providing immunity to superinfection by other Ff phage. This property has important consequences in the design of phagemid vectors which generate g3p fusions: since superinfection with helper phage is required for the production of viral particles, sequences encoding the N-terminal domain of g3p must be deleted from the phagemid otherwise superinfection will be prevented. This feature was incorporated in the first vector designed for Fab display, pComb3 (Barbas *et al.*, 1991) and is also found in pDH188 (Garrard *et al.*, 1991) and pEXmid3 (Soderlind *et al.*, 1992), which are also designed for Fab display. Several g3p phagemid systems designed for scFv display -

pHEN (Hoogenboom *et al.*, 1991), pSEX (Dubel, 1993), pCANTAB (Pharmacia) - utilise the entire g3p as a fusion partner. In these systems, additional steps in the selection process are necessary. For example, glucose must be added to shut down expression from the phagemid to allow superinfection. Removal of this solute by washing or dilution relieves catabolite repression, allowing expression for display. The key feature of all g3p-based strategies is their capacity for monovalent display – this has important consequences for the selection of antibodies of the highest affinity.

1.6 Antibody fragments used in library construction

Two major types of antibody fragments may be chosen for the construction of phage display libraries, Fabs or Fvs. Fab fragments, the natural antigen binding fragments of whole antibodies, retain the binding characteristics of the whole antibody with the exception of avidity. The Fv fragments consisting only of the V_H and V_L domains are the smallest immunoglobulin fragments to contain the whole antigen-binding site. They may have greatly reduced binding affinity for antigen as compared to the whole antibody (Bird and Walker, 1991). Fvs appear to have a lower energy of interaction between their two chains than Fab fragments, which are also held together by the constant domains C_H1 and C_L. Stable association of the V_H and V_L domains can be achieved with a linking peptide sequence (Huston *et al.*, 1988) or disulphide bridges (Glockshuber *et al.*, 1990).

In scFvs (single chain Fv fragments), peptide linkers of about 3.5 nm are required to span the distance between the C-terminus of one domain and the N-terminus of the other (Huston *et al.*, 1988). Both orientations, V_H-linker-V_L or V_L-linker-V_H can be used. The 15 amino acid linker $(G_4S)_3$ originally employed by Huston *et al.* is often used in V_H-V_L scFvs. Since scFvs are only half the size of Fabs, they have lower tissue retention times, more rapid blood clearance and better tumour penetration. They are also less immunogenic, and are amenable to fusion with other proteins and peptides. Potential applications include tissue imaging and the targeted delivery of drugs, toxins or radionuclides to a tumour site (Little *et al.*, 2000).

Generally Fv fragments by themselves are unstable because the V_H and V_L domains of the heterodimer can dissociate and this results in reduced binding affinity. Covalent connection of V_H and V_L by a flexible peptide linker results in scFv molecules (Bird *et al.*, 1988; Huston *et al.*, 1988) that are more stable than Fvs. Another approach for improving Fv stability is to introduce a disulphide bind between the two domains, forming a ds-Fv (Young *et al.*, 1995). Recombinant antibodies, especially scFvs, often

fail to fold properly and aggregate within the bacterial periplasm or 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 aggregetion can be alleviated by growing the cells at 30 or 25 °C (Somerville *et al.*, 1994), by using tightly regulated promoters (Clark *et al.*, 1997), or by co-expression of proteins that assist folding such chaperones (Hayhurst and Harris, 1999).

Other forms of Fvs include single antibody domains, antigen binding V_H domains (Ward *et al.*, 1989). However, these constructs carry a "sticky patch" which usually forms interactions with V_L domains. In the absence of a light chain partner, these residues adversely affect the solubility and stability of isolated V_H domains (Ward *et al.*, 1989; Davies and Riechmann, 1994). Even smaller antigen-binding peptides based on one CDR have been described, but they often have a low affinity for antigen and the tendency to cross react with unrelated antigens (Adams, 1998).

To increase the avidity of monovalent scFvs, they can be forced to form multimers by shortening the peptide linker. scFvs are predominantly monomeric (~30 kDa) when the V_H and V_L domains are joined by polypeptide linkers of more than 12 residues (Figure 1.1). A scFv molecule with a linker of 3 to 12 residues cannot fold into a functional Fv domain and instead associates with a second scFv molecule to form a bivalent dimer or "diabody" (~60 kDa; Holliger *et al.*, 1993). Reducing the linker length still further to below three residues can result in the formation for trimers (tribody, ~90 kDa) or tetramers (tetrabody, ~120 kDa). Diabodies can be provided with extra stability by joining the two dimerising parts with a peptide linker (Kipriyanov *et al.*, 1999). Under certain conditions, these four-domain chains dimerise again to form a fairly stable tetravalent tandem diabody, which is particularly interesting for the production of tetravalent bispecific molecules (Kipriyanov *et al.*, 1999). Diabodies have been produced using disulphide bridges (Adams *et al.* 1993), or by joining the two scFv fragments with a third polypeptide linker (Gruber *et al.*, 1994). Alternatively, the scFv can be genetically fused with protein dimerising motifs such as amphipathic helices (Pack and Plucktun, 1992) or immunoglobulin C_H3 domains (Hu *et al.*, 1996). In a similar fashion, tetravalent scFv have been produced by fusing them with the tetramerisation domain of the human transcription factor p53 (Rheinnecker *et al.*, 1996) or streptavidin (Kipriyanov *et al.*, 1996b).

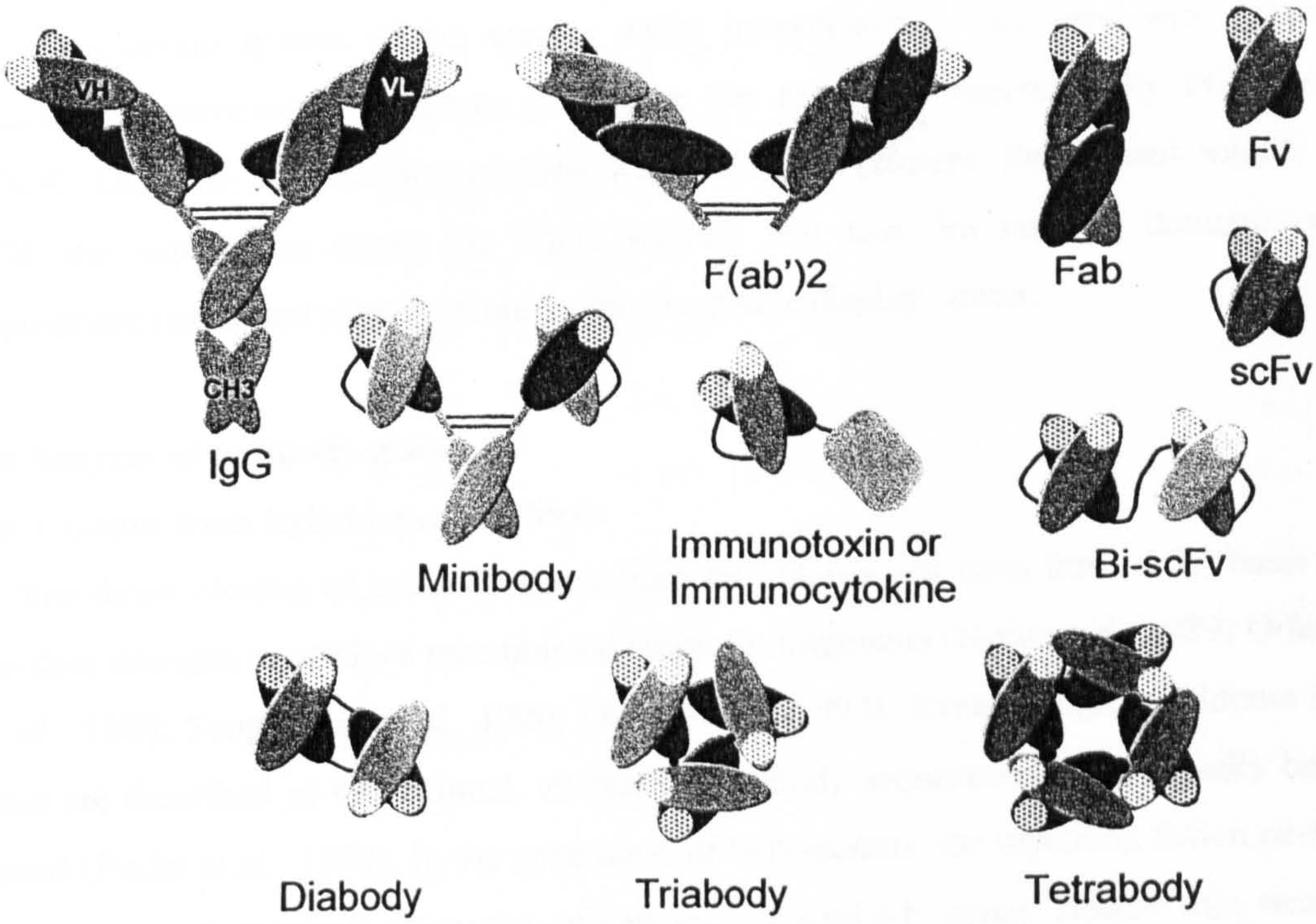


Figure 1.1 Schematic representation of some different formats of antibody fragments. The Fv fragment (V_H and V_L domains) possess the binding activity. The scFv corresponds the V_H linked to the V_L by a flexible peptide linker (black line). Dia-, tri- and tetrabodies can be obtained by using short linkers (represented by black lines or dots). (Chames and Baty, 2000)

1.7 Cloning strategies for library construction

Library construction begins with mRNA that is used as a template for cDNA synthesis. cDNA then becomes the template for the PCR. Thorough consideration of the end use of the antibody fragment should dictate the strategy to be taken. Protocols for the construction of Fab or scFv libraries are quite distinct. A major difference in the construction of scFv libraries is that there is no antibody constant region in this construct. This dictates that multiple J region primers – the number depends on the immunoglobulin system of the species under investigation – are used with primers against the framework sequences to recover the expressed repertoire by PCR from cDNA. Generally, the cloning of Fabs requires fewer primers. Subsequent rounds of PCR are required to create the linker regions that fuse V_H and V_L domains and appropriate restriction sites for cloning into the phage display vector.

1.8 Sources of antibody genes

1.8.1 Genes from hybridoma cell lines

The direct cloning of antibody genes from hybridoma cell lines formed the basis for the first attempts to produce recombinant antibody fragments (Huse *et al.*, 1989; Orlandi *et al.*, 1989; Songsivilai *et al.*, 1990; Dubel *et al.*, 1994). Even though hybridoma cell lines are described as monoclonal, alternative antibody sequences are continually being found (Fuchs *et al.*, 1997). In the generation of hybridomas, the myeloma fusion partner is chosen for its minimal expression of endogenous antibody genes. Despite this, weakly expressed pseudogenes, or other immunoglobulins can be amplified through PCR along with mutated sequences that have accumulated during extended cultivation of the hybridoma. It is therefore advisable to use hybridoma cultures that are freshly subcloned and tested as a source of cDNA for cloning. When soluble antigen is available, screening an immunoglobulin library by phage display is surely the method of choice. The genetic heterogeneity can be analysed by sequencing at least 10 clones per region. If different sequences appear, all possible combinations of light and heavy chains fragments should be produced separately and functionally tested (Breitling and Dubel, 1999).

1.8.2 Genes fragments from immune libraries

Producing human monoclonal antibodies with conventional hybridoma technology is very difficult and targeted immunisation of human patients is only possible in rare cases. Recombinant antibodies offer a way out of this dilemma by the use of human antibody

gene libraries. Especially interesting sources of antibody genes are people who have already developed an appropriate immune response resulting from exposure to an infectious agent, malignancy or other immunological trigger. An important area of use of immunised libraries is research into autoimmune diseases. Various potential autoantibodies could be identified with the help of patient libraries. Patient libraries therefore offer a way to access the antibodies that are otherwise difficult to obtain.

An interesting variation in the production of human antibodies is offered by the immunisation of SCID (severe combined immune deficiency) mice. These mice lack their functional immune system and can be temporarily populated with human peripheral lymphocytes. Antibody responses can be triggered amongst these cells by primary exposure to antigen; when they include memory B lymphocytes, it is possible to stimulate them into a second, strong immune response with antigen. Specific human antibody fragments can then be isolated from an antibody library produced as outlined above (Duchosal *et al.*, 1992). A further interesting source of material for the construction of human antibody libraries against specific agents are genetically modified lines of mice which have undergone germline reconstitution with human lymphoid material .

In non-human species, ethical constraints on specific immunisation are not so restrictive and so libraries can be constructed after antigen exposure has increased the frequency of antigen-specific B cells, and somatic hypermutation has matured the affinity of immunoglobulins for the targets of interest. These are features likely to increase the likelihood of success with *in vitro* phage selection techniques. (Hoogenboom and Chames, 2000). To date, antibody phage display techniques have been applied to a wide range of species including non-human primates, mice, rats, rabbits, sheep, cattle, camels, and chickens (O'Brien and Aitken, 2002).

1.8.3 Genes from nonimmune libraries

In the early 1990s, two sets of findings raised the possibility that specific immunisation could be bypassed in the search for antigen-specific immunoglobulins. First, it was shown that antibodies against many different antigens could be selected from a "naïve" library made from gene pools contributing to the IgM repertoire of nonimmunised, healthy individuals (Marks *et al.*, 1991). Second, libraries of germline segments synthetically diversified by oligonucleotide cloning (Hoogenboom *et al.*, 1991; Barbas *et al.*, 1992a) were shown to behave in a similar way to "naïve" antibody

libraries. It thus became possible to use "naïve" or "synthetic" antibody libraries for the isolation of antibodies against most antigens, indeed the diversity carried in these resources has proved sufficient to provide antibodies against molecules which are nonimmunogenic *in vivo*, toxic substances and targets which are conserved between species (reviewed by Winter *et al.*, 1994).

1.8.3.1 Antibody gene libraries from nonimmunised donors

'Naïve' antibody libraries have been more widely used than synthetically diversified resources. The libraries have been built from various lymphoid materials, including peripheral blood, bone marrow, spleen or tonsils. These libraries have harvested antibody binding sites which are assembled naturally (Marks *et al.*, 1991). To minimise bias by the inclusion of V-genes of immune responses on-going in the donor, the B cells can be enriched for IgM-IgD surface expression (Hawkins and Winter, 1992), or the variable heavy-chain gene can be amplified from IgM mRNA (Marks *et al.*, 1991).

Provided the repertoire captured in a library is diverse, larger libraries give rise both to many more different antibodies against a given antigen, and also to higher affinity antibodies (reviewed in Winter *et al.*, 1994). As a consequence, there have been several efforts to establish very large primary libraries by brute-force cloning (Vaughan *et al.*, 1996; Sheets *et al.*, 1998; de Haard *et al.*, 1999) or by site-specific recombination procedures (Griffiths *et al.*, 1994; Sblattero and Bradbury, 2000), to yield collections of 10^9 to 10^{11} potentially different binding sites. These library sizes are close to the total number of clones that can be effectively sampled using *in vitro* selection, and larger libraries are not expected to yield much better antibodies. Instead, the binding sites can be fine-tuned *in vitro* for binding to the antigen in a process that resembles the *in vivo* affinity maturation process.

1.8.3.2 Synthetic antibody gene libraries

Binding-site repertoires with naturally rearranged V-genes are potentially heavily biased towards certain sequences and clones, owing to evolutionary pressures and the immune history of the host. This will result in biases and redundancy in natural binding-site libraries. The use of laboratory-assembled variable-regions genes would provide complete control over library composition, allow engineering of the individual building blocks, and a choice of where and how the diversity could be introduced in these repertoires. It was thought that with these possibilities, antibody libraries could be built

with a high level of relevant diversity that might surpass that of naïve resources. (Hoogenboom and Chames, 2000).

In the first synthetic antibody library to be described (Hoogenboom and Winter, 1992; Nissim *et al.*, 1994), a large collection of human germ line gene segments was randomly diversified in CDR3 of the heavy chain by PCR. Later modifications introduced limited diversity in CDR3 for the light chain (Griffiths *et al.*, 1994). This design strategy reflects the limited natural germline diversity in the other CDR regions. From this and related libraries, antibodies to many different targets have been isolated with affinities into the nanomolar range (Griffiths *et al.*, 1994). Other synthetic designs have been described, varying from the use of one rearranged V_H and V_L segment with randomised CDR3 regions (Barbas *et al.*, 1992a), to segments with completely randomised CDRs (Garrard and Henner, 1993; Hayashi *et al.*, 1995; Soderlind *et al.*, 1995). Other design strategies have attempted to extend diversity beyond what is generated by the immune system *in vivo*. For example, diversification of the heavy chain CDR3 might provide a primary 'signature' for the antibody, which could be enhanced by directing further mutation to the natural hot spots for somatic hypermutation located in the other CDRs (Pini *et al.*, 1998). Libraries with non-natural CDR regions (*e.g.* mixed between species, artificially combined or with longer or shorter CDR loops [Jirholt *et al.*, 1998]) could well expand the structural scope of the repertoire, or might be used to build up the antigen combining site around the antigen (Lamminmaki *et al.*, 1999). The first binding-site libraries with a propensity for binding to a chosen antigen or antigenic epitope have been constructed (Kirkham *et al.*, 1999).

1.9 Selection

The selection step is a key feature of any molecular display technology. A major advantage of this *in vitro* procedure is that we can choose and manipulate the procedure and therefore the outcome of selection (reviewed in Hoogenboom, 1997).

Phage systems readily allow sorting of enormous libraries. This is a result of the linkage of recognition and replication; only those phage that express at their surface antibody capable of interacting with the target antigen propagate through a procedure known as panning. Surface display of antibody on the phage surface allows for the selection of clones by panning against antigen coated onto ELISA wells or immunotubes. Alternatively, the binding event can take place in solution. Antigen-bound phage can then be retrieved by incubation with streptavidin-coated magnetic beads

(biotinylated antigen)(Hawkins *et al.*, 1992; Schier and Marks, 1996) or other ligands that capture the antigen (*e.g.* antibodies). The phage display approach is not limited by a requirement for purified antigen and is sensitive to rather rare components, for example, in viral lysates, allowing for the discovery in principle of biologically active antibodies to unknown antigens (Burton and Barbas, 1994).

Many other sources of antigen have been used for selection, ranging from whole cells or cell lysates, tissue and protein blots, to bacteria that display recombinant antigens. When using such complex sources, selection of phage bearing antibodies against irrelevant antigens or epitopes has to be reduced, for example, by specific elution using a known ligand to the antigen. Depletion or subtraction procedures are also possible using an excess of irrelevant antigens, for example, by fluorescence-activated cell sorting or sorting with magnetic beads (Siegel *et al.*, 1997; Chang and Siegel, 1998).

Despite the availability of these elegant selection methods, it has been difficult to isolate high-affinity antibodies to some antigens, including carbohydrates and seven-transmembrane receptors. In some instances, the antibody repertoire might have a structural limitation in what antigen type it can recognise; in others, the phage antibodies might not be able to access the antigen (Hoogenboom *et al.*, 1999). Given the rapid advances made in phage display technology over the past decade, one might predict that solutions to these obstacles will emerge in due course.

1.9.1 Selection for function

The *in vitro* procedure permits selection for function as well as binding capabilities. Selection can be performed under conditions that mediate the selection of phage antibodies with particular characteristics, for example, under reducing conditions to retrieve disulfide free yet stable antibodies (Proba *et al.*, 1998), or in the presence of protease to select for successfully folded molecules (Kristensen and Winter, 1998). Antibodies might also be selected with or for a particular functional activity, for receptor cross-linking, signalling, gene transfer or catalysis. The availability of the cloned antibody genes further offers the possibility of combining phage selections (for binding) with other selections, such as a neutralising activity of the antibody when expressed in an intracellular environment (Gargano and Cattaneo, 1997; reviewed in Hoogenboom *et al.*, 1998).

1.9.2 Selection based on phage infectivity

Several selection methods have been described which are based on the restoration of phage infectivity using the antibody-antigen reaction. In this approach only phage antibodies that bind to the antigen would provide the phage with the necessary elements (*e.g.* functional g3p) for infectivity (Duenas and Borrebaek, 1994; Garmatikoff et al., 1995). In selectively infective phage (SIP) technology, the basic infectivity of the M13 filamentous phage is destroyed by deleting from the phage genome the N domains of the gIII. A peptide or protein library is fused N-terminally to some or all of the C domain of g3p. The infectivity of the phage can now only be restored by adding N domains which are absolutely required for infection. These domains are themselves fused or chemically coupled to a ligand which binds to the peptide or protein displayed on phage. These systems require a very high affinity interaction between antibody and antigen to be effective, and are thus not yet suited for applications with most primary phage libraries (reviewed in Jung *et al.*, 1999). With an increase in understanding of the phage infection process, more generally applicable infectivity-mediated selection procedures based on bacterial antigen display and phage antibody libraries, might be developed in the future (Borrebaeck 1998).

1.10 Eluting

After vigorous washing, phage bound to the immobilised target are enriched for those, which display antigen-specific antibodies. Elution of the phage is most generally achieved by acid elution. Alternatives to acid elution include base elution, antigen elution, trypsin elution (Goletz *et al.*, 2002) direct infection by simple addition of *E. coli,* reductive elution. The recovered virus are then amplified and reselected by further rounds of panning. Each step selects for antigen-specific clones as well as for clones of the highest affinity, at least in the monovalent display. In this way one can rapidly generate a panel of antigen-specific Fabs / scFvs (Burton and Barbas, 1994).

1.11 Objectives

The aims for this study can be divided into two parts: to characterise the bovine immunoglobulin J_H locus; to use the antibody phage display technique to generate recombinant antibodies against *Mannheimia haemolytica*, an important pathogen of cattle. The former would provide information required to interpret events during heavy chain gene rearrangement and aid the generation of recombinant antibody fragments.

The second part of study would help optimise the construction of bovine recombinant antibody libraries and the generation of antibodies against antigens of importance in bovine disease.

Chapter 2

Characterisation of the bovine J_H locus

2.1 Introduction

2.1.1 Structure of antibodies

Typically antibodies have a common core structure of two identical light chains and two identical heavy chains. One light chain is attached to each heavy chain, and two heavy chains are attached to each other. Both the light chains and the heavy chains contain a series of repeating, homologous units, each about 110 amino acids residues in length, which fold independently in a common globular motif, called an immunoglobulin (Ig) domain.

In humans, there are more than 1x 10^7, and perhaps as many as 10^9 structurally different antibody molecules in every individual, each with unique amino acid sequence in their antigen-combining sites. The extraordinary diversity of structure accounts for the extraordinary specificity of antibodies for antigens, because each amino acid difference may produce a difference in antigen binding. In theory, such extensive sequence diversity poses a structural problem because the three-dimensional structure of any protein is completely determined by its amino acid sequence and certain sequences are incapable of folding into soluble, stable proteins. In the evolution of antibody molecules, this problem has been avoided by confining the sequence diversity to three short stretches within the amino terminal Ig domains of the heavy and light chains. These amino terminal domains are called variable (V) regions to distinguish them from the more conserved constant (C) regions of the remainder of each chain (Wu and Kabat, 1970). The three highly divergent stretches within the V regions are called hypervariable regions (Kabat *et al.*, 1977), and they are held in place by more conserved framework regions (FR1-FR4). In an intact Ig, the three hypervariable regions of a light chain and the three hypervariable regions of a heavy chain can be brought together in three-dimensional space to form an antigen-binding surface. Because these sequences form a surface complementary to the three-dimensional surface of the bound antigen, the hypervariable regions are also called complementary-determining regions (CDRs). Proceeding from the amino terminus, these regions are called the CDR1, CDR2 and CDR3, respectively. CDR3 is the most variable of the CDRs and there are more genetic mechanisms for generating sequence diversity in this region than in CDR1 and CDR2. V region folding into an Ig domain is mostly determined by the framework regions adjacent to the CDRs. Within the framework regions, certain amino acid residues and certain structural features are very conserved. Other portions of the framework regions differ between kappa (κ) and lambda (λ) chains. When V regions fold into an Ig domain,

the CDRs are positioned on the surface as projecting loops. Sequence differences among the CDRs of different antibody molecules result in unique chemical structures being displayed by the projecting loops. Relatively few residues have been identified that, through their packing, hydrogen bonding or the ability to assume unusual conformations are primarily responsible for the variation in three-dimensional or canonical structure. These variations in surface structure account for specificity for binding to antigens (Chothia and Lesk, 1987). The structure of immunoglobulin molecule has been reviewed by Padlan (1994).

2.1.2 Mechanisms of antibody diversification

VJ and VDJ combinatorial association

The question of germ-line versus somatic generation of antibody diversity was long a central debate in immunology. The antibody repertoire is, in fact, generated during B cell development by DNA rearrangements (somatic rearrangement) that combine and assemble different segments from relatively small groups of sequences at each Ig locus. Diversity is further enhanced by the process of somatic hypermutation in mature B cells. In the case of light chains each variable domain is encoded in two separate DNA segments. The V_κ or V_λ segment encodes the first 95-101 (amino terminal) amino acids of the light chains; the second segment encodes the remainder of the variable domain and is termed a joining (J_κ or J_λ) gene segment. The joining during rearrangement of a V and a J segment creates a continuous piece of DNA encoding the whole of the light chain variable region. The heavy chain variable domains are encoded in three gene segments. In addition to the V and J gene segments (denoted V_H and J_H) a third segment is recruited to the coding unit during rearrangement called the diversity or D_H gene segment. The cluster of D_H segments lies between the V_H and J_H gene segments at the heavy chain locus. The process of recombination that generates a complete heavy chain variable region occurs in two stages. In the first, a D_H segment is joined to a J_H gene segment; then a V_H gene segment rearranges to DJ_H to complete a heavy chain variable region gene.

In the light chain, FR1 to FR3 (including the CDR1 and CDR2) and the amino terminus of CDR3 are encoded by the V gene segment. The carboxy terminus of CDR3 and FR4 are encoded by the J segment. Similar patterns operate in the heavy chain, although CDR3 largely is encoded by the D_H gene segment selected during

rearrangement.

There are multiple copies of all of the gene segments in germline DNA. The number of functional gene segments varies between species. It varies even between the individuals in each species because of insertion or deletion of gene segments by meiotic recombination, or because of mutations that transform a functional gene into a pseudogene.

Junctional and insertional diversity

The variability of CDR1 and CDR2 arises mainly from the sequence heterogeneity of the multiple germline V genes. In comparison with the factors that diversify CDR3, this variability is modest. Diversity in CDR3 is based on the multiplicity of D_H and J_H segments, and is further increased by imprecision in the joining process, the deletion of nucleotides from the ends of segments during joining, the addition of N nucleotides and use of all three reading frames in the D segment (Reynaud *et al.*, 1994; Corbett *et al.* 1997). The precise positions at which the genes for the V and J, or the V, D, and J, segments are fused together are not constant and imprecise DNA recombination can lead to changes in amino acids at these junctions and shifts in the reading frame of the D segment. Further diversification can arise through deletion as nucleases remove bases from the free ends of the rearranging segments. Furthermore, on the heavy chain, small sets of nucleotides may be inserted at the V-D, and D-J junctions through the action of terminal transferase. These extra bases are called N regions and are typified by an enriched G/C content (Alt and Baltimore, 1982; Roth *et al.*, 1988). They are rarely longer than about 15 nucleotides (Roth *et al.*, 1988).

Somatic gene conversion

Studies in some species, most notably in birds and rabbits, have revealed that these animals use a mechanism known as somatic gene conversion to generate a repertoire of diverse Ig specificities. In somatic gene conversion, part of a donor gene or genes is copied into an acceptor gene, but only the acceptor gene is altered. Typically the donor sequence is derived from an Ig pseudogene.

It is now clear that, in contrast to mice and humans, many species rely on somatic gene conversion and somatic hypermutation to generate diversity within the primary Ig repertoire, that is, before antigen stimulation. For example, chickens use somatic gene conversion as a major mechanism to generate the primary light chain and heavy chain

repertoires (Reynaud *et al.*, 1985; Reynaud *et al.*, 1987; Reynaud *et al.*, 1989). Other species such as rabbits (Becker and Knight, 1990; Knight *et al.*, 1995; Schiaffella *et al.*, 1999) and cattle (Parng *et al.*, 1996) use very limited V(D)J recombination plus somatic gene conversion and somatic hypermutation to generate their primary Ig diversity.

Somatic hypermutation

Mutations that occur in V genes of heavy and/or light chains during the lifetime of a B cell also increase the variety of antibodies produced by the B cell population. Generally an antibody of low affinity is produced in the primary response to antigen. As the immune response matures, especially after secondary stimulation by antigen, an increase in affinity of the antibody for the antigen occurs, and a divergence is found from the amino acid sequence that is encoded in germline DNA. This divergence results from point mutation in the recombined V(D)J unit, which results in changes in individual amino acids most frequently in the CDRs. This phenomenon is termed somatic hypermutation and is used in humans (Griffiths *et al.*, 1984; Berek and Milstein, 1987), mice and sheep (Reynaud *et al.*,1991b; Reynaud *et al.*,*1995*; Reynaud *et al.*,1997) for V-gene diversification. By increasing the affinity of interaction between antibodies and antigens, somatic hypermutation increases the functional diversity of the Ig repertoire.

Random assortment of heavy and light chains

As both the heavy and light chain variable regions contribute to antibody specificity, each light chain in the Ig repertoire can combine with many different heavy chains to give numerous different antibody specificities. The ability to create many different specificities by making many different combinations within a small gene pool is known as combinatorial diversity. In practice, combinatorial diversity is likely to be less significant in Ig diversification than we might expect from the theoretical calculations as not all of the functional V gene segments are used at the same frequency; some are common, while others are found only rarely (Cook and Tomlinson, 1995) . Moreover, not all V_H regions pair successfully with all V_L regions. It remains most likely that imprecise joining of V(D)J gene segments and somatic hypermutation are of greater significance in generating a diverse Ig repertoire.

2.1.3 Rearrangement of V, D and J gene segments

Non-coding regions flanking the different heavy and light chain V, D, and J gene segments have conserved sequences adjacent to the points at which recombination takes place. The sequences consist of a conserved block of seven nucleotides (heptamer) which is always contiguous with the coding sequence, separated by a spacer of roughly 12 or 23 base pairs from a second conserved block of nine nucleotides (nonamer). The spacer varies in sequence but its length is conserved. The heptamer-spacer-nonamer is often called a recombination signal sequence, RSS .

Recombination can only link a gene segment flanked by a 12mer-spaced recombination signal sequence to one with a 23mer-spaced RSS (the 12/23 rule [Sakano *et al.,* 1980]). Thus, for the heavy chain, a D_H gene segment can be joined to a J_H gene segment, but V_H gene segment cannot be joined to J_H segment directly, as both V and J gene segments are flanked by 23 base pair spacers and the D_H gene segments have 12 base pair spacers. Gene segments that lack conventional RSS are rendered non-functional and these pseudogenes fail to contribute to the Ig repertoire.

2.1.4 Human Ig variable regions

Heavy chains

The functional human V, D and J segments genes that encode heavy chain variable regions are located on chromosome 14q32.3 (Darlington *et al.,* 1982). The V_H locus contains approximately 95 V_H segments (Tomlinson *et al.,* 1992, Matsuda *et al.,* 1993; Cook *et al.,* 1994). Interestingly, 8 V_H segments and a cluster of D_H5 are located on chromosome 15q11.2 and 16 V_H segments have been mapped on chromosome 16.p11.2 (Nagaoka *et al.,* 1994; Tomlinson *et al,.* 1994). Some of these orphan genes are potentially functional but there is no evidence to date that they contribute to the functional repertoire.

The V_H segments can be grouped into families on the basis of similarity of DNA sequence and on this basis, they have been subdivided into seven families whose members are more than 80% homologous. The families can be further grouped into clans, made up of families that are more similar to each other than to families in other clans. Human V_H gene segments fall into three such clans. All of the V_H gene segments identified from other species also fall into the same three clans, suggesting that all may have evolved by gene duplication from three ancestral V gene segments (Cook and Tomlinson, 1995).

For the heavy chains of human, there are 51 functional V_H gene segments, 6 functional J_H gene segments (Ravetch *et al.*,1981) and approximately 27 D_H gene segments (Corbett *et al.*, 1997). Thus around 8000 different possible V_H regions are possible through rearrangement alone, emphasising the importance of other processes in the diversification of the repertoire.

Light chains

The human V_λ locus is located on chromosome 22q11.2 (de la Chapelle *et al.*, 1983) and contains 37 gene segments of which approximately 30 are functional (Williams *et al.*, 1996). The locus also carries 7 J segments, 4 of them functional, each with a slightly different copy of C_λ gene nearby. A V_λ segment may fuse to any of these alternative J_λ segments and the resulting V_λ/J_λ exon can then be transcribed together with the adjacent C_λ segment (Vasicek and Leder, 1990).

The genetic information needed to produce κ chains lies within a single locus on chromosome 2p11.2 (Malcolm *et al.*, 1982). This carries 77 V_κ segments and 55 truncated pseudogenes and relics (Lotscher *et al.*, 1986; Schable and Zachau, 1993; Kawasaki, *et al.*, 2001). Of the Vκ segments, 46 possess open reading frames, 37 genes rearrange with J_κ, 31 of these rearranged products are transcribed and finally only 29 genes produce functional proteins (Kawasaki *et al.*, 2001). The human J_κ cluster consists of five segments all of which are functional (Hieter *et al.*,1982).

2.1.5 Features of animal heavy chain variable regions

The V(D)J rearrangement process is common to all vertebrates but germline sequence diversity varies across species. The genetic mechanisms by which antibody diversity is generated have been studied extensively in mice and humans, but less so in other species. V_H gene families in mice and humans have been classified into three clans designated I, II and III. In each clan, FR3 is unique. Experiments with mouse V_H probes suggest that clan III is the closest to the primordial gene from which the vertebrate Ig gene arose. Ig genes from fish have close similarity to clan III and, to a lesser extent, to clan II mammalian Ig V_H genes, corroborating this idea. In contrast, clan I probes from mice hybridised only with members of Rodentia, suggesting this clan has diverged more between species. In mice, there are in excess of 100 V_H germline genes which group into fourteen families.

Birds and other mammals which have been studied show less variability in the use of V_H genes. For instance, in rabbits 80-90% of antibodies are derived from only one V_H gene, known as V_H1, although >100 V_H genes are present in the genome (Gallarda *et al.*, 1985; Currier *et al.*, 1988; DiPietro and Knight, 1990). Two other V_H segments, V_HX and V_HY, are used in most other rearrangement products (Friedman *et al.*, 1994). The primary repertoire arises mainly from gene conversion in which the other V_H genes donate genetic information to V_H1, as well as by somatic hypermutation (Knight and Becker, 1990; Schiaffella *et al.*, 1999). Preferential utilisation of D and J_H has also been observed, so of the 12 D and 6 J_H segments available, D2a, D2b, D5 and J_H4 are used most frequently (Friedman *et al.*, 1994; Tunyaplin and Knight, 1995).

Gene conversion is also involved in generating diversity in the chicken repertoire: the single functional V_H gene is diversified by gene conversion with the 100 V_H pseudogenes which lie upstream at the locus (Reynaud *et al.*, 1989). The chicken D_H cluster contains 16 D segments, with 15 of them being extremely homologous and 1 being divergent (D_X) D_X is judged to be poorly functional, since it has a low rearrangement frequency. The high incidence of D-D junctions observed (25% of DJ rearrangements) might represent the major functional contribution of this multigene cluster to a system in which diversity will be generated later by gene conversions (Reynaud *et al.*, 1991a; Reynaud *et al.*, 1994).

In swine, it appears there are <20 V_H genes belonging to a single gene family, which has identity to human V_HIII (Sun *et al.*, 1994; Butler *et al.*, 1996). Despite the close similarity of their Ig gene sequences to humans, swine belong to the group of animals that includes chickens, sheep and rabbits when classified on the basis of B cell development. This group, unlike rodents and humans, have a single V_H family, diversify the antibody repertoire in hindgut follicles early in life (rather than bone marrow throughout life) and achieve this through gene conversion (Reynaud *et al.*, 1989, Reynaud *et al.*, 1991b; Lanning *et al.*, 2000).

The germline of sheep contains nine V_H segments belonging to a single V_H family closely related to the human V_H4 gene family. Six of these genes are potentially functional and three are pseudogenes (Dufour *et al.*, 1996).

The presence of few functional germline V genes in chicken, rabbit, pig, sheep, and cattle restricts the combinatorial diversity encoded in the germline, but these species employ other strategies to achieve antibody diversification. For example, gene conversion is involved in diversification of the pre-immune antibody repertoire in the

bursa of Fabricius of chickens and in the appendix of rabbits. In sheep, somatic hypermutation occurs during B cell development in ileal Peyer's Patches (Reynaud *et al.*, 1989; Reynaud *et al.*, 1991b; Lanning *et al.*, 2000).

In most vertebrates studied to date including humans, mice, rats, rabbits, sheep and chickens, J_H gene segments encode the 3` part of CDR3 and the whole of FR4. In all species except the cartilaginous fishes, J_H segments are carried at a locus spanning 1.5 – 2.5 kilobases (kb) 5-8 kb 5' to the Cμ1 gene. The sequences of J_H segments and their adjacent RSS tend to be conserved, but the number of segments varies between species. Variability in the number of J_H segments may reflect duplication and/or deletion events during evolution. The utilisation of J_H segments also varies according to the species and developmental stage. Generally, all J_H segments are not used with same frequency, and one or a few J_H segments are preferentially expressed and found in the variable regions of heavy chains.

Unique structure of Camel Immunoglobulin

The majority of functional camel antibodies are homodimers consisting of only heavy chains, devoid of light chains (Hamers-Casterman *et al.*, 1993). Sequence analysis has shown that the V_H part of these heavy chain antibodies is very similar to human and mouse V_H (Hamers-Casterman *et al.*, 1993; Muyldermans *et al.*, 1994). Mouse V_H proteins have been reported to be sticky (Ward *et al.*, 1989; Davies and Riechmann, 1994) probably because removal of the V_L domain exposes a large hydrophobic surface (Chothia *et al.*, 1985; Stanfield *et al.*, 1993). In contrast, camel V_HHs were found to have a good solubility behaviour presumably due in part to several amino acid substitutions at this surface (Muyldermans *et al.*, 1994). The potential antigen-binding surface of the camel V_HH is increased due to CDR3 sequences that average 14 amino acids in length (Muyldermans *et al.*, 1994).

2.1.6 Animal light chain variable region

The processes, which diversify the heavy chain repertoire of an animal generally also, act upon immunoglobulin light chains. Chickens express light chains of the λ isotype. A single $V_\lambda 1$ gene is functional and this is located 1.8 kb upstream of a single J-C unit. Upstream of $V_\lambda 1$ lies a cluster of 25 V pseudogenes. Most of these pseudogenes lack recombination signal sequences, none of them have upstream regulatory elements, and

some of them are so severely truncated that they only encode part of the V coding sequence. Sequences of somatically rearranged $V_\lambda 1$ genes from embryonic and bursal cells post-hatching show that diversification of light chain sequences occurs during ontogeny by segmental gene conversion which takes place at a frequency of 0.05-0.1 per cell generation between the pseudogene pool and the unique rearranged functional V gene (Reynaud *et al.*, 1985; Reynaud *et al.*, 1987).

In rabbits, the limited VH gene repertoire sits in contrast with the diverse set of $V_\kappa J_\kappa$ sequences which are expressed. Sehgal *et al.* (1999) identified at least 39 rabbit germline V_κ genes of which at least 28 were expressed. As for V_H genes, the V_κ genes are further diversified by somatic hypermutation and gene conversion during clonal expansion of B cells (Sehgal *et al.*, 2000).

In sheep, foetal B cells express 12 distinct V_λ segments from 90-100 V_λ genes in the germline and 6 V_κ segments are present. Southern blot analysis using V_κ probes indicated that the sheep Igκ locus contains perhaps 10 V_κ genes (Reynaud *et al.*, 1995; Hein and Dudler, 1999). Antigen-independent hypermutation is the major process that provides diversity post-rearrangement in sheep light chains (Reynaud *et al.*, 1995).

In common with many domesticated animals, the light chain repertoire of cattle is dominated by expression of lambda chains (Butler, 1997). Investigation has revealed that the bovine light chain repertoire is dominated by a single gene family designated $V_\lambda 1$ with at least 14 members (Sinclair *et al.*, 1995). Studies of the bovine light chain repertoire have identified FR4 sequence which are closely related (Ivanov *et al.*, 1988; Jackson *et al.*, 1992; Sinclair *et al.*, 1995; Parng *et al.*, 1996) suggesting that V_λ segments rearrange with a single or a very limited number of J segments, although there may be more present at the lambda locus (Parng *et al.*, 1994). Cattle are unable to establish a diverse primary antibody repertoire from the process of rearrangement alone. Evidence for gene conversion and / or somatic hypermutation is being actively sought in several laboratories.

2.1.7 Bovine heavy chain variable region and the J_H locus

The most marked differences between the Ig genes of cattle and humans or mice are found amongst those sequences that contribute to the formation of the antibody variable region. All characterisations of the bovine heavy chain repertoire to date have concurred that it is founded upon expression of a single family of V_H segments (Bov_H1)

homologous to the murine Q52 family (Sinclair and Aitken, 1995; Berens *et al.*, 1997; Saini *et al.*, 1997; Sinclair *et al.*, 1997). Bov_H1 has highest identity to the single expressed sheep family (83%) and is assigned to the clan II cluster of Ig (Sinclair and Aitken, 1995; Berens *et al.*, 1997). There is evidence that alternative gene families are present in the germline (Berens *et al.*, 1997; Saini *et al.*, 1997) but they do not appear to be expressed for reasons which, at present, are not clear. It has been estimated that the dominant gene family comprises as few as 15 members; of greater significance is the very low levels of diversity which have been observed amongst these segments (Berens *et al.*, 1997; Sinclair *et al.*, 1997). As in the sheep (Reynaud *et al.*, 1991b; Reynaud *et al.*, 1994; Reynaud *et al.*, 1997), rearrangement would thus seem unable to impart significance diversity upon the primary heavy chain repertoire, an inference which is supported by analysis of foetal bovine heavy chain cDNA (Berens *et al.*, 1997).

Many studies have shown that bovine immunoglobulin heavy chain cDNA possesses highly conserved FR4 sequences with little or no mutation (Armour *et al.*, 1994; Sinclair and Aitken, 1995; Berens *et al.*, 1997; Saini *et al.*, 1997; Sinclair *et al.*, 1997) suggesting that one J_H segment is preferentially utilised in rearrangement. Supported by the similarity of sequences encoding FR4, this property is shared with the sheep, in which just two of six segments at the J_H locus are functional, one of which is predominantly expressed (Dufour and Nau, 1997). However, one striking feature of the bovine heavy chain repertoire which distinguishes it from the ovine system is the frequency with which CDR3 sequences arise encoding an average of 21 (Sinclair and Aitken, 1995; Berens *et al.*, 1997; Saini *et al.*, 1997; Sinclair *et al.*, 1997) and up to 61 amino acids (aa) (Saini *et al.*, 1999); ovine CDR3 segments are more typical in their length (beween 5-18 aa with an average of 13 aa; [Dufour *et al.*, 1996]). The basis of this property remains a matter of speculation (Aitken *et al.*, 1999) until such time as bovine D segments can be isolated.

2.1.8 Objectives

The objective of work presented in this chapter was to isolate and characterise the bovine J_H locus. It was then my intention to establish the evolutionary relationship between the bovine J_H system and loci from other mammals and to explain if possible the dominance of a single J_H segment in the bovine heavy chain repertoire.

2.2 Materials and methods

Preliminary data indicated close homology between bovine and ovine antibody cDNA sequences in framework 4 (Jackson *et al.*, 1992; Armour *et al.*, 1994; Sinclair and Aitken, 1995; Dufour *et al.*, 1996; Berens, *et al.*, 1997; Dufour and Nau, 1997; Saini, *et al.*, 1997; Sinclair *et al.*, 1997), suggesting that the J_H loci carry segments of related sequence. From this foundation primers can be designed to recover parts of the bovine J_H locus, basing these primers on ovine sequence.

The nucleotide sequences of primers used in this section are shown in Table 2.1.

2.2.1 Ovine JH1- JH4 amplicons

2.2.1.1 Amplification

Primer ovine JH1 primes upstream of the first segment in the ovine J_H locus. It was designed from the sheep J_H sequence (Dufour *et al.*, 1996). Primer JH4 was based upon the short stretches of bovine sequence lying about 80 base pairs (bp) downstream of a J_H segment (Aitken and Sinclair, unpublished data).

Bovine genomic DNA was isolated from liver obtained from a local slaughterhouse using Wizard DNA Purification Kit (Promega). The tissue was stored at –80 °C after collection. Small pieces of frozen tissue were ground to a fine powder in a mortar and pestle on dry ice. Approximately 20-40 mg of powdered tissue was transferred to a 1.5 ml eppendorf tube containing 600 µl of nuclei lysis solution. The mixture was incubated at 65 °C for 30 min. RNase was added to the viscous nuclear lysate to a final concentration of 20 µg/ml and the mixture was incubated at 37 °C for 15 min. Two hundred µl of protein precipitation solution was added and after vortexing the mixture for 20 sec and chilling on ice for 5 min, it was centrifuged at 13000 x g for 10 min. The supernatant was separated from the white protein pellet and was transferred to a 1.5 ml tube containing 600 µl of isopropanol at room temperature. The solution was inverted gently until thread-like strands of DNA became visible. The tube was centrifuged at 13000 x g for 1 min at room temperature and the supernatant was decanted. The DNA pellet was washed by adding 600 µl of 70% ethanol and inverting the tube. After centrifuging at 13000 x g for another 1 min, the ethanol was discarded and the pellet was air dried. DNA was then recovered using 100 µl of DNA rehydration solution (10 mM Tris-HCl [pH 7.4], 1mM EDTA [pH 8]) and incubation at 65 °C for 1 hr.

Table 2.1 The nucleotide sequences of primers used for recovery and characterisation of the bovine J_H locus*.

Ovine JH1	GCCCACTGTGACTATGCTGACTTCC
Ovine JH1R	GGAAGTCTGCATAGTCACAGTGG
JH2A	CCTGGTCACCGTCTCCTCAGG
JH4	GGACATATCAGAGGACCGGACAGTGATG
JH4F	CTGAGCATCACTGGTCCTCTGATGTGTCC
M13/pUC Forward❖	GTTTTCCCAGTCACGAC
M13/pUC Reverse❖	CAGGAAACAGCTATGAC
JH6:	GGTGAGGGCTTAGGTGAG
JH7	ATCATTGTCACTGTTTAACAACTGG
JH8	TGGGCCGGAGGAAAGGTGG
JH8R	CCACCTTTCCTCCCGCCCAGTCAGC
JH9	GACGGAAGCCCCTGGTCCTGCTC
JH10R:	TTGCTGGCCCTGACTCCCAT
EμR	AGACTCTCAAGTGGACATGCTTCCTGCCACC
Lambda Right Reverse	GTACGCATTACAGGTGATGCG
Lambda Right Reverse 2	CATATACATGGTTCTCTCCAGAGG
M13/pUC Forward*	GACGTTGTAAAACGACGGCCAG
M13/pUC Reverse*	TCACACAGGAAACAGCTATGA
DQ52	RGHRYCWGTGCTAACTGG**
VHF	CCCACTGTGGACCCTCCTCTTT
VH1A	AGTACTATAGTATACGGACCGAGCCTGGTGAAGCC CTCACAGACC

* All primers are shown 5' to 3'

❖ Used for sequencing of pZErO2 (1.5) and crude lysate screening (3.4)

* Used by MBSU for sequencing of pUC18 and pCR®4-TOPO®vector

** R=A or G, W=A or T, Y=C or T, H=A or C or T

1 μg of bovine genomic DNA was used as template to amplify the J_H locus by using the primers Ovine JH1 and JH4. PCR was performed in a reaction volume of 50 μl with final concentration of 750 μM of each dNTPs (Life Technologies), 600 nM of each primer in water, 40 μg/ml bovine serum albumin and 5 mM $MgCl_2$. The PCR buffer used for the reaction (Promega, M190) also provided 10 mM Tris-HCl (pH 9.0), 50 mM KCl and 0.1% Triton®X-100 (final concentrations). 2.6 units of Expand™ high fidelity polymerase containing thermostable Taq DNA and Pwo DNA polymerase (Boehringer Mannheim) was used for amplification. The reaction was heated to 93°C for 1.5 min and was then incubated for 35 temperature cycles of 93 °C for 30 seconds, 55 °C for 1 min and 68 °C for 2.5-3 min (5 cycles for each +5 seconds). A final incubation of 68 °C for 5 min completed the amplification reaction.

2.2.1.2 Filling the protruding ends of amplicons

Amplicons were purified from agarose gels by the QIAQuick® Gel extraction kit (QIAGEN) as described in Appendix 2. Since the amplicons were to be cloned in a blunt ended ligation protocol, their termini were polished. About 900 ng of the 1.8 kb amplicons recovered by PCR, were incubated with 5 units of Klenow fragment of DNA polymerase I (Promega), in a total volume of 20 μl containing 10 μM dNTPs and 30 μg/ml bovine serum albumin. Two μl of Klenow buffer (Promega) provided 50 mM Tris-HCl (pH 7.5), 10 nM $MgSO_4$ and 0.1 mM DDT (final concentrations). Reactions were incubated at 37 °C for 15 min and the enzyme was then inactivated at 75 °C for 10 min.

2.2.1.3 Cloning the polished amplicons

The vector pZErO2 (InVitrogen) was used for cloning of amplicons. It was cut with *Eco*RV (Life Technologies) to form blunt ends. Digestion was done at 37 °C for 30 min and then the reaction was heated at 65 °C for 5 min to inactivate the enzyme.

The blunt ended amplicons and 50 ng of vector were ligated in a ratio of 3/1. Ligation was done using 3 units of T4 DNA ligase (Promega) in a total volume of 20 μl at 16°C for 19 hrs. Two μl of ligase buffer (Promega) provided 30 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DDT and 1 mM ATP for the reaction.

2.2.1.4 Transformation of ligated DNA

Competent *E. coli* DH5α cells (100 µl; Promega) were thawed on ice from storage at –80 °C. Three µl of ligation reaction was added and the mixture was incubated for 30 min on ice. The bacteria were then heat shocked at 42 °C for 60 seconds. The transformed cells were incubated in 900 µl SOC media at 37 °C for 1 hr and 10 and 100 µl of culture then were plated on Luria agar (LA) plates containing 35µg/ml Kanamycin and 3mM IPTG. Plates were incubated overnight.

Of 23 colonies, 5 were selected for the isolation of plasmid DNA. Colonies were inoculated into 5 ml Luria broth (LB) and incubated at 37 °C overnight. The cultures were centrifuged and plasmid DNA isolated from the bacterial pellet using the QIAprep spin miniprep kit (QIAGEN) (discussed in Appendix 2). DNA was double digested with restriction enzymes *Eco*RI and *Xho*I (Life Technologies) at 37 °C for 1.5 hrs and then analysed on a 0.5 % agarose gel.

2.2.1.5 Sequencing of cloned fragment

A clone (2-4) that carried the amplicon was sequenced with M13/pUC forward and reverse primers by the PNACL DNA sequencing service at the University of Leicester. Since the sequencing data did not cover the whole length of the fragment, another 3 primers JH7, JH8 and JH9 were designed. Reactions with these oligonucleotides completed sequencing of the insert carried in 2-4.

2.2.2 JH2A-EµR ampilcons

2.2.2.1 Amplification

Since analysis of the sequences of Ovine JH1-JH4 amplicon revealed the location of a J_H segment expressed with low frequency in bovine antibodies (Berens *et al.*, 1997), more frequently expressed segments were sought in flanking regions. To amplify the downstream sequences, two primers JH2A and Eµ reverse (EµR) were used. The former had been designed in our laboratory based on the sequences of bovine antibody cDNA (Sinclair and Aitken, 1995; Sinclair *et al.*, 1997) and primes the sequence commonly observed in FR4, extending towards the Cµ region. The second primer sequence, EµR, was taken from the ovine immunoglobulin heavy chain 5' intronic enhancer sequence, selecting a region without significant repetition that was conserved across a number of species (bases 297-320; GenBank accession number Z98207)

(Figure 2.1). It was intended to anneal to complementary bovine enhancer sequences, extending towards the J_H region.

PCR was performed in a reaction volume of 50 µl with final concentration of 500 µM of each dNTPs (Promega), 800 nM of each primers and 5 mM $MgCl_2$. In addition, reaction buffer provided 50 mM KCl, 10 mM Tris-HCl (pH 9.0) and 0.1% Triton®X-100. Five units of Taq DNA polymerase (Promega) were used for amplification with the following conditions. The reaction was heated to 94°C for 5 min and was then incubated for 35 temperature cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min, and then a final incubation of 72 °C for 5 min.

2.2.2.2 Phosphorylation and polishing of amplicons

Dephosphorylated *Sma*I cut pUC18 (Pharmacia) was used to clone the 900 bp JH2A-EµR amplicons. Firstly, the PCR product was run in 1% agarose gel and the desired band was extracted using the QIAQuick® Gel extraction kit. The vector was supplied with dephosphorylated blunt ends. Thus in contrast to cloning into pZErO2 (subheading 2.2.1.3), amplicons were phosphorylated and polished prior to the ligation step. These modifications were performed concurrently in a 50 µl reaction containing about 1 µg DNA, 1mM ATP, 35 µM dNTPs, 20mM $MgCl_2$, 80 µM Tris-HCl (pH 7.6), 5 mM DTT, 10 units T4 polynucleotide kinase (Promega) and 5 units Klenow fragment of DNA polymerase I (Promega). The reaction was incubated at 37 °C for 40 min and the enzymes were then inactivated at 75 °C for 10 min. The DNA was precipitated with sodium acetate and ethanol, dried and rehydrated in 30 µl of distilled water. To determine the concentration of recovered DNA, 2 µl was run on a 1% agarose gel.

2.2.2.3 Cloning of amplicons

200 ng of each amplicon and vector were used in ligation reactions, providing a ratio of about 4/1 of insert/vector. Ligation was done at 16 °C for 18 hrs in a volume of 20 µl containing 10mM DDT, 400 units T4 DNA ligase (New England Biolabs) and 2 µl of ligase buffer that provided 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 10 mM DDT, 50 µM ATP and 25 µg/ml BSA.

```
                                        Eμ reverse primer binding site
                                   <--------------------------
                                      300                                              350
Ovine  Eμ  Z98207      CACCTGCAGCAGGT  GGCAGGAAGCATGTCCACTTGAGAGTCTGTTTTTGGAAGCAAGAAAAAA CAGTTGGTAAATTTATCGCTTCTGGTT
Human  Eμ  X97051.1    CACCCACAGCAGGT  GGCAGGAAGCAGGTCACCGCGAGAGTCTATTTTAGGAAGCAAAAAAACA CAATTGGTAAATTTATCACTTCTGGTT
Mouse      J00440.1    CACCTGCAGCAGGT  GGCAGGAAGCAGGTCATGTGGCAAGGCGATTTGGGGAAG
Rabbit     X13700.1    CACCTGCAGCAGGT  GGCAGGAAGCATGTCCACTTGAGAGTCTGTTTTTGGAAGC
Human Eα   X17116.1    CACCCACAGCAGGT  GGCAGGAAGCATGTCCACTTGAGAGTCTGTTTTTGGAAGCAAGAAAAAA CAGTTGGTAAATTTATCGCTTCTGGTT

                            400                                           450
Ovine  Eμ  Z98207      TGGT TTTGCCCAGCCCCACAGAACCGAAAGTGTTCCACTGAGCACAACAGCAC CTGGCTAATTTGCATTTCTAAAATAAGGCGCAGATGCTGACCGAAACTG
Human  Eμ  X97051.1    TGGT TTTGCCCAGGCCCA..GATCTGAAAGTGCTCTACTGAGCAAAACAACAC CTGGACAATTTGCGTTTCTAAAATAAGGC...GAGGCTGACCGAAACTG
Mouse      J00440.1                                         CTGAGCACAACAGCAC CTGGCTAATTTGCATTTCTAAAATAAG
Human Eα   X17116.1                                                      CAC CTGGACAATTTGCGTTTCTAAAATAAGGC...GAGGCTGACCGAAACTG
Rat        M13799.1                                                 AACAACAC CTGGATAATTTGCATTTCTAAAATAAG
```

Figure 2.1 Alignment of sequences of heavy chain enhancers from various species. Species and accession number are shown to the left of the alignment. The sequence selected for design of the primer Eμ reverse primer is indicated by an arrow.

2.2.2.4 Transformation of ligated DNA

E. coli XL-1 supercompetent cells (Stratagene) were thawed on ice from –80 °C and mixed with β-mercaptoethanol to a final concentration of 25 mM. After incubation on ice for 10 min, 4 µl of each ligation reaction was added, and the incubation continued for a further 30 min. The cells were then heat shocked at 42 °C for 45 sec, added to 950 µl of SOC media and then incubated at 37 °C with shaking. After 1.5 hrs 40 µl of culture was plated on LA containing 100 µg/ml ampicillin, 12 µg/ml tetracycline, forty µl of 4% X-gal and 40 µl of 25 mM IPTG and incubated at 37 °C overnight.

The following day, six white colonies were inoculated into 5 ml Luria broth (LB) and incubated at 37 °C overnight. Plasmid DNA was isolated using the QIAprep spin miniprep kit. Purified DNA was digested with *Eco*RI (Promega) to determine the size of the linearised molecule, and also with two flanking enzymes *Hin*dIII / *Sst*I (Life Technologies) to check the insert frequency. Digests were analysed on 1% agarose gels.

2.2.2.5 Sequencing of cloned fragment

A clone designated E2 carrying the expected size of insert was chosen for sequencing. The plasmid was sequenced at the Molecular Biology Support Unit (MBSU) at the University of Glasgow using primers M13/pUC forward and reverse.

2.2.3 Right reverse-JH8R amplicons

2.2.3.1 Amplification

To amplify the sequences upstream from the region recovered with primers Ovine JH1 and JH4, a phage designated clone 15 from a lambda phage library of bovine genomic DNA was used as template. This clone was a gift from Professor Katherine Knight (Department of Microbiology and Immunology, Loyola University, Chicago). It carries about 18 kb fragment spanning the J_H locus, the Sµ switch region and Cµ exons (Knight *et al.*, 1988). From the limited published data, it was predicted that the insert would carry sufficient sequence upstream from the J_H locus to include any homologue of the DQ52 segment present in the bovine genome. The amplification was performed using primers right reverse (RR) and JH8 reverse (JH8R). The primer RR primes sequences on the right arm of the lambda phage vector 230 bp from the insert. Preliminary experiments established that the genomic DNA carried in clone 15 is

orientated with the J_H locus proximal to the right arm of the vector. The JH8R primer anneals to sequences between the second and third J_H segments, revealed from sequencing of Ovine JH1-JH4 amplicons.

PCR was done in a reaction volume of 50 µl with final concentration of 500 µM of each dNTPs (Promega), 400 nM of each primers and 3 mM $MgCl_2$. The PCR buffer (Promega) also provided 50 mM KCl, 10 mM Tris-HCl (pH 9.0), and 0.1% Triton®X-100. Five units of Taq DNA polymerase (Promega) were used for amplification. The reaction was heated up to 94°C for 5 min and was then incubated for 35 temperature cycles of 94 °C for 1 min, 55°C for 1 min and 72 °C for 3 min, and then a final incubation of 72 °C for 5 min.

2.2.3.2 Phosphorylation and polishing of amplicons

Dephosphorylated *Sma*I cut pUC18 (Amersham Pharmacia Biotech) was used as vector for cloning of the 1.2 kb amplicons from this reaction. Protocols for purification, phosphorylation and polishing of PCR products are described in subheading 2.2.2.2. The ethanol precipitated DNA was dissolved in 20 µl of distilled water and 2 µl was run in 1% agarose gel to determine the concentration of recovered DNA.

2.2.3.3 Cloning of amplicons

300 ng of amplicons and 200 ng of pUC18 were used in ligation reactions providing a ratio of about 4/1 insert/vector. Ligation was performed at 16 °C for 18 hrs in a volume of 20 µl with reaction conditions described in subheading 2.2.2.3.

2.2.3.4 Transformation of ligated DNA

E. coli Sure®2 supercompetent cells (Stratagene) were used for transformation. Bacteria (50 µl) were thawed on ice from storage at –80 °C and β-mercaptoethanol added to a final concentration of 25 mM. After 10 min 4 µl of each ligation reaction was added, incubation on ice continued for 30 min and cells were then shocked at 42 °C for 30 seconds. SOC medium (950 µl) was added and after 1.5 hrs shaking at 37 °C, 30 µl of culture was plated on LA containing 100 µg / ml ampicillin, 40 µl of 4% X-gal and 40 µl of 25 mM IPTG. Plates were incubated at 37 °C overnight.

Of the white colonies observed the following day, 22 were picked to check for the presence of insert. This was achieved by preparing crude lysates by heating (protocol mentioned in Appendix 2: General Methods) and PCR with M13/pUC forward and reverse primers. Eight µl of each PCR reaction was then analysed on 1% agarose gels to identify clones carrying the insert of interest.

2.2.3.5 Sequencing of cloned fragment

A clone designated C15-10 with the desired insert was chosen for sequencing with M13/pUC forward and reverse primers. Since the first set of sequencing did not cover the insert in its entirety, a second round of sequencing was performed using primers Right reverse 2 (RR2) and JH10 Reverse. The former primes the right arm of the lambda vector 45 bp from the cloning site; the latter primes the region between the first and second bovine J_H segments. Sequencing reactions were carried out in the MBSU, University of Glasgow.

2.2.4 Comparison of unrearranged and rearranged DNA from one individual

The work described above failed to locate the J_H segment which is most frequently rearranged in bovine heavy chain cDNA. In all, germline genomic DNA was characterised from four different sources. To identify the J_H segment concerned, attempts were focused on isolating and sequencing the J_H locus from tissue in which rearrangement had taken place. In B cells, the V and D segments are fused to the J_H segment selected for rearrangement, so the distance between V and J segments is short enough to be amplified by PCR (Figure 2.2). By using primers specific for the V_H segment and the 3' terminus of the J_H locus, it was reasoned that the rearranged segment could be identified from its sequence and flanking regions.

2.2.4.1 Recovery of rearranged antibody sequences from B cell genomic DNA

I Amplification with VHF and JH4

B cell genomic DNA from an adult bull (gifted by Dr. S. Stephens, Institute for Animal Health, Compton) was used as template for PCR with primers VHF and JH4. VHF binds to the leader sequences in the 5' region of bovine V_H segments and JH4 binds to sequences at the 3' terminus of the J_H locus, about 80 bp downstream from the sixth J_H segment. In principle, the amplicon should cover the V, D and J segments, its size depending upon which J_H segment undergoes rearrangement (Figure 2.2).

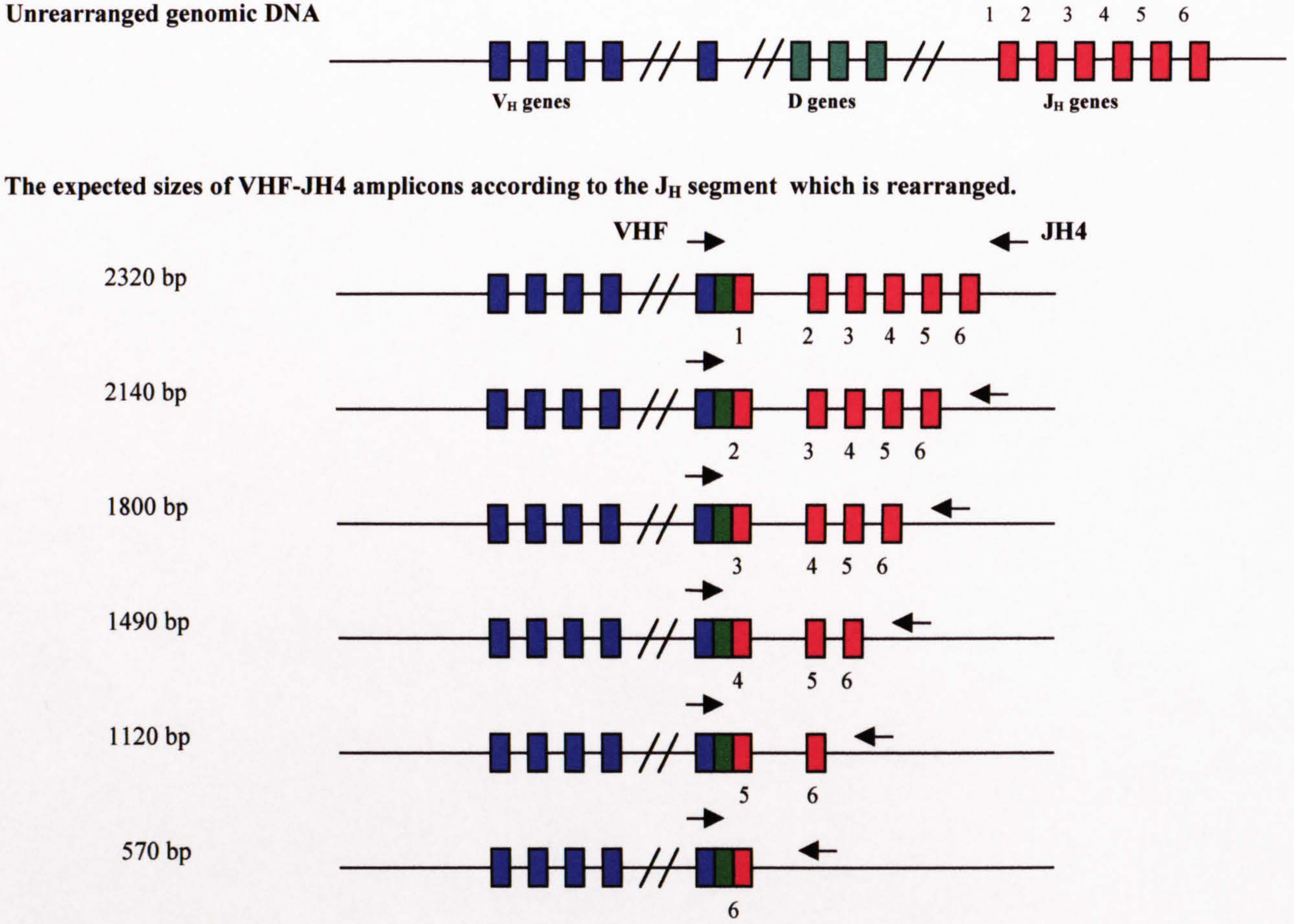


Figure 2.2 Organisation of V, D and J segments in genomic and rearranged DNA.

Three PCR reactions were performed using the conditions described in subheading 2.2.3.1 with $MgCl_2$ concentrations of 3.5, 4 and 4.5 mM and using Taq DNA polymerase. The annealing temperature for these reactions was 58 °C.

II Cloning

PCR products were run on 1% agarose gels. Analysis showed four bands with sizes of about 470, 600, 900 and 1500 bp. All bands were extracted using the QIAQuick Gel extraction kit. Firstly, amplicons of 470 and 600 bp were polished, phosphorylated and cloned into dephosphorylated pUC18 as described in subheadings 2.2.2.2 and 2.2.2.3. The sequencing of these amplicons revealed that they were irrelevant. The amplicons of 900 and 1500 bp were cloned into pCR4-TOPO vector using TOPO TA cloning Kit (Invitrogen). The pCR4-TOPO vector is a linearised vector carrying topoisomerase I molecules at the 3' termini. Topoisomerase I increases the efficiency of cloning. Four µl of the extracted DNA (900 and 1500 bp products), 1 µl (10 ng) of vector and 1 µl of salt solution, containing 1.2 M NaCl and 0.06 M $MgCl_2$ were mixed and the reaction was incubated on ice for 30 min.

III Transformation

Two µl of each ligation reaction was then added to thawed TOPO10 competent cells and after 30 min incubation on ice, the cells were heat shocked at 42 °C for 30 sec. The cells were left on ice for 2 min before addition of 250 µl of SOC media and incubation at 37 °C with shaking for 1.5 hrs. Fifty µl of each reaction were plated on LB agar containing 100 µg/ml ampicillin and the plates were incubated overnight at 37 °C.

The next day, 10 and 14 colonies from the 900 and 1500 bp amplicon cloning reactions were chosen to check the insert frequency. Crude lysates from these colonies were prepared as described in Appendix 2. PCR on crude lysates was performed with primers M13F and M13R to determine whether an insert was present.

IV Sequencing

Plasmids of clones carrying 900 and 1500 bp inserts were isolated (QIAGEN) for sequencing. Sequencing was carried out in the MBSU, University of Glasgow. The results showed that the 900 bp fragment was irrelevant but sequencing of a clone designated B12 carrying an insert of 1500 bp fragment with M13 forward and reverse

primers revealed that V_H and J_H sequences were present. Resequencing with primer JH6 recovered the entire sequence of the insert. The results showed that the VD genes were fused to J_H sequences located at the position of the fourth J_H segment. Also, the sequence corresponding to framework region 4 matched precisely that which predominates in bovine antibodies. To confirm the result, another 8 clones (designated B2, B5, B7, B9, B14, B21, B22 and B25) were sequenced with primers JH6 and/or M13 forward and reverse primers. The results showed again that sequence corresponding to predominant FR4 located at the position of the fourth JH segment, supporting the conclusion that the fourth J_H segment undergoes frequent rearrangement.

2.2.4.2 Unrearranged genomic DNA

The results from B cell genomic DNA conflicted with the sequence of the fourth J_H segment recovered from the germline of different individuals: the sequence of the rearranged J_H segment in B cell DNA matched the sequences that encodes the frequent expressed FR4. Therefore, genomic DNA from non-lymphoid tissue of the B cell donor was subjected to sequencing.

I Preparation of genomic DNA

Genomic DNA was prepared from peripheral blood lymphocytes using Wizard Genomic DNA Purification Kit (Promega). Twenty ml of heparinised blood from the B cell donor was divided to two aliquots of 10 ml and each was treated using the following procedures. Thirty ml of cell lysis solution was added to each 10 ml aliquot of blood in a 50 ml centrifuge tube to lyse red blood cells. The tube was inverted gently 6 times. The mixture was incubated at room temperature for 10 min, inverting every 2-3 min. The mixture was then centrifuged at 2000 x g for 10 min. The supernatant was discarded without the disturbing the white pellet and the pellet was then resuspended in the remaining liquid in the tube. Ten ml of nuclei lysis solution was added. Cell lysis was facilitated by repeated pipetting and incubation at 37 °C for 20 min. RNase was added to the viscous nuclear lysate to a final concentration of 20 μg/ml and the mixture was incubated at 37 °C for 15 min . Three point three ml of protein precipitation solution was added and after vortexing the mixture for 20 sec, it was centrifuged at 2000 x g for 10 min. The supernatant was separated from the dark brown protein pellet and was transferred to a 50 ml tube containing 10 ml of

isopropanol at room temperature. The solution was inverted gently until thread-like strands of DNA became visible. The tube was centrifuged at 2000 x g for 1 min at room temperature and the supernatant was decanted. The DNA pellet was washed by adding 10 ml of 70% ethanol and inverting the tube. After centrifuging at 2000 x g for another 1 min, the ethanol was discarded and the pellet was air dried. DNA then was recovered using 800 µl of DNA rehydration solution and incubation at 65 °C for 1 hr.

II Amplification

Isolated genomic DNA was used as template for PCR with primers JH8 and JH6. The primer JH8 binds to sequences between the second and third J_H segments and JH6 binds to sequences downstream of the fifth J_H segment. The amplicon would thus include the fourth J_H segment, resolving the apparent contradiction between the sequence of this region in B cell DNA from this donor and previously determined germline sequences. PCR was performed with conditions described in subheading 2.2.3.1 using 1 µl of genomic DNA, a final concentration of 3.5 mM $MgCl_2$ and an annealing temperature of 56 °C.

III Cloning and transformation

PCR products were run on an agarose gel and bands of about 1500 bp were extracted using the QIAQuick Gel extraction kit. The protocol for cloning and transformation of amplicons into the pCR4-TOPO vector and TOPO10 competent cells is described in subheading 2.2.4.1. II.

Plasmids from 10 colonies were isolated (QIAGEN) and were digested with *Eco*RI to assess which carried inserts. The cloning site in pCR4-TOPO is flanked by *Eco*RI sites.

IV Sequencing

A clone carrying an insert (designated BG1) was chosen for sequencing. Sequencing was carried out in the MBSU, University of Glasgow using M13 forward and reverse primers.

2.3 Results

2.3.1 Recovery and cloning of the J_H locus

2.3.1.1 Ovine JH1-JH4 amplicons

High molecular weight genomic DNA was recovered from homogenised liver for use as template in the PCR. In contrast to some reactions attempted in the course of this work, the Ovine JH1-JH4 primer set reproducibly yielded a 1.8 kb product without the need for substantial optimisation of the reaction conditions (Figure 2.3). This justified our strategy for recovery of the majority of the bovine J_H locus with primers designed from its ovine homologue (Dufour and Nau, 1997).

The vector pZErO2 was chosen for cloning the Ovine JH1-JH4 amplicon. This vector carries the *ccd*B gene which is lethal when expression from the *lac* promoter is induced with IPTG. It thus provided high-stringency selection for successful ligation of PCR products into the vector after digestion with *Eco*RV, without the need for further modification (*e.g.* dephosphorylation of the vector). In later work, a commercial source of *Sma*I-digested, dephosphorylated pUC18 was used, eliminating the need for repeated vector preparation and digestion. After ligation of the polished Ovine JH1-JH4 amplicon with pZErO2 and transformation into *E. coli* DH5α, 23 colonies were obtained from plating of 100µl of the transformation mixture onto selective plates containing IPTG. Plasmid DNA was isolated from 5 colonies, and digested with *Eco*RI and *Xho*I since the relevant recognition sequences flank the *Eco*RV site into which amplicons were ligated. Plasmid DNA from a clone designated 2-4 released fragments of 3 kb, about 1.5 kb and about 200 bp whereas other samples apparently lacked inserts (Figure 2.4). DNA from clone 2-4 was submitted for sequencing. Later analysis revealed that the insert contains two internal *Xho*I site and that in consequence, digestion with *Eco*RI and *Xho*I would be expected to release fragments of 3 kb (the vector backbone), 1.4 kb and two fragments of 200 bp.

2.3.1.2 JH2A-EµR amplicons

To recover sequence downstream from the main J_H locus, primers JH2A and EµR were used in PCR with liver genomic DNA. The sequence of primer EµR was taken from the published sequence of the ovine 5' intronic enhancer (GenBank accession number Z98207) (Figure 2.1). PCR with the JH2A-EµR primer set yielded an amplicon of about 900 bp (Figure 2.5). This was phosphorylated, polished and ligated with *Sma*I-

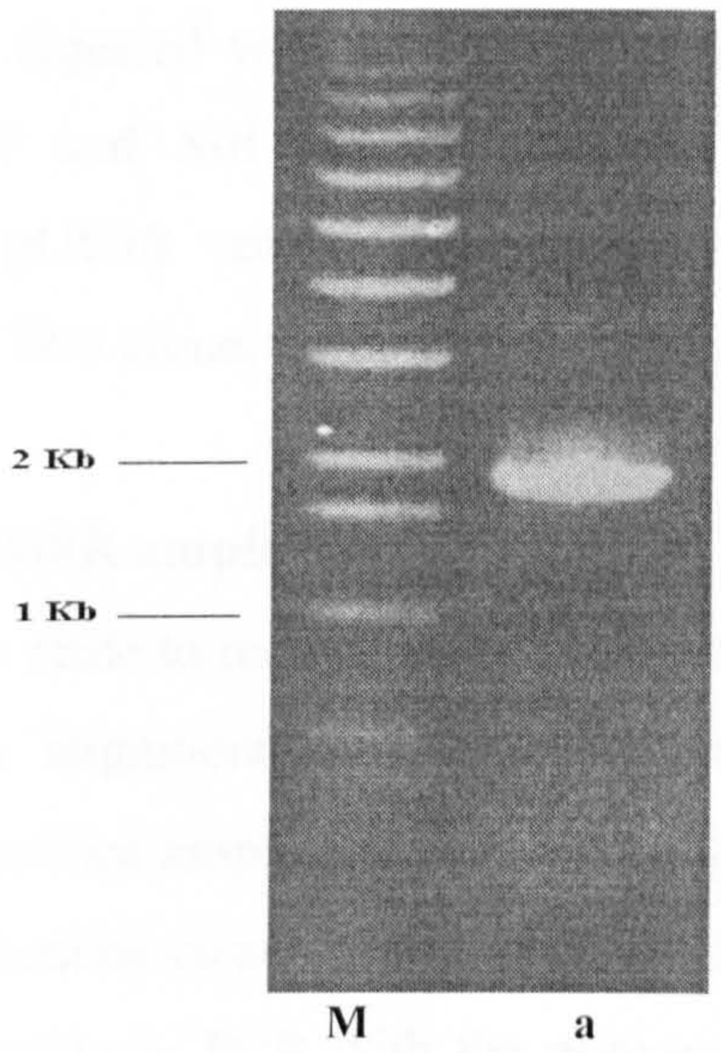


Figure 2.3 PCR from bovine genomic DNA with primers Ovine JH1 and JH4.

a: Ovine JH1-JH4 amplicon (c. 1.8 kb)
M: 1kb ladder (Life Technologies). The migration of markers of 2 kb and 1 kb is indicated

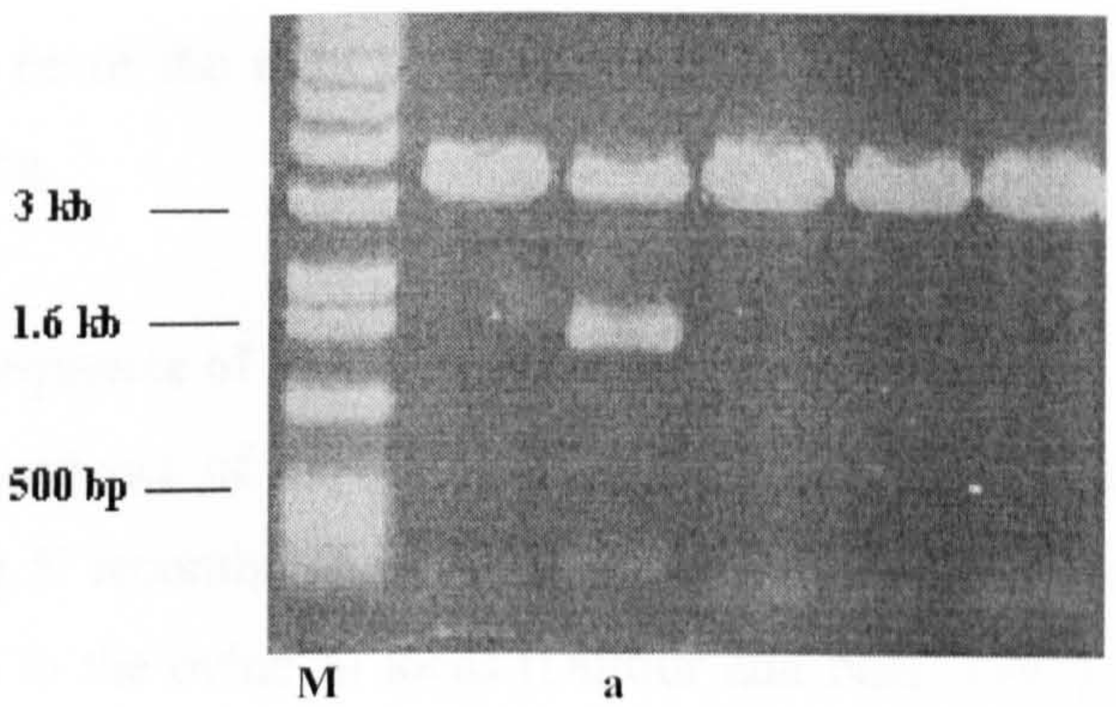


Figure 2.4 Restriction analysis of clone 2-4 with *Eco*RI / *Xho*I.

a: Digested sample of DNA from clone 2-4. Fragments of 3.3 kb (vector) and 1.5 kb (insert) are visible. A faint insert fragment of 200 bp was also detected.
M: 1kb ladder (Life Technologies). The migration of markers of 3 kb, 1.6 kb and 500 bp is indicated. Other lanes on the gel contain DNA samples from non-recombinant clones

digested, dephosphorylated pUC18. *E. coli* XL-1 cells were transformed and screened on selective indicator medium for a white phenotype. Plasmid DNA was isolated from 6 candidate colonies and digested with *Eco*RI to linearise the DNA. Further analysis by digestion with *Hin*dIII and *Sst*I showed that one released fragments of 2.7 kb (corresponding to the pUC18 vector) and another of the same size as the original amplicon (Figure 2.6). This clone, designated E-2, was sequenced.

2.3.1.3 Right reverse-JH8R amplicons

Many attempts were made to recover sequence upstream from the main J_H locus with primers designed from alignment of DQ52 segments from several species. These experiments failed to produce amplicons with bovine genomic DNA from the liver. To achieve this objective, lambda clone 15 was obtained from Prof K. Knight. Viral DNA was then used as a template in PCR with the primers Right reverse and JH8R. These anneal to the lambda right arm and within the J_H locus respectively. These reactions were successful and produced an amplicon of about 1.2 kb (Figure 2.7). The phosphorylated, polished product was ligated into *Sma*I-digested dephosphorylated pUC18. Transformed *E. coli* Sure2 cells were checked for a white phenotype on selective indicator medium. Candidate white colonies were screened by PCR, using crude bacterial lysates and primers flanking the *Sma*I site in the cloning vector. Of 22 samples, 19 contained inserts and 10 appeared to be of the right size (Figure 2.8). A clone designated C15-10 was chosen for sequencing.

2.3.2 Analysis the sequence of insert of clone 2-4

The completed sequence of the Ovine JH1-JH4 amplicon was scanned for potential J_H segments, seeking 5` recombination signal sequences (RSS), 3` donor splice sites and regions homologous to the ovine J_H locus (Dufour and Nau, 1997). Six segments were identified with these criteria. The sequence of these segments and the flanking elements including 5' nonamer, spacer and heptamer motifs and 3' donor splice site are shown in Figure 2.9. The segments span about 1850 bp with inter-segment regions ranging from 129 -500 bp. Nonamer motifs are variable in sequence as are the RSS spacers which vary in length from 20 to 23 bp. Heptamers are better conserved; only those associated with the second and third segments depart from the CACTGTG consensus. Segments are between 45 and 51 bp in length according to the criteria set out in the legend to Figure 2.9. Splice sites for RNA processing are present for all segments except the second but

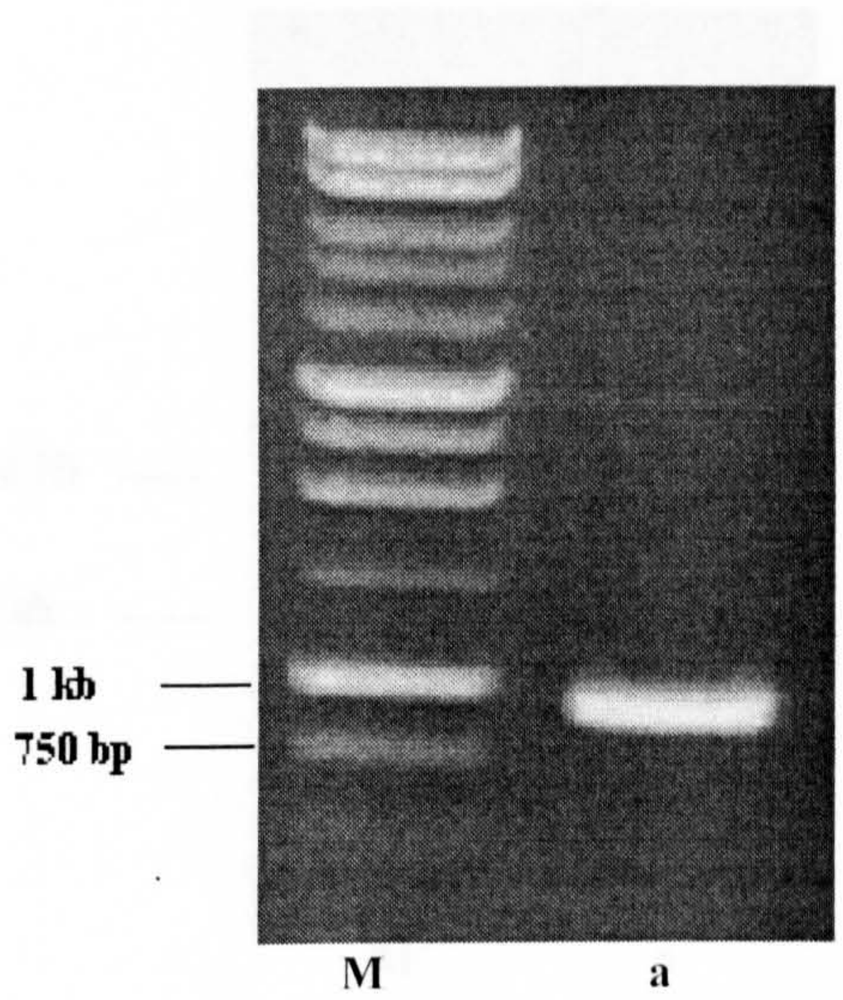


Figure 2.5 PCR from bovine genomic DNA with primers JH2A and EμR.
a: JH2A-EμR amplicon (c. 720 bp)
M: 1kb ladder (Promega). The migration of markers of 1 kb and 750 bp is indicated

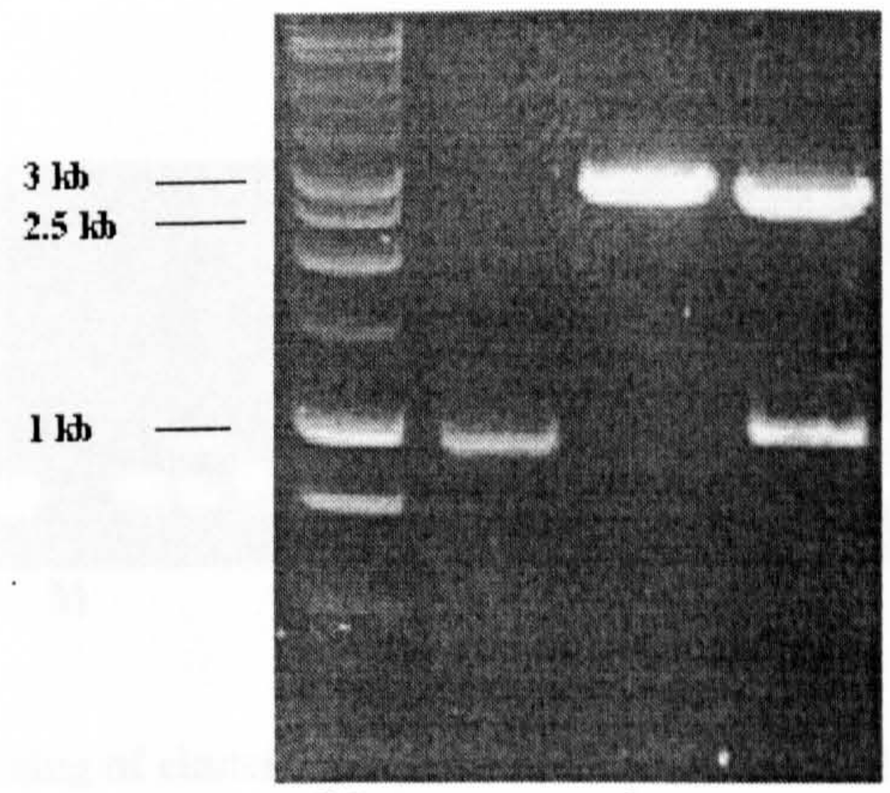


Figure 2.6 Restriction analysis of clone E-2.
a: JH2A-EμR amplicon
b: Sample of DNA from clone E-2 digested with *Eco*RI
c: Sample of DNA from clone E-2 digested with *Hin*dIII / *Sst*I.
M: 1 kb ladder (Promega). The migration of markers of 3 kb, 2.5 kb and 1 kb is indicated

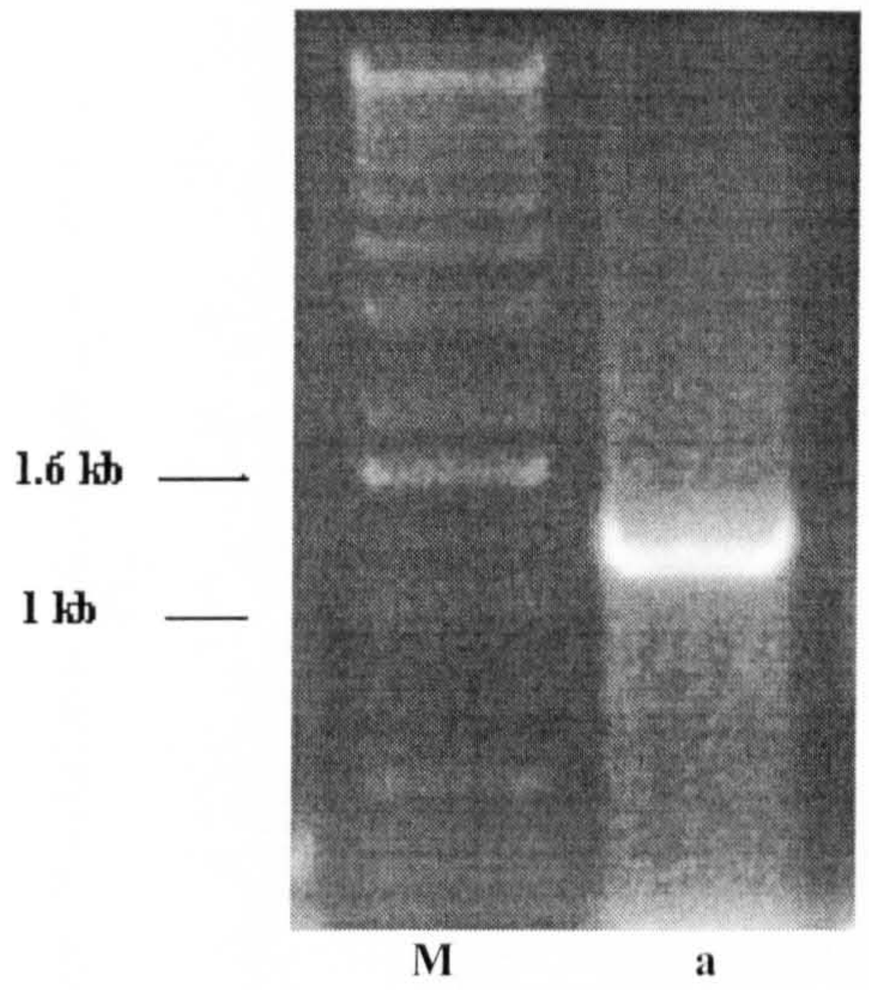


Figure 2.7 PCR from lambda clone 15 DNA using primers RR and JH8R.
a: RR-JH8 amplicon (c. 1.2 kb)
M: 1 kb ladder (Life Technology). The migration of markers of 1.6 kb and 1 kb is indicated

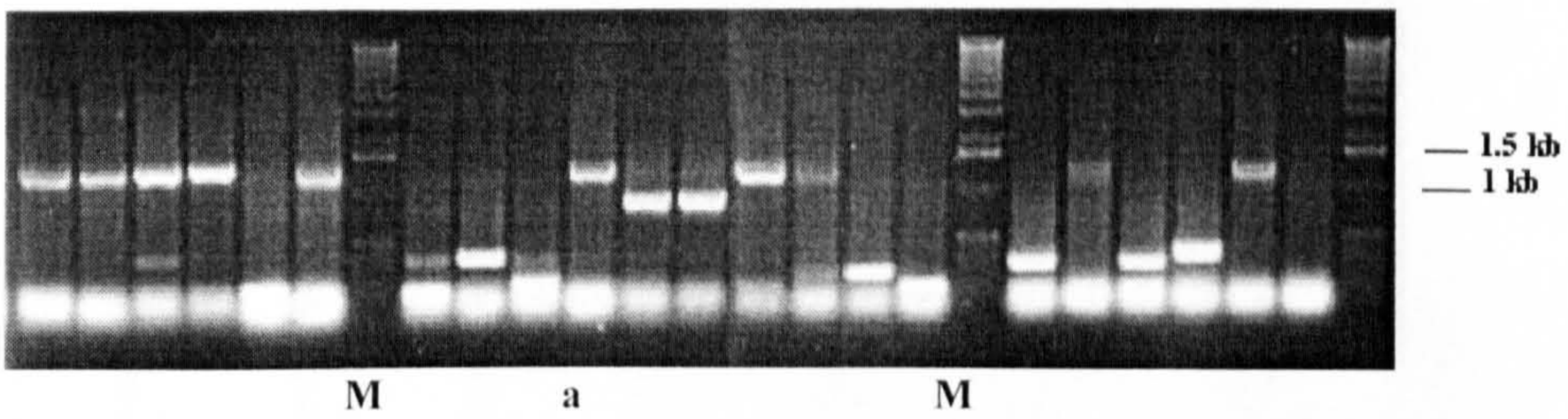


Figure 2.8 PCR screening of clones for insertion of the RR-JH8R amplicon.
PCR was performed with M13/pUC forward and reverse primers. Of 22 clones, 19 contained an insert, 10 were c. 1.2 kb
a: Clone with insert of the size sought (clone designated 15-10).
Smaller inserts are probably related to the degraded amplicon fragment.
M: 1 kb ladder (Life Technology). The migration of markers of 1.5 kb and 1 kb is indicated

	nonamer RSS	spacer	heptamer RSS		J_H segment	splice site
1st. segment	GGTTTCCGT	GCCTCCCAGCACAGGGCCAGCC 22	CACTGTG	AC	TATGCTGACTTCCATCTCTGGAGCCAGGCTGCCCTGGGCTGCCCTGCCTCAGGTGAA 51	
2nd. segment	TGTTTTTGT	GGAAAAGAATTAACAGAAGAGA 22	AGCCATG	C	TGCTGGGACTTGGATCTCTGGGGCCAGCGCACCCCGGTCATCGTGTCCTTG 51	
3rd. segment	GGTTCATGT	CTTGGGGGCAGCCGGGACTGCGT 23	CCCTCAG	C	AATGCTTTTGACTCCTGGGGCCAGCGCACCCCCATCTCCGCCTCCTCAGGTGAG 48	
4th. segment	GGGTTTTGC	ACAGCCCCTAGCGGGGCCCATGG 23	CACTGTG	A	CTATTCGACAACTGGGGCCCAGGAACCCAAAACACCGTCTCCTCAGGTGAG 45	
5th. segment	GCTCTTGCC	TGGGGTCACAGCATCATTGT 20	CACTGTG		TAACAACTGGCTCAAGCACTGGGGTCAGGAAGCCTGGGCACTGTCTGCTCAGCTGAG 51	
6th. segment	GGTTTTTGT	TGGGCGAGGCTGGAGATATCAC 22	CACTGTG	TT	TACTATGGTATAGACGCCTGGGGCCGAGGGCTCAGGGTCACCGTCTCCTCAGGTAAG 51	

Figure 2.9 Sequence of J_H segments and flanking regions. The segments are designated first to sixth according to their arrangement in the locus, 5' to 3'. Features of the recombination signal sequences (RSS) are indicated at the top of the figure with the number of bases in the spacer region placed under each sequence. The ovine J_H segments (Dufour and Nau, 1997) were used to predict the likely coding sequences, the length of which is indicated under each segment. Following the final predicted codon in each segment (TCA, where present), the likely RNA splice site and intronic sequence is underlined.

only the third and forth segments perfectly match the conventional sequence (C/A)AGGT(G/A)AG (Mount 1982).

Of the J_H segments identified in the Ovine JH1-JH4 amplicon, only the second would immediately appear to be a pseudogene on the basis of the features outlined above. Assessment of functionality for the others can best be judged from comparison of their coding sequences with FR4 sequences from bovine immunoglobulin cDNA (Figure 2.10). In this regard, segments one and three to six have similarity to the highly-conserved sequence observed in FR4 (Armor *et al.*, 1994; Sinclair *et al.*, 1995; Berens *et al.*, 1997; Saini *et al.*, 1997; Sinclair *et al.*, 1997) but none are a perfect match (Figure 2.11). The sixth J_H segment must be functional since the deduced amino acid sequence corresponds exactly to 17 amino acids of a rare FR4 sequence but one that can be detected in cDNA (Berens *et al.*, 1997; Figure 2.11). This segment possesses conventional 5' nonamer and heptamer RSS sequence, GGTTTTTGT and CACTGTG, with a 22 nucleotide spacer. The segment also possesses the functional 3' donor splice sequence AGGT (Mount 1982). The fourth segment is homologous to the most commonly expressed segment at the ovine locus. There are 7 substitutions within the segment itself but control sequences are almost identical - a single nucleotide substitution in the nonamer (bovine: GGGTTTTGC; ovine: GGTTTTTGC) and the bovine spacer is of the theoretically ideal length (23 bp *c.f.* 22 bp at the ovine locus)(Figure 2.21). However, there is no evidence from bovine immunoglobulin cDNA sequences that this segment is rearranged in cattle.

Overall, the sequence characterised in clone 2-4 is strikingly similar to the comparable part of the ovine J_H locus (Figure 2.12). There are limited stretches where deletion or insertions have occurred. For example, sequences are present in the ovine locus at positions 55 – 65 and 1258 – 1278 (numbering taken from ovine data) which are absent from the bovine. Similarly, a stretch of 27 bases is present at position 1878 – 1904 in the bovine locus which do not appear in the ovine. BLAST searches with these differences failed to make any significant matches.

2.3.3 Analysis the sequence of clone E-2

By using primers against the 5' intron enhancer, about 900 bp of additional sequence was recovered downstream from the sixth J_H segment (Figure 2.12). Again, significant similarity with the ovine locus is apparent with short runs of species-specific sequence that lack function as can be judged by BLAST searches (Figure 2.12). By comparing the

a


```
--- --- AG- --- --- --- --- --- --- --- --- --- --- --- ---
--T -C- --- --- --- --- --- --- --- --- --- --- --- --- ---
GG- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
--T -GA --- --- --- --- --- --- --- --- --- --- --- --- ---
--T -GA --- --- --- --- --- --- --- --- --- --- --- --- ---
--T TG- --- --- --- --- --- --- --- --- --- --- --- --- ---
GGG A-- --- --- --- --- --- --- --- --- --- --- --- --- ---
CCA CA- --- --- --- --- --- --- --- --- --- --- --- --- ---
GGT T-T TT- TTG --- --- --- --- --- --- --- --- --- --- ---
-G- --T --- --- --- --- --- --- --- --- --- --- --- --- ---
--- A-- --- -TT --- --- --G --- --- --- --- --- --- --- ---
--- A-- --A --- --- --- --- --- --- --- --- --- --- --- ---
C-A --- --- --- --- --T --- --- --T --- --- --- --- --- ---
--- --- --- --- --- --- --- --- A-- --- --- --- --- --- ---
--T CAT C-A --- --- --A --- --- --- --- --- --- --- --- ---
--- --- --- --- --- --- --- --- --- --- --- --- --T --- ---
GGA T-- --- --- --- --- --- --- --- --- --- --- --- --- ---
--- C-- --- C-- --- --- -T- --- --- --- --- --- --- --- ---
G-- --- --- --- --- --- --- --- --- --- --- -A- -G- --- ---
--- T-T --C TA- --- --- --- --- AC- --- --- --- --- --- ---
AT- -C- --- --- --- --- --- --- --- --- --- --- --- --- ---
-C- --- C-- --- --- --- --- --- --- --- --- --- --- --- ---
--T --- --C --- --- --- --- --- --- --- --- --- --- --- ---
--- --- ACC --- --- --- --- --- --- -A- --T --- --- --- ---
--- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
-T- CA- --- --- --- --- --- --- --- --- --- --- --- --- ---
--- A-- A-C --- --- --- --- --- --- --- --- --- --- --- ---

 Y   V   D   A   W   G   Q   G   L   L   V   T   V   S   S
TAC GTC GAT GCC TGG GGC CAA GGA CTC CTG GTC ACC GTC TCC TCA
```

b

1st segment

```
                                W   S   Q   A     A       L   G   C   P   A   S
TAT GCT GAC TTC CAT CTC TGG AGC CAG GCT   GCC     CTG GGC TGC CCT GCC TCA

                                W   S   Q   A     A       L   G   T   V   S   S
AT GCA GAC TTC CAT CTC TGG AGC CAG GCT   GCC     CTG GGC ACC GTC TCC TCA
```

2nd segment

```
                                W   G   Q   R     T       P   V   I   V   S   L
TGC TGG GAC TTG GAT CTC TGG GGC CAG CGC   ACC     CCG GTC ATC GTG TCC TTG
```

3rd segment

```
                                W   G   Q   R     T       P   I   S   A   S   S
GCT TTT GAC TCC TGG GGC CAG CGC   ACC     CCC ATC TCC GCC TCC TCA
```

4th segment

```
                                W   G   P   G   I/T       Q   N   T   V   S   S
CTA TTC GAC AAC TGG GGC CCA GGA A(T/C)C CAA AAC ACC GTC TCC TCA
```

5th segment

```
                                L   G   S   G     S       L   G   T   V   C   S
CAA CTG GCT CAA GCA CTG GGG TCA GGA   AGC     CTG GGC ACT GTC TGC TCA
```

6th segment

```
                                W   G   R   G     L       R   V   T   V   S   S
TAC TAT GGT ATA GAC GCC TGG GGC CGA GGG   CTC     AGG GTC ACC GTC TCC TCA
```

Figure 2.10 Comparison between the dominant sequence of bovine Ig framework four and the coding sequences of genomic J_H segments.

(a) Aligned sequences of framework four from bovine Ig cDNA showing the boundary between CDR3 and FR4 as denoted by the conserved W codon at position 103 (top of figure). A prototypical sequence for this region and its translated sequence is shown at the bottom of the figure. Above, the figure shows nucleotide variations to this sequence. Data prepared from Berens *et al.*, 1997.

(b) The nucleotide and predicted translated sequences of the six segments identified at the J_H locus. Differences between each segments and the dominant expressed sequence at the nucleotide and protein level are indicated with red letters. Two different sequences are presented for the first J_H segment. These are variants recovered from the genomic DNA of different individuals.

a

CDR3 —| |— FR4

```
                                                103
                                                 W   G   R   G   L   R   V   T   V   S   S
TAC TAT GGT ATA GAC GCC TGG GGC CGA GGG CTC AGG GTC ACC GTC TCC TCA
```

b

```
                                                 W   G   R   G   L   R   V   T   V   S   S
GGT ATA GAC GCC TGG GGC CGA GGG CTC AGG GTC ACC GTC TCC TCA
GGT ATA GAC GCC TGG GGC CGA GGG CTC AGG GTC ACC GTC TCC TCA
```

C

```
                                                 W   G   Q   G   L   L   V   T   V   S   S
TAC GTC GAT GCC TGG GGC CAA GGA CTC CTG GTC ACC GTC TCC TAC
```

Figure 2.11 Comparison between the less commonly expressed framework four sequence and the sixth J_H segment.

(a) The predicted coding sequence of the sixth J_H segment showing the boundary between CDR3 and FR4 as denoted by the conserved W codon at position 103 (top of figure). (b) The nucleotide and protein sequences of the less commonly expressed bovine FR4 region. (c) The nucleotide and protein sequences of the dominant FR4 region. Data in (b) and (c) prepared from Berens *et al.*, 1997. Note the R codons in (a) and (b) and the identical sequences upstream of the W codon.

Figure 2.12 Alignment of the bovine and ovine J_H loci. The ovine J_H sequence was assembled from NCBI (accession number Z71572 bases 1-2630; accession number Z98207 bases 2631-2764). The bovine J_H sequence is numbered from the DQ52 segment (see Fig. 2. 14). Overall homology between the two sequence is 89%. The location of six J_H segments (blue), and the immunoglobulin heavy chain 5' intronic enhancer (purple) is indicated, with RSS motifs (red). In view of the high level of identity, nucleotide differences are highlighted in the alignment with vertical lines. Where gaps have been introduced to improve the alignment, this is indicated with dots.

```
Ovine:     1     TCGAGGGTGG  GGGGCAAGCG  TCTTCAGGAA  GACAGACTTA  CAGCTCCCGG  GGGCTTCCTG
Bovine:    407   TGTCGGTTGG  GGGGCAAGTG  TCTTCAGGAA  GACAGACTTG  CAGC......  .....TCCTG

Ovine:     61    GGGGCTTCCT  GGGGACCCTG  AGTGGGTTTC  CATGCCCCCC  AGCACAGGGC  CAGCCCACTG
Bovine:    456   GGGGCTCCCT  GGGGACCCTG  GGTGGGTTTC  CGTGCCTCCC  AGCACAGGGC  CAGCCCACTG

Ovine:     121   TGACTATGCT  GACTTCCATC  TCTGGGACCA  GGGTGCCCTG  GTCACCGTCT  CCTCAGGAGA   1st JH
Bovine:    516   TGACTATGCA  GACTTCCATC  TCTGGAGCCA  GGCTGCCCTG  GGCTGCCCTG  CCTCAGGTGA   segment

Ovine:     181   ATCTCGTGTC  CAGTGACCAA  ATCAGGGGAC  AATGGGACTT  GGGTGGACTG  GGGATGGGAG
Bovine:    576   ATCTGGTGTC  TAGTGTCCAA  GTCAGGGGAC  AGCAGGACTT  GGGTGGACTT  GGGATGGGAG

Ovine:     241   TCAGGGCCAG  CAAGGAGGCA  GTGTGTGTTT  TTGTAGAAAA  GAAGCAGCAG  AATAGAAGCT
Bovine:    636   TCAGGGCCAG  CAAGGAGGTA  GGGTGTGTTT  TTGTGGAAAA  GAATTAACAG  AAGAGAAGCC

Ovine:     301   ACGCTGCTGG  GACTCGGGTC  TCTGGGGCCA  GCGCACCCCG  GTCACCGTGT  CCTTGGGCAG   2nd JH
Bovine:    696   ATGCTGCTGG  GACTTGGATC  TCTGGGGCCA  GCGCACCCCG  GTCACCGTGT  CCTTGGGGAG   segment

Ovine:     361   TGTCTCCCTT  GCAGTGCTGC  CTGGGCCTCG  GGCAGTTCCC  TGGG.TCTGTG  CAG.CCCACCA
Bovine:    756   TGTCTCCCTT  GCAGCACTGC  CTGGGCCTCG  GGCAGTTCCC  TGGGGTCCGTG  CAGCCCCACCG

Ovine:     421   TGCCCGGAGC  AAGGCAGGGT  CCTGGTCTAG  TGGGGTCCTG  CCCTTGGATC  TGCCAGAGCC
Bovine:    818   TGCCCTGAGC  AAGGCAGGGT  CCTGGTCCAA  CGGGGTCGCG  CCCTTGGATC  TGCCGGAGCC

Ovine:     481   CCTGGAGGTG  AGGCAGCTGA.  GCCTGAGAGG  AGGACAGGAG  CTG.CTGGGTG  GGAGGAAACG
Bovine:    878   CCTGGAGATA  AGCCAGCTGAG  GCCTGAGGGG  AGGACAGGAG  CTGACTGGGCG  GGAGGAAAGG

Ovine:     541   TGG.CAGAGCC  CGACGGGTCA  GGGTCTGTGT  GGTGAGGGCA  GGGGAGGCCC  CTGGGCCTGA
Bovine:    940   TGGGCAGAGC.  TGACGGGGCA  GGGTCTGTGC  AGTGAGGGCA  GGGGAGGCCC  CCGGGACTGG

Ovine:     601   CCTCCCAAGG  GTTCATGTCT  TGGGGACAGC  CGGGACGGCG  TCCTTCATCA  TTGCTTTTGA
Bovine:    1000  CCTCCCCAGG  GTTCATGTCT  TGGGGGCAGC  CGGGACTGCG  TCCCTCAGCA  ATGCTTTTGA

Ovine:     661   CTCCTGGGGC  CAGCGCGCCC  CGGTCACAGT  CTCCTCAGGT  GAGACGGCCC  TCTGCCCGCT   3rd JH
Bovine:    1060  CTCCTGGGGC  CAGCGCACCC  CCATCTCCGC  CTCCTCAGGT  GAGACGGCTC  TCTGCCCGCT   segment

Ovine:     721   CGGTCCTGGG  CTGGGAAAGA  AGTCTCCAGA  GGCCCCTCGG  TCTGTGGCAG  ACCCTCTGTG
Bovine:    1120  CCGTCCTGGG  CCGGGGAAGG  CGTCTCCAGG  GGCCCCTTGG  TCTGGGGCAG  ACCCTCCGTG

Ovine:     781   GCCCCTGGGG  GATCTGCCTC  TGAGGCCGTT  TGCCTCTCTG  CCCTGTTGAG  GTGGTGCCTC
Bovine:    1180  GTCCCTGGGG  GATCTGCGTC  TGAGGCCGTT  TGCCTCTCTG  CCCTGTTGAG  GTGGCGCCTC

Ovine:     841   TGCCTGTGGA  ATGTGGCCAG  GCAGAGTGGG  CTCTGTGGAC  CTGGGGGCCT  GGCTTTGCAT
Bovine:    1240  TGCCTGTGGA  ACGCGGCCAG  GCAGAGTGGG  CTCTGTG..C  CTGGGGGCCT  GGCTTTGTGT
```

Fig. 2.12 (continued)

```
Ovine:     901   GGGAGCAGAG  GCCGCCAAGC  CTTGGGTTTT  TGCACAGC.CC  TAACGGGGCC  CATGGCACTG
Bovine:   1298   GGGGGCAGAG  GACGCCAGGC  CTTGGGGTTT  TGCACAGCCCC  TAGCGGGGCC  CATGGCACTG

Ovine:     961   TGACTATATC  GACTACTGGG  GCCCAGGACT  CCTGGTCACC  GTCTCCTCAG  GTGAGCCCTC   4th JH
Bovine:   1359   TGACTAT.TC  GACAACTGGG  GCCCAGGAAT  CCAAAACACC  GTCTCCTCAG  GTGAGTCCTC   segment

Ovine:    1021   ACCAGCCTTC  TCTCCTCACT  CT..CTCTGAGT  TTTGGTGCAC  TTTGGGGGAA  ATCGAGGGTG
Bovine:   1418   AACAGCCCTC  TCTCCTCACT  CTGTCTCAGGGT  TTTGGTGCAC  TTTGGGGAAA  ATCGAGGGTG

Ovine:    1081   TCGGGTCTAG  AGGGCCTGGG  ACGGCCAGGG  GTCTGAGACG  GGGAAGGCCC  AGGGGCCCAG
Bovine:   1480   TCGGGTCTAG  AGGGCCTGGG  GCGG.CTGGG  ATCTGAGACA  CGGAGGACCC  AGGGGCCCAG

Ovine:    1141   GCTTACAGCA  GCAAGGAGCA  GAGGCTCCAG  GCACCCTCTC  CTCCTGGGCT  CTGTAGCCAG
Bovine:   1539   GCTTACAGCA  GCGAGGAGCA  GAGGCTCCAG  GCACCCTCTC  CTCCTGGGCT  CTGTAGCCAG

Ovine:    1201   GGCCTTCTCT  GAGGGCCTCA  GCCACCCTTG  GGCTCTGGAC  TCCCAAGGTC  CCAGCTGTGC
Bovine:   1599   GGCTTTCTCT  GGGGGCCTCA  GCCACCCTTG  GGCTCTGGAC  TCCCAAGGTC  CCAGCTG...

Ovine:    1261   TGGCCTCGTG  AGGCCACATG  TGAGGTAGTC  CGGAGGCCTC  AGCCGGCCAC  GAGCTCGTGC
Bovine:   1656   ..........  ........TG  TGAGGTGGTC  CGGAGGCCTC  AGCCGGCCAC  GAGCTCTTGC

Ovine:    1321   TTGGGGTCCC  AGCATCACTG  TCACAGTGTA  ACGACTGGCT  CAAGCACTGG  GGCCAGGGAC   5th JH
Bovine:   1698   CTGGGGTCAC  AGCATCATTG  TCACTGTGTA  ACAACTGGCT  CAAGCACTGG  GGTCAGGAAG   segment

Ovine:    1381   CCCGA.CGCTG  TCTGCTCAGC  TGAGCCCTCC  CCACCCCACT  TCACTGCACC  TGGGGAGGCC
Bovine:   1758   CCTGGGCACTG  TCTGCTCAGC  TGAGCCCTCA  CCACCCCACT  CCACTGCACC  TGGGGAGACC

Ovine:    1441   TGGGGTGTCA  GAGATCCAGG  GGCATTCTGG  AGGTCAAGAA  AGGGAGCTGG  GGAGAGGGT
Bovine:   1819   TGGAGCGTCA  GAGATCCAGA  GGCATTCTGG  AGGTCAAGAA  AGGGAGCTGG  GGAGAGGGT

Ovine:    1500   ..........  ..........  .......C
Bovine:   1878   TCTGGTAAAC  AGGCAGAGCC  AGACCTCC

Ovine:    1501   CCGCCCCAAG  GACACTGCAA  TGTGGGTATG  AGGCGGCTCC  TCTGGCGGGT  CTGGCTGTCT
Bovine:   1906   CTGCCCCAAG  GACACCACGA  TGTGGGTACA  AGGCGGCTCC  TTCGGTGGGT  CTGGCTGCCT

Ovine:    1561   GACTTGAGCA  GGACCGGGGG  CTTCCGTCGC  TGTCTGGGGC  AGGTGGCTGC  TCAAGGCTGG
Bovine:   1966   CACTTGAGCA  GGACCAGGGG  CTTCCGTCAC  TTTCTGGGGC  AGGCAGCTGC  TCGAGGCTGG

Ovine:    1621   ACTTAGGTGT  CTGTGGGTCA  CGGTCAGCTG  GTCCAGGCAG  GCACTGGTCT  GGCCTCTGGG
Bovine:   2026   ACTTAGGAGT  CTGTGGTTCA  TGGTCCGCCA  GCCCAGCCAG  GCAGTGGTCT  GGCCTCTGTG

Ovine:    1681   GGCCAAAATG  GGACATAGTG  TCTCTGGCAC  AGTCAGGTGG  GGCGGGGCCG  GCAGAGGGCC
Bovine:   2086   GGCCAGAATG  GGACATAGTG  TCTCTGGCAC  AGTCAGGTGG  GGTGGGGCCA  GCAGAGGGTC

Ovine:    1741   ACAGGCAAGC  GACTTTGACC  AGCGGCTTCC  CTGTGGTGCC  TGGAGATGGG  GTGGGGGCCC
Bovine:   2146   ACTGACAAGC  GACTTTGACC  AATGGTTTCC  CTGTGGCGCC  TGGAGATGGG  GTGGGGGCCC

Ovine:    1801   AGGTGCCTCG  AGCCTTGCCA  GGCTCCCGAG  GTTTTTGTTG  GGCGAGGCTG  GAGATAATCG
Bovine:   2206   AGGCGCCTCG  AGCCTCGTCA  GGCTCCCGAG  GTTTTTGTTG  GGCGAGGCTG  GAGATA.TCA
```

Fig. 2.12 (continued)

```
Ovine:    1861  CCACTGTGAT  TACTACGGTG  TAGATGTCTG  GGGCCGAGGA  CTCCTGGTCA  CCGTCTCCTC     6th JH
Bovine:   2265  CCACTGTGTT  TACTATGGTA  TAGACGCCTG  GGGCCGAGGG  CTCAGGGTCA  CCGTCTCCTC     segment

Ovine:    1921  AGGTAAGAGC  GGCC.ATACAG  AGCCTTTGCT  TTCTCTCCTA  TTCGTGGGAT  TTTTCTGAGC
Bovine:   2325  AGGTAAGAGC  AGCCCATCCAG  GGCCTTTGCT  TTATCTCAT.  TTCGTGCGAT  TTTTCTGAGC

Ovine:    1981  ATCACTG....GTC  CTCTGATGTG  TCCTTGTCCC  CTCCTCCCCG  GGGGGACTGG  GCAGACTGGC
Bovine:   2385  ATCACTGTCTGGTC  CTCTGATGTG  TCCTTGTCCC  CTCCTCCCCG  GGGGAACTGG  GCAGACTGGC

Ovine:    2041  CAGGAGGG.AC  CAGCTGCCCT  ATGCATTTCA  GAGTCTCT.AT  CTTCTGATAG  CTTTAAAAAA
Bovine:   2449  CAGGAGGGGAC  CAGCTGCCCT  ATGCATTTCA  GAGTCTCTTAT  TTTCCGATGC  CTTTAAAAAA

Ovine:    2101  CCAGAATCTT  GCTGGCATTC  AGAGGGGGCT  TGGGCAGGAA  GGGCCACCAG  TGGGGGAGTC
Bovine:   2511  TCAGAATCTT  GCTGGCATTC  AGAAGGGGCT  TGGGCGGGAA  TGGCCACCAG  TGGGGGAGTC

Ovine:    2161  CCAGGCCTCC  CTTGGCAGCA  G.........  ..........  ...GGCAACTTG  CTGTGGTCCT
Bovine:   2571  CCAGGCCCCT  CTTGGCAGCA  GGGCAGCTTG  GGATGCGGTG  GAAGGCAACTTG  CTGCAGTCCT

Ovine:    2201  AGCATCTGCG  GAGGAGCGTG  TCTGGATAAC  TTAGGGCCTC  AGGAGCGCCG  CCCGCAGTGG
Bovine:   2633  AGCATCTGCG  GAGGAGCGTG  TCTGGATAA.  TTAGGGCCTC  AGGAACGCTG  CCCGTGGCGG

Ovine:    2261  G.GCAGAGAAG  GCCCTCCTCG  GGTGAGGTTG  GCTCTGCACT  AGACTGTGTT  TAAAATTCTT
Bovine:   2692  GCGCAGAGAAC  GCCCCCCTCG  CGTGAGGTGT  GTTCTGCACT  AGACTGTGTT  TAAAATTCTT

Ovine:    2321  TATTGGGCAG  GAAGAGAATT  GTCTAGGTGA  GGAC.GACACG  CAGTGTCCCG  ACCGCGGCAA
Bovine:   2753  TATTGGGTAG  GAAGAGAATT  GTCTAGGTGA  GGACGGACATG  CAGTGTCCCG  ATCGTGGCGA

Ovine:    2381  GAGAGGGGAG  GCTGGGGAGG  TGACGGGCGC  TGGGCTTTGT  GAGGCCACTG  TAAGAGAAAG
Bovine:   2814  GAGAGGGGAG  CCCGGGGAGG  TGACGGGCGC  TGGGCTTTGT  GAGGCCAGTC  TAAGAGA..G

Ovine:    2441  AAAAGCTGTT  CGCCAGAGGA  GGTGTGCTTG  CGAATACCAA  GACAGGGCAT  CTTCAAAGCG     Enhancer
Bovine:   2872  AAAGGCCGTT  CGCCAGAGGA  GGTGTGCTTG  CGAACACCAA  GACAGGGCAT  CTCTGAAGCG

Ovine:    2501  ACTCCTGATA  GTCTGGAAAA  TTGAAACTTT  AAAAAGAGAG  ATGTTTAAAG  TATTTTAAAT
Bovine:   2932  ATTCCTGATA  GTCTGAAAAA  TTGAAACTTT  AAAAA..GAA  ATGTTTAAAG  TATTTTAAAT

Ovine:    2561  TTTTATCATT  TAATTAACAA  CTGCGAATCA  TGGCTTTGGA  GAGTTGAGTA  AG.....AGTTTGGC
Bovine:   2990  TTTTATCATT  TAATTAACAA  CCGCAAATCG  TGGCTTTGGA  GAGTTGAGAC  AGGTACAAGTTTGGC

Ovine:    2621  TGAAAAGTAC  TAACTAGGTT  CCATCGGCCC  TCGGCCCCAA  TTCAGGGCTG  TTTTGAGAAT
Bovine:   3055  CGAAAAGTAC  TAACTAGGTT  CCATCGGCCC  TCGGCCCCAA  TTCAGGGCTG  TTTTGAGAAT

Ovine:    2681  AATAAATTCA  GCTTATTTTT  TTAATGTAAT  TGGTGGTGCC  GAGTTAGTCA  AGATGGCCAC
Bovine:   3115  AATAAATTCA  GCTTATTTTT  TTAATGTAAT  TGGTGGTGCC  GAGTTAGTCA  AGATGGCCAC

Ovine:    2741  GGGCCAGACT  GACCACCTGC  AGCA  2764
Bonine:   3175  GGGCAGGACT  GACCACCTGC  AGCA  3198
```

ovine and bovine sequences from the first base of ovine J_H locus (Dufour and Nau, 1997; accession number Z71572) to the 296th nucleotide of immunoglobulin heavy chain enhancer (accession number Z98207), 89% homology can be calculated. Close scrutiny failed to reveal the presence of further J_H segments.

2.3.4 Analysis the sequence of clone C15-10

Recovery and cloning of the RR-JH8R amplicon from lambda clone 15 extended analysis of the bovine J_H locus about 500 bp upstream of the first J_H segment. Examination of the upstream flank identified the bovine DQ52 gene 410 bp upstream from the first J_H segment (Figures 2.13 and 2.14). As shown in Figure 2.13, the coding region consists of 14 nucleotides, somewhat longer than in other species (11 nucleotides in human, mouse, rat and rabbit and 10 in *Suncus murinus* (Chen *et al.,* 1996)). The coding region is flanked by RSS comprising nonamer and heptamer sequences with 12 nucleotide spacers, in accordance with the 23/12 rule. The J_H-distal RSS is probably functional given its similarity to consensus sequences. However, the heptamer present in the J_H-proximal RSS departs significantly from consensus (Figure 2.13) and it is therefore unlikely that rearrangement between the bovine DQ52 segment and J_H segment(s) takes place *in vivo*.

2.3.5 Consensus sequence of the bovine J_H locus

By assembling sequence from the clones characterised, a consensus for the bovine J_H locus can be derived (Figure 2.14). This is based upon 28 sequencing reactions with different primers on five different individual genomic DNA clones (Appendix 1). Restriction sites for the locus were mapped onto the sequence using DNAStar software (Fig 2.15). The locus consists of a DQ52 segment positioned a short distance upstream from the main cluster of J_H segments, as is typical for mammals. There are 6 J_H segments and a heavy chain enhancer all of which bear close similarity to equivalent sequences from the sheep. In contrast to the sheep, it is not immediately apparent from the data which J_H segment is responsible for the highly conserved FR4 sequence found in the large majority of bovine heavy chain cDNAs.

	5'nonamer	5'spacer	5'heptamer	coding region	3'heptamer	3'spacer	3'nonamer
Human DQ52	GTTTTTGGC	TGAGCTGAGAAC	CACTGTG	CTAACTGGGGA	CACAGTG	ATTGGCAGCTCT	ACAAAAACC
Mouse "	-G-----A-	-A---G---C--	---A---	---------AC	---G---	-CAC-TG----A	---------
Rat "	-G-----A-	-A---AA--C-T	---A---	---------AG	-------	-C-T-TG----A	---------
Rabbit "	-G----A--	-------G--G-	-T-----	---------AC	-------	-C-CAACA--G	TG----C--
Suncus "	-G-------	-------G--TA	---A---	T--C-----A	-------	---AATGTAGT-	---G-----
Llama "	-G-------	-------G----	-G-A---	--------A-C	-------	-C--A--A----	--------T
Cattle "	-G-------	CA---CAGA---	---A---	GAACTCGGTGGGGC E L G G N S V G T R W G	----AAA	----A--A--T-	---------

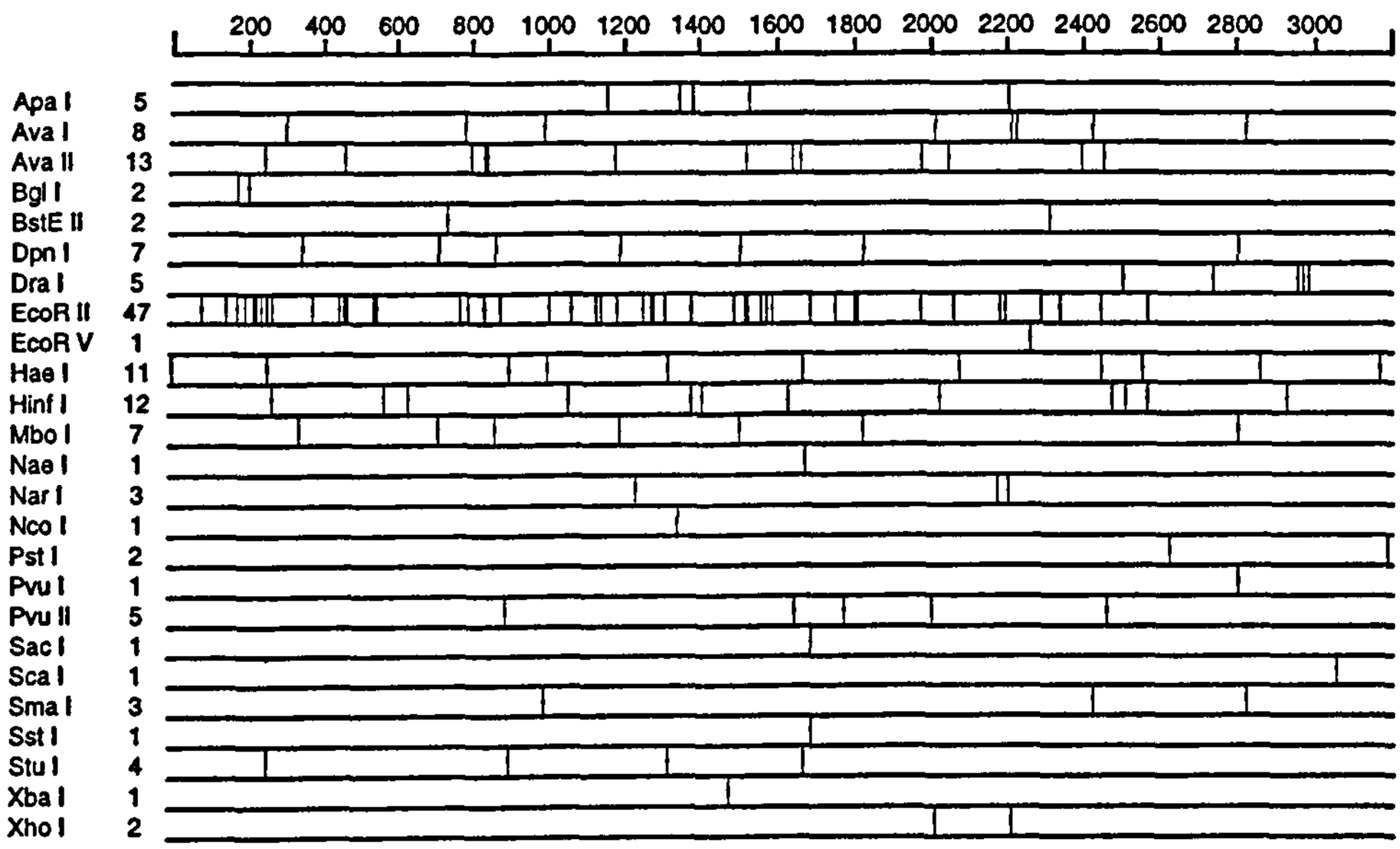
Figure 2.13 Comparison between DQ52 segments of cattle and other species. The coding sequence of each segment is flanked by heptamer and nonamer motifs as indicated at the top of the figure, with 12 base spacers. Identity with the human sequence is indicated with a dash. The three potential coding sequences of the bovine segment are indicated at the bottom of the figure.
Note the departure of the 3' heptamer motif from the consensus and the longer length of the coding sequence carried by the bovine segment.

Figure 2.14 Complete sequence of the bovine J_H locus. The sequence consists of 3282 nucleotides and contains a DQ52 segment (green), six J_H segments (blue) with RSS motifs (red) and part of the heavy chain enhancer (purple). These features are indicated on the right of the figure and are highlighted in different colors. Numbering begins from the first nucleotide of the 5' nonomer of the DQ52 RSS.

```
                                                    AATT  CCCCGGATCT  GTGGGCAGGG
          TTCCCAAGGG  GGTCGGTGGC  CGACCCCCCT  GGCCCTTGCT  CGCCTGCGGC  CAGCCCTAAG
    1     GGTTTTGGCC  AAGCCAGAAA  CCACAGTGGA  ACTCGGTGGG  GCCACAGAGA  TTGACAACTT      DQ52
   61     TACAAAAACC  CCTGAGCAAA  GTGGCCCCAG  GGAAGCCGGG  GGGGGTCTCA  CTTTGAGGGT
  121     TCCCTGTCAG  CGCACACGGG  GTCAGGTCAG  CCAGGAGCCT  GAAGGCACTG  GAAAAGAGCC
  181     ACCTGGGCTG  ACGGTGAAGG  GGCCTCCCAG  GCGGGCACGG  AGCCAGGCCC  AGCCCAGGCA
  241     AGGGGCCAGG  TCCCCAAGGC  CTGGGGTGCA  GGGAGTCCAG  GGAATTGGGG  GTTGAGGTGG
  301     AAAGCCTCGG  GAGGGGCAGG  GAGCATGTGA  CCTCATGGGA  ACAAAGCAGA  TCAGGAGACA
  361     GCAGACACTC  AAATCCCAGA  CCAGGGGCCT  GAGGCAGACA  GGTGCTTGTC  GGTTGGGGGG
  421     CAAGTGTCTT  CAGGAAGACA  GACTTGCAGC  TCCTGGGGGC  TCCCTGGGGA  CCCTGGGTGG
  481     GTTTCCGTGC  CTCCCAGCAC  AGGGCCAGCC  CACTGTGACT  ATGCAGACTT  CCATCTCTGG      1st
  541     AGCCAGGCTG  CCCTGGGCTG  CCCTGCCTCA  GGTGAATCTG  GTGTCTAGTG  TCCAAGTCAG      segment
  601     GGGACAGCAG  GACTTGGGTG  GACTTGGGAT  GGGAGTCAGG  GCCAGCAAGG  AGGTAGGGTG
  661     TGTTTTTGTG  GAAAAGAATT  AACAGAAGAG  AAGCCATGCT  GCTGGGACTT  GGATCTCTGG      2nd
  721     GGCCAGCGCA  CCCCGGTCAC  CGTGTCCTTG  GGGAGTGTCT  CCCTTGCAGC  ACTGCCTGGG      segment
  781     CCTCGGGCAG  TTCCCTGGGG  TCCGTCCAGC  CCCACCGTGC  CCTGAGCAAG  GCAGGGTCCT
  841     GGTCCAACGG  GGTCGCGCCC  TTGGATCTGC  CGGAGCCCCT  GGAGATAAGC  CAGCTGAGGC
  901     CTGAGGGGAG  GACAGGAGCT  GACTGGGCGG  GAGGAAAGGT  GGGCAGAGCT  GACGGGGCAG
  961     GGTCTGTGCA  GTGAGGGCAG  GGGAGGCCCC  CGGGACTGGC  CTCCCCAGGG  TTCATGTCTT
 1021     GGGGGCAGCC  GGGACTGCGT  CCCTCAGCAA  TGCTTTTGAC  TCCTGGGGCC  AGCGCACCCC      3rd
 1081     CATCTCCGCC  TCCTCAGGTG  AGACGGCTCT  CTGCCCGCTC  CGTCCTGGGC  CGGGGAAGGC      segment
 1141     GTCTCCAGGG  GCCCCTTGGT  CTGGGGCAGA  CCCTCCGTGG  TCCCTGGGGG  ATCTGCGTCT
 1201     GAGGCCGTTT  GCCTCTCTGC  CCTGTTGAGG  TGGCGCCTCT  GCCTGTGGAA  CGCGGCCAGG
 1261     CAGAGTGGGC  TCTGTGCCTG  GGGGCCTGGC  TTTGTGTGGG  GGCAGAGGAC  GCCAGGCCTT
 1321     GGGGTTTTGC  ACAGCCCCTA  GCGGGGCCCA  TGGCACTGTG  ACTATTCGAC  AACTGGGGCC      4th
 1381     CAGGAATCCA  AAACACCGTC  TCCTCAGGTG  AGTCCTCAAC  AGCCCTCTCT  CCTCACTCTG      segment
 1441     TCTCAGGGTT  TTGGTGCACT  TTGGGGAAAA  TCGAGGGTGT  CGGGTCTAGA  GGGCCTGGGG
 1501     CGGCTGGGAT  CTGAGACACG  GAGGACCCAG  GGGCCCAGGC  TTACAGCAGC  GAGGAGCAGA
 1561     GGCTCCAGGC  ACCCTCTCCT  CCTGGGCTCT  GTAGCCAGGG  CTTTCTCTGG  GGGCCTCAGC
 1621     CACCCTTGGG  CTCTGGACTC  CCAAGGTCCC  AGCTGTGTGA  GGTGGTCCGG  AGGCCTCAGC
 1681     CGGCCACGAG  CTCTTGCCTG  GGGTCACAGC  ATCATTGTCA  CTGTGTAACA  ACTGGCTCAA      5th
 1741     GCACTGGGGT  CAGGAAGCCT  GGGCACTGTC  TGCTCAGCTG  AGCCCTCACC  ACCCCACTCC      segment
 1801     ACTGCACCTG  GGGAGACCTG  GAGCGTCAGA  GATCCAGAGG  CATTCTGGAG  GTCAAGAAAG
 1861     GGAGCTGGGG  AGAGGGTTCT  GGTAAACAGG  CAGAGCCAGA  CCTCCCTGCC  CCAAGGACAC
 1921     CACGATGTGG  GTACAAGGCG  GCTCCTTCGG  TGGGTCTGGC  TGCCTCACTT  GAGCAGGACC
 1981     AGGGGCTTCC  GTCACTTTCT  GGGGCAGGCA  GCTGCTCGAG  GCTGGACTTA  GGAGTCTGTG
 2041     GTTCATGGTC  CGCCAGCCCA  GCCAGGCAGT  GGTCTGGCCT  CTGTGGGCCA  GAATGGGACA
```

Fig. 2.14 (Continued)

```
2101    TAGTGTCTCT GGCACAGTCA GGTGGGGTGG GGCCAGCAGA GGGTCACTGA CAAGCGACTT
2161    TGACCAATGG TTTCCCTGTG GCGCCTGGAG ATGGGGTGGG GGCCCAGGCG CCTCGAGCCT
2221    CGTCAGGCTC CCGAGGTTTT TGTTGGGCGA GGCTGGAGAT ATCACCACTG TGTTTACTAT   6th
                                                                           segment
2281    GGTATAGACG CCTGGGGCCG AGGGCTCAGG GTCACCGTCT CCTCAGGTAA GAGCAGCCCA
2341    TCCAGGGCCT TTGCTTTATC TCATTTCGTG CGATTTTTCT GAGCATCACT GTCTGGTCCT
2401    CTGATGTGTC CTTGTCCCCT CCTCCCCGGG GGAACTGGGC AGACTGGCCA GGAGGGGACC
2461    AGCTGCCCTA TGCATTTCAG AGTCTCTTAT TTTCCGATGC CTTTAAAAAA TCAGAATCTT
2521    GCTGGCATTC AGAAGGGGCT TGGGCGGGAA TGGCCACCAG TGGGGGAGTC CCAGGCCCCT
2581    CTTGGCAGCA GGGCAGCTTG GGATGCGGTG GAAGGCAACT TGCTGCAGTC CTAGCATCTG
2641    CGGAGGAGCG TGTCTGGATA ATTAGGGCCT CAGGAACGCT GCCCGTGGCG GGCGCAGAGA
2701    ACGCCCTCCT CGCGTGAGGT GTGTTCTGCA CTAGACTGTG TTTAAAATTC TTTATTGGGT
2761    AGGAAGAGAA TTGTCTAGGT GAGGACGGAC ATGCAGTGTC CCGATCGTGG CGAGAGAGGG
2821    GAGCCCGGGG AGGTGACGGG CGCTGGGCTT TGTGAGGCCA GTCTAAGAGA GAAAGGCCGT
2881    TCGCCAGAGG AGGTGTGCTT GCGAACACCA AGACAGGGCA TCTCTGAAGC GATTCCTGAT   Enhacer
2941    AGTCTGAAAA ATTGAAACTT TAAAAAGAAA TGTTTAAAGT ATTTTAAATT TTTATCATTT
3001    AATTAACAAC CGCAAATCGT GGCTTTGGAG AGTTGAGAC AGGTACAAGTT TGGCCGAAAA
3061    GTACTAACTA GGTTCCATCG GCCCTCGGCC CCAATTCAG GGCTGTTTTGA GAATAATAAA
3121    TTCAGCTTAT TTTTTTAATG TAATTGGTGG TGCCGAGTT AGTCAAGATGG CCACGGGCAG
3181    GACTGACCAC CTGCAGCA 3198
```



Aac I	**Bae II**	**BspJ106I**	**EcoDR 2**	**Nhe I**	**SmaA I**
Aat II	**BamH I**	**BspLu11**	**EcoR 3**	**Not I**	**Sna I**
Aca I	**Bbr I**	**BsrBR I**	**EcoK 1**	**Nru I**	**SnaB I**
Aca III	**Bcg I**	**BsrG I**	**EcoR I**	**Pau I**	**Spe I**
Acc I	**Bcg II**	**BssII II**	**EcoR124 I**	**Pfl 1108 I**	**Sph I**
Acc65 I	**Bci VI**	**Bst1107 I**	**EcoR124 II**	**Pfu I**	**Srf I**
AccEB I	**Bcl I**	**Bst 98 I**	**EcoRD 3**	**PinA I**	**Srl8D I**
Ace II	**Bco63 I**	**BstIIP I**	**Fbl I**	**Pme I**	**Sse232 I**
Aci II	**Bcu I**	**Btr I**	**Fse I**	**Ppi I**	**Sse8387 I**
Acs 1371 I	**Bgl II**	**CciN I**	**Fsp I**	**Ppu1253 I**	**Sse8647 I**
Afl III	**BmeT I**	**Chu II**	**Hal I**	**Ppu6 I**	**Sso I**
Age I	**Bna I**	**Cla I**	**HinJC I**	**PshA I**	**Ssp I**
Alo I	**Bpl I**	**Csp I**	**Hinc II**	**Psi I**	**StyIT III**
Ama I	**BsaA I**	**Csp45 I**	**Hind III**	**Rhc I**	**StySK I**
Aos I	**BsaF I**	**Eco VIII**	**Hpa I**	**Rrh4273 I**	**StySP.I**
Ape I	**BsaK I**	**DsaV I**	**Kpn 1**	**Rsr II**	**StySQ I**
Apu 16 I	**Bsb I**	**EciA I**	**Mxa879 I**	**Sac II**	**Swa I**
Asc I	**BscE I**	**Eco47 III**	**Mae II**	**Sal I**	**Tai I**
Ase I	**BsiW I**	**Eco52 I**	**Mfe I**	**Sap I**	**Taq II**
Asp52 I	**BsmG I**	**Eco72 I**	**Mlu I**	**Sdi I**	**Ttm I**
Asp5II I	**BsoD I**	**Eco82 I**	**Mlu113 I**	**SexA I**	**Vsp I**
AtuC I	**Bsp87 I**	**EcoA I**	**Mun I**	**Sfi I**	**Xma I**
Avr II	**BspD I**	**EcoB**	**Ncr I**	**Sgf I**	
Bae I	**BspH I**	**EcoD I I**	**Nde I**	**SgrA I**	

Figure 2.15 Restriction map of the bovine J_H locus. The sequence analysed comprises that presented in Figure 2.14.
Top: A selection of restriction enzymes in common use that cut the sequence. Numbering across the top is as used in Figure 2.14. The numbers to the right of the enzyme names show the number of sites present in the sequence. The location of sites is indicated with vertical lines.
Bottom: A list of enzymes taken from a database of 501 endonucleases that do not cut the sequence.
Analysis was performed using DNAStar software.

2.3.6 Sequencing of rearranged antibody genes in B cell genomic DNA

Rearranged genomic DNA prepared from B cells and was used as a template to amplify the sequence spanning the V_H and J_H segments and sequence downstream of the J_H locus. Four amplicons of about 470, 600, 900 and 1500 bp were obtained from PCR. Sequencing of clones carrying inserts of 470, 600 and 900 bp showed that they were irrelevant with no meaningful matches identified by BLAST searches of the GenBank database. Sequencing of one clone designated B12 carrying a 1500 bp insert was more informative.

Initial BLAST searches and close inspection revealed that the DNA carried bovine Ig sequence. At one terminus of the insert, the presence of the V_H intron confirmed that the amplicon was derived from genomic DNA (Figure 2.16).

Taking the data in detail from the downstream terminus (Figure 2.17), the rearranged sequences are near-identical to the germline downstream of the sixth J_H segment. Sequence homologous to the sixth segment is present but there are significant differences to the germline. In particular, a G to C substitution alters the W codon which is one of the most characteristic features of J_H segments. Upstream, further substitutions to the coding sequence are apparent but the RSS remains intact. Since the rearranged sequences were recovered from peripheral B cells, the loss of this match with J_H6 could be due to antigen-driven processes (*e.g.* somatic hypermutation) but the consistent occurrence of substitutions in the sixth J_H segment suggests an alternative explanation – that these sequences are drawn from a different genomic location to the germline locus described earlier.

Further substitutions to the germline can be detected between the fifth and sixth J_H segments. These are mainly single nucleotide substitutions but a deletion of 20 bases (2123-2149) can also be seen. Sequence could not be recovered for the majority of the fifth segment because of limits on the length of sequencing reads with the M13 primers but the matches with flanking regions are very strong, showing that this segment is present. Immediately upstream of the fifth segment, another interesting feature emerges: an insertion of 21 bp to the germline sequence (between 1655-1656). This shows some similarities with the comparable region of the ovine J_H locus, including some internal complementarity. It is unlikely that a feature of this size was introduce by somatic hypermutation.

Intergenic sequences between the fourth and fifth J_H segments are present but the frequency of substitutions to the germline seem to increase closer to the fourth segment.

```
                                                                                                                  Intron
Bovine V_H   5     CCCAC  TGTGGACCCT  CCTCTTTGTG  CTGTCAGCCC  CCAGAGGTGA  GTGTCTCTGG  GTCAGACATG  GGCACGTTGG  GAAGCTGCAT  CTGAGCCCAT

B12.M13R           -----  ----------  ----------  ----------  ----------  -------C--  ----------  -------G--  ---A------  ---------C
B14.M13R           -----  ----------  ----------  ----------  ----------  ----------  ----N-----  -------G--  --------C-  ---------C
B21.M13F           -----  ----------  ----------  ----------  ----------  ----------  ----------  -------G--  --------C-  ---------C
B22.M13F           -----  ----------  ----------  ----------  ----------  ----------  ----------  -------G--  --------..  -C-------C
B25.M13F           -----  ----------  ----------  ----------  ----------  ----------  ---G------  -------G--  -----C--C-  -T-------C


                                                                                                                  FR1
Bovine V_H 101 GGGTCACCGT  GCTTCTCTCT  CTCCACAGGG  GTCCTGTCCC  AGGTGCAGCT  GCGGGAGTCG  GGCCCCAGCC  TGGTGAAGCC  CTCACAGACC  CTCTCCCTCA

B12.M13R           ----------  ----------  ----------  ----------  ----------  --A-----GA  ----------  ----------  ----------  --N--T----
B14.M13R           ----------  C---------  ----------  ----------  ----------  ----------  -------N--  ----------  ----------  ----------
B21.M13F           ----------  ----------  ----------  ----------  --A-----T-  ---------A  ----------  ----------  ----------  ----------
B22.M13F           ----------  ----------  ----------  ----------  -N--------  ----------  ----------  ----------  ----------  --G-------
B25.M13F           ----T-----  ----------  ----------  ----------  ----------  ---C------  ----------  ----------  ----------  ----------
```

Figure 2.16 Alignment between the consensus for bovine V_H segments and sequences recovered from B cell DNA.
A consensus for bovine V_H segments was derived from 12 sequences (Sinclair *et al.*, 1997).The intron sequence is shown in green. The presence of the intron in samples from rearranged B cell DNA indicates their genomic origins.

Figure 2.17 Alignment of the ovine and bovine J_H loci with rearranged sequences recovered from B cell genomic DNA.

The numbering and alignment of the ovine and bovine loci (black) is taken from Figure 2.12, with vertical lines indicating nucleotide differences, dots indicting where gaps have been introduced. The green and red lettering show the germline sequence from the B cell donor and the rearrange sequence recovered from purified B cells respectively. Dashes show identity with the bovine germline sequence. Nucleotide differences to the bovine and ovine consensus are shown. Designations to the left of the figure identify the clone which was sequenced and the primer used for sequencing.

```
Ovine:   541  TGG.CAGAGCC CGACGGGTCA   GGGTCTGTGT   GGTGAGGGCA  GGGGAGGCCC  CTGGGCCTGA
                   |      | |      |            |   |                        |   |   |
Bovine:  940  TGGGCAGAGC. TGACGGGGCA   GGGTCTGTGC   AGTGAGGGCA  GGGGAGGCCC  CCGGGACTGG
BG1.M13F             >    ---------   ----------   ----------  ----------  ----------

Ovine:   601  CCTCCCAAGG  GTTCATGTCT   TGGGGACAGC   CGGGACGGCG  TCCTTCATCA  TTGCTTTTGA
                    |                       |            |      |   |   |
Bovine:  1000 CCTCCCCAGG  GTTCATGTCT   TGGGGGCAGC   CGGGACTGCG  TCCCTCAGCA  ATGCTTTTGA
BG1.M13F      ----------  ----------   ----------   ----------  ----------  ----------

Ovine:   661  CTCCTGGGGC  CAGCGCGCCC   CGGTCACAGT   CTCCTCAGGT  GAGACGGCCC  TCTGCCCGCT
                          |            ||   | | |                       |
Bovine:  1060 CTCCTGGGGC  CAGCGCACCC   CCATCTCCGC   CTCCTCAGGT  GAGACGGCTC  TCTGCCCGCT
BG1.M13F      ----------  ----------   --------AT   ----------  ----------  ----------

Ovine:   721  CGGTCCTGGG  CTGGGAAAGA   AGTCTCCAGA   GGCCCCTCGG  TCTGTGGCAG  ACCCTCTGTG
                   |          |   |   |   |          |          |      |           |
Bovine:  1120 CCGTCCTGGG  CCGGGGAAGG   CGTCTCCAGG   GGCCCCTTGG  TCTGGGGCAG  ACCCTCCGTG
BG1.M13F      ----------  ----------   ----------   ----------  ----------  ----------

Ovine:   781  GCCCCTGGGG  GATCTGCCTC   TGAGGCCGTT   TGCCTCTCTG  CCCTGTTGAG  GTGGTGCCTC
                   |                       |                                    |
Bovine:  1180 GTCCCTGGGG  GATCTGCGTC   TGAGGCCGTT   TGCCTCTCTG  CCCTGTTGAG  GTGGCGCCTC
BG1.M13F      ----------  ----------   ----------   ----------  ----------  ----------
BG1.M13R                                                      <   ----A--  ----------

Ovine:   841  TGCCTGTGGA  ATGTGGCCAG   GCAGA.GTGGG   CTCTGTGGAC  CTGGGGGCCT  GGCTTTGCAT
                         | |                                 ||                     ||
Bovine:  1240 TGCCTGTGGA  ACGCGGCCAG   GCAGA.GTGGG   CTCTGTG..C  CTGGGGGCCT  GGCTTTGTGT
BG1.M13F      ----------  ----------   -----.-----   ----.---..- ----------  ----------
BG1.M13R      ---T------  --------CA   --CANA-----   --T-T---..- ----------  ----------

Ovine:   901  GGGAGCAGAG  GCCGCCAAGC   CTTGGGTTTT   TGCACAGC.CC  TAACGGGGCC  CATGGCACTG
                   |          |   |         |              |          |
Bovine:  1298 GGGGGCAGAG  GACGCCAGGC   CTTGGGGTTT   TGCACAGCCCC  TAGCGGGGCC  CATGGCACTG
BG1.M13F      ----------  ----------   ----------   -----------   --G-------  ----------
BG1.M13R      ---.------  ----------   ----------   -----------   --G-------  ----------
```

Fig. 2.17 (continued)

Fig. 2.17 (continued)

```
Ovine:  1201 GGCCTTCTCT  GAGGGCCTCA  GCCACCCTTG  GGCTCTGGAC  TCCCAAGGTC  CCAGCTGTGC
                   |          |                                                |||
Bovine: 1599 GGCTTTCTCT  GGGGGCCTCA  GCCACCCTTG  GGCTCTGGAC  TCCCAAGGTC  CCAGCTG...
BG1.M13R     --------N-  ----------  ----------  ----------  ----------  -------...
B12.JH6      ----------  ----C-----  ----------  -A-------G  --G-------  -------TGC
B14.JH6      -A------N-  ----------  ATT-------  ----------  ----------  --GC--ACTT
B2.JH6       C-T-------  ----------  -TT-------  ATT----A-T  ----------  -------TAC
B5.JH6       C-T-------  ----------  -TT-------  ATT----A-T  ----------  -------TAC
B9.JH6                   --------    -TT-------  ATT-N--A-T  ----------  -------TAC
B21.JH6      ----C-----  ----------  A-T-------  ----------  ----------  --G----TTC
B22.JH6      ------T---  ------....  ......----  ----------  -G--------  -------TGT

Ovine:  1261 TGGCCTCGTG  AGGCCACATG  TGAGGTAGTC  CGGAGGCCTC  AGCCGGCCAC  GAGCTCGTGC
             ||||||||||  ||||||||              |                                |
Bovine: 1656 ..........  ........TG  TGAGGTGGTC  CGGAGGCCTC  AGCCGGCCAC  GAGCTCTTGC
BG1.M13R     ..........  ........--  ----------  ----------  ----------  ----------
B12.JH6      TCGCCTCATG  AGCCCACAGA  GA-C--G---  ----------  ----------  ---T------
B14.JH6      TCGTCTCACG  AGGCTACAG-  ---A-G-A--  .---------  ----------  ----------
B2.JH6       TGACCTCAGG  AGGCCACAG-  ----------  ----------  G---------  ----------
B5.JH6       TGACCTCAGG  AGGCCACAG-  ----------  ----------  G---------  ----------
B9.JH6       TGACCTCAGG  AGGCCACAG-  ----------  ----------  G---------  ----------
B21.JH6      TGGCCTCATG  AGGCCACAG-  --C---A---  ----------  ----------  ----------
B22.JH6      TGGCCTCATG  AGGCCACAG-  ---C------  ----------  --------G-  --C-------

Ovine:  1321 TTGGGGTCCC  AGCATCACTG  TCACAGTGTA  ACGACTGGCT  CAAGCACTGG  GGCCAGGGAC  5th JH
             |       |              |           |                       |     | |  segment
Bovine:1698  CTGGGGTCAC  AGCATCATTG  TCACTGTGTA  ACAACTGGCT  CAAGCACTGG  GGTCAGGAAG
BG1.M13R     ----------  ----------  ----------  ----------  ----------  ----------
B12.JH6      --------C-  -TG-------  ----C----T  -GGC------  ---     <
B14.JH6      --------C-  --      <
B2.JH6       --T-----C-          <
B5.JH6       --T-----C-          <
B9.JH6       --T-----C-          <
B21.JH6      -------TC-  -CT---N---  ----C-----  -A--------   <
B22.JH6      --------CA  --TG--G-C-  ----C---GT  -----C----  ----------  --    <

Ovine:  1381 CCCGA.CGCTG  TCTGCTCAGC  TGAGCCCTCC  CCACCCCACT  TCACTGCACC  TGGGGAGGCC
               | ||  |                          |                       |
Bovine: 1758 CCTGGGCACTG  TCTGCTCAGC  TGAGCCCTCA  CCACCCCACT  CCACTGCACC  TGGGGAGACC
BG1.M13R     ----------  --------C-  -A--------  ---   <
B12.M13F                           <    ---N---  --------T-  T-.-N----N-CN---A-----
B21.M13R                 < -----    C---------  -------G.-  T---A---G-  CA-A-A----
B22.M13R          < ---  -T--N---CT  -N-N-----N  --------T-  T--T----G-  C---A-----

Ovine:  1441 TGGGGTGTCA  GAGATCCAGG  GGCATTCTGG  AGGTCAAGAA  AGGGAGCTGG  GGAGAGGGT
               | |                           |
Bovine: 1819 TGGAGCGTCA  GAGATCCAGA  GGCATTCTGG  AGGTCAAGAA  AGGGAGCTGG  GGAGAGGGT
B12.M13F     CA-G-T----  N------G-G  ---------A  ----------  -----C----  -A-N---A-
B21.M13R     CAAGAT--A-  ---------G  ------N---  GC-----A--  ----GA----  -N-N-----
B22.M13R     CA-G-T---C  N--------G  ------T---  ----T--A--  ---A------  -A-------
```

Fig. 2.17 (continued)

```
Ovine:  1500 ..........  ..........  .......C
             ||||||||||  ||||||||||  |||||||
Bovine: 1878 TCTGGTAAAC  AGGCAGAGCC  AGACCTCC
B12.M13F     -N----N-N.  --CA-N--T-  --------
B21.M13R     -T-.-----.  -----.-A--  ---G----
B22.M13R     -T-----..-  --N--N----  --------

Ovine:  1501 CCGCCCCAAG   GACACTGCAA  TGTGGGTATG  AGGCGGCTCC  TCTGGCGGGT  CTGGCTGTCT
             |             || |        ||          || |        |
Bovine: 1906 CTGCCCCAAG   GACACCACGA  TGTGGGTACA  AGGCGGCTCC  TTCGGTGGGT  CTGGCTGCCT
B12.M13F     T---------   ---T--G-G-  -----C---G  ---T--G--T  --T--C----  ---------C
B21.M13R     TC-----C-C  ------G--T  N--------N  ---T------  --T--C----  ------C---
B22.M13R     TC--------  ----G-G-G-  ---------G  ---T------  --T--C----  ----------

Ovine:  1561 GACTTGAGCA  GGACCGGGGG  CTTCCGTCGC  TGTCTGGGGC  AGGTGGCTGC  TCAAGGCTGG
                         |           |           |           ||         |
Bovine: 1966 CACTTGAGCA  GGACCAGGGG  CTTCCGTCAC  TTTCTGGGGC  AGGCAGCTGC  TCGAGGCTGG
B12.M13F     GCT-------  --------A-  --------G-  ------A---  ----G-----  ----------
B21.M13R     N-G------G  ----------  --------G-  ---N------  ----G-A---  ----------
B22.M13R     AG--------  ----------  -C------G-  ---T------  -.--G---C-  --G-----.-

Ovine:  1621 ACTTAGGTGT  CTGTGGGTCA  CGGTCAGCTG  GTCCAGGCAG  GCACTGGTCT  GGCCTCTGGG
                   |           |     |   | ||    |   |       |                 |
Bovine: 2026 ACTTAGGAGT  CTGTGGTTCA  TGGTCCGCCA  GCCCAGCCAG  GCAGTGGTCT  GGCCTCTGTG
B12.M13F     ----------  -----C----  CC---A---G  ------G---  ---A------  .-------G-
B21.M13R     ----------  ----------  C----.C--G  ------G---  ----------  .C------G-
B22.M13R     ----------  ----------  C----A---G  ------G---  ----------  .-------G-

Ovine:  1681 GGCCAAAATG  GGACATAGTG  TCTCTGGCAC  AGTCAGGTGG  GGCGGGGCCG  GCAGAGGGCC
                   |                                                 |  |        |
Bovine: 2086 GGCCAGAATG  GGACATAGTG  TCTCTGGCAC  AGTCAGGTGG  GGTGGGGCCA  GCAGAGGGTC
B12.M13F     -A--------  -----.--C--  ---------G  -A-----...  ..........  .......---
B21.M13R     -A--------  ----------  -N-N-----G  -------...  ..........  .......---
B22.M13R     -A--------  ----------  ---------G  -------...  ..........  .......---
TGCGTGAC

Ovine:  1741 ACAGGCAAGC  GACTTTGACC  AGCGGCTTCC  CTGTGGTGCC  TGGAGATGGG  GTGGGGCCC
               | |                   ||   |        |
Bovine: 2146 ACTGACAAGC  GACTTTGACC  AATGGTTTCC  CTGTGGCGCC  TGGAGATGGG  GTGGGGCCC
B12.M13F     --AC-----T  ------C---  G----C----  ----------  ---------T  ----------
B21.M13R     ACGCA----T  ----------  G-C--C----  ----------  ---------C  ----------
B22.M13R     --AC-----T  ----------  G-C--C----  ----------  --N------C  ----------

Ovine:  1801 AGGTGCCTCG  AGCCTTGCCA  GGCTCCCGAG  GTTTTTGTTG  GGCGAGGCTG  GAGATAATCG
               |           |  |                                          |   |
Bovine: 2206 AGGCGCCTCG  AGCCTCGTCA  GGCTCCCGAG  GTTTTTGTTG  GGCGAGGCTG  GAGATA.TCA
B12.M13F     G---------  -----T-C--  -------A--  ---------C  C---------  ------.---
B21.M13R     G---------  -----T-C--  -------A--  ----------  ----------  ------.---
B22.M13R     G---------  -----T-C--  -------A--  ---C------  ----------  ------.---
```

Fig. 2.17 (continued)

```
Ovine:   1861 CCACTGTGAT   TACTACGGTG   TAGATGTCTG   GGGCCGAGGA   CTCCTGGTCA   CCGTCTCCTC  6th JH
                         |        |   |      | |              |      ||                      segment
Bovine:  2265 CCACTGTGTT   TACTATGGTA   TAGACGCCTG   GGGCCGAGGG   CTCAGGGTCA   CCGTCTCCTC
B12.M13F      --------A-   ------A--G   G-TTT-TT--   C---------   A--GA-----   ----------
B21.M13R      -------AA-   ------A---   --T-TATT--   C---------   A--GA---T-   ----------
B22.M13R      --------A-   ------A---   --T-T-TT--   C---------   A--GA-----   ----------
```

```
Ovine:  1921  AGGTAAGAGC   GGCC.ATACAG   AGCCTTTGCT   TTCTCTCCTA   TTCGTGGGAT   TTTTCTGAGC
                         |   |  |     |              |    | |         |
Bovine: 2325  AGGTAAGAGC   AGCCCATCCAG   GGCCTTTGCT   TTATCTCAT.   TTCGTGCGAT   TTTTCTGAGC
B12.M13F      ----------   G----------   ----------   G--------.   ----------   ---------
B21.M13R      ----------   G----G-----   ----------   ---------.   ----------   ---------
B22.M13R      ----------   G----------   ----------   T--------.   ----------   ---------
```

JH4 primer

```
Ovine:  1981 ATCACTG....GTC   CTCTGATGTG   TCCTTGTCCC CTCCTCCCCG GGGGGACTGG   GCAGACTGGC
                    ||||                                          |
Bovine: 2385 ATCACTGTCCGGTC   CTCTGATGTG   TCCTTGTCCC CTCCTCCCCG GGGGAACTGG   GCAGACTGGC
```

Upon reaching this part of the sequence, two features are readily apparent. Firstly, the reading frame of the segment is altered from the germline to that typically seen in Ig cDNA encoding framework region 4. The modification of codons for (I/T), Q and N (isoleucine, glutamine and asparagine) to L, L and V (leucine, leucine and valine)(sequence changes from A(T/C)C CAA AAC to CTC CTG GTC) is striking. Secondly, the sequence upstream to the W codon loses it match to the germline sequence of the J_H locus and eventually shows the features of bovine Ig framework region 3 (data not shown). This confirms that the fourth J_H segment has undergone rearrangement.

As shown in Figure 2.17, multiple clones carrying inserts of about 1500 bp were sequenced to confirm these results. Analysis supported the conclusion that the fourth J_H segment undergoes rearrangement (Figure 2.17). A variety of sequences can be seen downstream from the rearranged segment showing that several rearrangement events have been captured, but clones B2, B5 and B9 seem to be identical. These three clones carried VHF-JH4 amplicons of 1 kb. Sequencing of additional clones did not yield data of sufficient quality for presentation. Whilst there are some deviations in the nucleotide sequences of the rearranged J_H segment from that which predominates in Ig cDNA, the encoded protein sequences are remarkably similar (Figure 2.18).

2.3.7 Sequencing of germline DNA from the B cell donor

Given the apparent conflict between the germline sequence of the fourth J_H segment and that present in B cell genomic DNA, germline material from the B cell donor was sequenced through the majority of the J_H locus. This data is presented in Figure 2.17 as clone BG1. This showed a near-perfect match to the germline sequences previously gathered from other individual animals. This similarity spans the fourth J_H segment and the deleted region equivalent to positions 1258-1278 in the ovine sequence (Figure 2.17). Hence the differences observed between germline and rearranged DNA in the fourth J_H segment do not related to the genotype of the individual from which B cells were isolated.

2.3.8 Phylogeny of the J_H segments

The sequences of functional J_H segments and J_H pseudogenes characterised from cattle were compared to segments from humans, mice, rabbits, sheep and llama using DNAStar software. Using this package, phylogenic trees were prepared by maximum

```
                                  L   F   D   N   W   G   P   G   I   Q   N   T   V   S   S
Germline sequence                 CTA TTC GAC AAC TGG GGC CCA GGA ATC CAA AAC ACC GTC TCC TCA

                                  Y   V   D   A   W   G   Q   G   L   L   V   T   V   S   S
Frequent bovine FR4               TAC GTC GAT GCC TGG GGC CAA GGA CTC CTG GTC ACC GTC TCC TCA

                                  P   R   R   C   W   G   Q   ?   L   L   V   T   V   S   S
B12.JH6          CCA TCA TCT TNT CCG CGT CGG TGC TGG GGC CAA GNA CTC TTG GTC ACC GTG TCC TCA
B12.M13R         TCA CCA TCT TNT NCG CGT NGG TGC TGG GGC CAA AGA CTN TTG GTC ACC GTG TCC TCA

                                  P   R   R   C   W   G   Q   ?   F   L   V   ?   V   S   S
B7.JH6           TCA CCA TTT TCT CCG CGT CGG TGC TGG GGC CAA GNA TTT TTG GTC NCN GTG TCC TCA

                                  ?   N   ?   N   W   G   Q   G   L   L   V   T   V   ?   S
B2.JH6           TGN TCT TCA CGG TNC AAT ANA AAC TGG GGC CAG GNA CTC TTG GTC ACC GTC TCN TCA
B5.JH6           TGC TCT TCA CGG TCC AAT ANA AAC TGG GGC CAG GGA CTC CTG GTC ACC GTC TCN TCA

                                  C   F   L   R   L   G   Q   E   ?   L   V   T   V   S   S
B14.JH6          CTC AAG CGA TTG TGC TTT TTG CGC TTG GGC CAA GAA NTC TTG GTC ACC GTC TCC TCA

                                  *   C   R   C   W   G   Q   G   L   L   V   T   V   S   S
B21.JH6          AAC GGG TTG GCG TAA TGT CGA TGC TGG GGC CAA GGA CTC CTG GTC ACC GTC TCC TCA

                                  Y   *   H   R   W   G   Q   G   L   L   V   T   V   S   S
B22.JH6          GGG TTG TAC TTA TAT TAG CAT CGC TGG GGC CAA GGA CTC CTG GTC ACC GTC TCC TCA
```

Figure 2.18 Comparison of nucleotide and predicted amino acid sequences of the dominant framework four sequence with germline and rearranged J_H segments. Clone designation is shown to the left of the figure. Nucleotides shown in red have been reported by other researchers.

likelihood analysis (Figure 2.19) and percentage identities are shown in Figure 2.20. The closest relationships are between the ovine and bovine segments as noted at the outset of this work. For segments judged to be non-functional, percent identities range between 66% (the fifth segment present at each locus; bovine ps4 and ovine ps4) and 94% (the second segment present at each locus; bovine ps2 and ovine ps2). The sixth segment (bovine JH2 and ovine JH2) which in cattle seems to contribute infrequently to the Ig repertoire, are 84% identical at the nucleotide level. This study has shown that the fourth segment (bovine JH1) undergoes rearrangement but also some sort of modification takes place. In sheep, the sequence of the equivalent segment is identical in the germline and in Ig cDNA. The germline sequence of this bovine segment is 76% identical to its ovine counterpart, but this rises to 82% after rearrangement. Expressed J_H segments tend to encode relatively conserved protein sequences and this is reflected in a wider analysis of the homology of the bovine locus. Bovine JH1 is very similar in sequence to human segments JH2, JH4 and JH5 and rabbit JH1 and JH2 (>70% identity). Bovine JH2 matches human JH6 (86% identity) and murine JH4 (72 %). In several cases, modification the germline sequence of bovine JH1 to the rearranged sequence improves the level of identity. For example, human JH2 and the bovine germline JH1 segment are 69% identical, but this reaches 76% when taking the rearranged bovine sequence. Similar trends are seen when comparing with rabbit JH1 (72 to 77). Interestingly, in some instances the reverse is true. This is shown when comparing the sequences with llama JH3 (71% identity falls to 64%), mouse JH2 and JH4 (71% falling to 64%; 67% to 60%) and rabbit JH4 (76% to 71%).

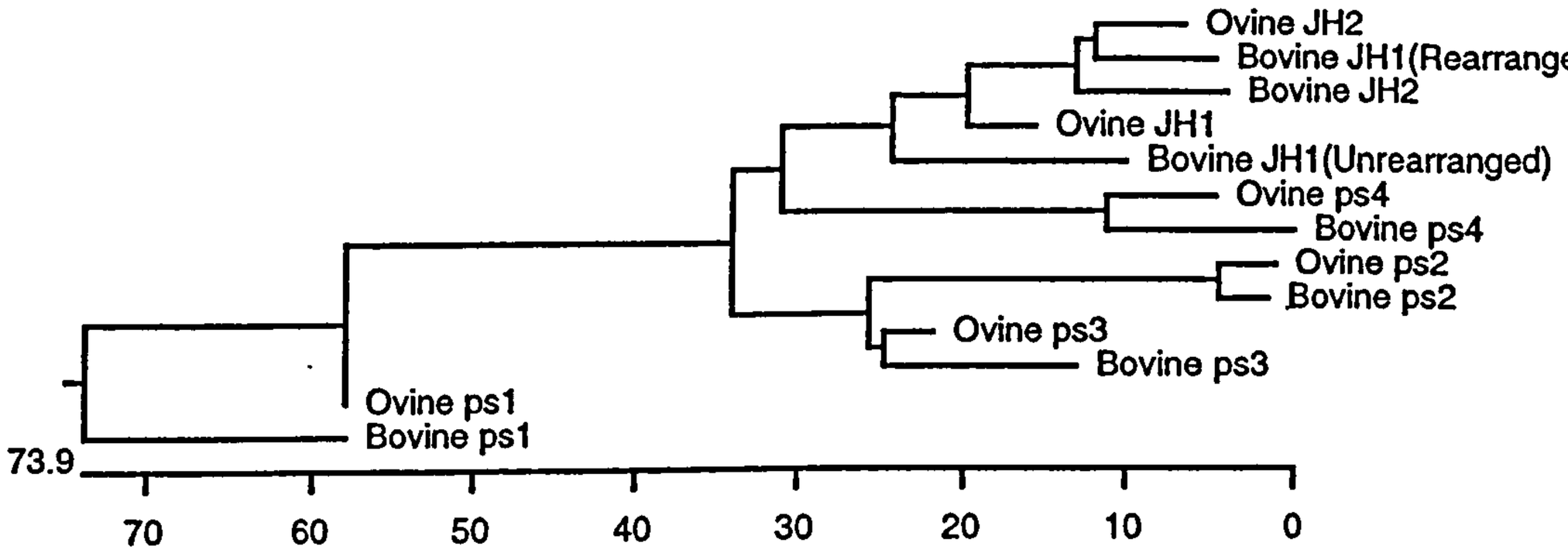


Figure 2.19 Phylogenetic relationships between bovine and ovine J_H segments. Having confirmed in previous figures that the fourth and sixth bovine J_H segments undergo rearrangement, these are now designated JH1 and JH2 respectively. Other segments are designated pseudogenes (ps) 1 (the first segment in the locus) to 4 (the fifth segment). Ovine segments are identified using the same convention (Dufour and Nau, 1997).

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	
1		64.4	75.6	53.3	62.2	57.8	51.1	73.3	75.6	66.7	73.3	73.3	64.4	31.1	46.7	26.7	60.0	64.4	64.4	55.6	68.9	73.3	64.4	57.8	60.0	82.2	82.2	73.3	57.8	64.4	35.6	76.9	77 8	71.1	71.1	66.7	66.7	1
2	36.9		60.0	40.0	55 6	60.0	53.3	68.9	68.9	64.4	75.6	75.6	66.7	33.3	48.9	26.7	53.3	64.4	71.1	60.0	64.4	68.9	71.1	57.8	66.7	75.6	64.4	62.2	48.9	62.2	48.9	71.8	75.6	68.9	75.6	66.7	68.9	2
3	19.0	36 4		35.3	47.1	53.3	43 8	51.0	62.7	64.0	60.4	62.7	86.3	27.5	41.2	25.5	47.1	64.4	66.7	51.1	66.7	66.7	60.4	52.1	72.5	76.6	84.3	56.9	45.1	60.0	36.2	69.2	68.8	64.7	64.4	56.2	76.5	3
4	54.6	91.7	82.8		51.0	46.7	39.6	58.8	56.9	46.0	52.1	47.1	39.2	29.4	45.1	25.5	37.3	44.4	48.9	44.4	57.8	49.0	39.6	33.3	33.3	40.4	43.1	76.5	47.1	48.9	40.4	46.2	54.2	51 0	51.1	52.1	31.4	4
5	46.3	50.3	65.1	55.2		66.7	29.2	62 7	74.5	54.0	66.7	58.8	62.7	29.4	54.9	29.4	49.0	60.0	66.7	64.4	68.9	68.6	60.4	45.8	54.9	51.1	54.9	64.7	94.1	71.1	31.9	61.5	66.7	68.6	68.9	70.8	52.9	5
6	47.1	36.1	42.7	68.6	33.3		31.1	66.7	60.0	66.7	73.3	71.1	60.0	40.0	48.9	24.4	46.7	64.4	68.9	57.8	71.1	55.6	66.7	48.9	66.7	57.8	53.3	57.8	60.0	82.2	35.6	66.7	75.6	73.3	68.9	68.9	60.0	6
7	110.0	114.1	149 3	350.0	103.4	75.2		56.2	45.8	39.6	56.2	66.7	41.7	20.8	62.5	35.4	43.8	53.3	46.7	44.4	46.7	37.5	41.7	50.0	41.7	59.6	47.9	47.9	31.2	44.4	66.0	53.8	47.9	47.9	51.1	52.1	45.8	7
8	26.0	34 0	33.0	36.9	27.1	36.7	110.4		72.5	68.0	81.2	70.6	58.8	33.3	56.9	21.6	60.8	71.1	73.3	71.1	82.2	64.7	70.8	64.6	58.8	72.3	56.9	78.4	62.7	71.1	55.3	74.4	77.1	74.5	82.2	77.1	56.9	8
9	25.6	29.6	29.6	69.8	26.9	42.1	72.6	28.3		68.0	79.2	74.5	69.8	26.4	56.6	36.5	71.2	73.3	68.9	71.1	75.6	83.0	72.9	64 6	60.4	72.3	72.5	76.5	73.1	71.1	42.6	82.1	79.2	76.5	82.2	77.1	66.7	9
10	29.8	40.7	29.3	65.8	36.1	50.7	121.8	33.7	38.4		72.9	68.0	68.0	24.0	42.0	28.0	48.0	77.8	71.1	66.7	75.6	68.0	66.7	72.9	70.0	61.7	62.0	70.0	46.0	68.9	31.9	79.5	83.3	74.0	75.6	79.2	62.0	10
11	26.7	27.9	28.7	50.0	28.9	24.0	90.8	16.6	34.1	31.5		89.6	75.0	29.2	54.2	25.0	62.5	77.8	84.4	77.8	86.7	72.9	85.4	77.1	72.9	83.0	70.8	72.9	62.5	77.8	48.9	76.9	72.9	77.1	86.7	81.2	70.8	11
12	19.9	27.1	29.7	45.7	26 6	26.9	103.7	20.6	31.3	37.8	8.9		74.5	31.4	58 8	25.5	64.7	77.8	82.2	73.3	80.0	68.6	75.0	72.9	68.6	80 9	70.6	66.7	54.9	75.6	55.3	82.1	79.2	84.3	77.8	85.4	74.5	12
13	23.9	26.4	15.5	69.3	38.0	33.0	108.2	34.4	24.4	29.5	24.4	28.3		25.9	42.6	25.0	57.7	75.6	75.6	60.0	71.1	77.4	75.0	60.4	77.8	74.5	78.4	60.8	63.5	66.7	38.3	74.4	66.7	70.6	73.3	75.0	84.3	13
14	78.8	82.4	73.4	76.3	75.6	69.7	108.4	88.4	68 7	106.6	87.0	65.4	89.5		31.5	23.1	21.2	31.1	31.1	26.7	37.8	22.6	25.0	27.1	25.9	29.8	29.4	33.3	32.7	35.6	38.3	30.8	43.8	35.3	31.1	31.2	33.3	14
15	88.5	93.2	139.3	111.6	96.1	75.9	34.1	91.3	81.1	123.2	76.7	80.5	99.4	120.1		23.1	48.1	51.1	53.3	55.6	57.8	50 9	45.8	41.7	42.6	44.7	45.1	47.1	55.8	55.6	42.6	53.8	50.0	56.9	51.1	58.3	47.1	15
16	59.6	114.5	91.7	148 6	145.6	128.5	120.7	76.3	71.1	106.2	95.7	93 6	121.2	143.1	203.7		30.8	33.3	28 9	24.4	33.3	23.1	25.0	29.2	21.2	19.1	21.6	31.4	28.8	26 7	21.3	33.3	27.1	27.5	24.4	29.2	21.6	16
17	26.9	37.8	42.0	58.6	40.1	36.9	78.8	23.5	19.8	35.3	18.9	14.7	28.3	93.8	62.2	76.2		62.2	60.0	62.2	57.8	63.5	54.2	47.9	50.0	61.7	56.9	54.9	46.2	57.8	42.6	61.5	60.4	60.8	60.0	58.3	52.9	17
18	26.3	40.2	28 9	76.2	37.6	41.4	75.8	30.2	33.0	26.6	24.5	20.8	26.6	87.0	83.1	78.0	15.9		73.3	73.3	73.3	64.4	66.7	68.9	73.3	64.4	62 2	66.7	55.6	75 6	40.0	71.8	77.8	77 8	71.1	77.8	73.3	18
19	29.8	28.1	30.4	63 8	38 8	25.3	119.0	20.3	33.0	24.5	10.2	20.6	23.5	91.1	99.9	70.3	29.7	22.2		77.8	82.2	66.7	73.3	73.3	73.3	73.3	66.7	71.1	64.4	73.3	42.2	74.4	71.1	80.0	71.1	77.8	71.1	19
20	32.7	35.6	45.6	82.5	40.7	33.4	92.5	24 8	29.7	32.4	16.3	27.6	36.5	121.2	72.7	75.2	13.4	20.1	13.7		71.1	57.8	68.9	80.0	60.0	62.2	57.8	62.2	57.8	68.9	37.8	69.2	68.9	75 6	66.7	75.6	60.0	20
21	19.9	40.7	28.9	49 1	30 2	27.6	74 5	20.3	30.4	24.2	12.3	15.5	23.5	85.5	76.2	101.0	25 9	24.6	13.0	20.0		71.1	73.3	77.8	82.2	64.4	66.7	80.0	75.6	75.6	40.0	74.4	75 6	80.0	80.0	75.6	68.9	21
22	25.6	30 0	23.3	79.4	33.0	48.6	96.1	44.1	19 3	35.4	44.8	44.6	14.5	101.2	101.3	127.9	35.7	44.2	36.5	52.8	37.1		72.9	58.3	62.3	68.1	66.7	66.7	65.4	62.2	36.2	74.4	72.9	66.7	77.8	66.7	66.7	22
23	41.3	34.8	29 8	93.8	40.4	24.5	93.6	28.2	33 6	33 0	17.4	30.5	22.1	94.5	89.2	144.0	36.5	36.4	26.5	28.7	31.4	33.7		70.8	68.8	70.2	58.3	60.4	56.2	75.8	42.6	69.2	66.7	66.7	80.0	70.8	64.6	23
24	32.7	44 0	50.1	104.9	50.7	56.0	123.5	43.2	37.1	19 8	28.1	36.2	40.8	110.5	99.8	109.9	29.7	27.1	27.6	21.2	24.8	48.1	36.5		58.3	63 8	54.2	56.2	43.8	57 8	40.4	69 2	62.5	66.7	68 9	72.9	60.4	24
25	34.0	18 4	29.2	92.0	40.8	29 8	109.2	33.7	33.0	34.7	18.8	26 1	16.7	90 5	102.1	121.9	35.1	35.5	25.2	39.8	31.5	28.4	22.7	36.1		70.2	62.7	58.8	51.9	68.9	36.2	69.2	70.8	70.6	64.4	64.6	72 5	25
26	20.6	22.1	19 0	59.3	50.8	41.6	112 7	22.6	28.9	32.4	13.4	16.3	28.2	75.3	86.4	82.2	33.0	33.2	22.7	28.9	26.7	32.7	29.5	37.6	25.0		78.7	63.8	51.1	64.4	48.9	76.9	74.5	66.0	77.8	70.2	76.6	26
27	13.3	32.4	15 5	58.2	51.6	48.1	149.3	26.4	17.4	25 6	22.1	26.9	20.9	74.0	128.1	68.6	31.5	34.0	34.0	37.2	25.9	23.3	36.5	42.0	34.0	15.8		64.7	51.0	60.0	40.4	76.9	68.8	66.7	68.9	70.8	74.5	27
28	27.6	50.1	40.2	22.8	30.2	49.7	350.0	13.7	36.8	33 8	22.6	19.6	37.6	93.8	103.2	106.2	27.3	37.8	28.9	38.9	21.0	50.9	47.4	52.7	46.8	27.3	25.3		62.7	68.7	40.4	71.8	77.1	70 6	75.6	75.0	56.9	28
29	51.6	55.6	71.4	64.4	6.3	36.4	106.8	33.0	32.8	43 4	31.8	29.2	40.6	78.1	85 6	151.4	44.4	45.5	38.8	44.0	25.6	39.1	44.5	54.1	40.8	56.4	56.6	33.3		64.4	31.9	61.5	60.4	64.7	68.9	68.7	51 0	29
30	33.8	40.0	31.3	67.0	25.9	14.7	91.5	29.7	30.4	42.0	17.9	20 6	26.4	62.0	90.8	84.1	25.6	30.4	19.0	22.6	21.2	40.4	18 4	42.0	26.3	29.5	35.3	31.9	28.9		35.6	71.8	80.0	77.8	73.3	73.3	62.2	30
31	95.2	129.3	155 4	130.2	121.1	112.9	17.3	114.6	76.1	128.8	161.0	134.6	123.8	97.9	34.7	144.9	113.4	98.1	177.1	136.8	96.8	99.2	151.8	350.0	145.6	118.4	155.4	134.3	125.0	107.7		41.0	42.6	48.9	40.0	48.9	40.4	31
32	30.5	36.6	34.7	66.7	42.6	45.7	88.5	20.6	28.0	24 0	31.7	27.7	35.6	58.6	82.4	113.7	35.2	30.4	29.5	26.3	22.1	36.7	45.6	30.5	42.3	26.6	30.5	32.8	37.0	35.7	68.5		92.3	87.2	79.5	87.2	87.2	32
33	16 3	23.9	25.3	54.1	26.3	38.9	97.2	19.0	21.6	18 9	29.2	22.2	33.6	69.9	86.0	86 6	21.8	23.3	24.6	23.7	21.2	30.4	34.0	27.3	29.3	16.3	18.9	25.5	33.3	31.3	82.4	8.2		87.5	80.0	81.2	72.9	33
34	19.4	33.9	37.1	53.1	21.2	34.3	75.6	22.7	28.5	24.4	25.5	18.1	31.6	82.1	82.4	106.2	17.6	17.4	20.3	21.5	14.9	44.2	42.0	28.9	34.3	26.1	30.4	25.2	27.3	27.3	79.0	17.5	11.2		75.6	91.7	72.5	34
35	30.7	22.1	26.4	59.2	34.7	31.8	105.5	17.4	17.6	18.9	15.8	23.5	17.5	67.3	90.8	72.8	28.1	28.9	24.0	26.1	22.4	23.9	23.3	37.8	28.4	19.4	20.4	25.9	38.9	24.8	109.4	15.5	13.3	26.9		75.6	73.3	35
36	26.3	37.6	41.3	48 6	18 0	32.3	79.7	21.6	18.9	17.4	23.5	18.9	24.6	71.1	68.8	91.8	17.6	18.9	23.3	19.6	15.7	33.6	42.0	24.0	34.3	29.8	26.9	20.6	21.2	25.2	82.6	17.5	14.9	6.7	23.5		75.0	36
37	28.9	25.2	25.5	70.0	47.0	31.8	80.7	23.3	23.3	22.0	22.1	20.4	17.7	62.9	74.4	91.7	42.0	18.9	27.1	33.2	22.4	26.8	29 6	34.0	30.1	22.1	29.2	32.7	51.6	31.4	75.4	12.0	13.0	23.5	17.5	20.4		37
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	

1 Bovine JH1(Rearranged)
2 Bovine JH1(Unrearranged)
3 Bovine JH2
4 Bovine ps1
5 Bovine ps2
6 Bovine ps3
7 Bovine ps4
8 Human JH1
9 Human JH2
10 Human JH3
11 Human JH4
12 Human JH5
13 Human JH6
14 Human ps1
15 Human ps2
16 Human ps3
17 Llama JH1
18 Llama JH2
19 Llama JH3
20 Llama JH4
21 Llama JH5
22 Murine JH1
23 Murine JH2
24 Murine JH3
25 Murine JH4
26 Ovine JH1
27 Ovine JH2
28 Ovine ps1
29 Ovine ps2
30 Ovine ps3
31 Ovine ps4
32 Rabbit JH1
33 Rabbit JH2
34 Rabbit JH3
35 Rabbit JH4
36 Rabbit JH5
37 Rabbit JH6

Figure 2.20 Percent identities and divergence between bovine J_H segments and those of other species. Bovine and ovine segments are designated as in Figure 2.19. Two pseudogenes present at the murine locus have been excluded from the analysis. Of the five segments present in the llama, it is no presently established which undergo rearrangement.

Consensus:	GGTTTTTG(T/C)		CACTGTG	(C/A)AGGT(G/A)AG
1ST ovine:	GGTTTCCAT	22	CACTGTG	CAGGAGAA
1ST Bovine:	GGTTTCCGT	22	CACTGTG	CAGGTGAA
2ND OVINE:	TGTTTTTCT	22	AGCTACG	-
2ND BOVINE:	TGTTTTTGT	22	AGCCATG	-
3RD OVINE:	GGTTCATGT	23	CCTTCAT	CAGGTGAG
3RD BOVINE:	GGTTCATGT	23	CCCTCAG	CAGGTGAG
4TH OVINE:	GGTTTTTGC	22	CACTGTG	CAGGTGAG
4TH BOVINE:	GGGTTTTGC	23	CACTGTG	CAGGTGAG
5TH OVINE:	GCTCGTGCT	20	CACAGTG	CAGCTGAG
5TH BOVINE:	GCTCTTGCC	20	CACTGTG	CAGCTGAG
6TH OVINE:	GGTTTTTGT	23	CACTGTG	CAGGTAAG
6TH BOVINE:	GGTTTTTGT	22	CACTGTG	CAGGTAAG

Figure 2.21 Comparison between consensus RSS motifs and transcriptional splice sites and features of bovine and ovine J_H segments. The segments are numbered according to their order at the loci. Deviations from the consensus sequences are indicated in red.

2.4 Discussion

2.4.1 Preliminary studies

Although this chapter describes the isolation of the bovine J_H locus using PCR methods, this was not the first strategy employed in the project. Initially, a cosmid library of bovine genomic DNA was screened to identify clones carrying the locus. The library was made with the cosmid vector cNEO (Southern and Berg, 1982) by Dr John Williams and colleagues at the Roslin Institute, Edinburgh, using genomic DNA from peripheral blood. To screen the library, a digoxigenin-labelled probe was made from clone L2-1 which carries the 3' region of the bovine J_H locus. The clone was generated using adapter-ligated fragments of bovine genomic DNA and PCR with primers designed from heavy chain framework 4 sequences (Aitken *et al.*, unpublished data). The screen identified several colonies that hybridised with the probe, but analysis of the bovine genomic inserts was complicated by the lack of a well-defined restriction map of the vector. Subcloning and sequencing of restriction fragments did not yield data with homology to the heavy chain loci of other species and so this approach was abandoned in favour of the strategy described in this chapter of the thesis.

2.4.2 PCR strategies for assembly of a contiguous sequence

Following attempts to screen the cosmid library, effort focused on amplification of the J_H locus directly from the germline. In the first step, the primers Ovine JH1 and JH4 were designed from the sequence of the sheep J_H locus and the limited sequence available from clone L2-1. This experiment recovered the majority of the bovine J_H locus (about 1.85 kb).

Since none of the J_H segments identified in the recovered sequence matched the more frequently expressed sequences in bovine framework 4, the downstream and upstream flanks were sought. To recover the downstream sequence, primers JH4 forward (JH4F) and Eμ reverse (EμR) were used. Analysis of two clones carrying the resultant amplicons revealed irrelevant sequences with the JH4 primer sequence at both termini. It was concluded that this primer bound to bovine genomic DNA non-specifically. Hence, the primer JH4F was replaced with JH2A that primes in the 6th J_H segment 100 bp upstream to the JH4F binding site. By using this primer and EμR, about 900 bp of sequence was determined. Although the heavy chain enhancer was

recognised in the terminus distal to the locus, additional J_H segments were not identified.

To recover regions of the locus lying upstream to the Ovine JH1-JH4 amplicon, different methods were used.

Using a primer to DQ52

In many species, the D segment DQ52 is located adjacent to the J_H locus and contributes to the heavy chain repertoire to an extent which varies between species (reviewed by Chen *et al.*, 1996). Hence a primer was designed from alignment of the sequences of DQ52 segments from humans and other species (Figure 2.13). About 30 PCR reactions were performed with this DQ52 primer and JH6, JH9 and JH8R. Only PCR with JH6 yielded distinct products but sequencing of amplicons of 1200 and 1900 bp showed that they were irrelevant DNA. Later work described below showed that though a bovine DQ52 segment exists, it differs significantly from the consensus which was used in primer design, explaining the failure of this approach.

Using VH1A primer

The primer VH1A binds to bovine V_H segments, about 80 bp downstream to the leader sequence. We reasoned that PCR with this primer and a primer to the J_H locus primers might amplify the rearranged V, D and J segment from lymphoid genomic DNA, enabling identification of the rearranged J_H segment. Four clones bearing VH1A-JH6 amplicons of 550, 650, 800 and 1500 bp and one clone possessing a 1.2 kb VH1A-JH8R amplicon were sequenced but all were irrelevant DNA fragments. Genomic DNA prepared from a lymph node and liver were used as templates in these experiments. Control reactions confirmed that under the experimental conditions employed, all primers would extend successfully from their binding sites. This experiment assumed that the template would contain sufficient rearranged DNA from B cells to prime the intended reaction. In reality, the sample would have contained DNA from many cells in which rearrangement of the heavy chain locus had not taken place (connective tissue, dendritic cells and other leukocytes, T lymphocytes, for example). Hybridisation / extension of primers to DNA from these sources might therefore have depleted the concentration available to prime amplification from the B cell DNA in the pool. Significantly, other experiments described below used genomic DNA from purified B cells to a similar objective; these experiments were successful.

Lambda genomic libraries

Work by other students in the laboratory isolated a clone from a lambda library of bovine liver DNA which hybridised with a J_H probe. It was considered possible that the clone might carry upstream flanking sequences and perhaps J_H segments not recovered by the PCR strategy. Using PCR, three parts of the locus characterised thus far were recovered from the lambda clone for sequencing, spanning about 1850 bp between the first and the sixth J_H segments. In addition, sequences representing short interspersed nucleotide elements (SINE) were detected around the JH8R binding site. Amplicons from a 3.6 kb *Sma*I fragment and *Dra*I fragments of 3.9, 4.3 and 5 kb were sequenced but all were irrelevant sequences, mainly with homology to alpha globin. It therefore seems likely that the lambda clone carries a fragment of the J_H locus that has been translocated to a different chromosome. Similar translocations have been described from the human heavy chain locus (Cook and Tomlinson, 1995).

From published studies (Knight *et al.*,1988), there were better grounds to suppose that a lambda clone obtained from Prof K. Knight (clone 15) would carry the upstream flanking sequences which would complete the characterisation of the J_H locus and perhaps identify that segment which most frequently forms the sequence of framework 4. Having established the orientation of the insert, PCR recovered about 500 bp of upstream flanking sequence. Although the amplicon carried the bovine DQ52 segment at the 5' terminus, it did not contain any J_H segments.

2.4.3 Organisation of the bovine J_H locus

The locus can be divided into three parts: the DQ52 segment, the J_H segments and the downstream flanking region. Each are discussed in turn below.

The DQ52 segment

DQ52 forms the 3' terminus of the D locus, separated from the first J_H segment by a short distance which varies between species. In the mouse, it lies 696 bp upstream of J_H1 (Sakano *et al.*, 1981) and is located within the J region in humans (Ravetch *et al.*, 1981). In rabbits it is localised about 800 bp upstream of J_H1 (Chen *et al.*, 1996). The segment makes a similarly variable contribution to the heavy chain repertoire. In humans, DQ52 is used preferentially during rearrangement in the foetal liver

(Schroeder and Wang, 1990) but it is rarely expressed in peripheral B lymphocytes of the adult (Yamada *et al.,* 1991). In mice, DQ52 is preferentially expressed in the foetal liver and in neonatal B cells (Bangs *et al.,* 1991), but its rearrangement can also be detected in thymocytes of foetal, newborn and young mice (Born *et al.,* 1988). In contrast, DQ52 in the rabbit is expressed very infrequently during foetal development (Chen *et al.,* 1996). Our study has revealed that in cattle, DQ52 is located 448 bp upstream of the first J_H segment. The segment possesses a 14 bp coding region that is longer than those reported from other animals (Figure 2.13) and also deviates from them in sequence. One unresolved issue concerning the formation of bovine Ig heavy chains is what underlies the exceptional length of the third CDR (Aitken *et al.* 1999). Perhaps the length of DQ52 indicates that other D segments will be atypically long, explaining this property. By comparing the sequence of DQ52 with other data (Berens *et al.*, 1997; Sinclair *et al.*, 1997; Saini *et al,.*1997), there is no evidence to show that DQ52 is used in DJ rearrangements in cattle during foetal development or in the adult Ig repertoire. This may be due to loss of recombination competence. The 5' heptamer and nonamer RSS match those of the mouse except for the eighth base in the nonamer. Similarly, the 3' heptamer matches that from humans, mice and rats, but the 3' nonamer differs in three bases (CACAAAA *versus* CACAGTG). It has been shown that when RSS deviate from the consensus sequence in the heptamer, spacer and nonamer regions, this can drastically diminish the recombination frequency (Hesse *et al.,* 1989, Akamatsu *et al.,* 1994). Although CAC at the first three positions are the most essential nucleotides, it has shown that the substitution of the 5th or 7th or both with A diminishes the recombination rate to 87%, 74% and 26% respectively (Hesse *et al.,* 1989).

The J_H segments

The cattle J_H locus appears broadly similar to that of the sheep (Dufour and Nau, 1997) with only three significant insertion / deletions between the species. Comparison of the loci by pairwise BLAST, excluding these three stretches, reveals 89% homology. The bovine J_H locus contains 6 J_H-like segments as in sheep (Dufour and Nau, 1997), rabbits (Becker *et al.,* 1989; Friedman *et al.,* 1994; Tunyaplin and Knight, 1995) and mice (Sakano *et al.,* 1980), whereas the chicken possesses a single J_H segment (Reynaud *et al.,* 1989), and there are nine segments at the human J_H locus (Ravetch *et al.,* 1981). In these species, two J_H segments are functional in sheep, 5 in rabbits, 4 in

mice, 1 in chickens and 6 in humans. Comparison between the bovine and ovine J_H segments sequences revealed the homology of the equivalent segments is 76.5%, 94.1%, 82.2%, 64.4%, 66% and 84.3% for the first to sixth segments respectively.

It is of obvious importance to assess which segments at the locus retain or have lost the ability to undergo rearrangement and thus contribute to the formation of Ig. The significance of sequence in RSS spacer regions has been addressed by Fanning *et al.*, (1996). In their work, mutation of the conserved fifth nucleotide of two 12 and 23-base spacers resulted in a relative reduction in the rate of recombination. Other articles suggest that mutation of 50% or even 100% of AT nucleotides in spacer regions to GC did not significantly reduce the level of recombination (Akira *et al.*, 1987; Wei and Lieber, 1993). Against this background, it is very difficult to assess the significance of anything other than nonamer, heptamer and RNA splice signals where well-defined consensus sequences exist. With regard to the first J_H segment, the heptamer motif matches the consensus CACTGTG but the nonamer departs from consensus and the 3' RNA splice site is non-functional. It is therefore likely that as in the sheep, this segment is non-functional. The second bovine J_H segment is likely to be a pseudogene for similar reasons. The third J_H segment possesses RNA splice sites but the RSS departs from consensus sequences. Inspection suggests the fifth J_H segment of cattle is a pseudogene because of non-functional heptamer and RNA splice sites and a spacer of just 20 bases.

In sheep the fourth and sixth segments are functional. At the bovine locus, all RSS motifs in segments four and six are identical to the consensus except that the nonamer of the fourth segment has a T to G substitution at the third base. In sheep the fourth J_H segment (JH1) is used in more than 90% of Ig cDNAs and the 6th segment (JH2) contributes to the structure of the remainder (Dufour and Nau, 1997). In cattle, a similar bias operates with a single sequence appearing in framework four very commonly in heavy chain cDNA (Armour *et al.*, 1994; Sinclair and Aitken, 1995; Berens *et al.*, 1997; Saini *et al.*, 1997; Sinclair *et al.*, 1997) and evidence of low-frequency rearrangement of an alternative J_H segment (Berens *et al.*, 1997). It is on this topic that the real significance of this characterisation of the bovine J_H locus emerges. Of the 6 segments detected, the sequence of the 3'-terminal segment (the 6th segment), matches precisely that of the less frequently detected framework sequence. Despite partial homology between the sequences of the remaining five segments and most commonly observed sequence in framework four, no segment matches exactly. A major challenge

was therefore to identify which J_H segment undergoes frequent rearrangement in cattle and to explain what processes might modify the germline sequence to create that observed in Ig cDNA.

The downstream flanking region

Sequence of the region downstream of the J_H locus has close homology to that of the sheep. There are regions of disparity ranging from 1 to 24 nucleotides but is difficult to establish if these represents insertions or deletions. The most notable of these features is a deletion of 24 nucleotides from the sequence present in sheep, corresponding to nucleotides 2592-2613 of the bovine sequence. Intriguingly, the ovine sequence possesses internal complementarity suggesting that it might form a short stem-loop structure when transcribed from upstream promoters (the so-called "sterile transcripts" or "germline transcripts" [Hesslein and Schatz, 2001]). The 3' terminus of the characterised regions carries about 300 bp of the 5' intronic heavy chain enhancer with 94% similarity to the ovine sequence.

2.4.4 Identification of the rearranging segment

At the outset of this work, it was expected that characterisation of the bovine J_H locus would lead to identification of two segments matching precisely the major and minor framework four sequences observed in Ig cDNA. Given that a high level of similarity was detected between the bovine and ovine loci and that this outcome emerged from studies of the sheep (Dufour and Nau, 1997), it therefore came as a considerable surprise to find that a J_H segment corresponding to the major framework four sequence could not be identified. To resolve this issue, PCR was applied to genomic DNA from purified peripheral B cells, using primers against the V_H segment and regions of the J_H locus. Sequencing of VHF-JH4 amplicons clearly implicated the fourth J_H segment but showed a series of substitutions had altered its germline sequence to that typically observed in Ig cDNA. Specifically, these modifications altered codons 105,107-109 through 6 or 7 substitutions. Downstream of the segment, the number of substitutions to the germline sequence becomes more pronounced. This would be expected since the B cells were isolated from the peripheral circulation of an adult animal and therefore are likely to have encountered antigen and undergone affinity maturation through somatic hypermutation. This process is known to modify the transcriptional unit, rather than the coding sequence (Hesslein and Schatz, 2001).

The most attractive explanation of these results is that at some stage during B cell development, the fourth J_H segment or the rearranged sequence encoding framework four undergoes modification. However, the following explanations were also considered.

Variation exists in the sequence of the fourth J_H segment

By chance, these studies may have isolated variants of the fourth J_H segment that carry an atypical sequence. This is unlikely. At least two independent preparations of genomic DNA from different animals returned the same sequence for the fourth J_H segment, a sequence which was identical to that from independent clones from different lambda libraries of bovine genomic DNA. Also, the sequence of the fourth segment was determined for a single animal from leukocytic (typical germline sequence) and peripheral B cell genomic DNA (typical Ig cDNA sequence). This reduces to a very low level the likelihood that allotypes or other variants of the fourth J_H segment exist, one rearranged to form framework region four and another (or others) that fails to undergo rearrangement / expression in cDNA. These considerations and the frequency with which a single framework four sequence has been described in cDNA isolated in independent studies (Armour *et al.*, 1994; Sinclair and Aitken, 1995; Berens *et al.*, 1997; Saini *et al.*, 1997; Sinclair *et al.*, 1997) rules out the possibility of allelic variations to segment 4 in the bovine population generally.

The germline sequence of the fourth J_H segment arises from a PCR artefact

The recovery of "cross-over" products when using PCR on templates with homologous sequences is well known (Sinkora *et al.*, 2000). This would tend to introduce a false impression of diversity which is not a feature of the data presented here. Also, the chance of artefacts arising from PCR from single clones of lambda DNA is significantly reduced. The fact that a consistent germline sequence emerged from the studies irrespective of the source of template argues that the result – the sequence of the fourth (rearranging) J_H segment fails to match the sequence of framework 4 - is not an experimental artefact.

The sequence recovered comes from a non-functional duplication of the J_H locus

This possibility is more difficult to reject. It is known that the bovine genome carries at least one translocated copy of sequence from chromosome 21 to 9 which

includes IgM constant regions (Tobin Janzen and Womack, 1992; Mousavi *et al.*, 1998). The distance between the J_H locus and the first Cμ exon is about 6 kb and therefore this translocation could also include some or all of the J_H locus. If the segments on this or other translocated fragments of the heavy chain locus carried sequences different to that on chromosome 21, the study could have inadvertently characterised these non-functional duplications. Against this, lambda clone 15 carries sequence from the DQ52 segment through the J_H locus to beyond the IgM constant region (Knight *et al.*, 1988). This has all the characteristics of a genomic fragment of about 18 kb derived from the heavy chain locus on chromosome 21, yet the sequence of the fourth J_H segment is consistent with data gathered by PCR. The lambda clone independently isolated in our laboratory also carries this sequence but the presence of a SINE element strongly suggests that this genomic fragment is from a non-functional duplication of the locus. It therefore seems likely that both functional and non-functional copies of the J_H locus carry the sequence reported for the critical fourth segment and there are no grounds to suppose that the sequence characterised as the J_H locus has been recovered from a non-functional translocation of this region of the genome.

2.4.5 Possible mechanisms of modification

Working from the conclusion that the fourth J_H segment undergoes rearrangement and modification in the formation of bovine antibodies, what processes might be responsible for altering its sequence?

Templated versus *non-templated modification*

Somatic hypermutation is known to diversify the sheep antibody repertoire (Reynaud *et al.*, 1995) and it has been speculated that the process might also operate in cattle to achieve the same outcome (Sinclair and Aitken, 1995; Berens *et al.*, 1997; Saini *et al.*, 1997; Sinclair *et al.*, 1997; Aitken *et al.*, 1999). This process has biases in the bases which are modified and the locations of the changes which are introduced (Wagner and Neuberger, 1996). Comparison of the germline and rearranged sequence of the fourth J_H segment does not suggest that this process is responsible. The consistency with which substitutions to a limited number of nucleotides occur are more typical of a templated process. Notably, the mutation of the (I/T), Q and N (isoleucine, glutamine and asparagine) codons (A[T/C]C CAA AAC) to the leucine, leucine and

valine codons (CTC CTG GTC) is striking since multiple other substitutions could have achieved the same protein sequence. These features are more typical of a templated process (*e.g.* homologous recombination or gene conversion). The conserved sequence of the insertion present on the upstream flank of the fifth segment, and consistent substitutions in the sixth segment are consistent with this mechanism.

Linkage with B cell development and diversification post-rearrangement

Since rather little data is presently available, it is difficult to assess the timing of the modification process relative to rearrangement, B cell development and the diversification of the antibody repertoire. One statement which can be made with some certainty is that modification of the germline sequence takes place during foetal development before significant diversification of the immunoglobulin repertoire. This can be seen in data from Berens *et al.* (1997). Here, the authors aligned antibody cDNA sequences from foetal tissue. It is clear from this that the sequence of framework four has achieved that typically seen in adult animals, yet there is very little diversity in the CDRs. Turning to the potential timing of modification in B cell development, the pathways of B cell development are poorly established in cattle. In human prenatal development, the foetal liver transiently plays the major role in the production of cells for the B lymphocyte lineage. Around 12 weeks into gestation, the production of B cells shifts to the bone marrow. Thereafter in mice and humans, the B cell repertoire is generated throughout life by continual rearrangement of Ig genes in each newly formed B cell in the bone marrow. B cell development in the bone marrow requires successful rearrangement at the heavy and light Ig loci and the surface expression of Ig, the B cell antigen receptor (BCR) (Retch, 1995). The first rearrangements, D-J and V-DJ, are seen in "early pro-B cells" and "late pro-B cells" respectively but rearrangement of the light chain occurs at a later stage in "small pre-B cells" (Melchers *et al.,* 1995). When pre-B cells undergo successful light chain rearrangement, they develop into immature B cells and express the mature BCR (Li *et al.,* 1993). Immature B cells produced in the bone marrow migrate into the periphery to complete the maturation process (O'Rourke *et al.,* 1997). Expression of the pre-BCR and BCR affords the possibility that the primary B cells repertoire is shaped and directed by continuous clonal selection beginning at the pre B cell stage of differentiation (Schwartz and Stollar, 1994). It is perhaps during this phase in cattle that modification takes place but one challenge for the future will be to derive a comparable understanding of B cell development in cattle.

For the present, the tissues in which the foundations of humoral immunity are laid remain uncertain (Lucier *et al.*, 1998).

Cis and trans-chromsomal rearrangement

Close inspection of the sequence of the bovine J_H locus failed to identify any candidates for templated modification (*e.g.* the non-functional segments present at the locus or remnants of J_H segments in the intergenic regions). The possibility therefore arises that the donated sequence may come from interlocus or *trans* rearrangement, in which the rearranged segments are from different loci (Baer *et al.*, 1985; Denny *et al.*, 1986; Kobayashi *et al.*, 1991). The participating loci can be situated on two chromatids, on two homologous chromosomes or even on non-homologous chromosomes. Knight *et al.*, (1974; 1995) showed that V_H and Cα allotypes used in rabbit IgA heavy chains were encoded by V_H and Cα genes in *trans* (from different parental chromosomes), whereas the V_H and J_H genes used in V(D)J rearrangements were from the same chromosome, indicating that the V_H, D and J_H gene rearrangements occurred in *cis*. They suggested that the trans-association between VDJ and Cα did not occur during VDJ rearrangement but during isotype switching. This idea supported previous studies performed on the V_H and C_H genes (Kipps and Herzenberg, 1986; Gerstein *et al.*, 1990).

Although interchromosomal VJ junctions have been detected in the TCR, they are rare, estimated to occur in only about 1 of every 10^5 thymocytes (Aster and Sklar, 1992). Interchromosomal recombination between the murine Ig κ and λ loci has been detected in Abelson virus transformed pre-B cells at a frequency which is about 1000-fold lower than rearrangements in *cis* (Baily and Rosenberg, 1997). It has been shown that the 12- and 23-RSSs can synapse and undergo coupled cleavage *in vitro* (Hiom and Gellert, 1998). In another experiment it was shown that 12- and 23-RSSs on separate transiently transfected plasmids could mediate relatively sufficient synapsis, coupled cleavage, and signal joint formation. This study reported a dramatic defect in coding joint formation if the two RSSs were in *trans*, which led to the proposal that mechanisms exist to selectively disfavour or inhibit coding joint formation in *trans* (Han *et al.*, 1999). In contrast, Tevelev and Schatz (2000) found that both coding and signal joint formation can occur relatively efficiently in *trans*. Inefficient as these processes might appear, it should be recalled that in humans, some 55 billion B cells

per day are lost in the bone marrow because they fail to make a functional immunoglobulin, or enter apoptosis because they are specific to self-antigens.

Given these finding, a hypothesis worth future investigations is that changes to the fourth J_H segment during B cell development occurs through a gene conversion process which operates post-rearrangement and utilises a donor sequence lying outwith the 3200 bp which has been sequenced in this study. One objective of this investigation would be to establish if modification is driven by processes operating *in cis* or *in trans*. In either case, this appears to be another highly unusual feature of the immunology of cattle as a group of vertebrates. These issues could be tackled by first identifying the genomic origins of the donor J_H segment by screening a genomic library for the inserted sequence introduced through rearrangement. In itself, this would assess the likelihood of a cis versus a trans-mediated process. Following from this, one would expect the distance between flanking marking to shorten in B cells if rearrangement were the driving force for introduction of the J_H segment. This parameter would remain unchanged and the sequence of the donating segment would not alter at the donating locus if gene conversion were at work.

Chapter 3

Construction of a library of recombinant bovine Fabs against *Mannheimia haemolytica*

3.1 Introduction

3.1.1 Overview of antibody phage display libraries

The processes of gene rearrangement, diversification and chain pairing creates sufficient antibody diversity to protect animals against most forms of challenge with antigen. This natural diversity can be captured by PCR and through phage display, antibodies against individual antigens can be conveniently isolated. Antibodies can be expressed as single chain Fvs (scFvs) or Fab fragments.

scFv libraries are generally constructed through the *in vitro* combination of V_L and V_H repertoires each recovered by PCR (Marks *et al.*, 1991; Cai *et al.*, 1995; Vaughan *et al.*, 1996; Muller *et al.*, 1997; de Haard *et al.*, 1998; Mousli *et al.*, 1999; Lorenzen *et al.*,2000). The V_H and V_L repertoires can also be linked into scFvs by recombination processes *in vitro* (Hogrefe *et al.*, 1993) or *in vivo* (Waterhouse *et al.*, 1993; Griffiths *et al.*, 1994). In some cases V_H and V_L gene fragments are separately and sequentially cloned into the phage display vector; these fragments may be either PCR products that are cloned directly, or released from an intermediate vector (Kipriyanov *et al.*, 1996a).

For construction of Fab libraries the V_H (-C_H1) and V_L (-C_L) fragments are cloned independently into the display vector. The fragments can be either PCR products digested with appropriate restriction endonucleases (direct cloning) (Barbas *et al.*, 1991; Ward *et al.*, 1996; Siegel *et al.*, 1997) or restriction fragments released from an intermediate vector (Yau *et al.*, 1998; de Haard *et al.*, 1999). The phagemids used for Fab library construction may possess C_L or C_H sequences; naturally, this will affect the strategies for designing primers for amplification of V_H(-C_H1) and V_L(-C_L) fragments.

Unlike humans and mice, the primary antibody repertoire of domesticated species often lacks great combinatorial diversity. Despite the apparent complexity of many light and heavy chain loci, the expressed repertoire may be founded on single families of V genes (*e.g.* cattle) or the rearrangement, diversification and expression of single V segment (*e.g.* chickens). Therefore, far fewer primers are required to recover the Ig repertoire by PCR from many livestock animals.

3.1.2 Phage display antibodies from animals

Hybridoma techniques have generally made little impact in the generation of monoclonal antibodies from livestock animals. In contrast, there are fewer constraints on the use of phage display and recombinant methods with these species and there are numerous potential benefits:

The application of phage display should speed the development of antibody-based therapies for species of veterinary and economic importance and could provide, through transgenesis (Sola *et al.*, 1998), or other novel methods of immunoprophylaxis (Lorenzen *et al.*, 2000) enhanced protection against disease. Other applications include passive immunomodulation of a range of physiological process and Ig-targeted drug or vaccine delivery (Wang *et al.*, 2000). By using antibodies of native sequence, anti-species immune responses would be avoided.

Antibody production from different species like chickens may be a useful way of producing antibodies recognising conserved epitopes on mammalian molecules which, because of self-tolerance, are not obtained by immunisation.

To take livestock as an example, phage-display technology will obviate the modelling of viral, bacterial or parasitic infections in rodent systems simply to obtain antibodies. This should eliminate potential artefacts arising from the limited ability of many veterinary pathogens to colonise laboratory animals or differences in antigenic recognition between natural and laboratory hosts.

In several important cases, human pathogens fail to establish in rodents, but relevant infection models are available in other animal species (*e.g.* HIV [Locher *et al.*, 2001]). Similarly, there are many human diseases with close parallels in veterinary medicine. The availability of antibodies from a wider range of species should increase the appeal of animals other than rodents for the study of human disease. The outbred characteristics of many of these mammals increase their value as models for human disease.

Although the majority of research in phage antibody techniques has been conducted using libraries of human origin, phage libraries have been produced from several livestock species.

Rabbits

Rabbits have been used for decades to produce polyclonal antibodies as diagnostic research tools; however, monoclonal antibodies from this species have not been produced *via* routine methods such as immortalisation of B cells. Monoclonal antibodies derived by phage display have been generated from rabbits in several cases (Ridder *et al.*, 1995; Lang *et al.*, 1996; Foti *et al.*, 1998; Li *et al.*, 2000; Steinberger *et al.*, 2000). These antibodies have been produced in Fab or scFv formats expressed in *E.coli* or *Pichia pastoris* and are derived from rabbits specifically immunised with

antigens of interest (detailed in Table 3.1).

Chickens

Although fusion partners for the production of chicken monoclonal antibodies have been described (Nishinaka *et al.*, 1991), hybridoma technology is not widely used. In the chicken, single functional Ig and joining gene segments at the heavy and light chain loci undergo V(D)J rearrangement. Diversity is subsequently introduced by gene conversion with upstream V region pseudogenes in such a way that practically all V regions have identical termini. This greatly simplifies the representative amplification of V region genes. Furthermore, the entire naïve repertoire of the adult chicken is produced in the bursa of Fabricius of young birds. These properties have been exploited in procedures to produce large phage display libraries of diverse chicken Ig genes. Most of the phage antibody libraries made from the chicken repertoire are from immunised individuals (Davies *et al.*, 1995; Yamanaka *et al.*, 1996; Andris-Widhopf *et al.*, 2000) although a naïve phage library has also been constructed and screened against a number of proteins (Cary *et al.*, 2000). The properties of these libraries are shown in Table 3.1.

Sheep

Genetic studies have shown that B cells of sheep express ten germline genes from a single V_H gene family, a homologue of the human V_H4 gene family (Dufour *et al.*, 1996) and that the germline carries a minimum of ten V_κ genes, classified into four families (Hein and Dudler, 1999). The diversity of the sheep Ig repertoire is generated by hypermutation of mature rearranged V genes, that is *via* postrearrangement diversification (Reynaud *et al.*, 1995; Reynaud *et al.*, 1997). Sheep polyclonal antibodies have been used as specific high affinity immunologic probes in a variety of immunochemical approaches for analytical and clinical uses. In contrast, ovine monoclonal antibody technology is poorly developed. Antibody phage display technology has allowed the generation of monoclonal antibody fragments from sheep. Libraries constructed *via* this manner are from immunised animal and express antibody fragments in scFv form (Charlton *et al.*, 2000; Li *et al.*, 2000).

Table 3.1 Characteristics of some of antibody phage libraries constructed from animal gene repertoires.

		MAb format	source of gene repertoire	immunisation	Ag that the library was screened against	vector/ library cells	size of library	vector/ expression cells	amount of Ab production
Rabbits	Ridder *et al.*,1995	scFv	spleen	+	human leukaemia inhibitory factor	pGEM-III/ *XL1-Blue*	–	pGEM-III/ *Pichia pastoris*	100 mg / L
	Lang *et al.*,1996	Fab	spleen, bonemarrow	+	human plasminogen activator inhibitor	pComb3H	2×10^7	pAraHA	–
	Foti *et al.*,1998	Fab	B lymphocytes	+	Keyhole limpet hemocyanin	pComb3/ *XL1-Blue*	–	pMF3/ *XL1-Blue*	–
	Li *et al.*,2000	scFv	spleen	+	mixture of four herbicide	pSD3/ *TG1*	–	pSD3/ *TG1*	1-5 mg / L
	Steinberger *et al.*, 2000	Fab	spleen, bonemarrow	+	human CCR5-N-GST	pComb3H / *TOP10*	–	pComb3X/ *TOP10*	–
Chickens	Davies *et al.*, 1995	scFv	bursal lymphocytes	–	HEL, BTG, BSA	fd-tet-DOG1/ *MC1061*	2×10^6	fd-tet-DOG1/ *MC1061*	–
	Yamanaka *et al.*, 1996	scFv	spleen	+	murine serum albumin	pPDS/ *XL1-Blue*	1.4×10^7	pPDS/ *XL1-Blue*	–
	Andris-Widhopf *et al.*, 2000	scFv, Fab	spleen, bonemarrow	+	fluorescein BSA fluorescein ovalbumin	pComb3H / *ER2537*	–	pComb3X/ *ER2537,TOP10F'*	–
	Cary *et al.*, 2000	scFv	spleen, bonemarrow	human clan III Ig	human and mouseIg	pComb3H/ *ER2537*	–	pARA/ *XL1-Blue*	–
Sheep	Charlton *et al.*, 2000	scFv	spleen	atrazin-BTG	was used for Ab diversity analysis	pDM1/ *TG1*	1×10^8	pDM1/ *XL1-Blue*	–
	Li *et al.*, 2000	scFv	spleen	HSA, CONA	HSA, CONA	pCD3a/ *TG1*	–	pCD3a/ *XL1-Blue*	–
Camel	Arbabi *et al.*, 1997	single domain antibody	blood lymphocytes	tetanus toxoid, lysozyme	tetanus toxoid, lysozyme	pHEN4/ *XL1-Blue, XL2-Blue*	1×10^7	pHEN4/ *WK6*	–
Cattle	O'Brien *et al.*, 1999	Fab	lymph node	GST/bovine PV-4 L2a fusion	GST	pComBov/ *XL1-Blue*	2×10^7	pComBov/ *XL1-Blue*	–

Abbreviations: HEL: Hen egg white lysozyme, BSA: Bovine serum albumin, BTG: Bovine thyroblobulin, CONA: Chicken egg coalbumin, GST: Glutathione S-transferase, HSA: Human serum albumin

Blanks: No data is available

Camels

The *camelidae* is the only taxonomic family known to produce an important fraction of their functional Ig as homodimers of only heavy chains, devoid of light chains (Hamers-Casterman *et al.*, 1993). Sequence analysis has shown that the V_H part of these heavy chain antibodies is very similar to human and mouse V_H. This opened the possibility of generation of single-domain antibody fragments from cloning of the V_H genes. An antibody phage library from camels has been constructed to select antibodies specific for tetanus toxoid (Arbabi *et al.*, 1997). Peripheral blood lymphocytes were used as source of mRNA for cDNA synthesis. The V_HH genes were amplified using two specific primers and amplicons then were cloned into pHEN4 (modified pHEN1 vector optimised for camel V_HH gene cloning). *E. coli* XL1 and XL2 were used for transformation of ligated materials and library generation. After selection of positive clones, antibody fragments were expressed as single domain monomeric V_HH by using *WK6* cells.

Cattle

A single gene family comprising 3 subfamilies dominates the bovine V_L repertoire. The bovine V_H repertoire is also dominated by a single gene family consisting of about 15 members, although there is evidence of other gene families at both the heavy and light chain loci. A Fab phage display library, comprising more than 2×10^7clones, has been constructed (O'Brien *et al.*, 1999). In this case, the lymph node of a calf vaccinated against glutathione S-transferase (GST) was used as the source of the Ig repertoire. After total RNA isolation and cDNA synthesis, the light chain and heavy chain repertoires were recovered by using one pair of primers for each. The amplicons were cloned into phagemid pComBov and the ligated materials were transformed to *E. coli* XL1-Blue cells. pComBov is a derivative of pComb3H vector that has been modified for bovine Fab phage library construction and expression. The characteristics of pComBov, the phagemid used in this phase of the project, have been discussed in the end of this section (subheading 3.1.4).

3.1.3 Complexity of antibody gene libraries

Theoretically, it is sufficient to produce an antibody gene library only once. This library should approximate to the complexity of the immune repertoire in which nearly every potential antibody is presented. For several reasons, however, this has proved

difficult. First, it is unlikely that all components of the starting material will amplify with equal efficiency in the PCR and successfully ligate with the chosen display vector. Next, the efficiency with which these recombinant molecules can be transformed is limited and this affects the complexity of resulting library. Once the display library is established, the selection system may be pushed to its limit in isolating rare components. Added to this, production and cultivation of very large libraries requires enormous effort, leading to logistical limitations.

According to the intended application and the affinity required for a particular antibody, libraries of different complexities (size) are necessary, ranging from just a few clones up to 10^{10} different sequences. For some applications, a pre-existing hybridoma line can deliver the antibody gene desired. If this source is not available, an antibody gene library must be produced, from which the desired antibody can be selected. Often for this purpose, a source of B lymphocytes (*e.g.* blood) from an immunised donor is used. This preselects for antigen-specific genes and hence libraries of several million independent clones are usually sufficient to achieve the desired recombinant antibody fragment. From immune libraries, antibodies can be obtained mainly against the set of antigens to which an immune response was provoked. In some instances (*e.g.* when high affinity is required or when antibodies are sought for multiple antigens), it is necessary to generate large ("single-pot") libraries. Since the natural primary antibody repertoire within B cells contains a large array of IgM antibodies that recognise a variety of antigens, they can be used for large naïve library construction. From naïve libraries, antibodies to any chosen antigens including autoantigens, non-immunogenic and toxic substances are selected. On the other hand, the diversity present in the IgM repertoire is potentially limited by unequal expression of the different V gene families or segments, and the unknown immune history of the B cell donor. These factors can influence the content and the quality of the library. The affinity of antibodies selected from a naïve library depends upon the size of that library; low affinity antibodies are usually selected from smaller-size libraries, but when large repertoires (10^{10}) are available, high affinity antibodies are isolated directly. Many naïve libraries have been generated in both Fab (de Haard *et al.*, 1999) and scFv (Vaughan *et al.*, 1996; Sheets *et al.*, 1998; Little *et al.*, 1999; Sblaterro and Bradbury, 2000) formats, using different sources of IgM mRNA including peripheral blood lymphocytes (Sheets *et al.*, 1998; Little *et al.*, 1999; Sblaterro and Bradbury, 2000), spleen (Sheets *et al.*, 1998; de Haard *et al.*, 1999), bone marrow and tonsil (Vaughan *et*

al., 1996).

In the other kind of single-pot libraries - synthetic antibody libraries - antibodies are generated by *in vitro* assembly of V-gene segments and D/J segments. Most of an antibody's binding strength resides in the six CDRs and most natural structural and sequence diversity is found in the loop most central to the CDR3 of heavy chain (Chothia *et al.*, 1989). This has been exploited to create antibodies entirely outside their natural host. To construct a synthetic antibody library, V-genes are assembled by introducing a predetermined level of randomisation to the CDRs (possibly also the bordering framework region) of germline V-gene segments (Hoogenboom and Winter, 1992; Griffiths *et al.*, 1994; Nissim *et al.*, 1994; de Kruif *et al.*, 1995) or rearranged V-genes (Barbas *et al.*, 1992a). One of the main advantages of synthetic repertoires over naïve repertoires is that the contents, local variability and overall diversity of the library may be controlled and defined.

Given the importance of library size, more efficient techniques such Cre-*lox* recombination have been developed for the construction of large antibody libraries (Waterhouse *et al.*, 1993). In this process, termed also combinatorial infection, the heavy and light chain repertoires are combined on the same replicon within the bacterium by Cre catalysed recombination at *loxP* sites, generating a large number of combinations, potentially as large as the number of bacteria that have been infected. Libraries with large numbers of synthetic Fabs (close to 6.5×10^{10}; Griffiths *et al.*,1994) scFvs (3×10^{11}; Sblaterro and Bradbury, 2000) have been constructed by using Cre-*lox* recombination and antibodies specific for many different proteins and other antigens have been isolated from them.

3.1.4 pComBov: a system for the production of recombinant bovine antibodies

The pComBov phagemid has been designed and optimised for the construction of combinatorial libraries of bovine Fab fragments (O'Brien *et al.*, 1999). The vector is a modified form of pComb3H, in turn, a modified version of the pComb3 vector (Barbas *et al.*, 1991).

3.1.4.1 Composition and properties of phagemids pComb3, pCom3H and pComBov

The phagemid vector pComb3 is derived from the phagemid pBluescript (Stratagene) (Barbas *et al.*, 1991). It contains both the origin of replication of the

multicopy plasmid ColE1 and the origin of replication of the filamentous bacteriophage f1. pComb3 contains two *lac* promoters, one for the transcription of the heavy chain fusion to gIIIp and one for the transcription of the light chain. In this phagemid, two *pelB* leader peptides direct independent transport of each antibody fragment into the periplasm where natural assembly forms the Fab fragment. The carboxyl-terminal methionine of the *pelB* leader is fused to sequences encoding AQVKL and AE which proceed the sites of heavy and light chain insertion respectively. Furthermore, pComb3 contains the gene for the enzyme beta-lactamase that confers ampicillin (carbenicillin) resistance to bacteria that harbour pComb3. Since the vector contains the origin for replication of phage f1 but lacks the genes required for replication and assembly of phage particles, superinfection with helper phage is necessary for replication, assembly and display of the cloned Fab at the viral surface with native gIIIp. Removal of the sequence encoding gIIIp from pComb3 can be achieved by cleavage with *Spe*I and *Nhe*I restriction enzymes. Induction of the *lac* promoters then drives expression and export of the Fab sequence alone into the bacterial periplasm.

The pComb3H vector was designed to provide a human consensus sequence at the amino terminus of the heavy chain, replacing sequences encoding QVKLLE with EVQLLE. Furthermore, the duplicated sequences in pComb3 were removed. This includes one of the *lac* promoters, creating instead a dicistronic operon comprising light and heavy chain inserts. The *pelB* leader linked to the light chain was replaced by an *ompA* signal sequence. Typically, the vector is provided as pComb3HSS designating that the vector carries stuffer fragments between cloning sites for the immunoglobulin inserts. The heavy chain stuffer is 300 bp and the light chain stuffer is 1200 bp in length. Like pComb3, a *Nhe*I / *Spe*I digest allows for the expression of soluble Fab.

In the pComBov phagemid, some changes were introduced to pComb3H to modify it for bovine Fab library construction. DNA downstream of the unique *Nru*I site located in the bacterial leader, was replaced, retaining the *ompA* reading frame and adding codons for QAVLTQPSS, the native amino terminus of the bovine λ light chain, downstream of the predicted cleavage point of signal peptidase. Introduction of *Sac*I and *Bst*EII restriction sites enabled the insertion of V_L as *Sac*I / *Bst*EII fragments. The remainder of the λ constant domain (C_L) was amplified from bovine cDNA and inserted downstream of the light chain cloning site.

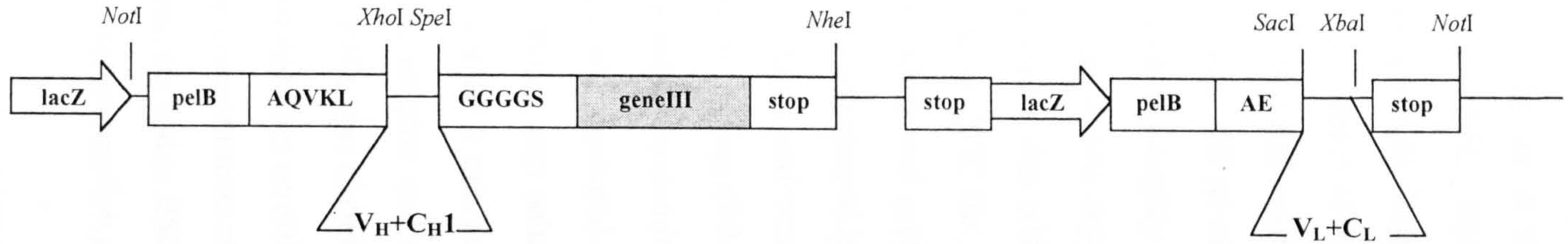
Sequences encoding the amino terminal residues of the heavy chain were replaced to provide the native bovine sequence QVQLRESGPS (Jackson *et al.*, 1992; Berens *et al.*, 1997; Saini *et al.*, 1997; Sinclair *et al.*, 1997). An *Rsr*II restriction site was embedded in the last three codons allowing insertion of V_H-C_H1 amplicons as *Rsr*II / *Spe*I fragments. As with pComb3H, digestion with *Spe*I and *Nhe*I removes the gIII sequence, allowing for expression of soluble Fab (O'Brien *et al.*, 1999).

Expression cassettes of pComb3, pComb3H and pComBov are shown in Figure 3.1.

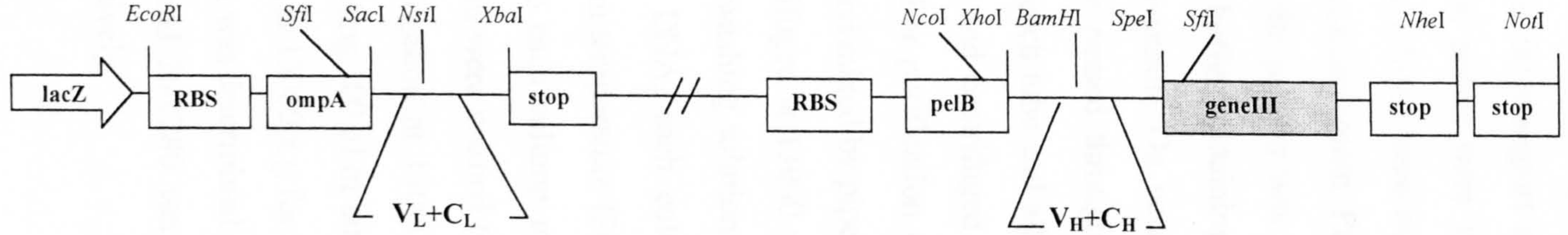
3.1.5 Objectives

The objectives of this aspect of the study were to improve upon existing methods for the construction of bovine Fab libraries in pComBov and to apply these techniques to construction and screening of a library from calves immunised against *Mannheimia haemolytica*. We envisaged that recombinant antibodies of defined specificity could be used to elucidate the contribution of individual factors to the pathogenic process through in *vitro* assay, organ culture or direct challenge studies.

pCom3



pCom3H



pComBov

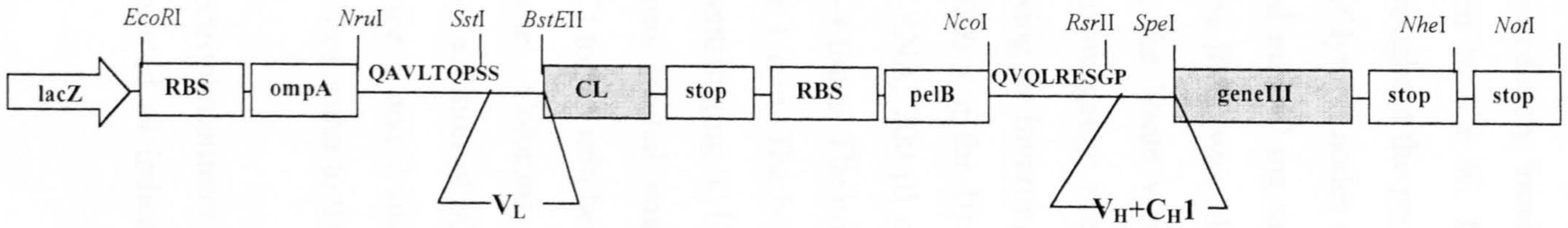


Figure 3.1 Schematic structure of phagemid vectors pCom3, pCom3H and pComBov. pCom3 is the backbone for construction of pCom3H and pComBov.

3.2 Materials and methods

3.2.1 RNA isolation

Total RNA was prepared by using the SV total RNA isolation kit (Promega) from seven lymph nodes of calves vaccinated against *Mannheimia haemolytica*. Tissue was packed in dry ice at slaughter for transport and immediately transferred to long-term storage at –70°C. The lymph nodes were provided by Dr W. Donachie, Moredun Research Institute, Edinburgh. Gloves were worn throughout the preparation of RNA to reduce the chance of RNase contamination. Pieces of lymph nodes were ground on dry ice by mortar and pestle and the powder was divided into 40 mg samples in eppendorf tubes. About 200 µl of lysis buffer containing 0.97% βME was added to each sample and mixed thoroughly by inversion. On occasions, the lysate was too viscous to be pipetted so it was repeatedly passed through a narrow gauge needle. 350 µl of SV dilution buffer was added to each tube and after mixing by inverting, the samples were incubated at 70°C for 3 min and centrifuged at 13000 x g for 10 min to remove the cellular debris and proteins. For purification of the RNA, 200 µl of 95% ethanol was added to each cleared lysate and mixed by pipeting 3-4 times. The mixture was loaded to a spin column and was centrifuged at 13000 x g for 1 min. The bound RNA was then washed by adding 600 µl of washing solution and centrifuging at 13000 x g for 1 min. To eliminate contaminating DNA, each column was treated with 50 µl of DNase solution and incubated at room temperature (20-25°C) for 15 min before addition of 200 µl of DNase stop solution to each column membrane. Columns were centrifuged at 13000 x g for 1 min and again were washed twice by addition of 600µl, then 250 µl of washing solution and centrifugation at 13000 x g for 1 and 2 min respectively. The bound RNA was eluted by adding 100 µl of nuclease-free water to the membrane of each column and then centrifuging at 13000 x g for 2 min.

The concentration of RNA was determined by spectrophotometry at 230, 260 and 280 nm and the ratios 260/230 and 260/280 were calculated as indices of guanidine and protein contamination respectively.

Concentration of RNA

To concentrate the RNA, NaCl was added to a final concentration of 0.1 M and after adding 2.5 volumes of cold absolute ethanol, the mixture was incubated at – 20°C for 30 min. It was then centrifuged at 10000 x g for 15 min at 4°C. The RNA pellet was

resuspended in nuclease-free water.

3.2.2 cDNA synthesis

Single and double strand cDNAs were prepared from RNA and were used as template to recover the light chain and heavy chain repertoires at different stages of the experiments described below.

Double strand cDNA synthesis

Double strand cDNA was synthesised using the Universal Riboclone cDNA Synthesis system kit (Promega). In all reactions, nuclease-free water was used to prevent degradation of the RNA or DNA. First strand synthesis was driven by avian myeloblastosis virus (AMV) reverse transcriptase using random hexamer and oligo(dT) primers in two separate reactions. In each reaction of 15 µl, 2 µl of primer (500 µg / ml) was added to about 20 µg of RNA (estimated to contain about 2 µg mRNA). The mixtures were heated to 70 °C for 10 min and then chilled on ice for 5 min. First strand cDNA synthesis was carried out in a 25 µl reaction containing 15 µl sample, 30 units of AMV reverse transcriptase, 40 units of RNasin ribonuclease inhibitor, 4 mM sodium pyrophosphate and 5 µl of first strand 5x buffer that provided 50mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM spermidine and 1mM of each dNTP. Reactions were incubated for 60 min at 37°C and 42°C for random hexameric primers and oligo(dT) primer respectively.

After incubation, 20 µl of each reaction was used in a second strand synthesis reaction of 100 µl. The reaction contained 23 units DNA polymerase I, 0.8 units RNase H and 40 µl of the second strand 2.5x buffer. The buffer provided 40 mM Tris-HCl (pH 7.2), 90 mM KCl, 3 mM $MgCl_2$, 3 mM DTT and 0.5 mg / ml bovine serum albumin. Reactions were incubated at 14 °C for 2 hrs and then were heated at 70°C to inactivate the enzymes. After centrifugation and incubation on ice for 5 min, 4 units of T4 DNA polymerase was added and the reaction temperature was raised to 37°C for 10 min. Ten µl of 200 mM EDTA was added to each sample to stop the reactions. cDNA was then isolated. An equal volume of TE-saturated phenol-chloroform-isoamyl alcohol was added to each reaction and mixed by inversion. To separate the aqueous phase, the tubes were centrifuged at room temperature for 2 min. cDNA was precipitated by using 0.1 volume 2.5 M sodium acetate and 2.5 volumes cold absolute ethanol. Mixtures were incubated at

–70°C for 30 min. The tubes were then centrifuged at high speed for 5 min. The pellets were washed with 0.5 ml cold 70% ethanol and were centrifuged again for 2 min. The supernatants were removed and the pellets were resuspended in 15 µl of TE buffer.

Single strand cDNA synthesis

Single strand cDNA was prepared by using the SuperScript II RNase H reverse transcriptase (Life Technologies). The general procedure was as described above for first strand cDNA synthesis with differences detailed as follows. One µl (500 µg / ml) of each oligo(dT) and random hexameric primers was used in separated 12 µl reactions, each containing 4 µg RNA and 1µl of 10mM dNTPs. Each mixture was incubated at 65 °C for 5 min and then on ice for 2 min. Four µl of 5x first strand reaction buffer, 2 µl of 100 mM DTT, 1 µl of RNasin ribonuclease inhibitor (40 units) were added to each reaction and after incubation at 42 °C for 2 min (reactions primed with random hexamers were held at 25 °C for 4 min before incubation at 42 °C), 1 µl of SuperScript II reverse transcriptase containing (200 units) was added to drive first strand synthesis. The 5x first strand buffer provided final concentrations of 50 mM Tris-HCl pH 8.3, 75 mM KCl and 3 mM $MgCl_2$. The reactions were incubated at 42 °C for 50 min with a preincubation of hexameric-primed reaction at 25 °C for 10 min. . Reactions then were inactivated at 70 °C for 15 min. To remove any remaining RNA, 2 units of RNase H was added, and reactions were incubated at 37 °C for 20 min.

3.2.3 Recovery of heavy (H) and light (L) chain repertoires

In different stages of this study, repertoires of the light chain variable region (V_L) and the heavy chain - the variable domain and most of the first constant region - were each amplified from cDNA prepared with oligo(dT) and random hexameric primers. PCR reactions were carried out with different polymerases including Taq DNA polymerase (Promega) and Pfu DNA polymerase (Promega). As discussed before, pComBov, the phagemid used for Fab library construction, contains a C_L gene and so only the V_L repertoire needs to be cloned. The vector also carries a stuffer fragment between cloning sites for the heavy chain inserts. In common with many domesticated animals, cattle use a limited range of V_L and V_H segments to establish the antibody repertoire. Thus few primers are required for amplification and recovery of the immunoglobulin repertoire. Primer sequences are shown in Table 3.9. The general conditions for PCR amplification

with Taq or Pfu are as follows. Where variation between the methods occurred, it is indicated.

Repertoire recovery with Taq DNA polymerase

PCR reactions were performed with 0.5-1 µl cDNA in a reaction volume of 50 µl with final concentrations of 500 µM dNTPs (Promega), 400 nM of each primer and 2-4 mM $MgCl_2$. In addition, the reaction buffer provided 50 mM KCl, 10 mM Tris-HCl (pH 9.0) and 0.1% Triton®X-100. Five units of Taq DNA polymerase (Promega) were used for amplification with the following cycling conditions. The reactions were heated to 94°C for 5 min and were then incubated for 35 cycles of 94 °C for 1 min, 55-57 °C for 1 min and 72 °C for 3 min, with a final incubation of 72 °C for 5 min.

Repertoire recovery with Pfu DNA polymerase

The V_L and heavy chain repertoires were amplified using Pfu polymerase with the following conditions. The PCR reactions, mostly with a volume of 50 µl, contained 1.5 units Pfu, final concentrations of 300 µM dNTPs, 400 nM of each primer, 1.5 µl cDNA, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1 mg/ml BSA, 0.1% Trition®X-100. The reactions were kept on ice prior to thermal cycling. Thermal cycling consisted of one cycle of 2-4 min at 94 °C for initial denaturing, 35 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 2.5 min, followed by final extension at 72 °C for 5 min .

3.2.4 Direct cloning of light chain segment

Firstly, direct cloning was chosen as strategy for cloning of light chain and heavy chain amplicons. Phagemid pComBov (O'Brien *et al.*,1999) was used as vector for Fab library construction.

Initially, efforts were made to clone the V_L segments. In total, five attempts were made to achieve this aim. In each attempt, potentially significant changes in protocol were introduced; these modifications are highlighted in the text which follows. In these attempts, the V_L repertoires were amplified from cDNA by using Taq DNA polymerase and primers VL1 and VL2. These primers were based on the known sequences of bovine light chains and contain recognition sites for *Sst*I and *Bst*EII respectively (O'Brien *et al,* 1999). After digestion with these enzymes, the amplicons could be cloned into

pComBov prepared with the same enzymes.

Procedures used in the first experiment are described in detail. For other experiments, only variations to this basic method are described. About 800ng of amplicons of the V_L repertoire and the pComBov vector were digested by 30 units of *Sst*I and *Bst*EII (Life Technologies). Digestion was done concurrently in buffer number 2 provided by manufacturer, incubating the reactions for 1.5 hrs at 37 °C and then 1.5 hrs at 60 °C. This should release the short sequence separating the light chain cloning sites in pComBov and also digest the termini of the V_L amplicons. After heat inactivation of the enzymes at 75 °C for 15 min, samples were run on agarose gels and pComBov and V_L DNA extracted from gel slices using extraction columns (Life Technologies) or QIAEXII resin (QIAGEN). They were then mixed and ligated.

In addition, pComBov DNA carrying a bovine light chain (pComBov-L) was digested with the same enzymes to provide control material. After digestion, a sample of the reaction was run on a gel to confirm that the pComBov-L had been digested successfully with each enzyme. The vector and light chain fragments were then isolated. Purified fragments and the pComBov-L digestion products prior to gel-purification were mixed in ligation reactions according to Table 3.2. Ligation was performed in 10-20 µl reactions with an insert/vector ratio of 2/1 ligations, 150 ng of vector DNA and 4 units of T4 DNA ligase (Life Technologies). Reactions were incubated at 16 °C for 16-20 hrs. Ligation reactions then were transformed into Epicurian Coli Solopack Gold supercompetent cells (Stratagene) using heat shock at 54 °C for 60 sec. After 1 hr incubation with shaking, 20 µl of each transformation reaction was plated onto LA containing 100 µg/ml carbenicillin, 12 µg/ml tetracycline and 1% glucose. Results showed that reaction C had significantly higher transformation efficiency than the other two reactions. Assessment for insert frequency was done by restriction analysis of plasmid DNA from 10 colonies from each transformation. Plasmids were isolated by QIAprep spin miniprep kit (QIAGEN) and were then cut by *Eco*RI (Promega). Digestion products were run on agarose gels and their sizes were compared with linearised pComBov and pComBov-L DNA. Insert frequency was also determined by PCR using Taq DNA polymerase and primers VL1 and VL2. Isolated PCR products from different clones were digested by *Eco*RII (Promega) to determine sequence diversity.

Colonies chosen from reaction A did not possess any inserts perhaps due to the incomplete digestion of pComBov and its subsequent religation. All colonies chosen

Table 3.2 Composition of ligation reactions for comparison of ligation efficiency with light chain fragments from two sources. + indicates the digest added to each ligation reaction.

	A	B	C
pComBov	+		
V_L amplicons	+	+	+
pComBov (released from pComBov-L)		+	+
Light chain fragment (released from pComBov-L)			+

from reaction C possessed insert and the *Eco*RII digestion profile indicated that 90% of them were identical, meaning that the majority of inserts were those released from pComBov-L, not the V_L amplicons prepared from lymph node cDNA. These data suggested a significant difference between the efficiency of restriction digestion between V_L amplicons and V_L fragments released from pComBov-L.

Dephosphorylation of vector

Since the first attempt to clone V_L amplicons into pComBov suggested vector religation was a complicating factor, we evaluated the effect of dephosphorylating pComBov after digestion with *Sst*I and *Bst*EII. Vector DNA was isolated from gel slices using the Concert™ gel extraction columns (Life Technologies). Dephosphorylation was performed in a 100 µl reaction using 1 µl of calf intestinal alkaline phosphatase (CIAP; Life Technologies) and incubation at 37 °C for 30 min. The reactions were stopped by addition of EDTA to a final concentration of 5 mM and heating to 56 °C for 30 min to inactivate the enzyme. DNA was extracted with phenol-chloroform and recovered in distilled water after ethanol precipitation. Ligations were set up with an insert/vector ratio of 3/1 using 12 units of T4 ligase (Life Technologies) for 16 hrs at 16 °C. Solopack Gold supercompetent cells (Stratagene) were used for transformation. These reactions failed to yield any transformants.

Variation to insert/vector ratio

In the next attempt, a range of insert/vector ratios - 1/1, 2/1, 3/1 and 4/1 - were tested. Twenty µl ligation reactions containing 9 units T4 ligase (Promega), 150 ng of pComBov (without any dephosphorylation treatment) were set up with digested V_L amplicons at the indicated ratios. Reactions were incubated at 16 °C for 18 hrs and then transformed into NovaBlue competent cells (Novagen). Again, these reactions failed to yield any transformants..

Study of the efficiency of restriction digestion of V_L amplicons

Following from the experiments described, we studied the efficiency with which V_L amplicons were digested.

- Cloning of light chain fragments released from plasmid DNA

In this attempt, light chain fragments released from a clone of pComBov-L were used for cloning. After digestion of pComBov and pComBov-L with *Sst*I and *Bst*EII, V_L fragments and vector fragments from each source were extracted separately using the Concert™ gel extraction system columns. Two series of reactions were set up for ligation of V_L fragments with each pComBov fragment using insert/vector ratios of 1/1, 1/2 and 5/1. In each series, an additional ligation reaction lacking the insert evaluated background due to the presence of vector cut at just one site. Transformation was carried out using NovaBlue competent cells (Novagen). This experiment revealed that the cloning problem could not be attributed to the pComBov vector.

- Elevated concentration of restriction enzymes

Finally, pComBov and 1µg of V_L amplicons produced with Pfu were digested with 150 units of *Sst*I and *Bst*EII. The digested DNA was run on an agarose gel and then isolated using QIAEXII resin. Insert and vector were added to 10 µl ligation reactions at ratios of 3/1 and 5/1. After overnight incubation at 16 °C, 2.5 µl of each ligation reaction was transformed into XL1 electrocompetent cells. The very low numbers of transformants isolated showed that the persistent resistance of V_L amplicons to digestion, perhaps due to the proximity of the restriction sites to the termini.

The difficulties encountered in cloning the V_L repertoire directly led us to change the strategy and to construct intermediate libraries of V_L and heavy chain fragments. In this way, release of the fragments after digestion guarantees their suitability for recloning into the phage display vector.

3.2.5 Construction of intermediate library

Dephosphorylated *Sma*I-cut pUC18 (Pharmacia) was chosen as the intermediate vector for the construction of libraries. Two separate pUC-V_L and pUC-H libraries were generated as described in the following protocols.

3.2.5.1 Intermediate V_L library

Amplification of the V_L repertoire

V_L repertoire was amplified from lymph node cDNA using Pfu DNA polymerase as

described in subheading 3.2.3. Twenty, 50 µl PCR reactions were set up for amplification of the repertoire using cDNA prepared with oligo(dT) and random hexameric primers. The PCR products were mixed and run on a 1% agarose gel. The band was excised and amplicons were extracted using columns from Concert gel extraction kits (Life Technologies). DNA was eluted in 400 µl of TE and its concentration was measured by spectrophotometry at 260 nm.

Phosphorylation and polishing reactions

The V_L repertoire was amplified with primers without 5' phosphate groups, and although Pfu polymerase should create blunt termini, it was judged wise to polish the amplicons. Phosphorylation and polishing were performed concurrently according to protocol described insubheading 2.2.2.2. with one variation: desalting of the final products was carried out with QIAEXII resin instead of ethanol precipitation.

Ligation

In total, six attempts were made to clone V_L amplicons into pUC18, each consisting of several ligation reactions of 10-20 µl, different ratios of insert/vector (5-50/1) and vector concentration of 20-50 ng/reaction. Since blunt end ligation requires about 50 times more T4 ligase than cohesive ligation, 1 µl of a high concentration product containing 400 units/µl (New England BioLabs) was used in each reaction. Ligation buffer provided final concentrations of 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 25 µg/ml BSA and 50 µM ATP. Following the supplier's recommendation, the concentration of ATP was reduced from 1mM to 50 µM to enhance ligation efficiency. Reactions were incubated at 15-16 °C for 18-24 hrs.

Transformation

For transformation of the ligated DNA, *E.coli* XL1-Blue electrocompetent cells were used. The cells were prepared according to a method described by Prof W. Barnes (http://barnes1.wustl.edu/wayne/electrocompetent.html). A few colonies of *E. coli* from an LA plate were picked into 30 ml of LB and media was incubated at 37 °C overnight with shaking. The following day, 10 ml of culture was inoculated in 1 litre 2xTY medium in a 2 litre flask and shaken at 37 °C until the optical density at 550 nm reached between 0.4 and 0.5. The culture then was chilled on ice to prevent further growth, it was

dispensed into six, sterile 250 ml bottles. After centrifugation at 6000 rpm, the supernatant was poured off and the pellets were resuspended in 1/3 initial culture volume of cold 10 % glycerol in Millipore water with minimal force. The mixture was again spun and washed 3 more times with successively smaller volumes of cold 10 % glycerol until the volume of suspension was twice the cell pellet volume. All washing steps and centrifugations were carried out on ice and at 4 °C respectively. Cells were divided into 100 µl aliquots and were transferred into liquid nitrogen for storage at -80 °C.

For transformation by electroporation, the prepared competent cells (100µl) were gently thawed on ice. 1-4 µl of ligation reaction was added to the cells and after mixing, the suspension was added to the bottom of a cold, 1 mm electroporation cuvette (Filtron). The cuvette was placed in the electroporator (BioRad) and pulsed once at 25 µF, 1.7 kV and 200 Ω. Nine hundred µl SOC medium was immediately added to the cuvette. After mixing, cells were transferred to a 1.5 ml eppendorf tube, and incubated at 37 °C with shaking for 1 hr. From 1 ml of culture, 10-20 µl was plated on LA containing 100 µg/ml ampicillin, 40 µl of 4% (w/v) X-gal and 40 µl of 25 mM IPTG and incubated at 37 °C overnight. The size of the library was measured according the number of white colonies recovered from each transformation.

Assessment of intermediate pUC-V_L library

To assess the insert frequency in the pUC-V_L library, plasmids from 20 white colonies were isolated as described in Appendix 2. DNA was digested with *Eco*RI (Promega) and run on 1 % agarose gels. The size of the plasmids was compared with the size of cut pUC18 and control pUC-V_L plasmids known to contain inserts. In addition, insert frequency was determined by PCR using M13/pUC forward and reverse flanking primers (sequences shown in Table 2.1). PCR was performed with Taq DNA polymerase under the conditions described in subheading 3.2.3 with a final concentration of 3.5 mM $MgCl_2$ and an annealing temperature of 56 °C. The PCR products were run on 1.2% agarose gels to check the size of inserts.

The diversity of inserts carried in the pUC-V_L was determined by *Eco*RII digestion of Taq amplicons from clones picked at random. DNA profiles were analysed on 3% agarose gel for comparison.

3.2.5.2 Intermediate H fragment library

For construction of the pUC-H library, PCR conditions, phosphorylation and polishing, ligation, transformation, were identical to those described for the pUC-V_L library. Primers VH1 and VH2 were used for amplification of heavy chain repertoire. These carry recognition sequences for *Rsr*II and *Spe*I respectively. To characterise the library, 40 colonies were analysed.

3.2.6 Recloning of V_L and H fragment repertoire into pComBov

After construction of pUC-V_L and pUC-H intermediate libraries, we planned to release the V_L and heavy chain repertoires for recloning into pComBov.

3.2.6.1 Recloning of the V_L repertoire

Growth of the pUC-V_L library

Transformation mixtures containing the pUC-V_L library were mixed together and the volume adjusted to 200 ml with 2xTY media containing 150 µg/ml ampicillin and 1% glucose. The culture was incubated at 37 °C with shaking for 2 hrs. Ten ml of the culture was taken and after adding 5 ml of 50% sterile glycerol, it was stored at -80 °C as a stock library. The remaining culture was centrifuged at 6000 rpm for 10 min, the supernatant was discarded and the pellet was resuspended in 200 ml of 2xTY containing 200 µg/ml of ampicillin and 1% glucose. Culture was incubated overnight at 37 °C with shaking.

Preparation of the V_L repertoire and pComBov DNA for ligation

The following day, plasmid DNA was isolated using a Midiprep plasmid purification system (Life Technologies). A sample of the isolated DNA was run on an agarose gel to check its concentration. Since some smearing was evident, 2 ml of DNA was loaded to a large preparative gel and the band corresponding to pUC-V_L was extracted with QIAQuick® Gel extraction kit (QIAGEN). The concentration of the prepared DNA was measured by spectrophotometry.

Restriction digestion

Plasmid DNA from the pUC-V_L library and pComBov were digested with *Sst*I and *Bst*EII to obtain the V_L fragment and the cut phagemid. Firstly, about 7 µg of pUC-V_L

plasmid was digested with 70 units *Bst*EII (Life Technologies) in a volume of 380 µl. In a separate reaction of 300 µl, 70 units of *Bst*EII was used to cut about 7.5 µg of pComBov. Buffering was according to the manufacturer's recommendation. Reactions were incubated at 60 °C for 3.5 hrs. *Bst*EII and *Sst*I (Life Technologies) work in the same buffer. Therefore, 200 units of *Sst*I and 10 µl of reaction buffer were added to the pUC-V_L reaction and volume was increased to 485 µl with distilled water. Similarly, 100 units of *Sst*I, 10 µl reaction buffer and 85 µl distilled water were added to the pComBov reaction. Reactions were incubated at 37 °C for another 3.5 hrs. After inactivation at 70 °C, the digested samples were run on 0.7% agarose gels and the bands (released V_L 350 bp, pComBov 4 kb) extracted using QIAQuick® Gel extraction kit. The concentrations of extracted DNA were estimated by running 3 µl of each sample on an agarose gel.

Cloning of the V_L repertoire into pComBov

To clone the V_L repertoire into *Bst*EII / *Sst*I cut pComBov, two series of ligation reactions were set up. The first series consisted of two ligation reactions as controls for religation of the vector with different amounts of T4 DNA ligase. T4 DNA ligase was used at concentrations of 6 units/reaction (Promega) and 400 units/reaction (New England BioLabs). In the second set, 4 and 6 reactions were allocated to the cloning of V_L fragments into pComBov, with T4 DNA ligase at 6 units/reaction and 400 units/reaction respectively. About 150 ng of cut pComBov was used in all reactions with about 50 ng of V_L fragment to provide an insert/vector ratio of about 5/1. (Table 3.3). Reactions were incubated at 16 °C for 18 hrs.

Transformation of ligated materials

E.coli XL1-Blue electrocompetent cells were prepared and transformed by electroporation as described in subheading 3.2.5.1. Two µl of each ligation reaction was transformed into 100 µl of competent cells and after adding 900 µl SOC medium, 20 µl was plated on LA containing 50 µg/ml carbenicillin and 15 µg/ml tetracycline. Plates were left at 37 °C overnight and next day the colonies were counted. There was a marked difference between cloning transformations and controls for vector self-ligation (Table 3.14). Hence, further cloning transformation were performed.

Table 3.3 Composition and volume of reagents (µl) used for cloning V_L fragments into pComBov to construct pComBov-V_L library. Each series of ligation reactions were performed at two concentrations of T4 DNA ligase.

	Self ligation control		V_L / pComBov ligation	
T4 DNA ligase 3 U / µl	2	-	2	-
T4 DNA ligase 400 U / µl	-	1	-	1
Appropriate 10 x reaction buffer	1	1	1.6	1.5
V_l fragment ~6-7 ng / µl	-	-	9	9
pComBov ~40-50 ng / µl	3.5	3.5	3.5	3.5
Insert/ Vector ratio	-	-	5 / 1	5 / 1
Distilled water	3.5	3.5	-	-
Number of ligation reactions	1	1	4	6

Characterisation of the pComBov-V_L library

Fifteen colonies from the pComBov-V_L library were picked to determine insert frequency. They were inoculated into 5 ml LB and incubated at 37 °C overnight with shaking. Next day, plasmid DNA was isolated using the QIAprep spin miniprep kit as described in Appendix 2. Seven µl of each plasmid was digested with *Eco*RI at 37 °C for 1.5 hrs. Insert frequency was determined by running the digested DNA on a 0.7% agarose gel with linearised plasmid from a confirmed pComBov-V_L clone as control. In addition to this, all isolated plasmids and the control pComBov-V_L sample were subjected to PCR using VL1 and VL2 primers, using an annealing temperature of 56 °C.

3.2.6.2 Recloning of H fragment into pComBov-V_L library

Having recloned the V_L repertoire into pComBov, the last stage of Fab library construction required release of the heavy chain repertoire from the pUC-H library and recloning into the pComBov-V_L library. Several attempts were made to achieve this objective: protocols for preparing DNA, digestion, ligation conditions and transformation are described in detail in the following sections. Variations to these conditions then follow.

Growth of the pUC-H and pComBov-V_L libraries

Transformation mixtures of the pUC-H and pComBov-V_L libraries were mixed, and their volumes adjusted to 265 ml and 250 ml respectively with 2xTY and glucose to a final concentration of 1%. Ampicillin and carbenicillin were added to the pUC-H and pComBov-V_L cultures respectively to a final concentration of 75 ng/ml. Cultures were incubated at 37 °C for 2 hrs with shaking. From each library, 10 ml was sampled and 5 ml of 50% sterile glycerol was added to each before storage at -80 °C as stocks. The remaining cultures were centrifuged at 6000 rpm for 10 min, supernatants were discarded and the pellets were resuspended in 200 ml 2xTY containing 100 ng/ml carbenicillin and 1% glucose. They were then incubated at 37 °C overnight with shaking. Stock libraries were resampled for subsequent plasmid isolations.

Preparation of pUC-H and pComBov-V_L DNA for ligation

Plasmid DNA from the pUC-H and pComBov-V_L libraries was isolated using QIAprep spin miniprep, Miniprep and Midiprep plasmid reagents. To improve the thoroughness of the purification and to concentrate the isolated material, DNA was run

on 0.7% agarose gels, the relevant bands were excised and DNA was extracted using Concert gel extraction system columns. The concentration of DNA was estimated by running 5 µl samples on a 0.7% agarose gel.

Restriction digestion

In separate reactions, about 13 µg of pUC-H and 6 µg of pComBov-V_L DNA were digested concurrently with *Rsr*II and *Spe*I (Life Technologies). For digestion of pUC-H and pComBov-V_L, 120 units and 100 units of *Rsr*II and 80 units and 70 units of *Spe*I were used respectively. Reaction conditions were controlled with buffer 5 with a final concentration of 1 mM dithiothreitol (DTT) (Table 3.4). According to the manufacturer's information, *Spe*I cuts DNA with 75% efficiency under these conditions. The reactions were incubated at 37 °C and after 4.5 hrs, samples were assessed on an agarose gel to verify that the DNA had cut as expected. Since the heavy stuffer in pComBov carries an *Rsr*II site, digestion of pComBov-V_L was expected to release fragments of 100 and 250 bp after digestion with *Rsr*II and *Spe*I. pUC-H should release a heavy chain fragment of about 660 bp with these enzymes. After confirmation, each entire reaction was run on a 0.7 % agarose gel and the bands of interested - the fragment corresponding to the heavy chain repertoire and the prepared pComBov-V_L vector - were isolated using Concert gel extraction reagents. The concentration of each DNA was estimated by running of 2 µl of eluted DNA on an agarose gel before setting up ligation reactions.

Ligation

Two sets of ligations, with low and high concentrations of T4 ligase, were set up for cloning the heavy chain fragments into the pComBov-V_L library (Table 3.5). In the first set, 6 units of T4 ligase (Promega) was used to ligate 160 ng of pComBov-V_L and about 100 ng of the heavy chain repertoire in 3 reactions of 16 µl. The second set comprised 7 reactions of 15 µl containing the same amounts of vector and insert but with 100 units of T4 ligase (New England BioLabs). Each set of reactions also included a control reaction containing cut vector but no insert to check for vector religation. Ligation reactions were incubated at 16 °C for 18 hrs.

Table 3.4 Composition and volumes (µl) of reagents used in restriction digests required for recloning of H fragment into pComBov-V_L library.

	pUC-H library	pComBov-V_L
DNA	**360**	**200**
***Rsr*II 20 U/µl**	**6**	**5**
***Spe*I 10 U/µl**	**8**	**7**
Reaction buffer No 5	**46**	**27**
DTT 10 mM	**46**	**27**
Reaction volume	**466**	**266**

Table 3.5 Composition and volume of reagents (µl) used for cloning heavy chain fragments into pComBov-V_L library. Each series of ligation reactions were performed at two concentrations of T4 DNA ligase.

	Self- ligation control		H / pComBov-V_L ligation	
T4 ligase 3 U/µl	**2**	**-**	**2**	**-**
T4 ligase 400 U/µl	**-**	**1**	**-**	**1**
Appropriate 10x reaction buffer	**1.6**	**1.5**	**1.6**	**1.5**
Heavy chain fragment 15 ng/µl	**–**	**–**	**6.4**	**6.4**
pComBov-V_L 25 ng/µl	**6.6**	**6.6**	**6.6**	**6.6**
Insert / vector ratio	**-**	**-**	**4 / 1**	**4 / 1**
Distilled water	**6.4**	**6**	**-**	**0.5**
Reaction volume	**16.6**	**15.1**	**16.6**	**16**
Number of ligation reactions	**1**	**1**	**3**	**7**

Transformation

E. coli XL1-Blue supercompetent cells (Stratagene) were used for transformation. After thawing the cells on ice from -80 °C, 0.85 µl of 1.42 M of β-mercaptoethanol provided with the cells was added to each 50µl of cells giving a final concentration of 25 mM. Cells were kept on ice before adding 2.5 µl of each ligation reaction. They were then incubated on ice for a further 30 min. Transformation with 0.1 ng of circular pUC18 plasmid (provided with the cells) was used to estimate transformation efficiency. After 30 min incubation on ice, the cells were heat shocked by transfer to a water bath at 42 °C for 45 sec, then return to ice for 2 min before addition of 950 µl SOC medium. The cultures were incubated in 37 °C with shaking for 1 hr and then 20 µl of each was plated on LA containing 50 µg/ml carbenicillin, 12 µg/ml tetracycline and 1% glucose. Plates were incubated at 37 °C overnight and then checked for the appearance of colonies.

Analysis of ligation reactions by gel electrophoresis

Aside from transformation, samples from some ligations were run on an agarose gel in an attempt to identify a link between the migration of DNA and transformation efficiency.

As mentioned above, several attempts were made to clone the heavy chain fragment. In each attempt, one or more reaction parameters were changed as described in the following sections.

Variation in the source of restriction enzymes

Since the first attempts at cloning were unsuccessful, *Rsr*II and *Spe*I from New England Biolabs were used to digest the DNA. The experiments assessed the impact of the source of enzymes on digestion efficiency. The protocols for preparation of DNA, purification, digestion conditions, ligation and transformation were as described above except that the ratio of insert/vector was 3/1.

Variation in competent cells

In this experiment, *E.coli* XL1-Blue supercompetent cells (advertised transformation efficiency of >1 x 10^9 cfu/µg of pUC18 DNA) were replaced by Epicurian Coli Solopack Gold supercompetent cells (Stratagene). One µl of β-mercaptoethanol was added to 50

µl of cells and the cells were heat shocked at 54 °C for 60 sec. The restriction enzymes *Rsr*II and *Spe*I were from Life Technologies and at ligation, the ratio of insert/vector was 3/1. Ligation was performed using 100 and 6 units (from New England Biolabs and Promega respectively) of T4 DNA ligase in separate reactions. Reactions were incubated at 16 °C for 19 hrs. The ligation reactions also were run on an agarose gel to analyse the profile of ligation products.

In experiments performed up to this stage, cloning attempts were unsuccessful. Analysis of the profile of ligation products showed the presence of bands of higher molecular weight than linearised pComBov-V_L, a band corresponding to a dimer of the heavy chain fragment (about 1300 bp), but no evidence of circular DNA which might be pComBov-V_L containing a heavy chain insert. An experiment was set up to characterise the 1.3 kb band. Two ligation reactions were separately digested with *Rsr*II or *Spe*I (Life Technologies). Each 10 µl reaction contained 6 µl of ligation mix, the reaction buffer provided by Life Technologies, and 10 and 20 units of *Rsr*II and *Spe*I respectively. The digests were incubated at 37 °C for 2.5 hrs and the reactions were then run on an agarose gel for analysis. Results indicated that the dimer was cut by *Spe*I but not by *Rsr*II.

Variation to insert/vector ratio

Since gel analysis showed the tendency for heavy chain dimer formation, further ligations were set up with an insert/vector ration of 1.5/1, as opposed to the ratios of 5/1 and 3/1 used in previous attempts. In addition, a control ligation reaction was prepared. This contained DNA linearised by digestion with *Eco*RI. The DNA was isolated from a clone known to carry a pComBov construct with both V_L and heavy chain inserts. XL1-Blue supercompetent cells were used for transformation. Although gel analysis revealed the disappearance of the heavy chain dimer and evidence of re-circularisation of control DNA, only a single colony was obtained and this was from the re-circularisation reaction.

The effect of pComBov derivatives on survival of bacterial cells

After observing very low transformation efficiencies with ligation of linearised pComBov carrying V_L and heavy chain inserts (pComBov-V_LH), a control experiment was arranged to assess if these constructs had an adverse effect on cell growth. About 50

ng of the pComBov-V_L library and a pComBov-V_LH clone were transformed into 50 µl of XL1-Blue supercompetent cells. The high level transformation efficiency confirmed that pComBov derivatives containing the bovine light chain or both light and heavy chains do not affect cell survival.

The effect of gel extraction methods

The tendency of heavy chain fragments to form dimers only at the *Spe*I-cut termini, and the low ligation efficiency of linearised pComBov-V_LH suggested a basic problem of ligation between vector and insert. One potential explanation was degradation of the termini during fragment isolation. Hence different gel extraction methods were studied with control DNA. In two separate digestion reactions of 130 µl, about 2.5 µg of pComBov DNA was cut with *Eco*RI (Promega) or *Pvu*II (Life Technologies) in reaction buffers supplied by the manufacturer. These digests produced protruding and blunt-ended fragments respectively. After inactivation at 65 °C for 20 min, each reaction was divided to 3 parts. The first sample from each reaction was precipitated with sodium acetate and ethanol, the second sample was extracted using QIAEXII resin and the third sample was run on an 0.7% agarose gel before reisolating with extraction columns (Life Technologies). DNA was eluted in water of 30, 35 and 40 µl respectively. Four µl of each purified sample was run on an agarose gel to determine the concentration of DNA. About 200 ng of the three samples prepared from *Eco*RI-cut DNA were religated using 6 units of T4 ligase (Promega). The same amounts of *Pvu*II-cut DNA were religated with 6 units (Promega) and 400 units (New England BioLabs) T4 ligase in separate reactions (Table 3.6). Seven and half µl of each ligation reaction was run on an agarose gel to analyse the changes arising from ligation. Two µl of each of the *Eco*RI-cut samples was transformed to XL1-Blue cells. Since digestion of pComBov with *Pvu*II yielded five fragments, these reactions were not transformed. The profile of ligation products from these reactions revealed differences between the religation of column extracted DNA and other isolation methods in both low and high concentrations of T4 ligase. Similarly, there were marked differences in the numbers of colonies arising from ligation of column-extracted DNA and the other two methods (details are discussed in Results section).

Following from this experiment, another attempt was made to clone the heavy chain repertoire into the pComBov-V_L library. Plasmid DNA from pUC-H and pComBov-V_L libraries was digested with *Rsr*II and *Spe*I (Life Technologies) using conditions

Table 3.6 Volume and composition of reactions to religate *Eco*RI and *Pvu*II-cut pComBov.

	Religation of *Eco*RI and *Pvu*II cut DNA	Religation of *Pvu*II cut DNA
T4 ligase 3 U/µl	2	-
T4 ligase 400 U/µl	-	1
Related reaction buffer	1.5	1
pComBov ~35 ng/µl	6	6
Distilled water	5.5	2
Reaction volume	15	10

described above. DNA fragments for this experiment were extracted from gel slices using QIAEXII resin. Ligation reactions were set up with an insert/vector ratio of 3/1 and high and low concentrations of T4 ligase. Ligation products were transformed into XL1-Blue supercompetent cells. The experiment again failed to yield transformants.

Gel composition and extraction methods

In this experiment, multiple purpose agarose was replaced with low melting point (LMP) agarose to ease the dispersal of gel containing the desired DNA fragments. Linearised DNA was prepared by digesting pComBov-V_L with *Sst*I (Life Technologies). Digestion products were run on a 0.7% LMP agarose gel. Gel slices were divided to two parts: one was extracted with QIAEXII resin; to the second, two volumes of TE buffer were added and after heating to 50 °C, the solution was extracted with phenol-chloroform extraction and finally precipitated with ethanol. Two high and low concentrations of T4 ligase were used to religate the linearised DNA. After transformation into XL1-Blue supercompetent cells, no significance difference between the two extraction methods could be observed.

Although the numbers of colonies recovered in this experiment was much lower than expected, another attempt was made to clone the heavy chain repertoire into pComBov-V_L library, using electrophoresis through LMP agarose, phenol-chloroform extraction and ethanol precipitation to isolated DNA fragments for ligation. Analysis of the ligation reactions showed a strong dimer band and no evidence of successful insertion of the heavy chain fragment into pComBov-V_L. Restriction analysis of the dimer again revealed it was cut into two fragments of about 650 bp by *Spe*I but not by *Rsr*II.

Temperature-cycle ligation

It has been reported that temperature-cycle ligation (TCL) can achieve higher ligation efficiency than constant temperature reactions by balancing the different requirements for high enzyme activity and DNA annealing (Lund *et al.*, 1996). This was evaluated by seeking religation of DNA from known pComBov-V_L after digestion with *Eco*RI (Promega). The linearised DNA was divided into three fractions. Two samples were extracted with QIAEXII resin or ethanol precipitation. The third samples was run on an LMP agarose gel and then isolated with Wizard reagents (Promega). About 130 ng of each extracted DNA was religated using 6 units of T4 ligase (Promega) and overnight temperature cycling of 30 sec at 10 °C, 1 min at 13 °C and 16 °C and 30 sec at 19 °C, 20

°C and 22 °C. After overnight thermal cycling, the ligation reactions were run on an agarose gel to evaluate the profile of DNA fragments and also transformed into XL-1 Blue supercompetent cells. Gel electrophoresis showed evidence of circular DNA and the number of colonies from transformation showed that high religation efficiency had been achieved in each of the three methods.

Given these results with the religation of linearised pComBov-V_L, it was decided to use this method in another attempt to clone the heavy chain repertoire into the pComBov-V_L library. DNA from pUC-H and pComBov-V_L libraries was digested by *Rsr*II and *Spe*I (Life Technologies), the samples were run on an LMP agarose gel and bands were extracted using Wizard reagents. Ligation was carried out at insert/vector ratios of 3/1 and 4/1, with 100 ng and 150 ng of vector in each reaction and 6 units of T4 ligase (Promega). Thermal cycling was carried out overnight, each cycle comprising 20 sec at 10 °C, 1 min at 13 °C and 16 °C and 30 sec at 19 °C, 22 °C and 25 °C. Ligation reactions were run on an agarose gel. Since the DNA profile showed the presence of heavy chain dimer but no evidence of circularised pComBov-VLH, transformation was not carried out.

During these experiments, it was noted that digestion of DNA from the pUC-H library with *Rsr*II and *Spe*I yielded a band corresponding to the heavy chain fragment and a second of about 100 bp. Restriction analysis revealed that a high number of clones in the pUC-H library carried inserts with *Spe*I sites at each terminus and an *Rsr*II site in the middle of the insert. Although this would not affect release of the *Rsr*II / *Spe*I heavy chain fragment, 3 clones from the pUC-H library were sequenced. The data showed complete identity between the 3 inserts. This led us to conclude that certain clone(s) dominated the pUC-H library perhaps due a marginally shorter generation time during repeated amplification of the library in preparing DNA for the experiments described above.

This factor, coupled with the failure to construct a pComBov-V_{LH} library via a pUC-H intermediate step, led us to a re-evalutation of the direct cloning strategy.

3.2.7 Direct cloning of the heavy chain repertoire into the pComBov-V_L library

As described at the beginning of this chapter, attempts to clone the bovine V_L repertoire directly into pComBov were not successful. It was concluded that the close proximity of *Sst*I and *Bst*EII sites to the termini of the V_L amplicons (7 and 10 bp

respectively) probably reduced substantially the efficiency of digestion. However, attempts to clone the heavy chain repertoire from an intermediate pUC-H library into the pComBov-V_L library failed. Therefore, a new approach to the direct cloning of heavy chain amplicons was adopted with the aim of increasing the digestion efficiency of *Rsr*II and *Spe*I. Two new heavy chain primers (VH1A and VH2A) were designed, extending the 5' ends of VH1 and VH2 respectively. The efficiency of action of *Rsr*II and *Spe*I on heavy chain amplicons was evaluated before starting further cloning.

Efficiency of restriction digestion of VH1A / VH2A amplicons

RNA isolation and single strand cDNA preparation were performed as described in subheadings 3.2.1 and 3.2.2 respectively. cDNA and a single pComBov-V_LH clone carrying light and heavy chain fragments were used as templates for Taq DNA polymerase amplification using different pairs of primers VH1, VH1A, VH2, VH2A and gIII. PCR products were desalted and concentrated by running on an agarose gel and then extracted using Concert gel extraction columns. Recovered DNA samples were digested with *Rsr*II and *Spe*I separately or concurrently (Table 3.7) and were run on a 3% agarose gel with 10 bp ladder DNA markers (Life Technologies).

Cloning of heavy chain repertoire amplicons

Since the termini heavy chain amplicons recovered with primers VH1A and VH2A were digested successfully by *Rsr*II and *Spe*I, these Taq amplicons were used to construct the pComBov-V_LH library. DNA from the pComBov-V_L library was prepared as described above. The heavy chain amplicons and pComBov-V_L DNA were cut with *Spe*I and *Rsr*II sequentially. First 6 µg of each DNA was digested with 50 units of *Spe*I (Life Technologies) for 2 hrs at 37 °C and 1 hr at 50 °C. The digested DNA was run on a 1% LMP agarose gel and the desired bands were extracted using Concert gel extraction columns. The extracted DNA was then cut with 80 units *Rsr*II (Life Technologies), run on a 1.5% LMP agarose gel and extracted again. The efficiency of each digestion was assessed by electrophoresis. In addition, DNA from a single clone of pComBov-V_L was cut with *Eco*RI to use as a control for ligation efficiency. Ligation reactions contained about 150 ng of vector with an insert/vector ratio of 3/1, 100 units T4 ligase (New England BioLabs) and 1x reaction buffer. An additional control ligation was performed under the same conditions but without the insert. After 38 hrs incubation at 15 °C, 4 µl of

Table 3.7 Digestion of heavy chain amplicons recovered with different primers combinations. + shows successful digestion was carried out with the restriction enzyme indicated; a blank cell indicates that the digest was not performed.

			Restriction enzyme		
		Amplicon	*Rsr*II	*Spe*I	*Rsr*II-*Spe*I
Template	cDNA	VH1-VH2	+	+	+
		VH1A-VH2	+	+	+
		VH1A-VH2A			+
	pComBovV_LH	VH1-VH2	+	+	+
		VH1A-VH2	+	+	+
		VH1A-VH2A		+	
		VH1-gIII		+	

each reaction was run on a 1% agarose gel to evaluate the DNA profile and 4 µl of each mixture was transformed into XL1-Blue supercompetent cells. Although the number of colonies recovered from cloning the heavy chain repertoire was significantly less than that from religation of linearised control DNA, 8 colonies were characterised to assess the insert frequency. This was done by digesting plasmid DNA with *Eco*RI. Since non of the clones contained insert and the efficiency of ligation was low, conditions were modified as described below.

Heavy chain inserts from plasmid DNA as controls

A comparison was made between the efficiency of cloning heavy chain repertoire amplicons and a heavy chain fragment released from a circular vector. Concurrent digestion with *Rsr*II and *Spe*I (Life Technologies) was used to release the heavy chain fragment from a single clone of pComBov-V_LH, to prepare DNA from the pComBov-V_L library and to cut amplicons of the heavy chain repertoire. Digestion products were run on an agarose gel and the desired bands were extracted using Concert™ gel extraction columns. These bands included the amplicons of the heavy chain repertoire, vector fragment from the pComBov-V_L library, the vector fragment from the single pComBov-V_LH clone, and its released heavy chain fragment. To guarantee that the vectors were successfully digested, they were run on a second gel and extracted again. Ten µl ligation reactions were set up with two low (Promega) and high (New England BioLabs) concentrations of T4 ligase. Each reaction contained 120 ng of vector and 60 ng of insert to provide an insert/vector ratio of 3/1. Details are shown in Table 3.8. The ligation reactions were incubated at 16 °C for 23 hrs before transformation into XL1-Blue supercompetent cells.

Variation to restriction enzymes, extraction methods and competent cells

The failure to recover transformants in the previous experiment despite high transformation efficiencies with control pUC18 DNA showed an obstacle to ligation existed. In an attempt to define this problem, amplicons of the heavy chain repertoire and pComBov-V_L library DNA were cut concurrently with *Rsr*II and *Spe*I (New England BioLabs) in buffer number 2 provided by the manufacturer. After inactivation at 65 °C, DNA samples were run on a 1.3% LMP agarose gel and the desired bands were extracted using QIAEXII resin. Ligations were set up in two 20 and 185 µl reactions

Table 3.8 Content of ligation reactions for comparison of cloning efficiency of heavy chain fragments from two different sources.

	Control ligation	Heavy chain repertoire amplicons		Heavy chain fragment released from pComBov-V_LH clone	
T4 DNA ligase 3 U / µl	-	-	1	-	1
T4 DNA ligase 400 U / µl	1	1	-	1	-
Appropriate 10 x reaction buffer	1	1	1	1.2	1.2
Insert	-	4	4	6	6
pComBov-V_L ~30 ng / µl	4	4	4	4	4
Insert/ Vector ratio	-	3 / 1	3 / 1	3 / 1	3 / 1
Distilled water	4	-	-	-	-
Number of ligation reactions	1	4	4	4	4

with 400 and 800 units T4 ligase (New Engand BioLabs), 225 and 2400 ng of vector respectively and an insert/vector ratio of 3/1. After 18 hrs incubation at 16 °C, DNA from the larger volume reaction was extracted with phenol-chloroform and precipitated with ethanol. Epicurian coli SURE2 supercompetent cells (Stratagene) were used for transformation. One µl of β-mercaptoethanol provided by the manufacturer was added to each 50 µl aliquot of thawed cells and after 10 min, ligation products from all reactions were added. After 30 min incubation on ice, the cells were heat shocked at 42 °C for 30 sec, incubated on ice and recovered in 950 µl of SOC medium. After 2 hrs shaking at 37 °C, 30 µl from each transformation reaction was plated on two LA media, one containing 120 µg ampicillin, 12 µg tetracycline and 1% glucose and the second containing 120 µg ampicillin, and 1% glucose. Since no colonies were recovered after overnight incubation, the entire transformation reactions were then plated onto single plates.

Table 3.9 Sequences of primers used for amplification of bovine V_L and heavy chain fragment repertoires and restriction sites on them.

VL1:	TAGAGCT*CCGTGTCCGTSWMYCTGGG *SstI*
VL2:	GTAGAGGTAG*GTCACCGAAGGTGGGGACTTGGG *BstEII*
VH1:	ATATATACG*GACCGAGCCTGGTGAAGCCCTCACAGA *RsrII*
VH1A:	AGTACTATAGTATACG*GACCGAGCCTGGTGAAGCCCTCACAGACC *RsrII*
VH2:	TGGGCAA*CTAGTAACAGCCTTGTCCACCTTGGTGC *SpeI*
VH2A:	TGATGGTCTGCATGTA*CTAGTAACAGCCTTGTCCACCTTGGTGC *SpeI*
gIII:	TCGGCATTTTTCGGTCATTAGC

* All primers are shown 5' to 3'

3.3 Results

3.3.1 RNA isolation

Total RNA was prepared from the lymph nodes of an immunised calf using the Promega SV total RNA isolation kit. The recoveries of RNA were measured by spectrophotometery at 260 nM. In Table 3.10, the concentrations of RNA from different samples are expressed in µg/100 µl of recovered solution and the ratios of absorbances at 230, 260 and 280 nm (A260/A230 and A260/A280) are shown as indicators of the quality of the RNA. The amount of total RNA recovered from two samples of bovine liver - controls for the efficiency of the isolation method – are also shown in the table. The manual for the isolation kit does not indicate the average yield of RNA from lymph nodes. However, in Table 3.11 average recoveries in this study are compared with typical data presented in the manual for a selection of mouse tissues. Recoveries of RNA from 30-40 mg of bovine lymph node tissue compared reasonably well with results considered typical by the kit manufacturer. This weight of tissue perhaps undergoes more complete cell destruction in the lysis solution than larger amounts. Similarly, losses may have less impact on final yield than when smaller starting amounts of tissue are used.

The quality of RNA isolated was monitored by A260/A230 and A260/A280 ratios. Samples prepared with higher amounts of bovine lymph node were of better quality by these criteria than from smaller amounts of starting material. The expected ranges of A260/A230 and A260/A280 are 1.8-2.2 and 1.7-2.1 - lower values may indicate guanidine or protein contamination respectively. However it should be noted that the variation between starting materials and procedures from day to day can impact significantly (Table 3.10). Overall, the A260/A230 and A260/A280 ratios obtained for the bovine RNA isolation were considered acceptable and samples of higher concentration and quality were chosen for cDNA synthesis.

3.3.2 cDNA synthesis

For the synthesis of cDNA, oligo(dT) and random hexameric primers were used in separate reactions of 15-20 µl final volume. Each batch was assessed separately by PCR using heavy chain and light chain primers. The successful recovery of PCR products was taken as the criterion of the presence of cDNA from each synthesis reaction. Virtually all preparations of cDNA met this measure.

Table 3.10 Quantitative and qualitative data on recoveries of RNA from bovine tissue.

	Amount of tissue	Dilution factor	Absorbance measurements			Quality estimates		Concentration of RNA µg/100 µl prep
			230 nm	260 nm	280 nm	A260/A230	A260/A280	
Liver	6 mg	50	0.087	0.166	0.084	1.908	1.976	33.2
Lymph node	10 mg	50	0.106	0.222	0.113	2.094	1.965	44.4
1	5-10 mg	50	0.046	0.078	0.046	1.696	1.696	15.6
2	"	50	0.061	0.094	0.048	1.540	1.958	18.8
3	"	50	0.061	0.049	0.029	0.803	1.689	9.6
4	"	50	0.030	0.031	0.025	1.033	1.240	6.2
5	"	50	0.053	0.112	0.044	2.113	2.545	22.4
6	"	50	0.037	0.075	0.035	1.973	2.086	14.6
7	40 mg	50	0.062	0.103	0.047	1.661	2.191	20.6
8	50 mg	50	0.065	0.137	0.067	2.108	2.045	27.4
9	30-40 mg	20	0.336	0.370	0.170	1.100	2.18	29.6
10	"	20	0.359	0.495	0.240	1.38	2.06	39.6
11	"	20	0.435	0.590	0.270	1.36	2.19	47.2
12	"	20	0.412	0.664	0.320	1.61	2.08	53.12
13	"	20	0.525	0.575	0.307	1.1	1.81	46
14	"	20	0.317	0.667	0.312	2.1	2.14	53.36
15	"	20	0.160	0.240	0.154	1.5	1.56	19.2
16	"	20	0.544	0.494	0.209	0.91	2.36	39.52
17	"	20	.455	1.156	0.607	2.55	1.9	92.48
18	"	20	0.262	0.581	0.283	1.99	2.05	46.48
19	35 mg	10	0.429	0.843	0.404	1.97	2.08	33.7

Table 3.11 Summary of yields of bovine RNA and comparison with reported yields from mouse tissues.

		Amount of tissue mg	Average yield µg/100 µl prep	Average yield µg/mg tissue	Average A260/A230	Average A260/A280
Bovine	Lymph node	5-10	14.5	1.93	1.53	1.87
		30-40	45.5	1.30	1.83	2.25
		40-50	24	0.53	1.82	2.12
	Liver	6-10	38.8	4.85	2	1.97
Mouse (data from Promega)	Liver	30	131	4.4	2.4	1.9
	Kidney	20	44	2.2	2.1	1.9
	Spleen	15	79	5.3	2.3	1.9
	Brain	60	39	0.65	2.1	2.1
	muscle	30	22	0.73	1.8	2.1

3.3.3 Recovery of heavy (H) and light (L) chain repertoires

Recovery using Taq DNA polymerase

In order to amplify the bovine V_L and heavy chain repertoires from lymph node cDNA with Taq DNA polymerase, magnesium concentrations were titrated and annealing temperatures for the reactions were optimised. PCR products were achieved with $MgCl_2$ concentrations ranging between 2 and 4 mM and annealing temperatures of 55-57 °C (Figures 3.2 and 3.3). Amplification of the V_L and heavy chain repertoires was accomplished from both oligo(dT) and random hexamer-primed cDNA synthesis. Although the design of primers for these reactions underwent some changes during the course of the study, all appropriate combinations resulted successfully in amplicons. To achieve a greater level of purification, PCR products were routinely run on agarose gels and the desired bands were extracted from gel slices.

Recovery using Pfu DNA polymerase

PCR products of the V_L and heavy chain repertoires were also amplified with Pfu DNA polymerase. Amplicons were obtained with ease with primers VL1 / VL2 and VH1 / VH2 from both oligo(dT) and random hexamer-primed cDNA synthesis. Attempts to amplify the heavy chain repertoire with VH1A / VH2A did not yield the desired amplicons. Despite extensive attempts to optimise the concentration of $MgCl_2$, amplicons could not be obtained by PCR from cDNA or control plasmid DNA from a pComBov-V_LH clone known to carry a heavy chain V_HC_H1 fragment.

3.3.4 Direct cloning of light chain amplicons

Direct cloning of the bovine antibody repertoire into pComBov was initially chosen as our strategy for construction of a bovine Fab phage display library. To achieve this objective, amplicons of the V_L repertoire were digested with *Sst*I and *Bst*EII and attempts made to ligate them into pComBov cut with the same enzymes (ligation A). As controls, two further ligations were set up: a reaction containing pComBov and a mixture of equal amount of V_L fragment released from a single clone of pComBov-L and digested V_L amplicons (ligation C); the vector fragment released from pComBov-L and digested V_L amplicons (ligation B) (Table 3.2). The results of this cloning are shown in Table 3.12.

Despite the high number of transformants obtained from ligation A, non of the 10 colonies screened for the presence of insert carried the desired fragment. This might be

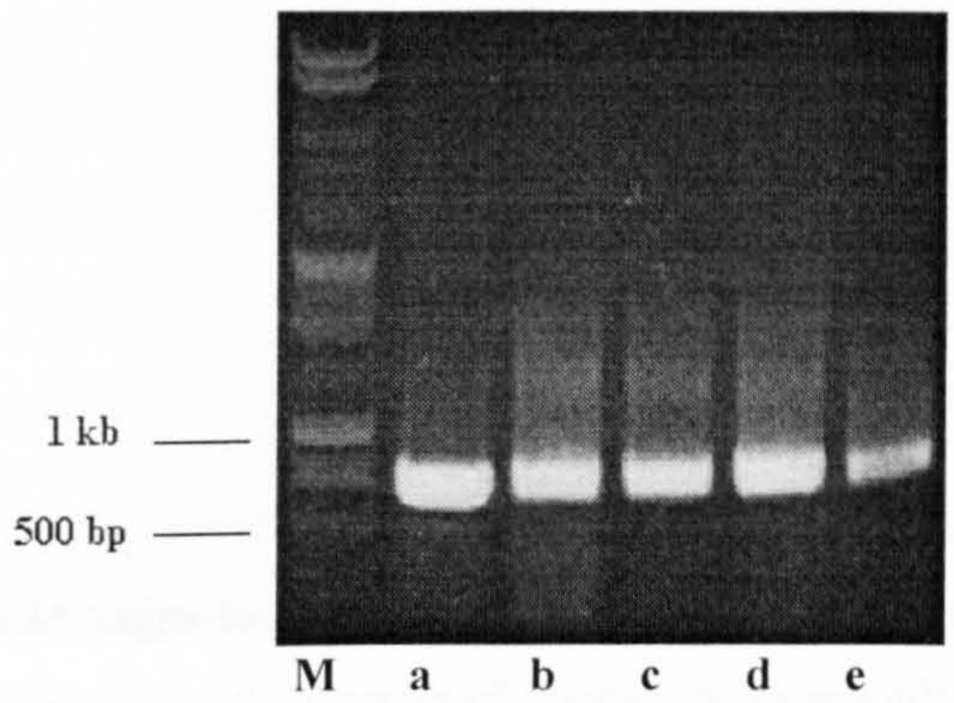


Figure 3.2 PCR recovery of the bovine heavy chain repertoire.

a: Amplicons from control pComBov-VLH template
b: Amplicons from cDNA template (random hexamer-primed synthesis)
c-e: Replicated reactions from cDNA template (oligo(dT)-primed synthesis)
M: 1 kb ladder (Promega). The migration of markers of 1 kb and 500 bp is indicated

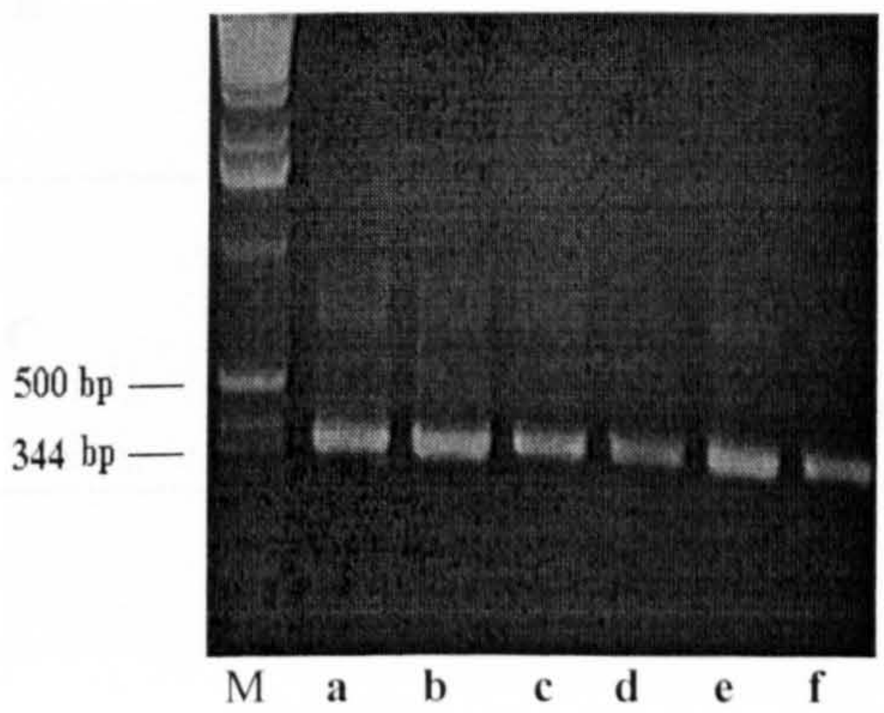


Figure 3.3 PCR recovery of the bovine V_L repertoire.

a-c: Replicated reactions from cDNA template (oligo(dT)-primed synthesis)
d-f: Replicated reactions from cDNA template (random hexamer-primed synthesis)
M: 1kb ladder (Life Technologies). The migration of markers of 500 bp and 344 bp is indicated

Table 3.12 Results of initial attempts to clone the V_L repertoire and an isolated V_L fragment into pComBov. The table shows the number of colonies recovered after plating of 20 µl samples from 150 µl transformation reactions onto selective agar medium.

	Composition of ligation reaction	Number of colonies
Transformation control	Circular pUC18	>2000
Ligation A	pComBov vector and amplicons of the V_L repertoire	>2000
Ligation B	pComBov vector isolated after digestion of pComBov-L and amplicons of the V_L repertoire	2
Ligation C	pComBov vector isolated after digestion of pComBov-L and a mixture of an isolated V_L fragment and amplicons of the V_L repertoire r	850

due to the incomplete digestion of pComBov and so the presence in the ligation reaction of vector cut by just one restriction enzyme. In contrast, ten colonies selected from ligation C and analysed by restriction analysis with *Eco*RI all carried insert. This outcome was confirmed by PCR on the plasmids using primers VL1 and VL2. Plasmid DNA from transformants with ligation C were analysed by digestion with *Eco*RII, a restriction enzyme which cuts immunoglobulin sequences with high frequency. This revealed that 9 of the 10 clones were identical (Figure 3.4) showing that most of the inserts were those released from the pComBov-L clone, not V_L amplicons. Results of ligations B and C showed the difference in efficiency of ligation of V_L amplicons and a V_L fragment released from pComBov-L back into the vector. This difference in ligation efficiency was evaluated in the next series of experiments.

Dephosphorylation of vector

All ten colonies sampled from cells transformed with ligation A – the reaction containing pComBov and amplicons from the V_L repertoire amplicons – did not carry an insert. Dephosphorylation of the digested vector was therefore used to prevent the religation of pComBov cut with a single enzyme. The ligation reaction contained 800 ng of dephosphorylated pComBov with an insert/vector ratio of of 3/1. About 100 ng of ligated DNA was then transformed into Solopack Gold supercompetent cells. The transformation efficiency achieved with control DNA – closed circular pUC18 – was high (1.35×10^8 cfu/µg) but no colonies were obtained with the test transformation.

Variation to insert/vector ratio

To evaluate the effect of insert/vector ratios on ligation efficiency, pComBov and V_L amplicons were digested with *Sst*I and *Bst*EII and four 20 µl ligation reactions set up with insert/vector ratios of 1/1, 2/1, 3/1 and 4/1. Again, transformation efficiencies with control pUC18 DNA were high (1.8×10^8 cfu/µg) but no colonies grew after plating 20 µl samples from 300 µl transformation cultures.

Study of the efficiency of restriction digestion of V_L amplicons

Since experiments to this point failed to yield recombinant transformants, attempts focused on the efficiency with which V_L amplicons were digested.

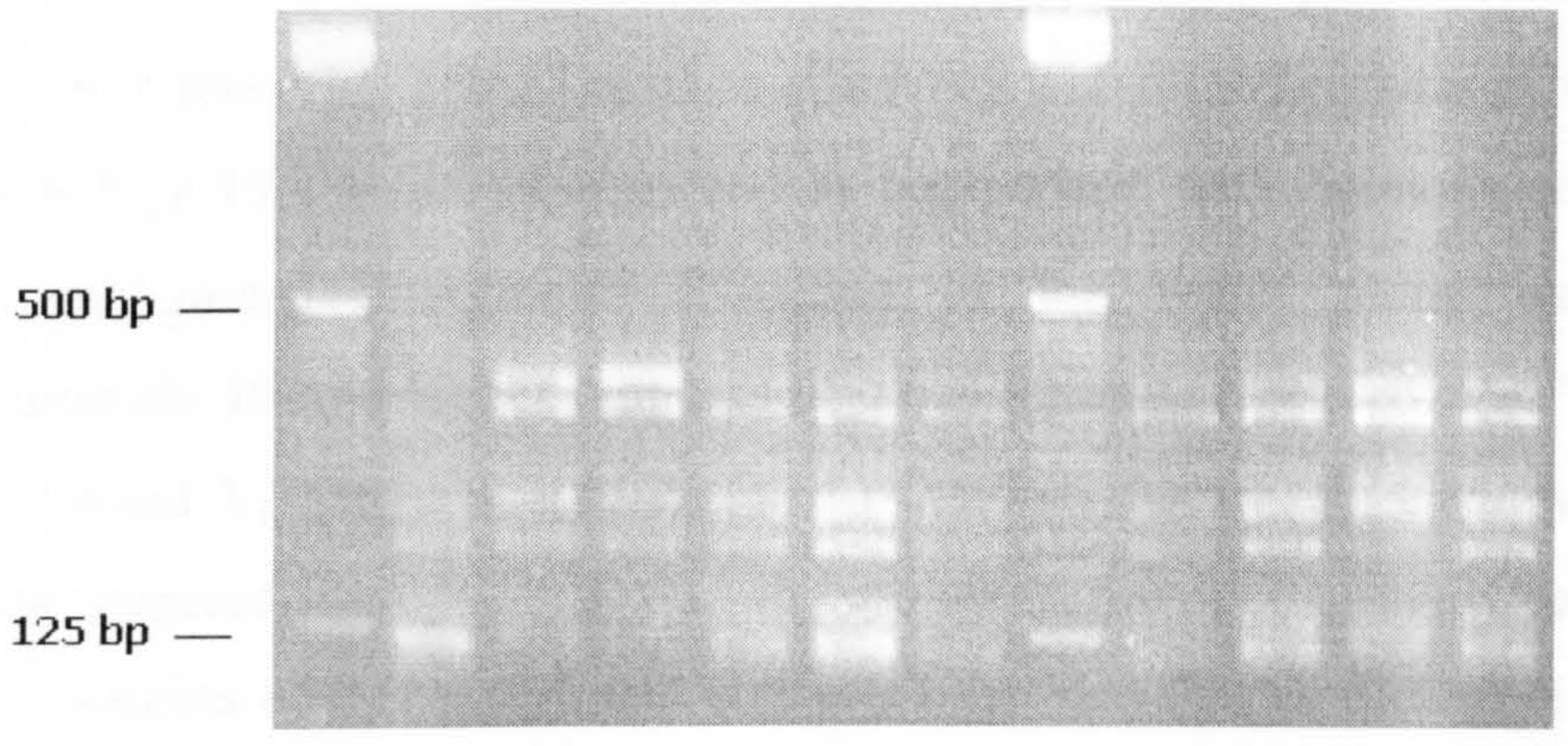


Figure 3.4 *Eco*RII restriction analysis of V_L inserts in pComBov.
The figure shows digests of 10 amplicons of which only one (a) differs from the remainder. Markers (M) were a 25 bp ladder (Life Technologies). The migration of markers of 500 bp and 125 bp is indicated.

- Cloning of a light chain fragment released from circularised vector

In this experiment, a V_L fragment was released from a characterised clone of pComBov-L by digestion with *Sst*I and *Bst*EII. Attempts were then made to clone the isolated fragment into the pComBov vector also isolated in this procedure and a second sample prepared by digestion with *Sst*I and *Bst*EII. Insert and vector fragments were mixed in different ratios for ligation and the products were transformed into NovaBlue competent cells. The recovery of colonies from 40 of the 300 µl transformation mixture is shown in Table 3.13. Transformation efficiency with closed circular pUC18 DNA was relatively high (5.2×10^7 cfu/µg) and in comparison with ligations containing both vector and insert, background religation of the vector alone was infrequent. Approximately 10-fold higher numbers of transformants were recovered from ligations of the isolated V_L fragment into digested pComBov *versus* religation with the vector fragment prepared from pComBov-L. In both cases, the optimal insert/vector ratio was 2/1. The success of this experiment supported the idea that inefficient digestion of the termini of V_L amplicons with *Sst*I and *Bst*EII was preventing ligation of the light chain repertoire into pComBov.

- Using high concentration of restriction enzymes

In general, the amount of a restriction enzyme required for digestion at a site close to the terminus of a piece of DNA is several times more than that required to cut at an internal site. This amount varies for different enzymes, some endonucleases being relatively efficient in this application. Although the amounts of *Sst*I and *Bst*EII used in experiments performed to this stage was higher (40 units/µg DNA) than those required for digestion of closed circular DNA, concentrations were increased further, up to 150 U/µg amplicon. After digestion and isolation from an agarose gel, the desired bands were extracted by QIAEXII resin to minimise potential degradation of the DNA termini. The isolated fragments were ligated with digested pComBov using 10 and 100 units of T4 ligase. Although the transformation efficiency for control pUC18 DNA was 4.7×10^8 cfu/µg, only two transformants were recovered with the test ligations.

3.3.5 Construction of intermediate library

Since attempts to clone directly amplicons comprising the V_L repertoire into pComBov failed, the cloning strategy was modified to achieve this aim. Firstly, it was

Table 3.13 Optimisation of ligation ratio for cloning V_L fragments into pComBov. The table shows the number of colonies recovered after plating of 40 µl samples (5 µl from the pUC18 control) from 300 µl transformation reactions onto selective agar medium.

Composition of ligation reaction	Insert/vector ratio	Amount of vector used in transformation (ng)	Number of colonies recovered	Cfu/µg DNA
Circular pUC18	-	0.1	87	5.2×10^7
Isolated V_L fragment and digested pComBov vector	1/1	88	922	7.9×10^4
	2/1	88	1264	1.1×10^5
	5/1	88	630	5.4×10^4
	Vector alone	88	34	2.9×10^3
Isolated V_L fragment and pComBov vector released from pComBov-L clone	1/1	127.5	115	6.8×10^3
	2/1	127.5	129	7.6×10^3
	5/1	127.5	67	3.9×10^3
	Vector alone	127.5	28	1.6×10^3

planned to clone the amplicons into an intermediate vector. By digesting the plasmid DNA with *Sst*I and *Bst*EII, it was envisaged that the desired fragments would be excised efficiently and their release would be a reliable indication of their suitability for cloning. For construction of intermediate libraries of the V_L and heavy chain repertoires, dephosphorylated pUC18 cut with *Sma*I was chosen as vector.

3.3.5.1 Intermediate V_L library

V_L repertoire amplification, phosphorylation and polishing of the amplicons

The bovine V_L repertoire was recovered from lymph node cDNA by amplification with Pfu DNA polymerase. After running the reactions on an agarose gel, products were isolation using extraction columns. In total about 260 µl of solution was recovered with a DNA concentration of 60 ng/µl. The termini of the V_L amplicons were polished with Klenow for thoroughness and phosphorylated using polynucleotide kinase. After phosphorylation of about 2.34 µg of PCR product, the DNA was extracted into TE buffer using QIAEXII resin. Measurement of the DNA concentration by spectrophotometry showed recovery of about 2.24 µg of DNA.

Ligation and transformation

In total, 6 ligations were performed to clone the 350 bp V_L amplicon into pUC18 (detailed in subheading 3.2.5.1). Forty transformations were carried out to generate the pUC-V_L library. Ligation products were electroporated into competent cells prepared in the laboratory. The size of the final pUC-V_L library was calculated from the number of colonies obtained from plating samples from each transformation. In total, 3.3×10^5 colonies were generated with an average of 8250 colonies per transformation. The transformation efficiency of different batches of electrocompetent cells using control circular pUC18 DNA was variable, ranging between 4.6×10^7 and 1.1×10^9 cfu/µg. With one of the best batches of cells, 27700 transformants were obtained from ligation of phosphorylated V_L DNA into pUC18.

Assessment of the intermediate pUC-V_L library

Plasmids of twenty white colonies from the pUC-V_L library were digested with *Eco*RI to determine the insert frequency. The size of the linearised DNA was compared with pUC18 and a known control pUC-V_L plasmids. Nineteen from 20 plasmids were the

same size as the control pUC-V_L construct. The plasmids including the pUC-V_L control were further analysed by PCR with flanking pUC18/ M13 forward and reverse primers to confirm the presence of inserts. Gel analysis of the amplicons revealed that 18 of the 19 candidates possessed inserts of the expected size. The amplicons were then digested with *Eco*RII to assess their diversity. Fourteen of the 18 inserts (~ 77%) showed unique patterns of fragments when run on a 3% agarose gel (Figure 3.5).

3.3.5.2 Intermediate H fragment library

Heavy chain repertoire amplification, phosphorylation and polishing of the amplicons

The bovine heavy chain repertoire was recovered from lymph node cDNA by PCR with Pfu DNA polymerase. After running the reactions on an agarose gel, the desired bands were excised and the DNA extracted with QIAEXII resin. Following procedures developed in the cloning of V_L amplicons, the heavy chain products were polished and phosphorylated for cloning into pUC18. About 7.8 µg of isolated amplicon was obtained from seven 50 µl PCR reactions and of this, 3 - 3.2 µg of phosphorylated DNA was obtained from each of 2 phosphorylation reactions.

Ligation and transformation

Seven ligations were performed to clone the 660 bp heavy chain amplicon into pUC18. The ligation conditions were essentially the same for all reactions. Sixty transformations were carried out to generate the pUC-H library. Again, electroporation was used for transformation of the ligated DNA into competent cells prepared in the laboratory. A library with 1.1 x 10^6 members was achieved with an average of 18333 colonies per transformation. Transformation efficiency for control pUC DNA was variable (1.9 x 10^7 to 1.32 x 10^9 cfu/µg) and the maximum yield for a single transformation of ligation products was 71500 colonies.

Assessment of the intermediate pUC-H library

Forty white colonies from the pUC-H library were picked to determine the insert frequency. Their plasmids were cut with *Eco*RI and the size of the digested DNA was compared with pUC18 and a known control pUC-H plasmid. Thirty from 40 plasmids were larger than the control pUC plasmid. Inserts were recovered from all plasmids including the pUC-H control by PCR with flanking pUC18/ M13 forward and reverse

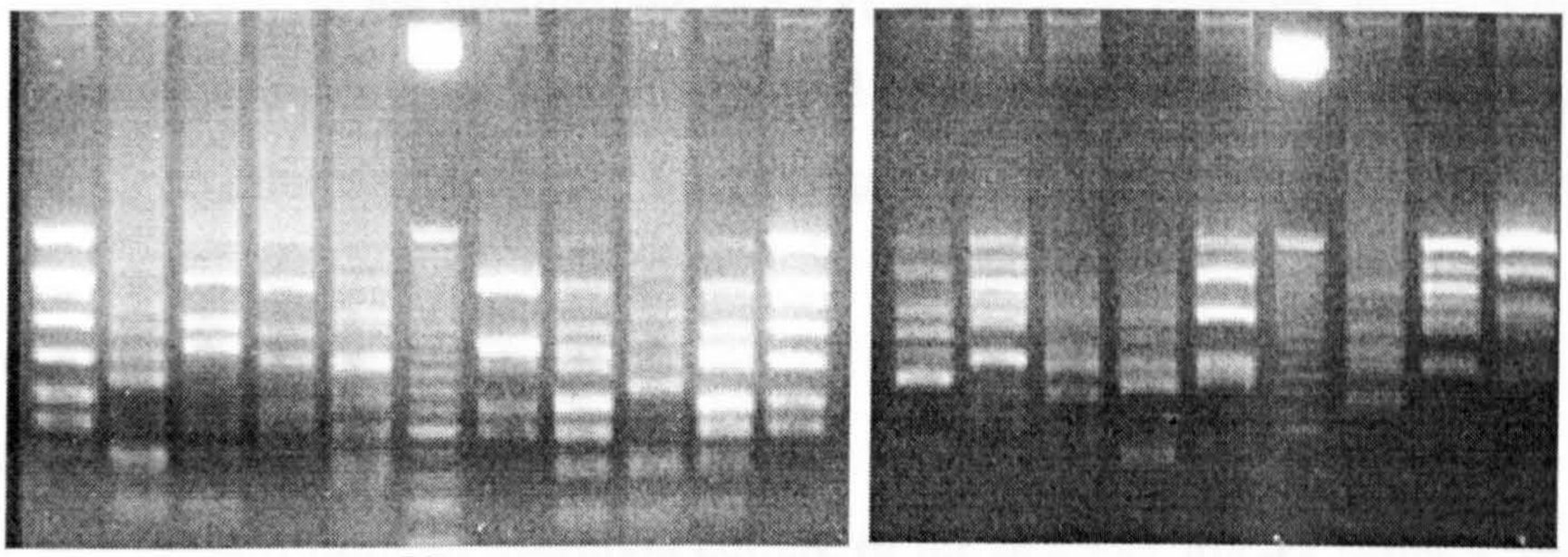


Figure 3.5 *Eco*RII restriction analysis to determine the diversity of the pUC-V_L library.
Samples were run on two separate gels (left and right panels). Fourteen of the 18 samples analysed showed unique digestion patterns.
M: 25 bp DNA marker (Life Technologies)

primers. Gel analysis of the amplicons on a 1.2% agarose gel revealed that 24 of the 30 candidate clones possessed inserts of the expected size. The amplicons then were digested by *Eco*RII to determine their diversity. Ninteen of twenty seven inserts (70%) showed unique digestion patterns when analysed on a 3% agarose gel.

3.3.6 Recloning of V_L and H repertoires into pComBov

After construction of the intermediate pUC-V_L and pUC-H libraries, the intention was to isolate the repertoires from each library and clone them separately into pComBov. Firstly, the V_L repertoire was released for construction of the pComBov-V_L library.

3.3.6.1 Recloning of the V_L repertoire

Preparation of the V_L repertoire

DNA was isolated from an overnight culture of the pUC-V_L library using a Midiprep plasmid purification system. Since gel electrophoresis of a sample of the isolated DNA showed some smearing, a 2 ml sample was run on a 0.7% agarose gel and the required band was extracted by QIAQuick gel extraction kit. DNA was eluted in 600 µl of elution buffer at a concentration estimated as 40 ng/µl.

Restriction digestion

pUC-V_L and pComBov DNA samples were cut with *Sst*I and *Bst*EII sequentially and were then run on a 0.7% agarose gel. The V_L fragment (350 bp) and pComBov vector (about 4 kb) were isolated using the QIAQuick gel extraction kit. Seven hundred ng of V_L fragment and about 7 µg of pComBov were harvested from digestion of 7 and 7.5 µg of pUC-V_L library and pComBov DNA respectively.

Cloning and transformation

For ligation of the V_L fragments into pComBov, two concentrations of T4 ligase were used for control and cloning reactions. Control reactions contained ligase and vector without insert evaluated the background due to the presence of undigested or single cut vector. For transformation, commercial XL1-Blue supercompetent cells and electrocompetent cells prepared in the laboratory were used. The results of transformation are shown in Table 3.14. The size of the pComBov-V_L library was estimated to be 4.6×10^5 transformants. This was achieved through 21 transformations.

Table 3.14 Results of transformations to construct the pComBov-V_L library. Transformations were carried out with samples of ligation reactions estimated to contain 20 ng of vector DNA

	Amount of T4 ligase	Number of colonies per transformation		Sum total of colonies from all transforma-tions	Transformation efficiencyof ligated DNA (cfu/µg)		Number of colonies from control reactions (vector alone)
		Average	Maximum		Average	Maximum	
XL1-Blue electrocompetent cells prepared in the laboratory *	6 U	10217	21583	102170	5.1×10^5	1×10^6	0
	400 U	18532	26220	148261	9.3×10^5	1.3×10^6	55
Commercial XL1-Blue electrocompetent cells **	6 U	10150	14600	20300	5×10^5	7.3×10^5	–
	400 U	49025	56000	196100	2.5×10^6	2.8×10^6	–

Competence calculated by electroporation with pUC18 DNA: * 2.45×10^8 and 4.2×10^8 cfu/µg; ** 1.98×10^9 cfu/µg

Blanks: No control reaction was est

Transformation with DNA from the control ligation reaction yielded only small numbers of colonies indicating a low level of vector religation.

Characterisation of the pComBov-V_L library

Fifteen colonies from the pComBov-V_L library were evaluated to establish the insert frequency. This was performed by digestion of plasmid DNA with *Eco*RI and also by PCR with primers VL1 and VL2. Gel electrophoresis of digestion products and PCR showed that all colonies possessed an insert of the expected size (350 bp).

3.3.6.2 Recloning of H fragment into pComBov-V_L library

The final phase of construction of the pComBov-V_LH library entailed release of the heavy chain repertoire from pUC-H and cloning into the pComBov-V_L library.

Preparation of DNA

Plasmids from the pUC-H and pComBov-V_L libraries were isolated from overnight cultures separately. The harvested DNA was purified and concentrated by running in an agarose gel and extraction with the QIAQuick gel extraction kit. In all, 650 and 500 µl of pUC-H and pComBov-V_L at concentrations of 30 ng / µl were obtained. Ten µg and 6 µg samples of pUC-H and pComBov-V_L were digested by *Spe*I and *Rsr*II concurrently. After electrophoresis on an agarose gel and verification that the desired fragments had been released, DNA was extracted with the QIAQuick gel extraction kit. pUC-H DNA released two fragments, the pUC vector and the 660 bp heavy chain insert. pComBov-V_L released fragments of 100 and 250 bp from the heavy chain stuffer and the vector (about 4 kb) .

Ligation and transformation

Ligation of the heavy chain repertoire into pComBov-V_L was performed at two concentrations of T4 ligase. In each set, an additional control reaction contained digested vector without the intended insert fragment. Transformation of ligated DNA into XL1-Blue supercompetent cells did not result in colony formation. The transformation efficiency for control circular pUC18 was 2.6×10^8 cfu/µg.

Gel analysis of ligation reactions

Since no colonies were recovered from ligation of the heavy chain repertoire into pComBov-V_L library, the ligation reactions were run on an agarose gel to assess the profile of DNA fragments. There was no evidence of circular pComBov-V_LH DNA but a band of about 1.3 kb was present in reactions set up with different concentration of T4 ligase. The size suggests that this fragment results from ligation of heavy chain fragments to each other.

Since the desired result was not achieved at the first attempt, efforts were made to find the reason for failure and to solve it.

Variation in the source of restriction enzymes

In this experiment, the endonucleases *Rsr*II and *Spe*I from Life Technologies were replaced by enzymes from New England BioLabs to check if variation in quality was responsible for the initial failure to complete library construction. Two concentrations of T4 DNA ligase (6 units and 100 units per reaction) were again employed. Although the transformation efficiency with pUC18 DNA was again high (4 x 10^8 cfu/µg), neither ligation produced transformants.

Variation to competent cells

XL1-Blue supercompetent cells were replaced with Solopack Gold supercompetent cells to rule out the possible effect of host genotype on plasmid stability. In this experiment, enzymes from Life Technologies were used to prepare DNA fragments which were again ligated with two concentrations of T4 ligase. Gel analysis of the ligation products showed a band corresponding to a heavy chain dimer (1.3 kb) in reactions each concentration of T4 ligase. No band which might represent circular pComBov-V_LH could be observed. Transformation efficiency for pUC18 in this experiment was 5.2 x 10^8 cfu/µg. No colonies were recovered from experimental ligations.

Characterisation of the 1.3 kb ligation product

The common presence of a 1.3 kb band in previous ligations, was investigated by restriction digestion to establish its origin. The band was excised from agarose gels and

was digested separately with *Rsr*II and *Spe*I. The results showed that the band was cut by *Spe*I, dividing it into fragments corresponding to the size of heavy chain. It was resistant to digestion to *Rsr*II, indicating that the 1.3 kb band was dimer of heavy chains ligated *via* their *Spe*I cut ends (Figure 3.6).

Variation to the insert/vector ratio

In order to check the effect of the insert/vector ratio on the cloning results, a ligation reaction was performed at a lower ratio (1.5/1 in comparison to previous attempts at ratios of 3/1 and 5/1). Transformation of these ligations into XL1-Blue supercompetent cells yielded no colonies although gel analysis of the ligation reaction revealed that the heavy chain dimer had disappeared. As a control, 60 ng of a known pComBov-V_LH construct, linearised by digestion with *Eco*RI, was ligated and transformed. This yielded only 50 colonies, a recovery lower than expected and a result which suggested that some degradation of DNA termini might be taking place during fragment isolation or other processing steps. Alternatively, we speculated that the construct might be toxic to bacteria in spite of attempts to suppress expression by inclusion of glucose in the selective media.

Checking the effect of pComBov derivatives on cell survival

For this experiment, 50 ng of each closed circular DNA from the pComBov-V_L library and a known pComBov-V_LH clone were transformed into XL1-Blue supercompetent cells. About 1 x 10^5 and 2.25 x 10^5 colonies were obtained from pComBov-V_L and pComBov-V_LH respectively. Transformation efficiency for control pUC 18 was 1 x10^9 cfu/μg. The difference in size between the pComBov constructs and the pUC control may explain to some extent the difference in transformation rates. However, the experiment eliminated the possibility of a significant toxicity effect between pComBov-V_L and pComBov-V_LH constructs.

The effect of gel extraction methods

To study the effect of fragment isolation using extraction columns, a series of religation reactions were set up. pComBov DNA was linearised by *Eco*RI and *Pvu*II and reisolated by three methods: QIAEXII resin, ethanol precipitation, and extraction from gel slices with QIAQuick gel extraction columns. The three batches of *Eco*RI fragments were religated with 6 units of T4 ligase; blunt ended *Pvu*II fragments were religated with

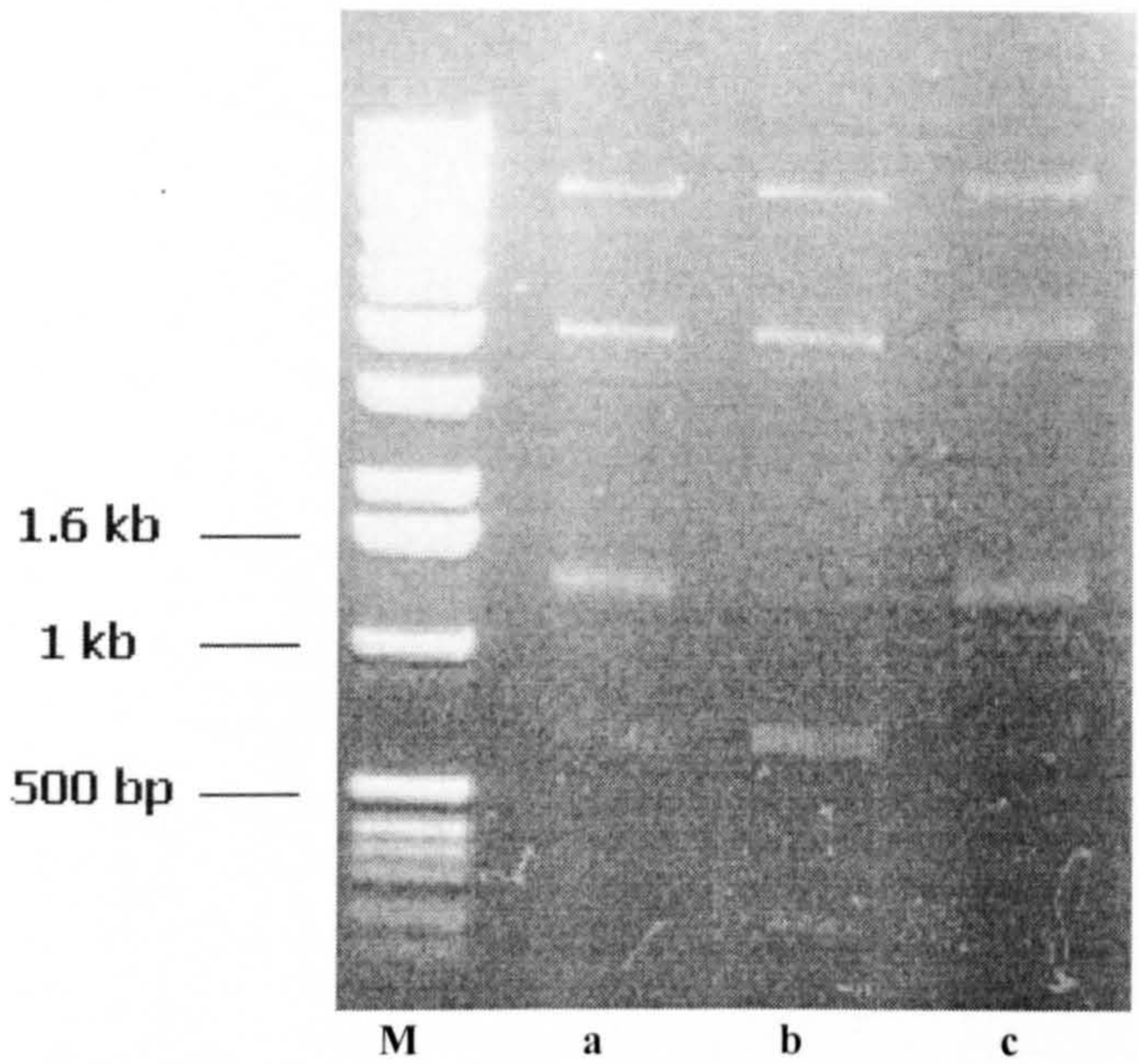


Figure 3.6 Restriction analysis of the 1.3 kb DNA fragment observed in ligation reactions during cloning of heavy chain fragments into the pComBov-V_L library.

a: Ligation products before digestion

b: Ligation products after digestion with *Spe*I

c: Ligation products after digestion with *Rsr*II

M: 1 kb markers (Life Technologies). The migration of markers of 1.6 kb, 1 kb and 500 bp is indicated.

6 units or 100 units of enzyme (Table 3.6). The profile of DNA fragments was studied by gel electrophoresis revealing differences between column-extracted DNA and samples isolated by the other two methods (Figure 3.7). Ligation reactions with the *Eco*RI-treated DNA were transformed into XL1-Blue supercompetent cells. Transformation of 25 ng DNA from ligations of resin-processed ethanol precipitated material yielded 80000 and 85000 colonies respectively. Equivalent amounts of DNA ligated after isolation from gel slices by extraction columns only yielded 250 colonies. In comparison, transformation efficiency for pUC18 was 7.5 x 10^8 cfu/µg. These data showed that the columns used for extraction of DNA from gel slices might have a significant effect on the integrity of the DNA termini.

Accordingly, another attempt was made to construct the pComBov-V_LH library. DNA from the pUC-H and pComBov-V_L libraries was digested with *Rsr*II and *Spe*I and run on an agarose gel. The desired fragments were isolated using QIAEXII resin. Attempts to transform the ligated DNA fragments into XL1-Blue supercompetent cells failed to yield any colonies whereas the transformation efficiency for control pUC18 DNA was 4.33 x 10^8 cfu/µg. Gel analysis of the ligation reaction showed again the presence of a 1.3 kb heavy chain dimer that could be cut with *Spe*I but not with *Rsr*II.

Variation in gel composition and alternative extraction methods

Further experiments were set up to optimise the extraction of DNA fragments. To achieve this, pComBov-V_L DNA was cut with *Sst*I. The linearised DNA then was divided into two parts, and each was run on an LMP agarose gel. The two samples were then extracted by different methods: one was extracted with QIAEXII resin, TE buffer was added to the second, and after dispersing the gel at 50 °C, DNA was isolated by phenol-chloroform extraction and ethanol precipitation. The two samples were then ligated using low (6 units) and high (400 units) concentrations of T4 ligase and samples corresponding to 70 ng of DNA were transformed into XL1-Blue supercompetent cells. Results are shown in Table 3.15. The transformation efficiency obtained with ligation products was low but sufficiently encouraging to prompt another attempt at pComBov-V_LH library construction. pUC-H and pComBov-V_L libraries DNA were digested by *Rsr*II and *Spe*I, the digestion products were run on an LMP agarose gel and slices containing the fragments of interest were processed as above (phenol-chloroform). Ligation was done at two concentrations of T4 ligase. Transformation of about 70 ng of

Table 3.15 The effects of extraction method and ligase concentration on religation of linearised DNA isolated from LMP agarose gel.

DNA and extraction method		Number of colonies per reaction	Transformation efficiency cfu/μg
pUC18			3.75×10^8
Resin extracted pComBov-V_L	6U T4 ligase	1150	1.65×10^4
	400 U T4 ligase	6450	9.2×10^4
Phenol-chloroform extracted and ethanol precipitated pComBov-V_L	6 U ligase	2750	3.9×10^4
	400 U ligase	5350	7.64×10^4

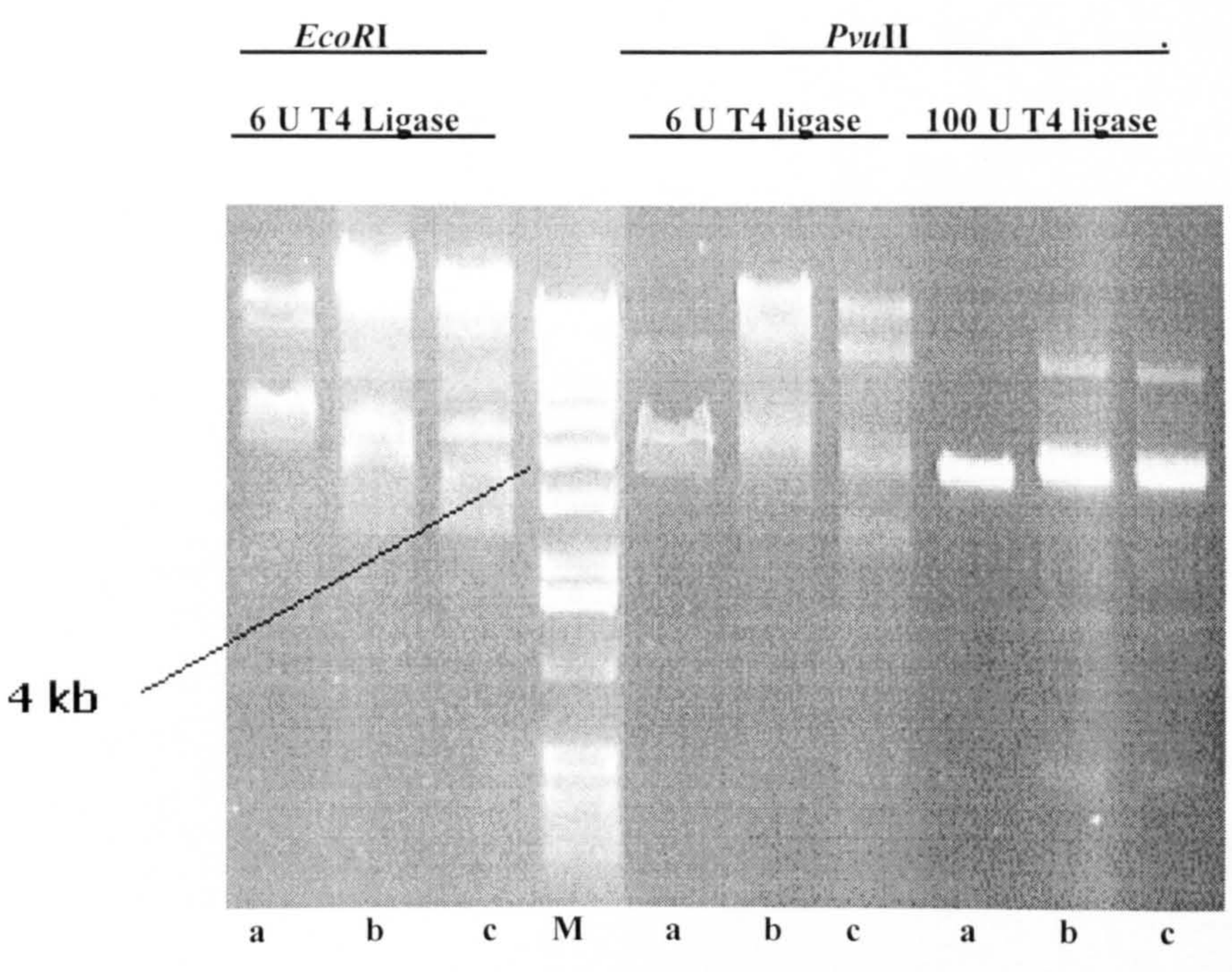


Figure 3.7 Gel analysis of pComBov after digestion with *EcoR*I or *Pvu*II and religation.

The Figure is in three sections showing plasmid digested with *EcoR*I (left) and *Pvu*II (right), and religation with 6 U (left and centre) and 100 U of ligase (right). Digested DNA was isolated for ligation by

a: spin columns

b: ethanol precipitation from the restriction digest

c: QIAEXII resin

M: 1 kb ladder (Life Technologies). The migration of the 4 kb marker is indicated.

ligation products resulted in only 50 (6 units ligase) and 150 (400 units ligase) colonies. The transformation efficiency of pUC18 DNA in this experiment was 1 x 10^9 cfu/µg.

Temperature-cycle ligation

Temperature cycling was studied in an attempt to employ more efficient ligation conditions. A mixture of DNA from known pComBov-V_L clones was cut with *Eco*RI and divided into 3 parts. DNA was then isolated using QIAEXII resin, ethanol precipitation and extraction from slices from a LMP agarose gel. Religation used 6 units of T4 ligase and thermal cycling. About 80 ng of ligated DNA was then transformed into XL1-Blue supercompetent cells. Results (Table 3.16) showed that the isolation of linearised DNA from LMP agarose gels using a Wizard extraction kit did not affect the efficiency of religation in comparison to direct reisolation methods. Gel electrophoresis of the ligated mixtures also showed the presence of circular DNA (Figure 3.8).

These results prompted another attempt at construction of the pComBov-V_LH library. Digested DNA from the pUC-H and pComBov-V_L libraries was run in an LMP agarose gel and then extracted by Wizard extraction kit. Thermal cycling was used for ligation and the products were evaluated by gel electrophoresis. As there was no evidence of circular DNA, transformation was not carried out.

Overall, the project failed to establish the conditions required for cloning the bovine heavy chain repertoire from an intermediate library into the pComBov-V_L library. In addition, sequencing of 3 colonies from the pUC-H library showed them to be completely identical, throwing into doubt the diversity of the intermediate library. Therefore, the strategy for cloning the heavy chain repertoire into pComBov-V_L shifted again to direct cloning approaches.

3.3.7 Direct cloning of the heavy chain repertoire into the pComBov-V_L library

First attempts at direct cloning of the V_L repertoire into pComBov were not successful, perhaps due to the inefficiency of restriction endonucleases to act close to the termini of the amplicons. Therefore, for direct cloning of the heavy chain repertoire into the pComBov-V_L library, new primers - VH1A and VH2A - were designed, carrying extensions at the 5' termini of primers VH1 and VH2. Definitive data on the properties of *Rsr*II are not available but it was reasoned that this modification should assist the action of the restriction enzyme.

Table 3.16 The effects of extraction method and thermal cycling on religation of linearised DNA.

Extraction method	Number of colonies per reaction	Transformation efficiency cfu/μg
pUC 18 control		3.6×10^8
Resin extraction	80000	1.14×10^6
Ethanol precipitation	32000	4.6×10^7
Wizard column extraction from LMP agarose	80000	1.14×10^6

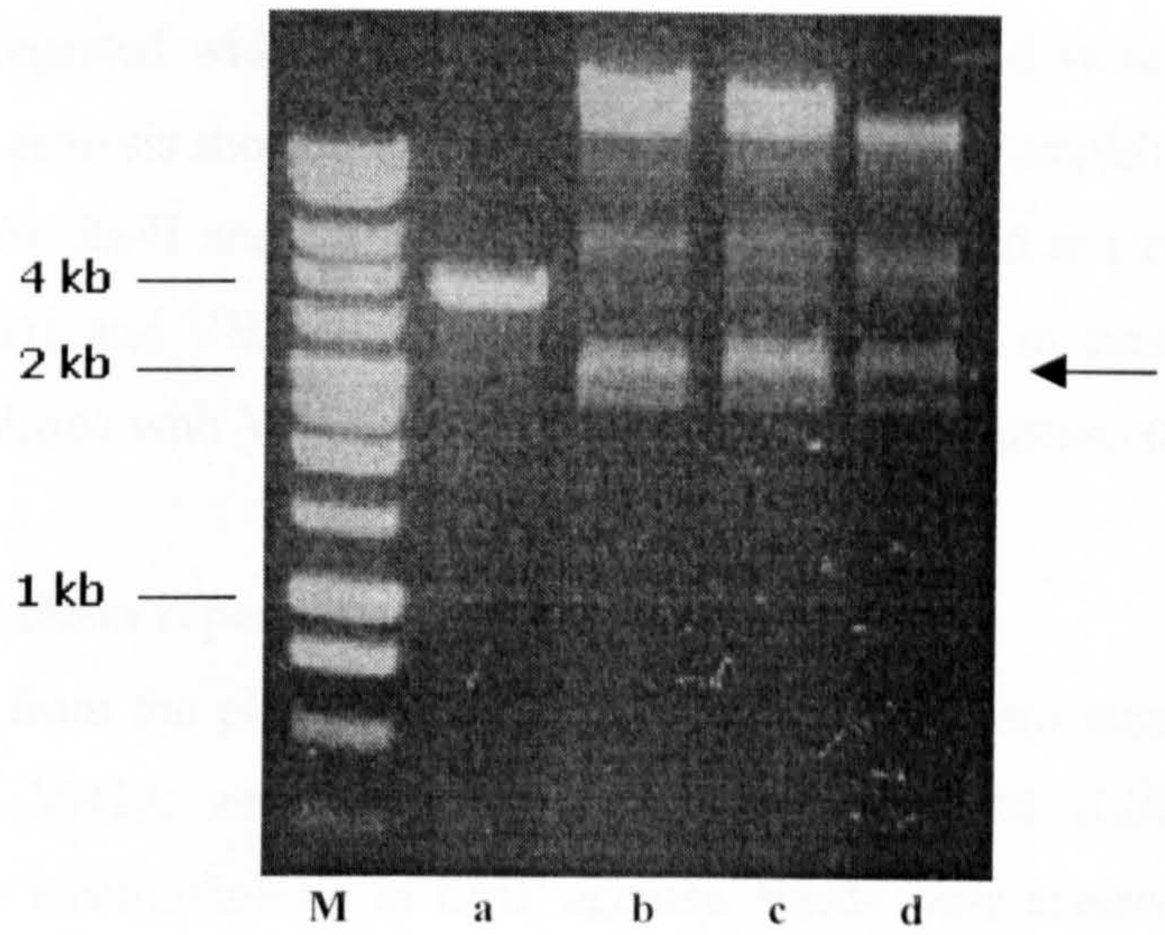


Figure 3.8 Gel analysis of pComBov after digestion with *Eco*RI, extraction by different methods and religation.

a: pComBov digested with *EcoR*I (no religation)
b: DNA extracted by ethanol precipitation and religated
c: DNA extracted by QIAEXII resin and religated
d: DNA isolated from LMP agarose using Wizard columns and religated.
Circular DNA are indicated by arrow.
M: 1 kb ladder (Life Technologies). The migration of markers of 1 kb, 2 kb and 4 kb is indicated.

Efficiency of digestion of VH1A / VH2A amplicons

To assess the ability of restriction enzymes to digest the termini of PCR products produced with VH1A and VH2A, reactions were set up with different pairs of primers from the following pool: VH1, VH1A, VH2, VH2A and gIII. Bovine lymphoid cDNA and plasmid DNA from a known pComBov-V_LH clone were used as template. The amplicons were digested with *Rsr*II, *Spe*I or both enzymes and were analysed on 3% agarose gels. Gel analysis showed that the termini of amplicons amplified by VH1A and VH2A were cut by *Rsr*II and *Spe*I, but that these enzymes did not cut PCR products amplified with VH1 and VH2. To confirm the action of *Spe*I in these experiments, a PCR product amplified with VH1A and gIII was successfully digested (Figure 3.9).

Cloning of heavy chain repertoire amplicons

Plasmid DNA from the pComBov-V_L library and heavy chain amplicons produced with VH1A and VH2A were digested with *Spe*I and *Rsr*II (Life Technologies) sequentially. After electrophoresis in LMP agarose, bands were excised and DNA was extracted with the Concert gel extraction system. Ligation was performed using 400 units of T4 ligase at 16 °C. Two control reactions were set up: the first contained the digested vector without insert; the second contained pComBov-V_L DNA linearised by digestion with *Eco*RI to monitor the efficiency of the ligation reaction. Ligated materials were transformed into XL1-Blue supercompetent cells. Thirty µl of each transformation reaction was plated to select for transformants. Religation of linearised pComBov-V_L DNA yielded >2000 colonies, while 95 were obtained from ligation of double-digested vector in the absence of insert. Two ligation reactions containing vector and insert produced 71 and 126 colonies respectively. Restriction analysis of plasmid DNA isolated from 8 colonies picked at random from the cloning reactions showed that none contained a heavy chain insert. The results again revealed a fundamental problem in ligation of the bovine heavy chain repertoire into pComBov-V_L. They also prompted further consideration of how best to separate vector cut with *Rsr*II and *Spe*I from incompletely digested DNA in order to suppress the appearance of false positive colonies.

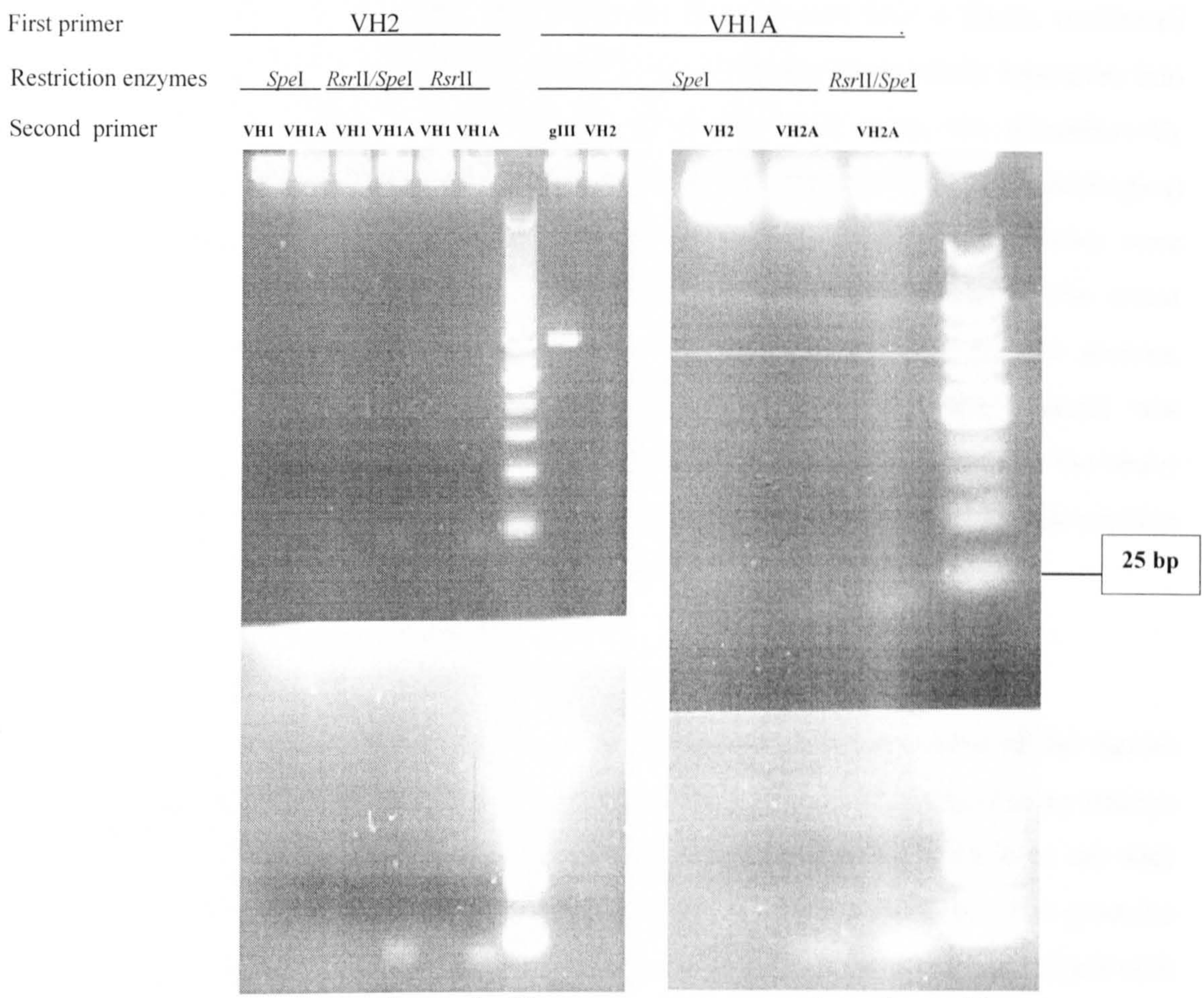


Figure 3.9 Restriction digestion of bovine heavy chain amplicons.

PCR products were generated with VH2 and VH1 or VH1A (left panel, lanes to left of markers [M]), or VH1A and gIII or VH2 or VH2A (left panel, lanes to right of markers [M]; right panel). The products were then digested with *Rsr*II and / or *Spe*I. Primer combinations are specified on the first and third lines above the Figure. Restriction enzymes used for the analysis are specified on the second line. Upper and lower panels show the same gel with different exposure conditions to demonstrate fragments released from the amplicons, as indicated by *. pComBov-LH was used as template for all PCR reactions except where indicated by #: in this case cDNA was used.

M: 25 bp ladder (Life Technologies). The migration of the 25 bp marker is indicated.

Using heavy chain fragments released from circularised vector as controls

In this experiment, the heavy chain fragment was released from a single, confirmed clone of pComBov-V_LH for use as a control in cloning the heavy chain repertoire into the pComBov-V_L library. DNA from the pComBov-V_LH clone, the pComBov-V_L library and heavy chain amplicons were cut with *Rsr*II and *Spe*I (Life Technologies) concurrently. The heavy chain fragments and vector from pComBov-V_L library were isolated from agarose gels using the Concert gel extraction system columns. The vector was used in two ligation reactions with heavy chain fragments from the two sources. Ligation was performed using two concentration of ligase and the material was transformed into XL1-Blue supercompetent cells. Irrespective of the source of the heavy chain insert, no colonies were obtained. The transformation efficiency for the reaction was checked with pUC18 DNA and was 2.6×10^8 cfu/µg.

Variations to restriction enzymes, extraction methods and competent cells

In this final attempt at construction of a pComBov-V_LH library, most of the factors involved in digestion, extraction, ligation and transformation were changed in an attempt to identify the problem. Heavy chain amplicons and pComBov-V_L DNA were cut with *Rsr*II and *Spe*I concurrently with enzymes from New England BioLabs. The products were then were run in an LMP agarose gel and the bands of interest were extracted with QIAEXII resin. The amount of vector DNA was increased to 225 and 2400 ng in two reactions of 20 and 185 µl volume. Four hundred and 800 units of T4 ligase were used for 20 and 185 µl reactions respectinely. After incubation at 16 °C for 18 hrs, DNA from the 185 µl reaction was concentrated using phenol-chloroform extraction and ethanol precipitation. Epicurian coli SURE2 supercompetent cells were used for transformation of samples from all of the ligation reactions. Samples from the transformation mixtures were plated onto LA containing ampicillin and glucose, with or without tetracycline. No colonies were recovered from any of the plates, even when the entire remaining transformation mixture was spread to the selective medium.

3.4 Discussion

The central goal of the experiments described in this chapter was to construct a library of recombinant bovine Fab antibodies from animals immunised against *Mannheimia haemolytica*. The intention was to screen this library for antibodies against outer membrane proteins from the pathogen. Antibodies isolated in this way would be more useful than antibodies generated in other species (*e.g.* conventional murine monoclonals) for immunological or pathological investigations of bovine pasteurellosis.

To achieve this aim, cDNA was prepared from an immunised animal and used to amplify the antibody light and heavy chain repertoires. This was achieved successfully with Taq and Pfu polymerases, confirming that this aspect of the work was feasible. Aside from work in cattle (O'Brien *et al.*, 1999), investigators have recovered the Ig repertoire from a range of different livestock species using PCR for further characterisation (rabbits, chickens, sheep and camels; see Introduction to this chapter). Oligonucleotide primer sets to achieve this objective have been recently compiled (O'Brien and Aitken, 2002).

Attempts to clone the V_L repertoire directly to phagemid pComBov failed although this is a strategy frequently employed in the construction of phage display libraries (Clark, 2002). Several variations were made to protocols to resolve the problem including variation to ratio of insert/vector fragments, dephosphorylation of vector ends, varying concentrations of restriction enzymes (*Sst*I and *Bst*EII), but the situation could not be resolved. By releasing a V_L fragment from closed circular plasmid DNA – a pComBov construct previously characterised – with these enzymes and using it as a control for ligation efficiency, it was established that the problem related to low efficiency digestion of the termini of amplicons with *Sst*I and *Bst*EII. It is known that restriction enzymes cleave DNA with a wide range of efficiencies when their recognition sequences are located near the termini of the duplex. A tabulation of the characteristics of several restriction enzymes is available from New England Biolabs as an appendix to the company catalogue. The data was gathered from two experimental approaches: the cleavage of short synthetic duplexes in which the restriction site is flanked by progressively longer stretches of random sequence; the cleavage of plasmid DNA linearised by digestion with other enzymes at sites progressively further from the site of interest. Both sets of data are useful, but neither is entirely comparable to cleavage close to the termini of a PCR product of several

hundred bases. Published data on this issue is limited (Popa *et al.*, 1995). From New England Biolabs data, it is known that the efficiency with which *Bst*EII cleaves a recognition sequence placed 2 bases from the terminus of the duplex is only 10% after 20 hrs digestion. Amplification of the V_L repertoire with primer VL1 placed the *Bst*EII site 10 bases from the terminus. On this basis, it seemed reasonable to expect considerably higher efficiency digestion to occur. There is no equivalent data for *Sst*I but given that this strategy was used successfully by O'Brien *et al.* in the construction of a bovine phage display library (O'Brien *et al.*, 1999), direct cloning was a valid starting point for this phase of the project.

The problems encountered in direct cloning led us to the alternative strategy described in this chapter, the cloning of amplicons into an intermediate vector. It was reasoned that this would increase the efficiency of restriction enzyme action and also would facilitate monitoring through gel electrophoresis the release of fragments for isolation and cloning into pComBov. This strategy has been adopted in the construction of Fab and scFv libraries by some investigators. In each case, the intention was to enhance the limited efficiency of direct cloning rather than to overcome a fundamental obstacle such as was encountered here. Sheets *et al.* (1998) constructed a large non-immune scFv phage library comprising 6.7×10^9 clones *via* the cloning of V_L and V_H repertoires into independent, intermediate plasmids. Inserts were released by restriction digestion, fused to create scFvs and recloned into pHEN1 to create the final phage display library. The intermediate V_L and V_H libraries in this work were 3×10^7 and 2.3×10^8 respectively. de Haard *et al.* (1999) also used this strategy in the construction of a large non-immune Fab libraries. The main library of 4.3×10^{10} (3.7×106 clones with full-length Fab inserts) was assembled from intermediate H, Vλ and Vκ libraries of 1.75×10^8, 5.2×10^7 and 9.4×10^7 members respectively.

In our experiments, intermediate libraries of the light and heavy chain repertoires were constructed by cloning amplicons into pUC18 as blunt ended fragments. The V_L repertoire was then released for cloning to pComBov by digestion with *Sst*I and *Bst*EII. The ease with which this was achieved confirmed the suspicion that direct approaches failed because of inefficient digestion of the termini with these restriction enzymes.

In contrast, an intermediate library of heavy chain fragments was constructed but difficulties were encountered in recloning *Rsr*II / *Spe*I fragments into pComBov-V_L. It

was confirmed that a fragment of about 660 bp could be release from the intermediate heavy chain library; the obstacle arose at ligation. A thorough investigation of experimental conditions attempted to find a solution but without success. These experiments covered the insert / vector ratio for ligation, temperature-cycle ligation (Lund *et al.,* 1996), the source of restriction enzymes, the host genotype, and the toxicity of the expressed products. In studying the effect of different DNA extraction methods, QIAEXII resin showed some advantage over spin-columns, at least for the religation of linearised DNA, but application of this method to the cloning of the heavy chain repertoire into pComBov again failed. The use of LMP agarose gels and extraction with phenol-chloroform yielded some recombinant colonies but the efficiency was far too low for use in library construction. Other authors have reported that the termini of DNA fragments can be damaged by some isolation methods with detrimental effects on cloning efficiency (Fransen *et al.*, 1998). Whether this effect is more pronounced with the 3 base extensions produced by enzymes such as *Rsr*II than the 4 base extensions typically produced by restriction endonucleases is not known.

After the failure of indirect cloning of the heavy chain repertoire into a pComBov-V_L library, attention returned to a direct cloning strategy. Using revised primers, the distance between the restriction sites and the termini of heavy chain amplicons was increased. Restriction analysis showed that the termini of amplicons were efficiently digested by *Rsr*II and *Spe*I. Attempts to clone the digestion products were unsuccessful, reiterating previous findings. It was also significant that when a heavy chain insert was released from a pComBov construct carrying a bovine Fab insert, this could not be religated into the releasing vector. This is in marked contrast to comparable control experiments with the V_L fragment. This finding clearly points to a failure of some aspect of the ligation. During the study, dimers of the heavy chain fragment could be identified which were ligated *via* their *Spe*I-digested termini. This implicates a failure of the *Rsr*II termini to undergo ligation.

In principle, there is no reason why DNA fragments prepared with *Rsr*II should not undergo ligation although Life Technologies report a religation efficiency about one-third of that of more commonly-used restriction enzymes. In the literature, several investigators report the use of this enzyme in cloning (Ye and Huang, 1993; Pfleiderer *et al.,* 1995) and bovine Fab libraries in pComBov were successfully prepared using *Rsr*II by O'Brien *et al.* (1999). *Rsr*II is a orthodox type II restriction enzyme that recognises and cleaves the heptanucleotide palindromic sequence 5' CGG(A/T)CCG

3' (O'Connor *et al.*, 1984; O'Connor *et al.*, 1987). Orthodox type II restriction endonucleases are typically homodimers comprising 30 kDa monomers, which recognise defined palindromic sequences 4 to 8 bp in length. In the presence of Mg^{2+}, the endonuclease cleaves the two strands of the target DNA sequence within or adjacent to the recognition site, generating termini which bear 5' phosphate and 3' hydroxyl groups. Many type II restriction endonucleases do not conform to this narrow definition, making it necessary to define subdivisions (Pingoud and Jeltesch, 2001). As the recognition sequence of *Rsr*II can possess degeneracy in its middle base (A or T) it was of obvious importance to ensure that fragments prepared with this enzyme possessed the same base at the central position to enable religation to take place. In this study, the *Rsr*II recognition sites on both the primer VH1A and pComBov were designed with A in this position. With regard to other factors, it is known that adenine methylation can reduce the efficiency of cohesive ligation (Levin and Boeke, 1990). Digestion with *Rsr*II can be inhibited if DNA has undergone methylation *in vitro* (Sambrook and Russell, 2001) but as the enzyme's recognition sequence does not contain the motifs targeted by the *E. coli* Dam (5' GATC 3') or Dcm (5' CCAGG 3' or 5' CCTGG 3') methylation systems, this seems an unlikely explanation for the findings reported in this chapter. It seems equally unlikely that the results can be attributed to local DNA structure (Merenkova *et al.*, 1993; Dolinnaya *et al.*, 1994), or sequence dependent effects (Merenkova *et al.*, 1992) since O'Brien *et al.* (1999) successfully constructed Fab libraries by ligating heavy chain amplicons into pComBov as *Rsr*II / *Spe*I fragments. In spite of attempts in this study to replicate as carefully as possible O'Brien's methods – extraction techniques, competent cells, source and concentration of enzymes – it therefore seems most likely that a minor difference had a substantial impact upon the ligation of fragments prepared by digestion with *Rsr*II.

One factor which could be investigated in future studies concerns the buffering conditions for the ligation step. The assumption was made in this chapter that standard buffers would suffice for the ligation of DNA prepared with *Rsr*II since the enzyme generates DNA fragments with 5' overhanging termini. It may be significant that the overhang is 3 nucleotides in length, compared with 4 from many other restriction enzymes. Perhaps under standard conditions this difference results in less stable association between fragments prior to the ligation reaction. To test this hypothesis, the addition of condensing agents such as polymeric polyethylene glycol as frequently

used in blunt ligation could be attempted (Sambrook and Russell, 2001). Also, it is known that the efficiency of T4 DNA ligase is dependent to some extent on the ionic strength of the buffer (Sambrook and russell, 2001). There are minor differences between the composition of ligation buffers from New England Biolabs, Promega and Life Technologies. Reagents from Promega were used for the repeated attempts to ligate heavy chain fragments into pComBov-V_L. It is not known whether the buffers used for ligation in this study differed in their composition from those used by O'Brien but this could potentially have influenced the results and may merit further investigation in the future.

Clearly, this issue needs to be resolved if the pComBov vector system is to be used for the routine isolation and expression of recombinant bovine antibodies. The *Rsr*II restriction site was chosen to maintain the native protein sequence in framework region 1 (5' (TC)C GGA CCG 3'; codons encoding the sequence SGP). In view of the difficulties encountered here, the *Bsp*EI site also encoded in this sequence might be utilised (5' TCC GGA 3') since the enzyme produces a 4 nucleotide 5' extension. Although *Bsp*EI is blocked by overlapping Dam methlylation, the two bases following the restriction site are CC which fails to complete the GATC motif recognised by the Dam methlyase (Sambrook and Russell, 2001). Alternatively, it might be argued that the *Rsr*II site should be replaced completely with one recognised by a more conventional restriction enzyme, sacrificing the native sequence of framework 1. It would be wise to assess the impact of amino acid substitution either by modelling on the known crystal structure of the heavy chain variable domain (Padlan *et al.*, 1989) or by direct investigation before undertaking lengthy studies. To take one example however, pComBov presently contains a single *Bam*HI site. If this were eliminated by site-directed mutagenesis, the recognition sequence could be reintroduced using a synthetic duplex (O'Brien *et al.*, 1999) to replace the *Rsr*II site. This would change 5' (TC)C GGA CCG 3' (encoding SGP) with 5' (TC)G GAT CC(G) 3' (encoding SDP). *Bam*HI fragments are easily ligated and it is known that the enzyme can cut close to the terminus of a DNA duplex (data from New England Biolabs), which is an added advantage.

In summary, these attempts to generate a phage display library of bovine Fab fragments failed and it was not possible to progress to screen for recombinant antibodies against the outer membrane proteins of *Mannheimia haemolytica.* In the

following chapter, this objective is addressed by an alternative strategy, the use of a large, semi-synthetic phage display library obtained from other investigators.

Chapter 4

Screening of Griffin.1 scFv library against outer membrane proteins of *Mannheimia haemolytica*

4.1 Introduction

4.1.1 Application of phage display antibodies to studies of infectious diseases

The phage display system provides a means to identify monoclonal binding ligands to pathogen antigens and virulence factors. Most studies of pathogenesis have tended to use conventional hybridoma methods to generate antibodies. In comparison, phage display offers several significant advantages. To begin with, phage antibody libraries are typically much larger than the number of hybridoma clones that could be easily propagated and screened and therefore they offer a much larger repertoire of antibodies from which useful ligands can be selected. Secondly, the flexibility of phage display enables selection strategies to be designed which preferentially isolate antibodies likely to be of relevance to the end use. For example, immobilisation of target molecules at an inert surface for panning directly leads to the isolation of antibodies with an affinity for these targets. Finally, the ligands isolated, scFv or Fab fragments, are readily produced *in vitro* for application as biological probes, affinity reagents, diagnostic tools or therapeutic agents. A selection of examples in which phage display has been used in the study of infectious disease are discussed briefly in the following sections.

4.1.1.1 Parasites, fungi and moulds

Haemonchus contortus

A scFv library was constructed from the abomasal lymph node of an immune sheep after challenge with the gastrointestinal nematode parasite *Haemonchus contortus* (White *et al.*, 2001). scFv were selected by panning the library against a purified outer membrane protein but only low affinity antibodies were isolated from nearly 960 clones processed by screening. The authors attributed this outcome to limitations in their selection strategy. Given the success of other investigators in generating ovine phage display libraries and isolating high affinity scFvs (Charlton *et al.*, 2000), this seems the most likely explanation of the findings of White *et al.*(2001).

Plasmodium falciparum

Phage antibodies were isolated from a phage library binding to the merozoite surface protein 1 (MSP1), a major candidate for blood-stage malaria vaccine. An antibody library was constructed from the blood lymphocytes from 10 patients with clinical malaria (Sowa *et al.*, 2001). After isolation of phage antibodies against MSP1, the authors concluded that the strategy was useful for both the characterisation of the

antigenic composition of the parasite and the nature of the human response to malaria infection.

Candida albicans

Haidaris *et al.* (2001) selected two scFv clones from an antibody phage library, which detected *Candida albicans* antigens by indirect immunofluorescence assay, ELISA and Western blotting. The phage library was prepared from peripheral blood lymphocytes of about 100 donors. Antibodies such as these can be used to either purify native antigen for biochemical characterisation, or evaluation as protective antigens. They can also be used to derive peptide mimetics. In this context, a scFv was first generated as a mimetic of the yeast killer toxin of *Pichia anomala.* Two recombinant strains of *Streptococcus gordonii* were then generated which either secreted the scFv or displayed it at the bacterial surface. These strains stably colonised the vagina of experimental rats and were evaluated as treatments against *Candida albicans* infection. Three weeks after challenge, 75% and 35% of the rats treated with the secreting and displaying bacteria, respectively, were cured of the infection in comparison to animals treated with a bacterial strain expressing an irrelevant scFv (Beninati *et al.*, 2000).

Aflatoxin

Phage antibodies specific for Aflatoxin-B1 were isolated by panning of two phage antibody libraries, a human lymphocyte library and a semi-synthetic library, on a BSA-toxin conjugate (Moghaddam *et al.*, 2001). This experiment is amongst the few to report isolation of a scFv against a low molecular weight hapten from a naïve phage antibody library. Work by Porter's group in Aberdeen has also tackled this objective for small microbial toxins (McElhiney *et al.*, 2000). A wide range of polyclonal and monoclonal antibodies to Aflatoxins have been prepared for routine screening for these products destined for animal and human consumption. Potentially, recombinant antibodies have greater sensitivity for these purposes.

4.1.1.2 Viruses

There are numerous reports in the literature of the generation of recombinant antibodies against viruses using phage display presumably because of the pressing need for novel antiviral therapies. The antiviral activity of antibodies have been recently reviewed (Parren *et al.*, 2001). A selection of examples of recombinant antiviral

antibodies is presented below.

Bluetongue virus

A scFv antibody specific to VP7 of bluetongue virus (BTV) was constructed through the cloning of V_L and V_H fragments of an anti-BTV myeloma antibody in a phage vector (Nagesha *et al.*, 2001). This scFv showed similar binding specificity to the parental antibody when tested against VP7.

HIV-1

By application of combinatorial library technology, scFv antibodies were constructed against the major capsid protein p24 of HIV-1. A library of scFvs was made using B cells taken from the spleen of mice immunised with a viral lysate. These scFv antibodies may form the basis for a sensitive assay for p24 as a measure of viral load (de Haard *et al.*, 1998).

Respiratory syncytial virus

A human Fab recombinant antibody was constructed against respiratory syncytial virus (RSV). The antibody bound to the viral F glycoprotein and neutralised the infectivity of virus. Anti-viral protection could be demonstrated by delivering the Fab directly into the lungs of RSV-infected mice (Barbas *et al.*, 1992b; Crow *et al.*, 1994). Nguyen *et al.*(2000) reported a combined effort of applying hu-PBL-SCID mice and scFv phage display library to boost human immune responses against RSV, and to clone a panel of RSV neutralising scFvs, respectively. The resulting scFvs were shown to be derived from different human V_H families with mutated CDR1 nucleotide sequences and some of them exhibited high RSV-F binding affinity associated with RSV neutralising activity *in vitro*. These data suggest that the generated scFv have clinical potential to treat severe RSV infection and that the combined approach may be widely applicable for cloning high antigen-specific and neutralising human monoclonal antibodies.

Rabies virus

Phage display technology was used to produce a scFv antibody from a hybridoma secreting an anti-glycoprotein antibody. The scFv was found to be identical in specificity and neutralising capacity to the full-length monoclonal from which it was derived. This antibody could be used in a cocktail of multivalent or multispecific

neutralising antibodies for passive therapy (Muller *et al.*, 1997).

4.1.1.3 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.

Bordetella pertussis

A scFv phage library was derived from the peripheral blood lymphocyte of two patients recently recovered from pertussis infection. The library was screened on pertussis toxin, an important virulence factor, but by no means the only one produced by this pathogen (Locht *et al.*, 2001). Six of the isolated scFvs bound to pertussis toxin and 3 were able to neutralise the toxin as determined by its effects on cells in tissue culture (Williamson and Matthews, 1999).

Bacillus anthracis

In this example, the starting resource was a naïve scFv phage library (Sheets *et al.*, 1998). The protein chosen for screening was PA83, the protective antigen from the toxin of *Bacillus anthracis.* Several unique clones were isolated with high affinity for different epitopes of PA83 of which one could inhibit receptor-mediated binding of PA to target cells. Aside from its potential as a therapeutic, the authors proposed another use for these scFvs might be as recognition elements for a biosensor (Cirino *et al.*, 1999).

Clostridium botulinum

Botulinum toxin is widely regarded as the most potent natural toxin known. Several groups have attempted to use phage display as an alternative to conventional hybridoma methods (Pless *et al.*, 2001) to understand better its function and to develop potential immunotherapeutics (Amersdorfer *et al.*, 1997; Chen *et al.*, 1997). In one recent example, three single chain Fv antibodies specific to the heavy chain binding domain of botulinum neurotoxin serotype A (BoNT/A Hc) were selected from phage libraries. The libraries were constructed from mice immunised with BoNT/A Hc or humans immunised with pentavalent botulinum toxoid. These antibodies were coated onto a

solid phase and phage libraries were panned against them in order to map epitopes of functional importance in toxin action (Mullaney *et al.*, 2001).

Pseudomonas aeruginosa

A Fab recombinant antibody was constructed from a conventional monoclonal antibody against the O antigen of *Pseudomonas aeruginosa*. The parent antibody bound to the O antigen of organisms of serotype O6, the serotype of most clinical relevance. Immunotherapy with 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).

Streptococcus suis

Our own efforts to extract anti-bacterial scFvs from the Griffin.1 synthetic library are described later in the chapter. This library has also been 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 for panning of the library. Three clones were identified in each panning which bound to the antigens of interest (de Greeff *et al.*, 2000).

Moraxella catarrhalis

Boel *et al.* have described the isolation of scFvs which recognised the high molecular weight outer membrane protein (HMW-OMP) of complement-resistant strains of *Moraxella catarrhalis* (Boel *et al.*, 1998), using a synthetic phage library as a starting point (de Kruif *et al.*, 1995). 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

4.1.2 Selection of scFv antibodies against *Mannheimia haemolytica* from Griffin.1 library

Since experiments performed in the previous chapter to construct a phage display library from cattle immunised against *Mannheimia haemolytica* failed, our attempts turned to use of the Griffin.1 library in order to select antibodies against outer membrane

proteins (OMPs) of this pathogen. *Pasteurella haemolytica* biotype 1 has been recently recategorised into the *Mannheimia* (Angen *et al.*, 1999).

The Griffin.1 library is a human synthetic scFv library with highly diversified V_H and V_L repertoires. It was developed at the Medical Research Council Centre for Protein Engineering, at the University of Cambridge. The repertoires were prepared from the *lox* library constructed by Griffiths *et al.*, 1994 and cloned into the phagemid pHEN2. The original Griffiths library expressed Fab fragments derived from commonly expressed human V_H and V_L families. In the Griffin.1 library, these repertoires were recloned as scFvs with fusion of the V_L gene to gIII allowing for expression of scFv on rescued phage. An amber codon between the antibody gene and gIII allows expression of soluble scFv fragments on induction with IPTG in a non-suppressor *E. coli* strain such HB2151. In amber suppressing strains such TG1, induction with IPTG causes production of a scFv-pIII complex, which embeds into the bacterial inner membrane before capping of the emerging phage particle (Figure 4.1).

4.1.3 The genus *Pasteurella*

Species of *Pasteurella* are small gram-negative, non-motile, non-spore-forming, facultative anaerobic rods or coccobacilli. These organisms have adapted to parasitic life on the oral and upper respiratory epithelia of apparently healthy animals and occasionally humans. Most strains have marked host species specificity, being associated almost exclusively with one or two specific hosts. Infection is more common than disease, which usually occurs as consequence of stress such as overcrowding, chilling, transportation or concurrent infection. Nevertheless, the "pasteurelloses", as these diseases are called, are of major pathologic and economic significance in veterinary medicine. Principal hosts of some *Pasteurella* species are shown in Table 4.1.

The pathogenicity characteristics of *Mannheimia haemolytica* are described below using its previous designation as *Pasteurella haemolytica.*

4.1.4 Pasteurella haemolytica

P. haemolytica is a commensal of the nasopharynx, and confined almost exclusively to ruminants such as cattle, sheep and goats. Serotypes are classified on the basis of immunological recognition (*e.g.* using passive haemagglutination or rapid plate

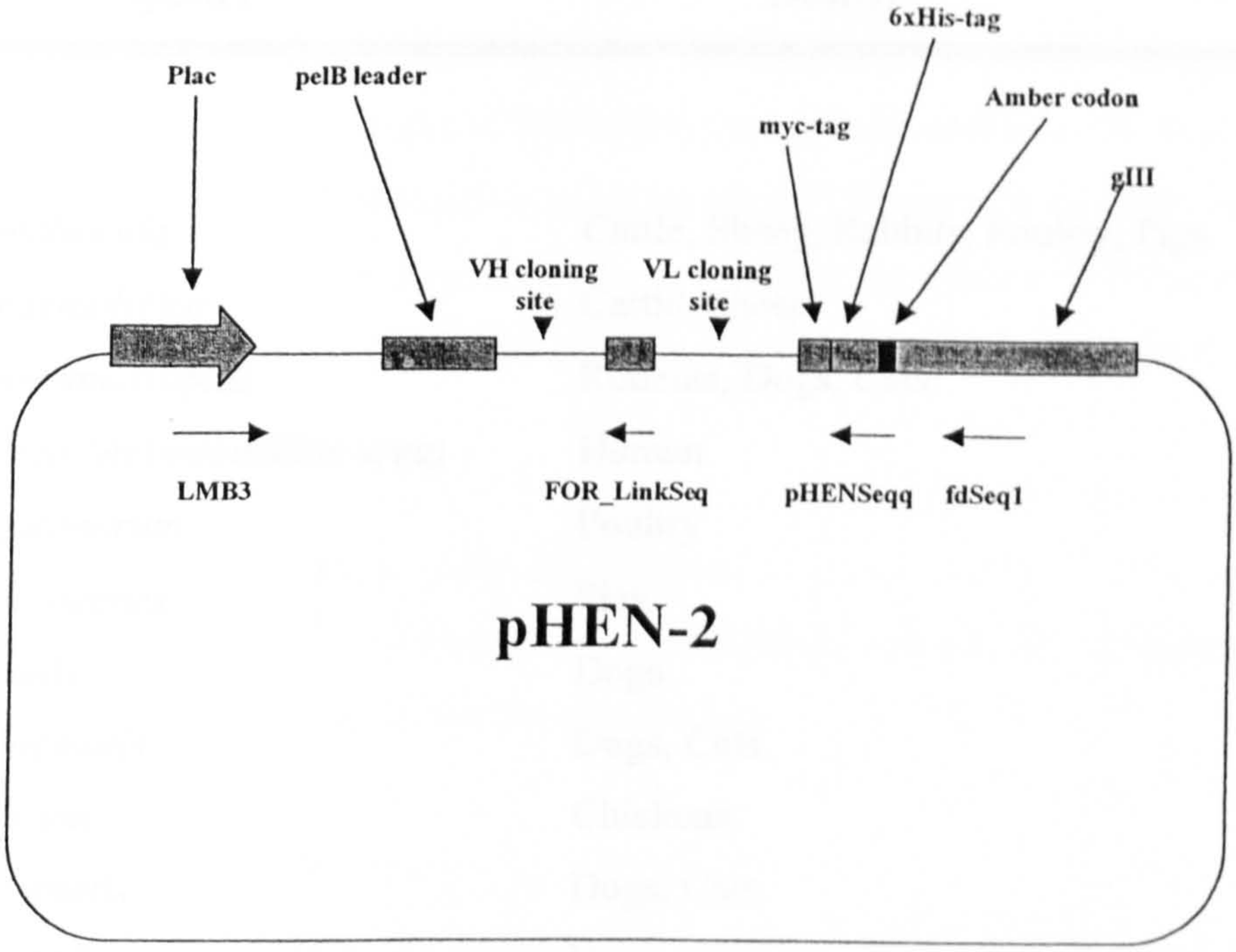


Figure 4.1 Structure of phagemid pHEN-2.

The main features of the vector are indicated. Transcription takes place from a *lac* promoter (shaded arrow) and in a suppressor strain of *E. coli*, the translated product comprises a PelB leader, the single-chain antibody created by insertion of V_H and V_L sequences at the sites indicated separated by a linker (shaded box), peptide tags, and the phage pIII protein. The binding sites of primers and their polarities are indicated by arrows.

Species	Host(s)
P. multocida	Cattle, Sheep, Rabbits, Poultry, Pigs
P. haemolytica	Cattle, Sheep
P. pneumotropica	Rodents, Dogs, Cats
P. urea (Actinobacillus urea)	Human
P. gallinarum	Poultry
P. aerogenes	Pigs
P. canis	Dogs
P. dagmatis	Dogs, Cats
P. avium	Chickens
P. stomatis	Dogs, Cats
P. caballi	Horses
P. anatipestifer	Ducks, Chickens, Turkeys, Pheasants
P. volantium	Chickens
P. anatis	Ducks
P. granulomatis	Cattle
P. langaa	Chickens
P. testudinis	Turtles, Tortoises

Table 4.1. Principal hosts of the *Pasteurella* species.

agglutination tests) of soluble or extractable surface antigens.

There are two distinct biotypes, A and T, a distinction based on fermentation of arabinose and trehalose respectively. The classification has biological significance since biotype A strains are associated with ruminant pneumonia, septicaemia in young lambs, and ovine mastitis, while biotype T strains are usually associated with septicaemic infection in older lambs and sheep. Both biotypes and all serotypes are found on the nasal and oral epithelia of clinically normal ruminants. Pneumonia of cattle and sheep are, economically, the most important *P. haemolytica* infections and the economic losses result both from acute fatalities and poor productivity of chronically affected animals.

4.1.4.1 Pathogenesis of *Pasteurella haemolytica*

The pathogenicity of *M. haemolytica* is incompletely understood but several of the most important virulence factors are described below.

Lipopolysaccharide (LPS)

As in other gram-negative bacteria, the cell wall of *P. haemolytica* contains lipopolysaccharide (LPS) or endotoxin. Intravenous injection of purified LPS in calves triggers the release of thromboxan A2, prostaglandins, serotonin, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) (Emau *et al.*, 1987). All or some of these mediators may be responsible for the clinical signs associated with endotoxic shock.

Leukotoxin (LKT)

All established serotypes of *P. haemolytica* produce a soluble, heat-labile leukotoxin with specificity for leukocytes of ruminants. At low concentrations, this toxin impairs phagocytosis and lymphocyte proliferation; higher concentrations result in cell death due to lysis. Lysis results from formation of 0.9-1.2 nm transmembrane pores in the membrane of alveolar macrophages and other target cells. Both the enzymes released following cytolysis and the LKT itself are chemotactic for other leukocytes and augment lung damage by increasing cell recruitment into the inflammatory focus. This toxin is also lytic for ruminant platelets, lysis of which may induce the pulmonary vascular thrombosis and fibrin exudation typically associated with shipping fever pneumonia.

Polysaccharide capsule (CPS)

Each serotype produces a characteristic polysaccharide capsule that is anti-phagocytic and may mediate adhesion to respiratory epithelium. Anaphylaxis and an IgE response following vaccination with purified CPS have suggested that this antigen has the potential to exacerbate pneumonia through induction of pulmonary hypersensitivity on subsequent exposure to live organisms (Conlon and Shewen, 1993).

Outer membrane proteins

The major outer membrane proteins (OMPs) of gram negative bacteria serve a range of structural and functional roles (*e.g.* many are porins and function to regulate the permeability of the outer membrane) but are also often targets for the host antibody response. In addition, OMPs isolated from *Pasteurella haemolytica* induce alterations to the biological activity of bovine polymorphonuclear leukocytes. A dose-dependent reduction of the capacity of adherence to nylon wool *in vitro* has been observed. OMPs also act as chemotaxins (Iovane *et al.*, 1998). Several of the OMPs expressed by *P. haemolytica* are regulated by conditions experienced *in vivo* (*e.g.* iron concentration) and have proved important antigens for the development of protective immune responses in cattle. OMPs with surface exposed domains may be targets for antibodies that function in bovine host defence mechanisms such as complement-mediated killing (MacDonald *et al.*, 1983) and phagocytosis and killing by neutrophils or macrophages (Czuprynski *et al.*, 1987; Chae *et al.*, 1990).

Outer membrane proteins involved in iron-acquisition

The ability to acquire iron in the host is necessary for most pathogens to successfully establish an infection. A number of different iron acquisition systems have evolved, including the production of siderophores and the presence of specific receptors for iron-containing glycoproteins such as lactoferrin and transferrin. *P. haemolytica* A1 produces several novel outer-membrane proteins in the absence of iron, and two of these (TbpA and TbpB) have been shown to bind bovine transferrin and mediate acquisition of iron from this source (Ogunnariwo *et al.*, 1997).

4.1.5 Objectives

As described in the previous chapter, our attempts to construct a phage display library from cattle immunised against *Mannheimia haemolytica* were unsuccessful. One might

expect that a Fab library from immunised cattle would be enriched in antibodies of relevance to this goal but the size and diversity of some phage display libraries is such that they contain antibodies against many natural or synthetic chemical targets. The aim of work presented in this chapter was therefore to select antibodies against outer membrane proteins (OMPs) of *Mannheimia haemolytica* from the Griffin.1 library, a large synthetic scFv library based upon commonly expressed human V_H and V_L gene families.

4.2 Materials and methods

4.2.1 *Manneheimia haemolytica* strain

Manneheimia haemolytica isolate 30 was obtained from Dr R. L. Davies, University of Glasgow. The organism was propagated on blood agar plates, grown at 37 °C. After overnight growth, the colonies were surrounded by haemolytic zone.

4.2.2 Preparation of outer membrane proteins (OMP) using Sarkosyl

The following method was performed essentially as described by Davies *et al.* (1991) except that EDTA was not incorporated in the Tris buffer. It exploits the differential solubility of the cytoplasmic and outer membranes in the detergent sodium *N*-lauroylsarcosine (Sarkosyl). Ten ml of Brain Heart Infusion broth (BHIB) was inoculated with *M. haemolytica*, and incubated overnight on the shaker at 37°C with loosened cap to ensure an aerobic environment. Four hundred µl of this overnight culture was added to 400 ml of pre-warmed BHIB in a two litre non-dimpled flask and incubated on an orbital shaker (120 rpm) at 37°C for six hrs. Bacteria were harvested in 250 ml centrifuge bottles by centrifugation at 10000 rpm for 20 min at 4°C. The pellet was resuspended in 20 mM Tris-HCl (pH 7.2) to a total volume of 50 ml and was kept on ice overnight. The next morning, the bacteria were washed by collecting the cells after centrifugation at 10000 rpm for 30 min at 4°C, and resuspending them in 7 ml of ice-cold 20 mM Tris-HCl. The cells were then sonicated on ice for 5-8 min in a sonication vial at a setting of 12 microns amplitude. To avoid frothing, the probe was immersed into the suspension to a depth of 1-1.5 cm. The sonicated sample was placed on ice for 10 min and then centrifuged at 10000 rpm for 30 min to remove unbroken cells. The supernatant was carefully removed and transferred to a 10 ml ultracentrifuge tube and centrifuged at 35000 rpm at 4°C for 1 hr to pellet the cell membranes. The supernatant was discarded and the gelatinous pellet was resuspended in 7 ml of 0.5 % sodium *N*-lauroylsarcosine (Sarkosyl) and left at room temperature for 20 min to solubilise the cytoplasmic membranes. The sample was centrifuged again at 35000 rpm at 4°C for 1 hr to pellet the outer membrane. The gelatinous pellet was resuspended in ice-cold 20 mM Tris-HCl (pH 7.2) and was centrifuged again at 35000 rpm at 4°C for 1 hr. The final pellet was resuspended in 1 ml of 20 mM Tris-HCl (pH 7.2). The prepared OMP was kept at – 80°C. Protein concentration was assayed and samples of the OMP were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to determine

their composition before use as a target for screening the phage display library.

4.2.2.1 Protein assay of OMPs

The concentration of protein in the OMP was measured using the BCA Protein Assay Kit (Pierce). Serial dilutions of a standard protein (BSA) were prepared and 0.1 ml of each dilution and the OMP were added to 2 ml of working reagent, before incubation at 37°C for 30 min. The absorbance of each sample was then measured at 562 nm and standard curve prepared. From this, the protein concentration of the OMP was determined.

4.2.2.2 SDS-PAGE analysis of OMPs

Polyacrylamide gel electrophoresis was carried out to standard protocols (Laemmli, 1970). Briefly, 25 µl of OMP was boiled in sample buffer containing βME for two min and then was loaded to one lane of a 12.5% SDS-polyacrylamide gel. Samples were run at 110 V and 30-40 mA current. The gel was stained with Coomassie Brilliant Blue stain for about 5 min and then destained until the bands became visible.

4.2.3 Selection of anti-OMP antibodies from Griffin.1 scFv phage library

4.2.3.1 Growth of library and preparation of VCS-M13 helper phage

Aliquots of the library and VCS-M13 helper phage were provided by Dr. P. Ogston, University of Glasgow. The library was originally supplied by the Medical Research Council Centre for Protein Engineering, University of Cambridge. Aliquots of library and helper phage were amplified according to the protocols provided by staff at the Centre (http://www.mrc-cpe.cam.ac.uk/phage/g1mtaf.html).

4.2.3.2 Panning the library against *Mannheimia haemolytica* OMP

First round of selection in immunotubes

One immunotube (NUNC) was coated by rotation overnight at room temperature with 4 ml of OMP at a protein concentration of 50 µg/ml in PBS. Next day, the tube was washed 5 times with PBS to remove unbound proteins. Blocking was performed with 4 ml of 2 % skimmed milk in PBS (MPBS) at 37°C for 2 hrs. The tube was then washed 5 times with PBS.

From the Griffin.1 scFv library, phage were added to the coated tube in a volume of 4

ml of 2 % MPBS and the tube was incubated for 30 min at room temperature, rotating continuously, and then stood for a further 90 min. The supernatant was discarded and the tube was washed 10 times with PBS containing 0.1 % Tween 20, and then 10 times with PBS to remove unbound phage antibodies. To elute the bound phage, 1 ml of 100 mM triethylamine (TEA) in water was added to tube and tube was left rotating for 10 min at room temperature. The eluted phage were mixed with 0.5 ml of 1 M Tris-HCl (pH 7.4) and stored at 4°C. After elution, a further 200 µl of 1 M Tris-HCl (pH 7.4) was added to the immunotube to neutralise the remaining TEA and the tube was kept at 4°C to be used in the next step.

Infecting E. coli with eluted phage antibodies

To provide exponentially growing *E. coli* cells ready for infection with phage, 50 µl of an overnight culture of *E. coli* TG1 in 2 x TY was inoculated to 50 ml of 2 x TY and the culture was incubated at 37°C with shaking until the absorbance reached 0.5 at 600 nm. From 1.5 ml of phage eluted from the first round of selection, 0.75 ml was added to 9.25 ml of exponentially growing bacteria. Also, 3.8 ml of bacterial culture was added directly to the immunotube containing 200 µl of buffer. To determine the number of input phage, 1 ml of the culture of TG1 was infected with 2 µl of a 1/500 dilution (in PBS) of phage stock. The three cultures were incubated in a water bath at 37°C without shaking for 30 min to allow infection to take place. After incubation, the 10 and 4 ml samples of infected TG1 cells were pooled as output and five, 100-fold serial dilutions (in 2xTY) were prepared. The same dilutions of TG1 infected with input phage were also prepared. Titration of input and output phage was performed by plating 100 µl of each dilution on TYE plates containing 100 µg/ml ampicillin and 1% glucose, and incubating the plates at 30°C overnight.

The remaining 14 ml of infected TG1 cells were centrifuged at 3300 g for 10 min and the bacterial pellet was resuspended in 1 ml of 2 x TY. This was then divided between two 20 x 20 cm plates of TYE containing 100 µg/ml ampicillin and 1 % glucose, and spread to dryness. Plates were incubated at 30°C overnight. The next day, 96 single colonies were picked from the large plates and inoculated into wells in a 96 well U bottom Nunc cell culture plate, each well containing 120 µl of 2 x TY. After 3hrs incubation at 37°C with shaking, 60 µl of sterile 50 % glycerol was added and plate was kept at –80°C.

Rescue of selected phage antibodies using helper phage

The colonies which appeared on the two large selection plates were washed into 40 ml of 2xYT. Seventy µl of this culture was added to 100 ml of 2 x TY containing 100 µl/ml ampicillin and 1% glucose. The culture was incubated at 37°C with shaking until the absorbance at 600 nm reached 0.57. To rescue the phage antibodies, about 4.4×10^{11} t.u. of helper phage (VCS-M13) were added to 10 ml of the culture. Cells were left at 37 °C for 30 min without shaking and the culture then was centrifuged at 3300g for 10 min to recover the infected bacteria. The pellet was resuspended in 50 ml of 2 x TY containing 120 µg/ml ampicillin and 30 µg/ml kanamycin and was incubated at 30°C with shaking overnight. Forty-five ml of overnight culture was centrifuged at 3300 g for 30 min to remove the bacteria. To harvest the rescued phage antibodies, 9 ml of PEG/NaCl was added to the supernatant and the mixture was left at 4°C for 1 hr. The suspension was then centrifuged at 5300 g for 30 min to precipitate the aggregated phage. The supernatant was discarded and the pellet was resuspended in 2 ml of PBS and then centrifuged again at 11600 g for 10 min to remove the remaining bacterial debris. The supernatant containing the rescued phage was kept at 4 °C before titration and use in the second round of selection.

Titration of phage rescued from the first round of selection

Titration was performed by infecting aliquots of an exponentially growing culture of *E. coli* TG1 with 10-fold serial dilutions of rescued phage as described for titration of the input phage.

Second round of selection

In the second round of selection, two immunotubes were coated with 4 ml of 12 µg/ml or 120 µg/ml of OMP in PBS, overnight. Selection at the lower concentration was to be eluted with TEA, aiming to preferentially select for phage antibodies with a high affinity for OMP. Phage binding in the second tube (120 µg/ml) were to be eluted by addition of a hyperimmune anti-OMP serum as a competitive ligand. Washing and incubation conditions were as described for the first round of selection. 1×10^{14} PEG precipitated phage from the first round of selection were added to each tube in 3% MPBS. After incubation and washing to remove the unbound phage, TEA was used to

recover bound phage from the first tube. To the second tube, 1 ml of a 1/100 dilution of anti-OMP antiserum (provided by Dr. R. L. Davies) was added and the tube was incubated at 37 °C with rotation for 1.5 hrs. After incubation, the contents of the tube were added to 10 ml of an exponentially growing culture of *E. coli* TG1. Four ml of bacterial culture was also added directly to the tube to recover by infection any remainder phage. Remaining procedures including isolation of infected bacteria, superinfection with helper phage, recovery of phage using PEG precipitation and titration were as described for round one.

Third round of selection

For the third round of selection, two immunotubes were again coated with OMP for elution with TEA and anti-OMP antiserum. Concentrations of OMP were reduced 10-fold for each tube, giving concentrations of 1.2 and 12 µg/ml for TEA and antiserum elution reactions respectively. 1×10^{13} PEG precipitated phage from the second round of selection were added to each tube. Procedures were as described for previous rounds of selection.

4.2.4 Screening phage antibodies by ELISA (monoclonal phage ELISA)

After each round of selection, 96 transfected colonies were picked at random into the wells of microtitre plates (see above). From these bacterial lines, phage were prepared and screened for binding to the *Manneheimia haemolytica* OMP antigens using ELISA. Since each preparation of phage is derived from a single infected bacterial colony, this is termed "monoclonal phage ELISA".

Preparation of phage antibodies for ELISA

From each well of the stock microtitre plates, 15 µl of bacterial suspension was transfered to a second 96 well plate containing in each well 70 µl of 2 x TY with 100 µg/ml ampicillin and 1% glucose. The second plate was incubated with shaking for 3-4 hrs at 37 °C. Five µl from each well was transferred to a third microtitre plate containing in each well 150 µl of 2 x TY with 100 µg/ml ampicillin and 1% glucose. Growth continued with shaking for 2 hrs at 37 °C before addition to each well of 1×10^{10} t u VCS-M13 helper phage in 20 µl of the same media. The plate was left standing in an incubator at 37 °C to allow the helper phage to infect the *E.coli* cells, and then it was

transferred to shaker incubator for a further 1.5 hrs. In order to rescue the phage antibodies, the plate was centrifuged at 1800 g on a specialist plate carrier, the supernatant was aspirated and the pellet in each well was resuspended in 220 µl of 2 x TY containing 100 µg/ml ampicillin and 50 µg/ml kanamycin. The plate was incubated overnight at 30 °C with shaking. The next day, the plate was again centrifuged at 1800 g and the supernatant containing phage antibodies was recovered.

Coating of antigen

The wells of ELISA plates were coated with 100 µl of OMP in PBS at a protein concentration of 50 µg/ml. Since milk had been use during phage selection, a second series of plates were coated with 100 µl of 2% MPBS to control for the specificity of the selected scFvs. Plates were left at room temperature overnight to allow protein binding.

Blocking

The following day, plates were flipped to discard excess liquid and washed 3 times with PBS to remove any unbound antigens. Remaining binding sites on the plastic were blocked by adding 200 µl of 3% bovine serum albumin in PBS (BSA-PBS) to each well and then incubating at room temperature for 2 hrs.

Adding the single phage antibodies

After washing the plates 3 times with PBS, 100 µl of each phage antibody under test was added to wells of the test (OMP coating) and control plates (MPBS coating). Plates were incubated for 2 hrs at room temperature to allow binding.

Detection of bound phage antibodies

After incubation, plates were washed 3 times with 0.05% Tween 20 in PBS and ten 3 times with PBS to remove unbound phage antibodies. One hundred µl of a 1000-fold dilution of HRP-conjugated anti-M13 antibody (Amersham Pharmacia Biotech) in 3% BSA-PBS was added to each well. This antibody recognises pVIII, the dominant component of the coat of filamentous phage. Plates were incubated at room temperature for 1.5 hrs.

Developing

After washing the plates 3 times with 0.05% Tween 20 in PBS and 3 times with PBS to remove unbound anti-M13 antibodies, 100 µl of developer was added to each well. This solution comprised 0.1 M citric buffer containing 1 mg / ml o-phenylenediamine (OPD; Sigma) and 0.4 µl / ml 30% hydrogen peroxide. The colorimetric reaction was stopped by addition of 100 µl 1M sulphuric acid. Absorbance was read at 450 nm on an ELISA reader (Rosys).

For each round of selection, the OD measurement for a given phage clone on the OMP-coated plate was corrected by subtraction of the OD measured on plates coated with milk. Values were then ranked. From those clones with strongest signals, 15 were chosen form the first round of selection, 15 each from TEA and anti-OMP elution at round two, and 24 and 15 from the final round after elution with TEA and anti-OMP antiserum respectively. These clones were then characterised further.

4.2.5 Optimisation of ELISA

Before starting the phage ELISA and during subsequent analysis, the following experiments were performed to check and optimise the reaction conditions. Aside from the variations highlighted in the sections which follow, all conditions (volumes, temperature and duration of incubations, washing steps and development) were as described in the previous section for the monoclonal phage ELISA.

Binding of OMP to ELISA plates

To check the ability of ELISA plates to adsorb the OMP antigens, 2 sets of 4 wells of an ELISA plate (Iwaki) were coated with 50 and 25 µg / ml OMP. After overnight incubation at room temperature, the plate was blocked with 3% BSA-PBS and then dilutions of 1/100, 1/500, 1/1000 and 1/2000 of a rabbit anti-OMP antiserum (donated to the study by Dr R. L. Davies) were added to each row. An HRP anti-rabbit conjugate (Sigma) was used at a dilution of 1/2000 as a secondary antibody and reactions were developed using OPD. To refine this experimental set-up and define factors contributing to background reactions, further assays omitted the OMP coating layer, coated wells with the rabbit anti-OMP antiserum, and defined levels of binding of the detecting antibodies to wells coated only with the BSA blocking protein (Table 4.2).

Table 4.2 ELISA to check the adhesion of OMPs to ELISA plates.

		Coating material	Primary antibody	Secondary antibody
A		OMP	anti-OMP	IIRP anti rabbit
B		PBS	anti-OMP	IIRP anti rabbit
C	1	Anti-OMP antiserum	-	IIRP anti rabbit
	2		-	-
D	1	BSA	anti-OMP	IIRP anti rabbit
	2		-	IIRP anti rabbit
	3		-	-

3% BSA in PBS was used for blocking.

Optimal dilution of anti-M13 phage antibody

Four rows of 4 wells were coated with 10 fold serial dilutions of VCS-M13 helper phage from a starting concentration of 2×10^9 phage particles per well. After incubation and blocking, HRP-conjugated anti-M13 antibody was added in 2 fold serial dilutions from 1/500 across the plate to create a matrix defining the optimal concentration of antibody to detect phage bound to the wells. An additional row of 4 wells was coated with BSA and then equivalent concentrations of the HRP-conjugated anti-M13 antibody were added (1/500, 1/1000, 1/2000 and 1/4000). The OD of these reactions showed the level of background attributable to binding of anti-M13 antibodies to the blocked plastic.

Comparison between BSA and milk

- Helper phage coated wells

A series of experiments compared the qualities of BSA and milk as blocking or diluent agents. Two sets of wells, each consisting of four rows of 4 wells, were coated with VCS-M13 helper phage. The first row of each set was coated with 2×10^{12} phage particles per well with succeeding rows receiving 10-fold serial dilutions. The first set was blocked with 3% BSA-PBS, the second with 2% MPBS. HRP anti-M13 antibody in 3% BSA-PBS was then added at concentrations of 1/500, 1/1000, 1/2000 and 1/4000 to each row of the first set. The second series of wells were tested with the detecting antibody diluted in 2% MPBS.

-OMP coated wells

Three rows of 4 wells were set up with different combinations of MPBS and BSA-PBS as blocking or diluent for phage ELISA (Table 4.3). All wells were coated with OMP at a concentration of 50 µg/ml. In two columns, remaining binding sites on the plastic were blocked by addition of 2% MPBS. In the other two columns, 3% BSA-PBS was used as blocker. Phage antibodies comprising a mixture of PEG precipitated phage from 7 clones known from previous experiments to be reactive with OMP, were added to the first row, the combined supernatant from cultures of the same clones, containing phage antibodies at a lower concentration, was added to the second row, and finally PEG precipitated phage antibody from a clone which failed to react with OMP in previous assays was added to the third row. The diluent for HRP anti-M13 antibody was varied; the first and third columns received the conjugate diluted in 2% MPBS while the diluent from columns two and four was 3% BSA-PBS. Since the titre of PEG precipitated phage

Table 4.3 Comparison of skimmed milk and BSA as blocking agents for phage ELISA.

Code for reaction	MM	MB	BM	BB
Blocking Phage source Diluent of anti M13	Milk PEG precipitated Milk	Milk PEG precipitated BSA	BSA PEG precipitated Milk	BSA PEG precipitated BSA
Blocking Phage source Diluent of anti M13	Milk Supernatant Milk	Milk Supernatant BSA	BSA Supernatant Milk	BSA Supernatant BSA
Blocking Phage source Diluent of anti M13	Milk Negative clone Milk	Milk Negative clone BSA	BSA Negative clone Milk	BSA Negative clone BSA

antibodies is higher than those in the supernatant from infected cultures, the OD values obtained in this experiment can only be compared for like treatments to judged the effect of BSA and milk on the assay. However, results consistently showed that milk particularly when used as a blocking agent, affected significantly the results of phage ELISA (Figure 4.14).

Comparison of two ELISA plates

This experiment compared the properties of ELISA plates supplied by Nunc and Iwaki. The rows of a plate from each supplier were divided into two parts. One was coated with OMP at 50 µg/ml, the other with 2% MPBS. Blocking was carried out with 3% BSA-PBS. In the next step, 100 µl samples of supernatant from overnight cultures of 12 positive colonies, selected from the third round of panning by elution with anti-OMP antiserum, was added to the 12 wells of the first row of each section on each plate. Ten-fold serial dilutions of each sample were then made into the next 3 wells in each column. HRP anti-M13 antibody was added in BSA-PBS and OPD was used as developer. The results revealed that the Nunc ELISA plates were superior for phage ELISA.

4.2.6 Analytical PCR for the detection of full-length scFvs

Various investigators, ourselves included, have observed that panning can isolate phage from antibody libraries which are reactive with the target antigen, but which lack either the V_H or the V_L components of the scFv. Presumably, these constructs possess sufficient affinity for the target to emerge from selection. To assess the presence of V_H and V_L in clones selected on OMP, crude bacterial lysates of 24 and 12 clones selected from the last rounds of selection, eluted by TEA and anti-OMP antiserum respectively, were analysed by PCR. Amplification was performed with primers LMB3 and FOR_LinkSeq, or LMB3 and pHEN-SEQ or Fdseq1 (Table 4.4). Primers LMB3 and FOR_LinkSeq amplify across the cloning sites for the heavy chain fragment, whereas LMB3 and pHEN-SEQ or Fdseq1 should recovers both light and heavy chain sequences (Figure 4.1). The approximate expected size of amplicons produced with these primer combinations is shown in Table 4.5 PCR was performed in a reaction volume of 25 µl with final concentrations of 500 µM of each dNTPs (Promega), 400 nM of each primer and 3.5 mM $MgCl_2$. The reaction buffer provided 50 mM KCl, 10 mM Tris-HCl (pH 9.0) and 0.1% Triton®X-100. 2.5 units of Taq DNA polymerase (Promega) were used for

Table 4.4 Primers used for characterisation of phage clones

LMB3:	CAGGAAACAGCTATGAC
Fd seq1:	GAATTTTCTGTATGAGG
FOR_LinkSeq:	GCCACCTCCGCCTGAACC
pHEN-SEQ:	CTATGCGGCCCCATTCA

Sequences are present 5' to 3'

Table 4.5 Predicted size of amplicons from PCR analysis of pHEN-2.

Primers	Possible scenarios	Approximate expected size (bp)
LMB3 - FOR_LinkSeq	No insert present	190
	V_H insert present	490
LMB3 - pHEN-SEQ	No insert present	320
	V_L or V_H insert present	620
	Both V_L and V_H present	920
LMB3 - Fdseq1	No insert present	360
	V_L or V_H insert present	660
	Both V_L and V_H present	960

amplification with the following conditions. The reaction was heated to 94°C for 5 min and was then incubated for 35 temperature cycles of 94 °C for 50 sec, 56 °C for 1 min and 72 °C for 3 min. There was a final extension step of 72 °C for 7 min. Products were analysed on 1% agarose gel. The results showed that most clones possessed only V_L inserts. Two clones, one with both V_L and V_H and another with only a light chain, were sequenced using primers LMB3 and pHEN-SEQ.

4.2.7 Western blotting

The OMP preparation from *Mannheimia haemolytica* contained a wide range of proteins. Western blotting was therefore used to determine the specificity of the phage antibodies selected by panning. The general procedures for Western blotting are described in the Appendix of General Methods. Variations and specific features employed in this phase of the project are highlighted in the following sections.

4.2.7.1 Western blotting using phage antibody and anti-M13 antibody

About 160 µg of Sarkosyl-prepared OMP was mixed with sample buffer and heated in boiling water for 3 min. The sample was then divided between 8 wells cast in a 12.5% SDS-polyacrylamide gel. Electrophoresis was done using 100 volts and 40 mA until the bromophenol blue dye reached the end of the gel. The proteins were transferred to nitrocellulose membrane (Amersham Pharmacia Biotech). Briefly, a nitrocellulose membrane was cut to the same size as the gel and soaked in transfer buffer. It was carefully located onto the gel which was then sandwiched between 4 layers of filter paper and fibre pads of the same size. The assembly was put into the blotting cassette and loaded to the electrophoresis tank, placing the gel in the anode (-) side and the membrane in the cathode (+) side. After 1.5 hrs of transfer at 100 volts, 40 mA current, the membrane was peeled off the gel and stained with Ponceau red dye to confirm that the proteins had been transferred. Ponceau binds to proteins temporarily and is easily removed by washing with distilled water. After confirming protein transfer had taken place, the membrane was blocked with 3% milk in Tris-NaCl (pH 7.4) overnight. The membrane was then washed with Tris-NaCl and was cut vertically into eight strips each carrying the separated OMP proteins. Each strip was incubated with supernatant from a single phage antibody clone isolated from the third round of selection. Incubation was performed in individual sealed pockets of polythene for 1 hr at 37 °C with shaking. After incubation and washing, the strips were incubated with HRP anti-M13 antibody for 1 hr

at 37 °C with shaking. The strips were washed and developed with 4-chloro-1-naphtol. This general procedure failed to detect binding by any of the phage antibodies under test. The experiment was repeated with clones isolated in different rounds of selection, with PEG precipitated phage, with extended incubation times and variations to the temperature of incubation, and with more sensitive light-emitting enzymes substrates. No variation was identified which would produce a signal from Western blotting.

4.2.7.2 Western blotting using scFv and anti-pIII antibody

Since the use of phage antibodies for Western blotting with OMP antigens did not succeed, we considered using scFvs. In this method, scFvs were expressed as a fusions to pIII by inducing with IPTG. Conventionally, scFvs are expressed as soluble proteins in amber non-suppressing strains of *E. coli* such as HB2151, exploiting the presence of the amber stop codon between the scFv insert and the gIII sequence in vectors such as pHEN2 (McCafferty *et al.*, 1990). By inducing expression in *E. coli* TG1, the scFv-pIII fusion protein accumulates in the cytoplasmic membrane from which it can be released with detergents and detected with anti-pIII antibodies (Tesar *et al.*, 1995; Mersman *et al.*, 1998) (Figure 4.2). Before using this system in Western blotting, it was necessary to verify that scFv-pIII proteins were successfully expressed and that they could be detected with an anti-pIII monoclonal antibody. This was assessed through dot blotting and ELISA.

Dot blotting

The procedures leading to the preparation of a bacterial cell lysate are shown in Figure 4.3. Two clones were chosen from the third round of selection showing the highest signal in anti-OMP phage ELISA. Fifteen µl of a suspension of each clone were added to 5 ml of LB containing 100 µg/ml ampicillin and 1% glucose and the cultures were incubated at 37 °C with shaking, overnight. Next day, 100 µl from each culture was added to 10 ml of LB containing 100 µg/ml ampicillin and 1% glucose. The cultures were incubated at 37 °C with shaking until the OD at 600 nm reached to 0.55 to 0.6. Each culture was then divided to 5 ml aliquots and centrifuged at 5100 rpm. One pellet from each clone was resuspended in 5 ml of LB containing 100 µg/ml ampicillin and 1 mM IPTG and the another pellet from each clone was resuspended in the same media containing 0.1 mM IPTG (Kipriyanov *et al.*, 1997). Cultures were left shaking at 30 °C

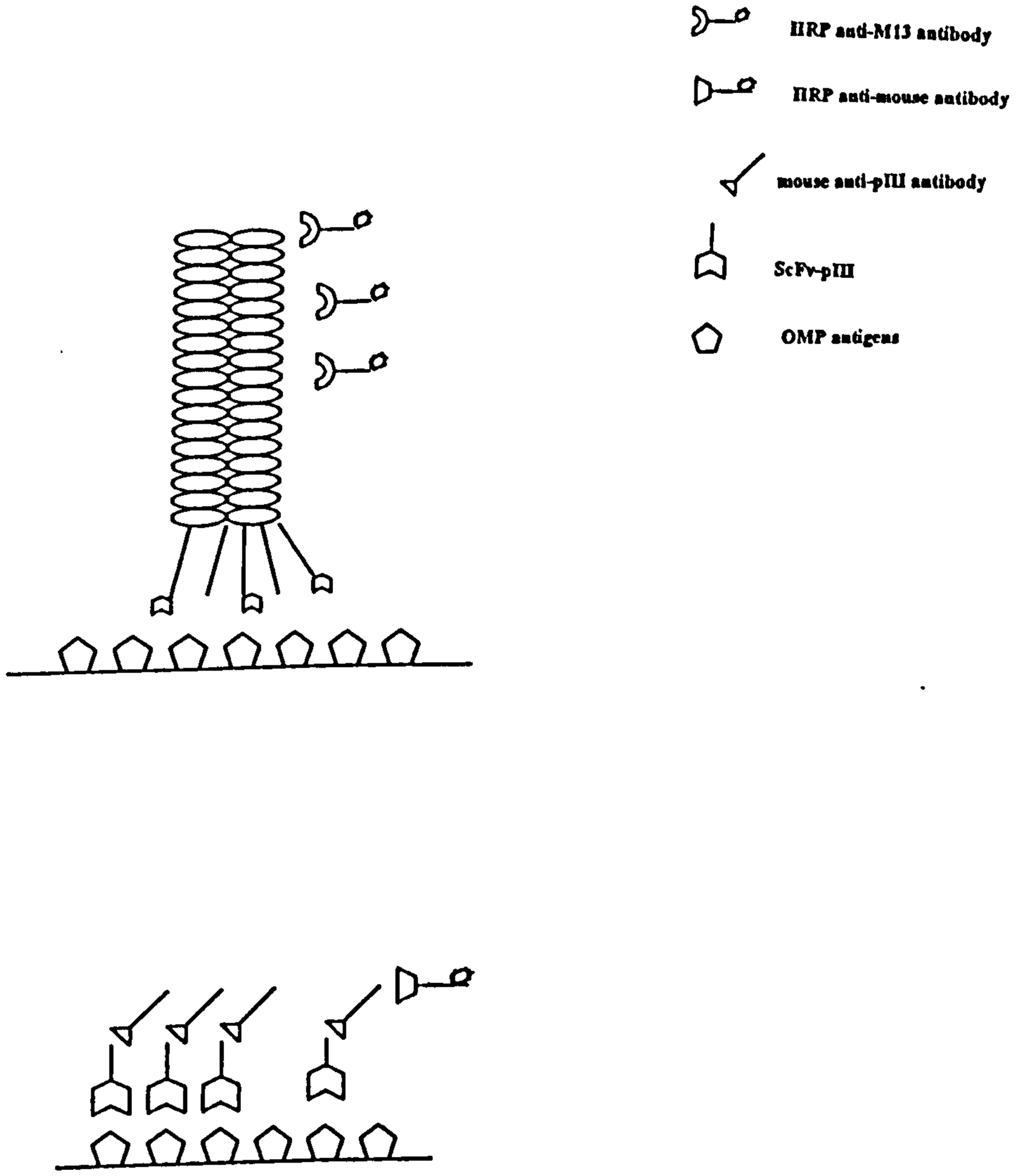


Figure 4.2 Two methods for detection of anti-OMP antibodies.

A: Phage carrying an anti-OMP scFv recognises the antigen coated to a solid phase. Anti-M13 antibodies conjugated to horseradish peroxidase (HRP) binds to the phage coat, most likely to pVIII, the most abundant component of the capsid.

B: pIII-scFv expressed by bacteria binds to the OMP-coated surface. It is then detected by a monoclonal anti-pIII antibody and HRP-conjugated anti-mouse reagents.

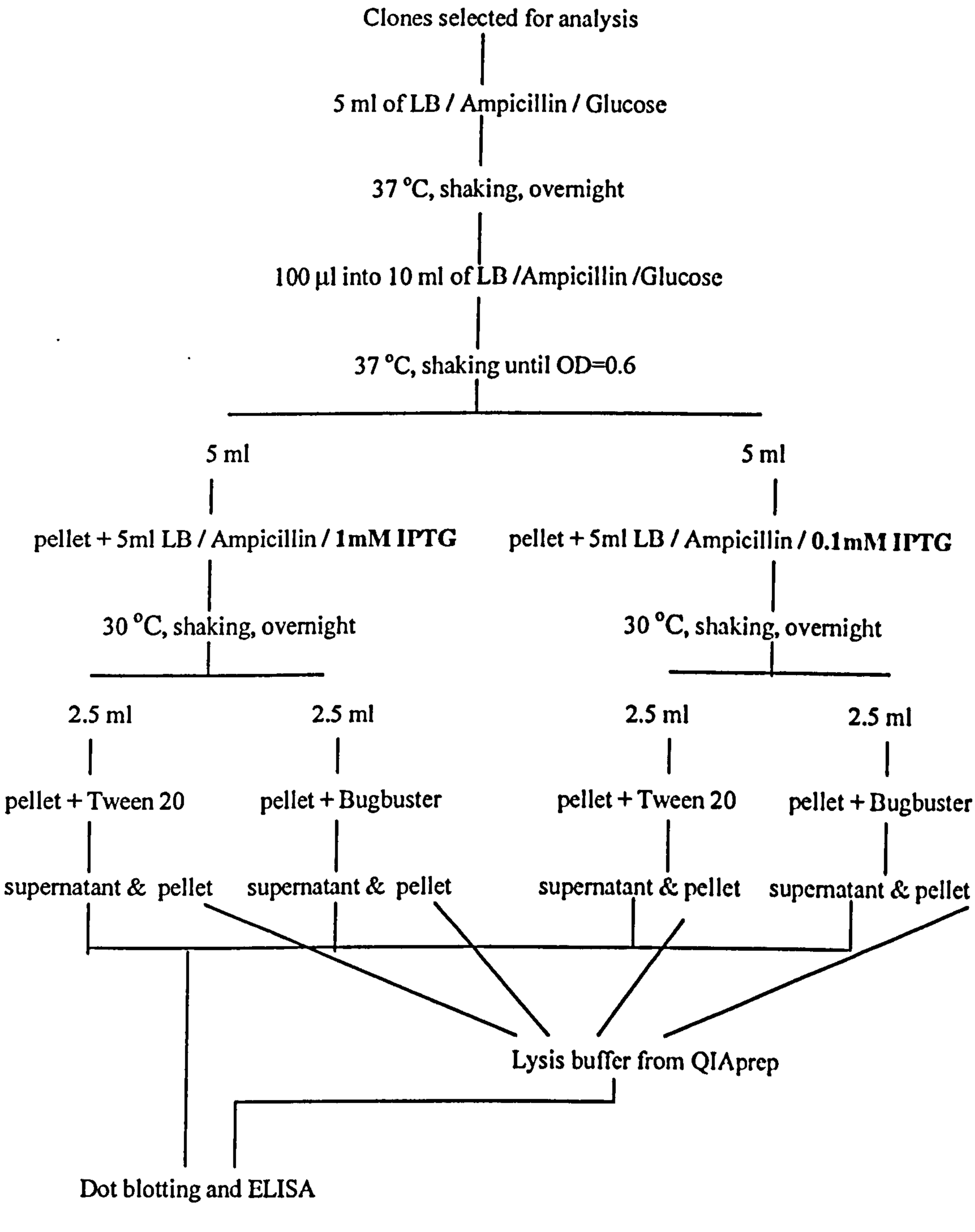


Figure 4.3 Procedure for the preparation of scFv-pIII used in dot blotting and ELISA.

overnight to allow expression of the scFv-pIII fusion. The following day, each 5 ml culture was divided into two parts for lysis with different detergents. After centrifugation, one pellet from each 2.5 ml aliquot was resuspended in 200 µl of LB containing 110 µl of 0.05% Tween 20; the other was resuspended in 200 µl of LB containing 110 µl of Bugbuster solution (Novagen). The lysis reactions were incubated with shaking at 30 °C for 4.5 hrs and were then centrifuged at 7000 g for 10 min. Supernatants predicted to contain scFv-pIII were collected and the pellets were treated with 50 µl of P1 and P2 solutions (resuspension and lysis solutions) from QIAprep spin miniprep kit to assess the efficacy of lysis.

A nitrocellulose membrane was used to spot out the lysates in a pattern shown in Figure 4.16. Two µl of the lysates prepared from each clone, after induction with 1 and 0.1 mM IPTG were spotted onto the membrane and left at room temperature until dry. Two µl of a PEG precipitated phage preparation was spotted as a positive control, and as negative controls, aliquots of Bugbuster and Tween 20 detergents were added. The membrane was blocked with 3% BSA-PBS for 2.5 hrs and it was then washed 3 times with PBS. The membrane was then incubated with a 1/1000 dilution of a mouse anti-pIII monoclonal antibody (Mo Bi Tec) diluted in 3% BSA-PBS. Incubation was for 1.5 hrs and after washing, a 1/1000 dilution of an HRP-conjugated anti-mouse antibody was added for a further 1.5 hrs. After the last round of washing, the dot blot was developed with 4-chloro-1-naphtol substrate. The result showed that the anti-pIII antibody recognised a ligand (pIII) on the blot (Figure 4.16).

ELISA

The efficacy with which anti-pIII could detect scFv-pIII fusions was checked by ELISA. Two rows of 4 wells were coated with 100 µl of 50 µg/ml OMP and two rows were coated with 2% MPBS as a control. After overnight coating at room temperature and blocking with 3% BSA at 37 °C for 2 hrs, wells from each coating were allocated for lysates produced from the clone analysed by dot blotting after induction with IPTG at 1 mM and 0.1 mM. One hundred µl samples of lysates produced with Tween 20 and Bugbuster and the Qiaprep lysates from each set of cell pellets were added to designated wells. After incubating the plate at room temperature for 2 hrs and washing, 100 µl of a 1/1000 dilution of mouse anti-pIII (Mo Bi Tech) was added to all wells for incubation at room temperature for 1.5 hrs. The plate was then washed and 100 µl of a 1/1000 dilution

of HRP anti-mouse antibody was added to each well and plate was incubated for 1.5 hrs incubation at room temperature. After final washing, OPD substrate was added and the OD was recorded at 450 nM. The results demonstrated the expression of scFv-pIII and recognition by the anti-pIII antibody.

Western blotting

After confirmation that scFv-pIII fusion was produced by the selected clones and that it could be detected by immunochemistry, further Western blotting experiments were set up to define the specificity of selected phage antibodies. All procedures were performed as described before. The nitrocellulose membrane carrying separated OMP proteins was cut into 10 strips and each was incubated with a scFv-pIII prepared by Bugbuster lysis from 10 clones from the third round of selection. The anti-pIII and anti-mouse antibodies were diluted to 1/1000 and 4-chloro-1-naphthol substrate was used. This experiment resulted in faint signals. Repeating the experiment with a mixture of lysate of 12 clones again resulted in a stronger signal at the same location.

4.2.8 Identification of major protein reacting with antibodies

To identify the component of the OMP preparation which was reactive with selected recombinant antibodies in Western blotting, the relevant band was sliced from a Coomassie stained acrylamide gel. The molecular weight of this protein was about 40 kDa. Since the reactive band migrated close to another OMP of similar molecular weight, care was taken to avoid contamination from this species. The band was despatched to the Department of Molecular and Cell Biology, University of Aberdeen where it was digested with trypsin and analysed by mass spectrometry. The molecular weights of the tryptic fragments were screened by database searches to identify the target protein.

4.2.9 Native acrylamide electrophoresis.

We reasoned that the weak reaction of scFvs in recognising of their target OMP proteins on Western blots (*c.f.* efficient detection in ELISA) might be due to conformational changes after exposure to SDS in acrylamide gel electrophoresis or the separation of multimeric proteins into their monomers. To address this, native acrylamide gel electrophoresis was chosen as an alternative separating technique. To achieve, this acrylamide gels were prepared with the same composition described before but without SDS. Two sets of 4 wells were prepared for samples analysis. In one well,

the OMP sample (25 μg) was loaded intact in a loading buffer without mercaptoethanol or heating. In other wells, 1.5 μl of 0.1% solutions of the following detergents were added; CHAPS, sodium deoxycholate and Tween 20. Electrophoresis was performed at 110 volts and 30-40 mA current using a transfer buffer from Western blotting of pH 8.7. After running, one set of samples was stained to determine protein migration. Only proteins prepared with addition of sodium deoxycholate and Tween 20 had undergone weak migration. The second set of samples was subjected to Western blotting. Protein transfer was performed using conventional transfer buffer with pH 8.1. Blocking was done with 2% skimmed milk in Tris-NaCl and the membrane was then incubated with the scFv-pIII fusions from two ELISA positive clones, prepared as Bugbuster lysates. After incubation with anti-pIII and anti-mouse antibodies, 400 μl of the sensitive ECL substrate was used. Signals could be detected from samples prepared with sodium deoxycholate and Tween 20.

4.3 Results

4.3.1 Protein assay of *Mannheimia haemolytica* OMP preparations

Based on standard curves, the concentrations of protein in different batches of OMP were determined with the BCA protein assay kit to be between 1.4 and 2.4 mg/ml.

4.3.2 SDS-PAGE analysis of *Mannheimia haemolytica* OMP preparations

The protein composition of isolated OMPs from *M. haemolytica* was analysed by SDS-PAGE. Three major bands could be observed along with numerous less abundant proteins (Figure 4.4). One of the dominant components ran with an estimated molecular weight of about 31 kDa. The other two migrated close together at around 40 kDa. The overall appearance of these gels was similar to those described by others (Morton *et al.*, 1996; Brennan, *et al.*, 1997) using bacteria of the A biotype.

4.3.3 Panning the Griffin.1 library against *Mannheimia haemolytica* OMPs

Anti-OMP antibodies were isolated from the phage antibody library using two strategies. The first was conventional: OMPs were bound to plastic immunotubes and phage selected by successive rounds of binding, washing and elution with TEA. In the other approach, phage recovered from the first round of conventional selection were applied to coated immunotubes but then eluted by adding an anti-OMP serum. This competitive elution was used in two rounds of selection. In both strategies, the concentration of OMPs applied to the selecting surface was reduced round-on-round: from 120 µg/ml (round 1), to 12 µg/ml (round 2), to 1.2 µg/ml (round 3) for elution with TEA and from 120 µg/ml (rounds 1 and 2), to 12 µg/ml (round 3) for elution with anti-OMP antiserum. The numbers of input and output phage at each of round of selection for the two methods are shown in Table 4.6 . For selections in which TEA was used as eluting agent, enrichment was not observed at round 2 but it rose sharply to 52 for the third round of selection. Similar patterns were observed when anti-OMP antiserum was used for elution: no enrichment was detected at round 2 (value 0.03) but 15-fold enrichment occurred at round 3.

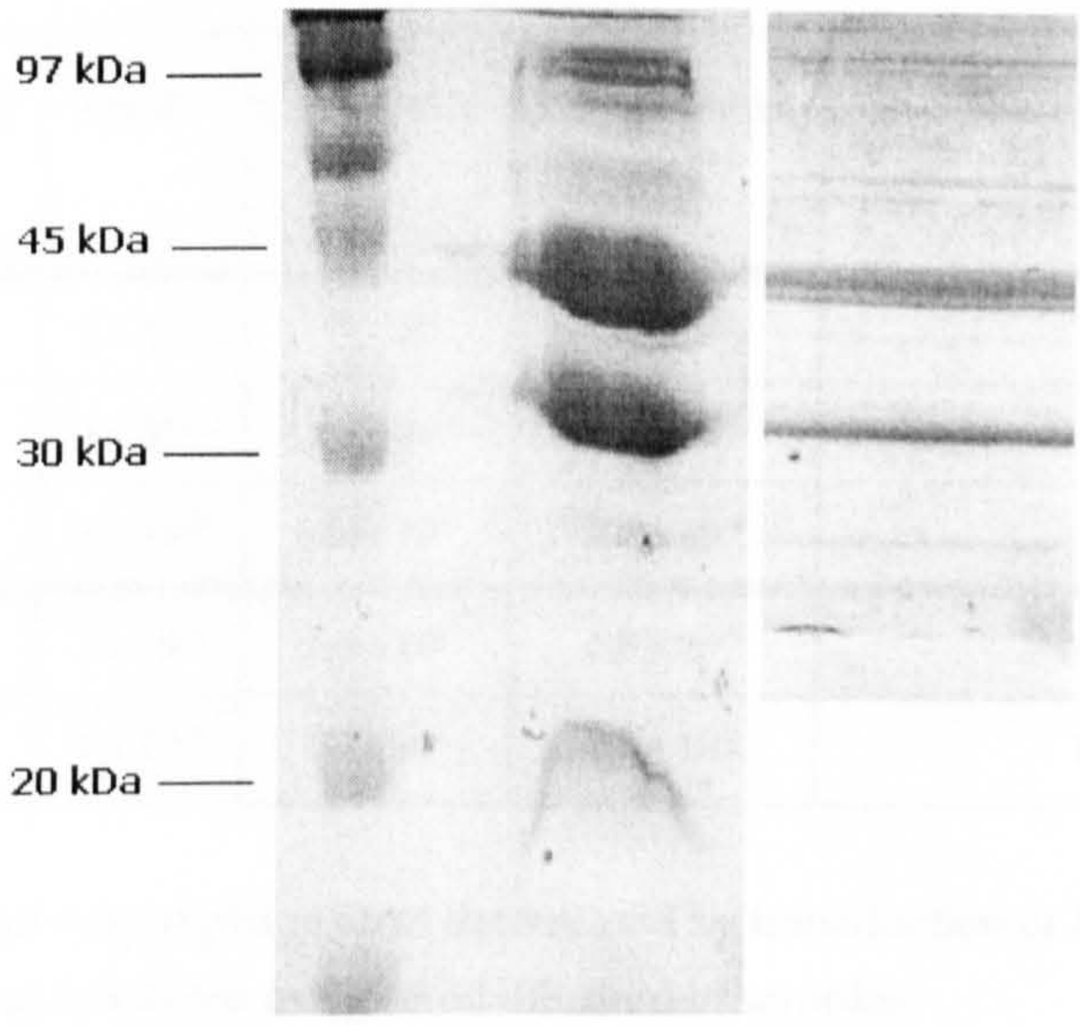


Figure 4.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) profile of outer membrane proteins of *Mannheimia(Pasteurella) haemolytica*.
Major proteins migrated with molecular weights of 31 kDa and 40 kDa. The right panel shows resolution of two bands from the 40 kDa component. Resolution was improved in this instance by loading sample across a broad well cast into the gel
M: Rainbow protein molecular weight markers (Pharmacia). The migration of standards of 97 kDa, 45 kDa, 30 kDa and 20 kDa is indicated.

Table 4.6 Recovery and enrichment rate of phage in each round of selection.

Elution agent	Round	Input [a]	Output [a]	% recovery x10^{3} [b]	Enrichment [c]
TEA	1	1×10^{13}	9×10^{6}	9×10^{-2}	-
	2	1×10^{14}	8.4×10^{5}	8.4×10^{-4}	0.009
	3	1×10^{13}	4.4×10^{6}	4.4×10^{-2}	52
Anti-OMP	2[d]	1×10^{14}	2.8×10^{6}	2.8×10^{-3}	0.03
	3	1×10^{13}	4.3×10^{6}	4.3×10^{-2}	15

[a] Input and output phage titres determined by transduction of *E. coli* to ampicillin resistance using serial dilutions of samples

[b] Percent recovery determined by division of output phage titre by input titre and multiplication by 100. Data expressed after further multiplication by 1000

[c] Enrichment calculated by division of percent recovery by percent recovery achieved in previous round of selection

[d] Round 1 for this experiment was round 1 with TEA elution

4.3.4 Screening recombinant antibodies by monoclonal phage ELISA

Ninety six colonies were picked after each round of selection and analysed by monoclonal phage ELISA to determine which showed highest reaction with the OMP preparation. Phage clones were screened on OMP-coated plates and on plates coated with skimmed milk, the blocking agent used in selection, to check for specificity. The OD measurement achieved for a given phage clone on the OMP-coated plate was corrected by subtraction of the OD measured on plates coated with milk and the values were then ranked. Results of these phage ELISAs for each round of selection are shown in Figures 4.5 to 4.9. Monoclonal phage ELISA was then repeated to compare the 80 clones showing strongest reactivity with OMPs. The results are shown in Figure 4.10. The data shows a progressive increase in reactivity of individual clones towards OMPs with the selection round from which they were recovered. This supports the hypothesis that progressive reduction in the concentration of the OMPs used for selection should drive the recovery of clones which react best with the target.

4.3.5 Optimisation of ELISA

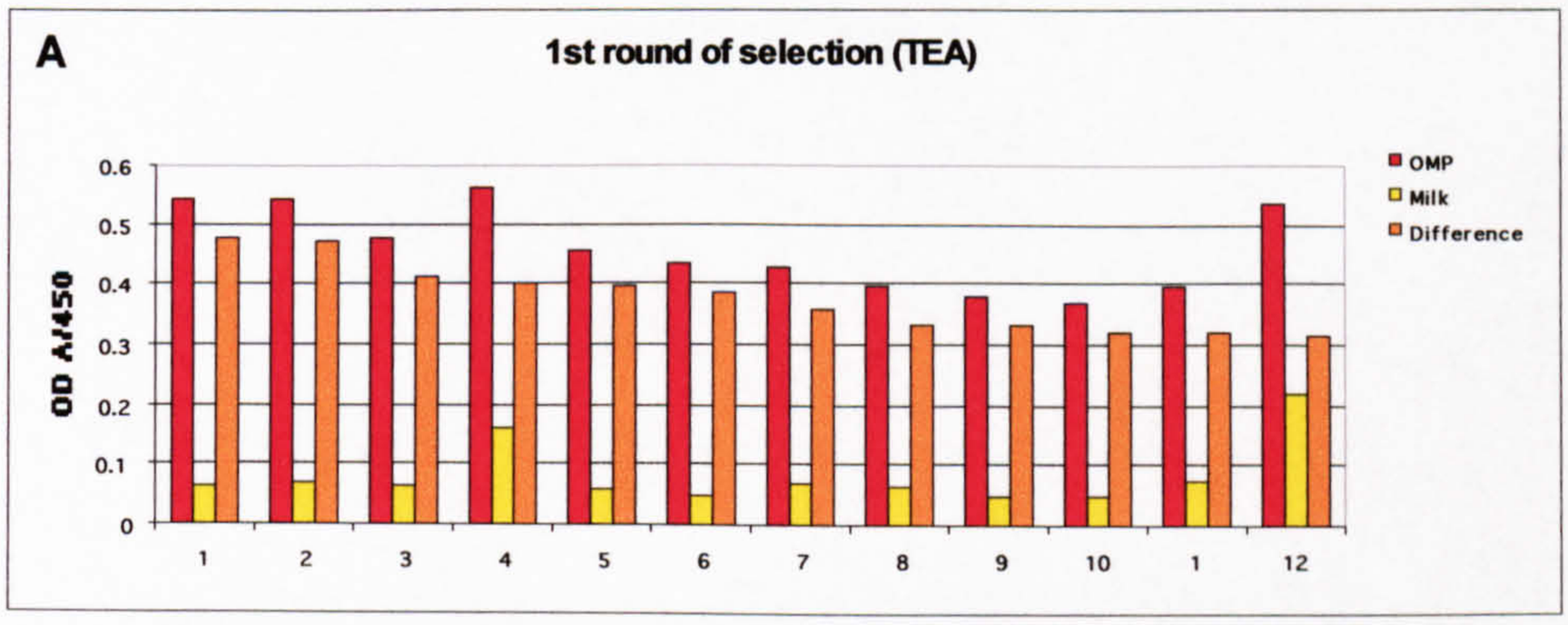
To achieve these results, substantial efforts were made to optimise the ELISA methods.

Binding of OMP to immunoplate

To determine the efficiency with which OMPs bound to the plastic surface, an ELISA experiment was set up with OMP antigens and anti-OMP antiserum. Controls also checked to what extent background readings from the ELISA could be attributed to the different reagents used in the assay. Results of this experiment, shown in Figure 4.11, confirmed that the OMPs prepared with Sarkosyl could bind to the microtitre plates. ELISA results with different coating proteins and combinations of other reagents confirmed that the absorbance values were specific for the interaction between OMPs coated to the plates and the test antiserum, and not due to the background.

Optimal dilution of anti-M13 phage antibody

Binding of anti-M13 antibody to phage was assessed by coating serial dilutions of PEG-precipitated phage to microtitre plates and then using HRP-conjugated anti-M13 antibody. Results of this experiment are shown in Figure 4.12. revealed that the anti-M13 conjugate recognised the phage.



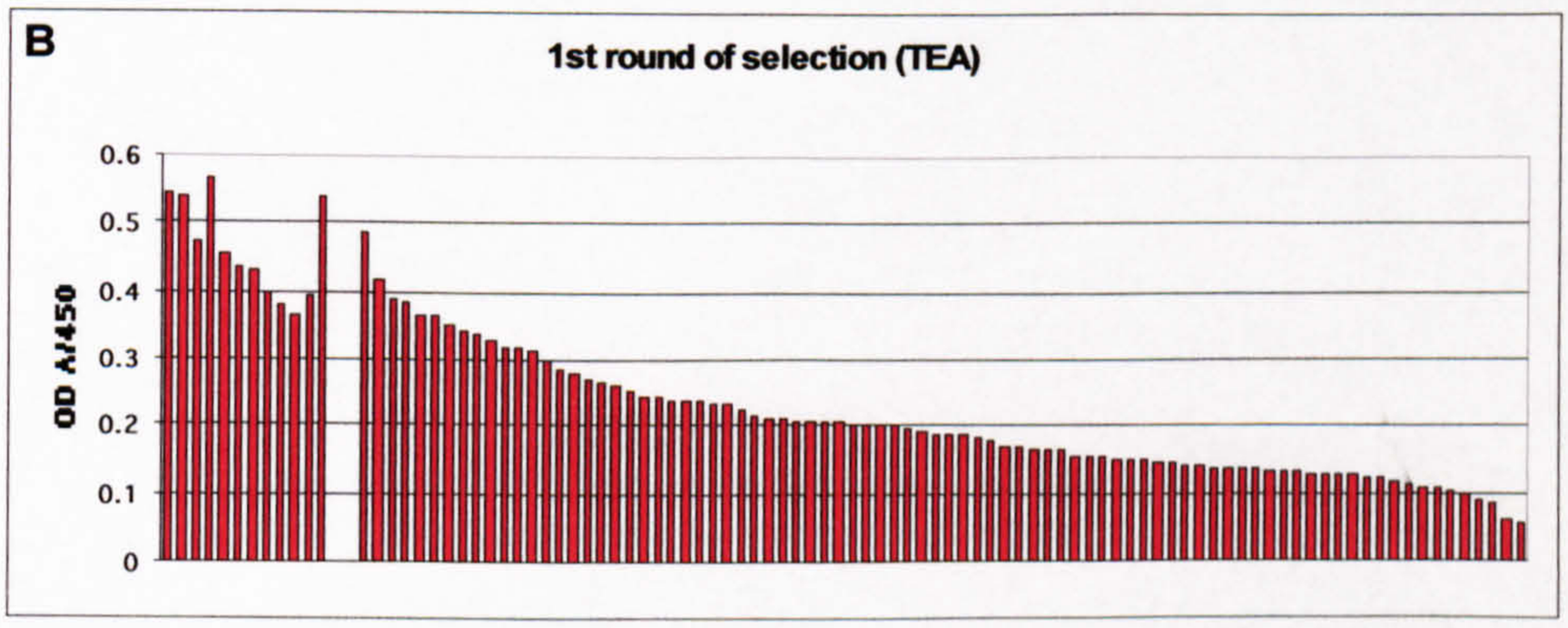
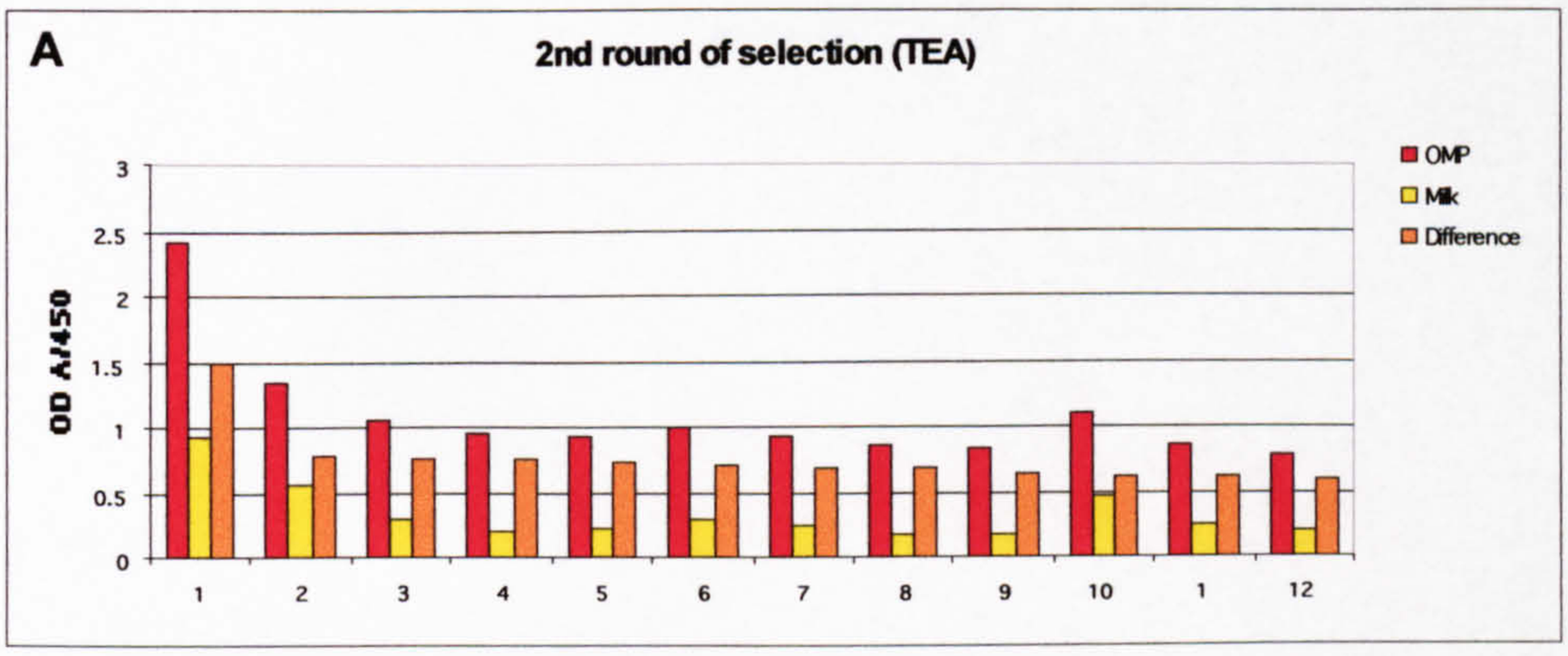


Figure 4.5 Phage ELISA of clones from the first round of selection and eluted with TEA. (A): ELISA results from 12 clones ranked by difference in reactivity in OMP *versus* milk coated wells. Note the differential reactivity ranges between 0.5 and 0.3. (B): ELISA reactivity towards OMPs of 96 clones picked at random from the first round of screening. Results for 12 clones with the strongest reactivity are separated from the rest.



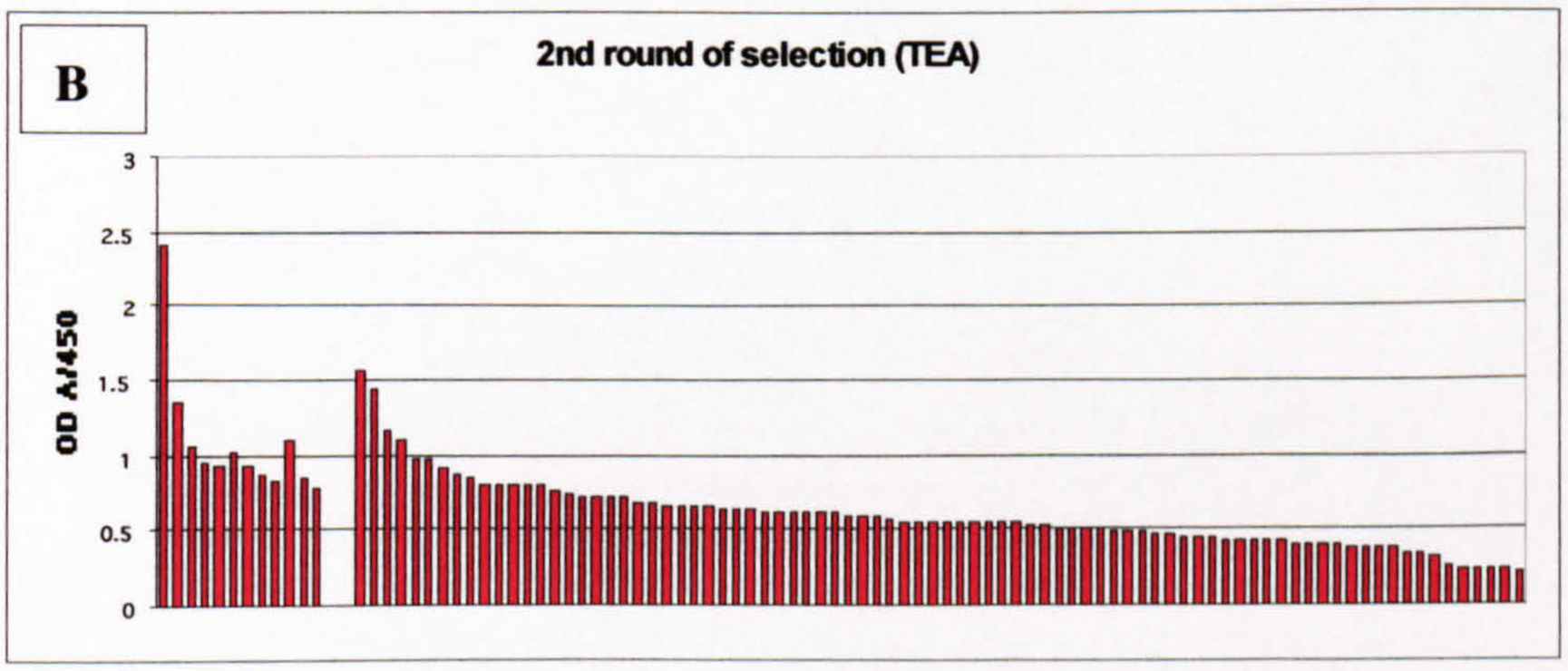
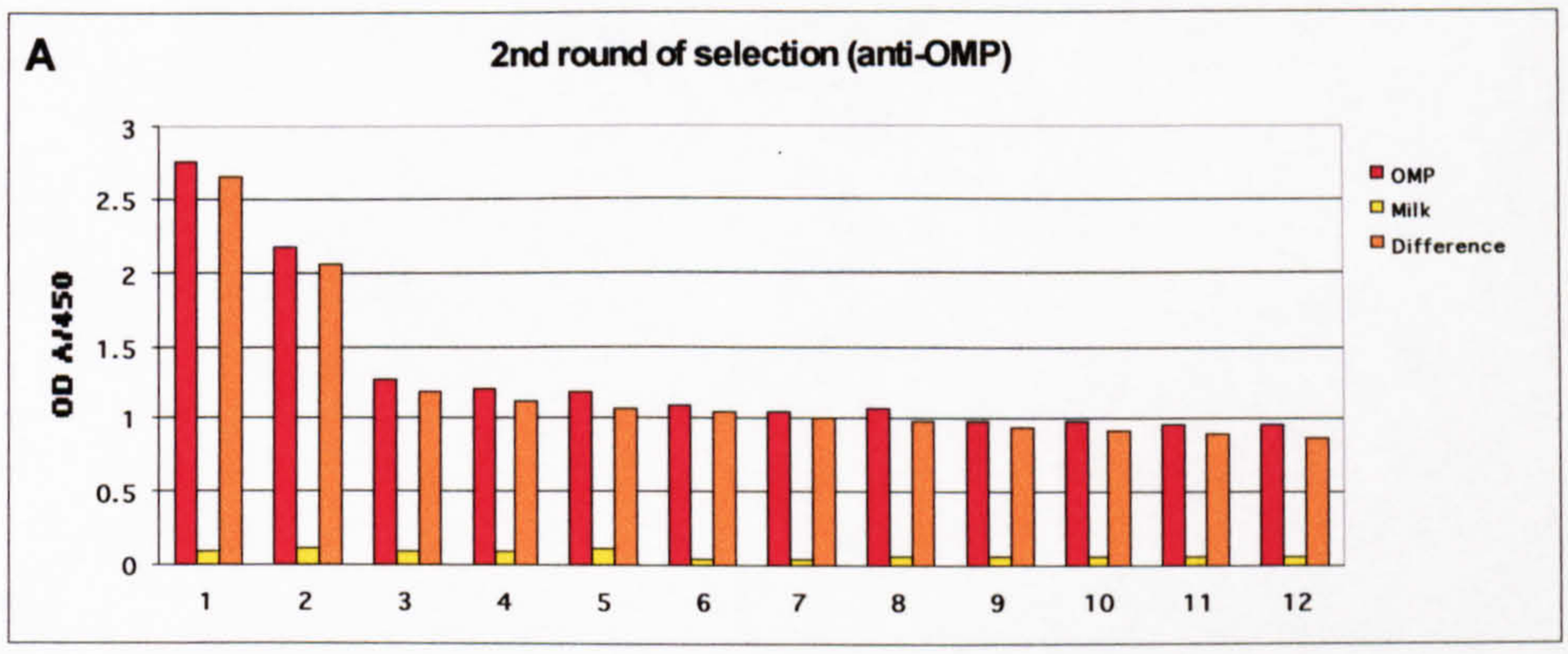


Figure 4.6 Phage ELISA of clones from the second round of selection and eluted with TEA. (A): ELISA results from 12 clones ranked by difference in reactivity in OMP *versus* milk coated wells. Note the differential reactivity ranges between 1.5 and 0.5. (B): ELISA reactivity towards OMPs of 96 clones picked at random from the second round of screening. Results for 12 clones with the strongest reactivity are separated from the rest.



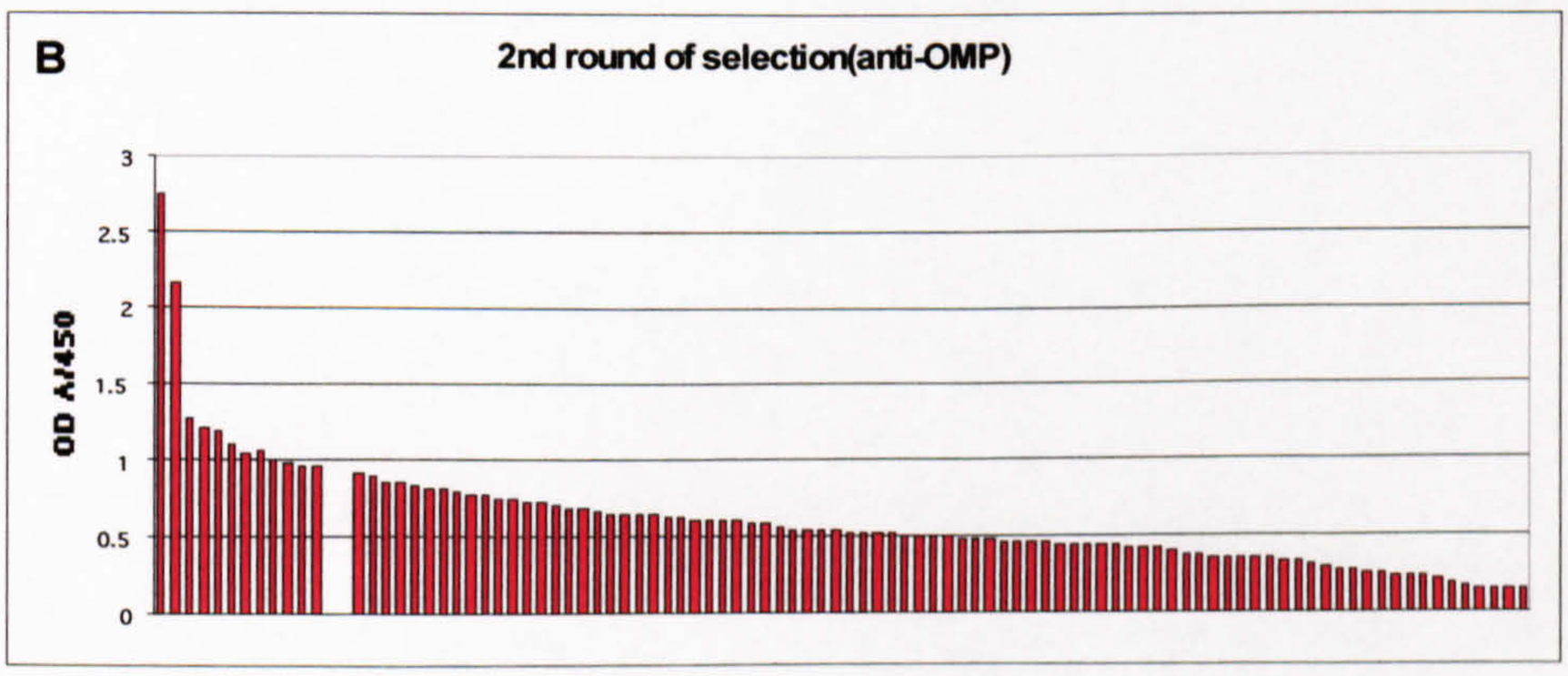
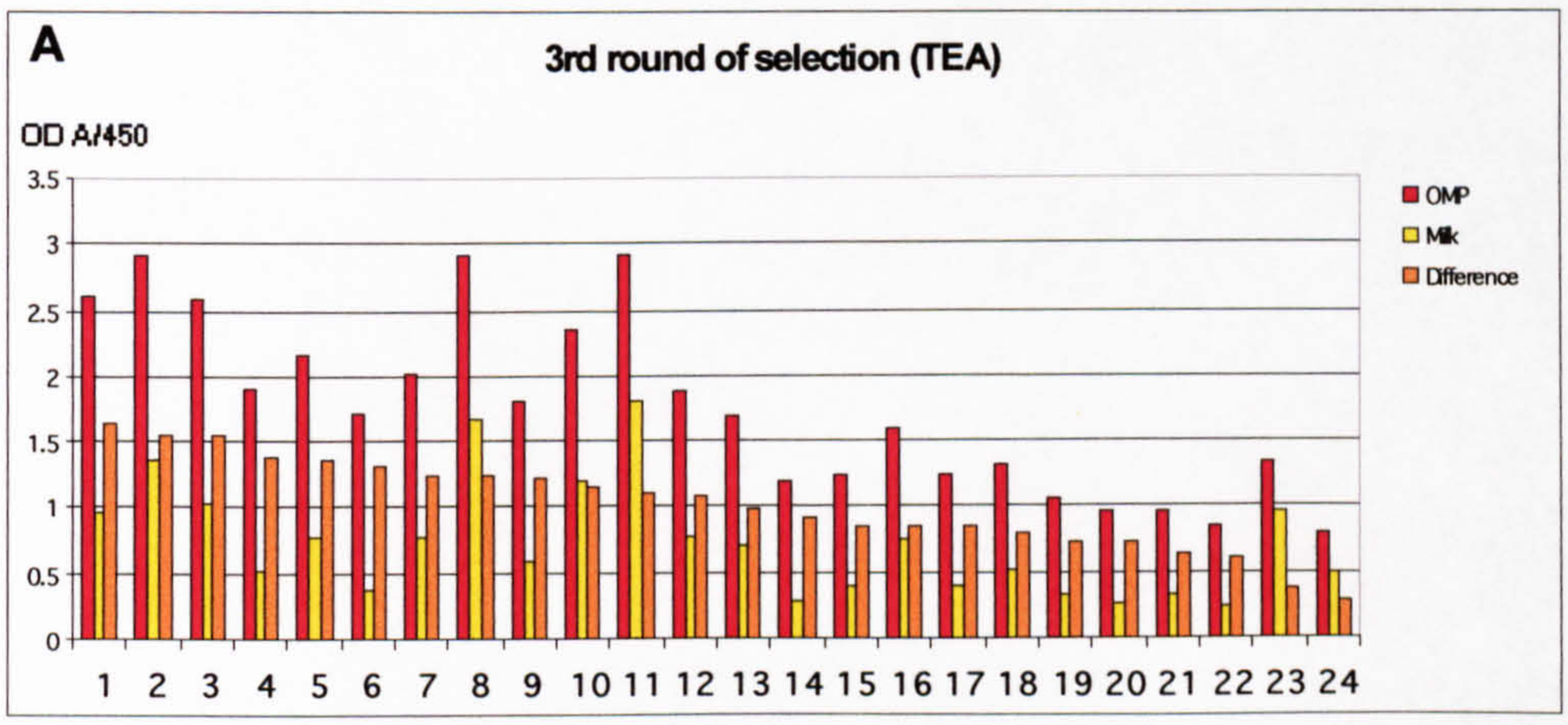


Figure 4.7 Phage ELISA of clones from the second round of selection and eluted with anti-OMP serum.

(A): ELISA results from 12 clones ranked by difference in reactivity in OMP *versus* milk coated wells. Note the differential reactivity ranges between 2.6 and 0.9. (B): ELISA reactivity towards OMPs of 96 clones picked at random from the second round of screening. Results for 12 clones with the strongest reactivity are separated from the rest.



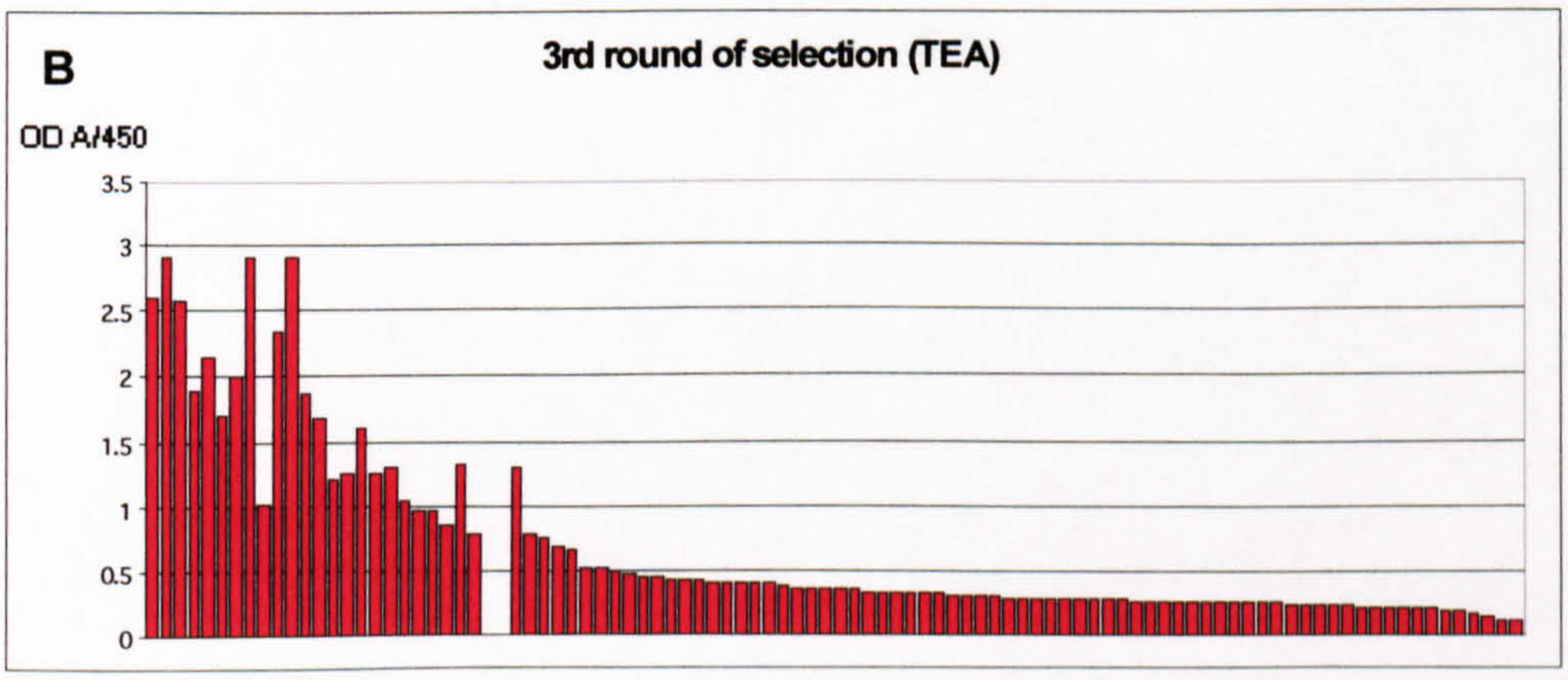
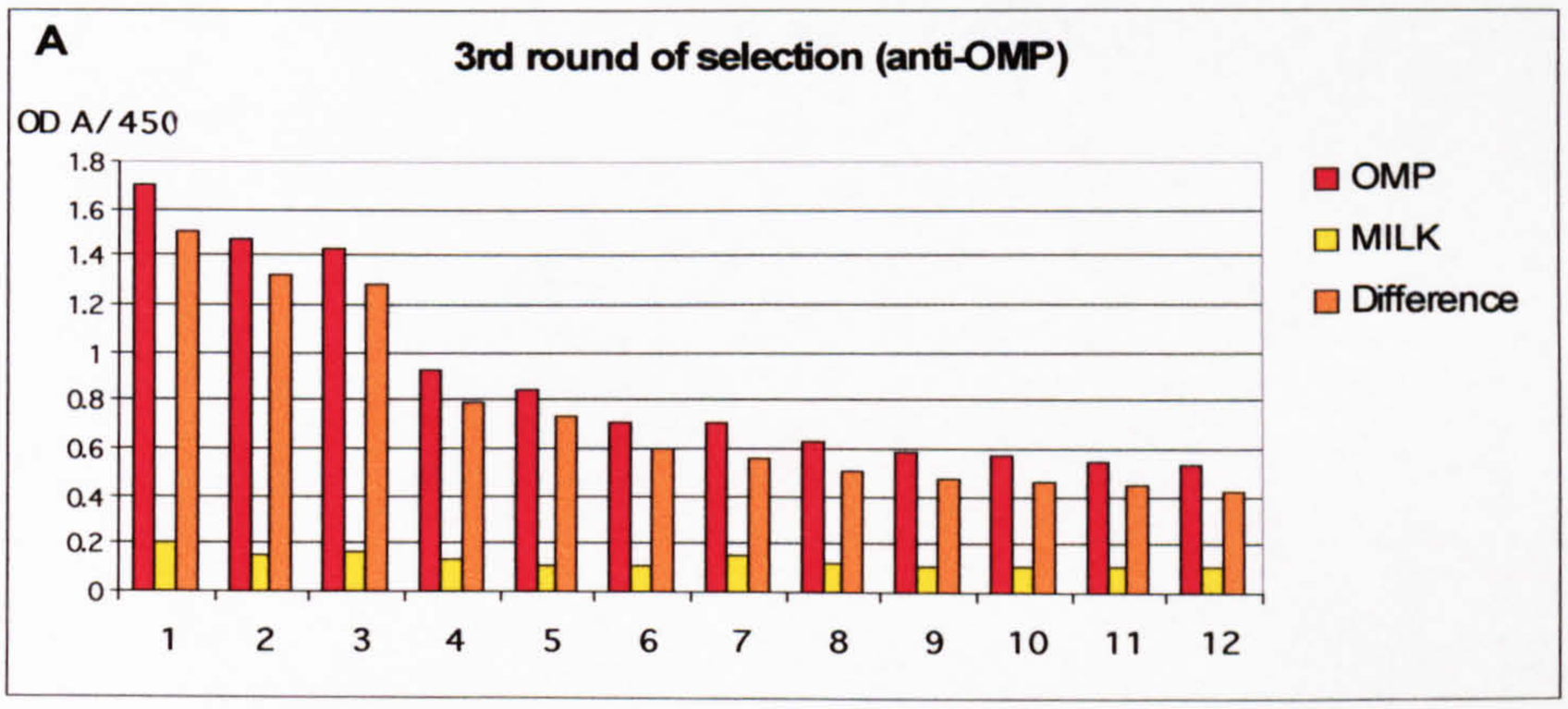


Figure 4.8 Phage ELISA of clones from the third round of selection and eluted with TEA.

(A): ELISA results from 24 clones ranked by difference in reactivity in OMP *versus* milk coated wells. Note the differential reactivity for the first 12 ranges between 1.6 and 1.1.

(B): ELISA reactivity towards OMPs of 96 clones picked at random from the third round of screening. Results for 24 clones with the strongest reactivity are separated from the rest.



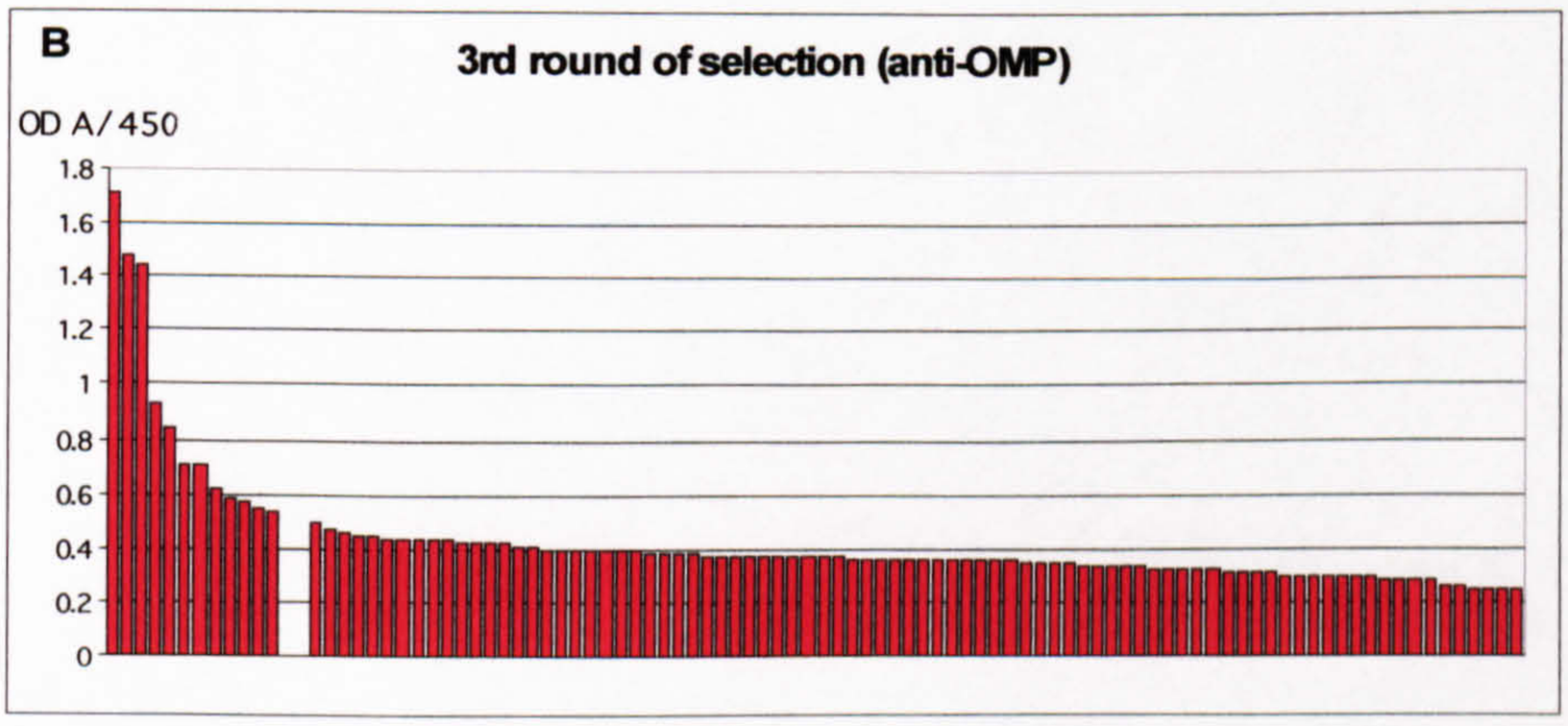


Figure 4.9 Phage ELISA of clones from the third round of selection and eluted with anti-OMP serum.

(A): ELISA results from 12 clones ranked by difference in reactivity in OMP *versus* milk coated wells. Note the differential reactivity ranges between 1.5 and 0.4.

(B): ELISA reactivity towards OMPs of 96 clones picked at random from the second round of screening. Results for 12 clones with the strongest reactivity are separated from the rest.

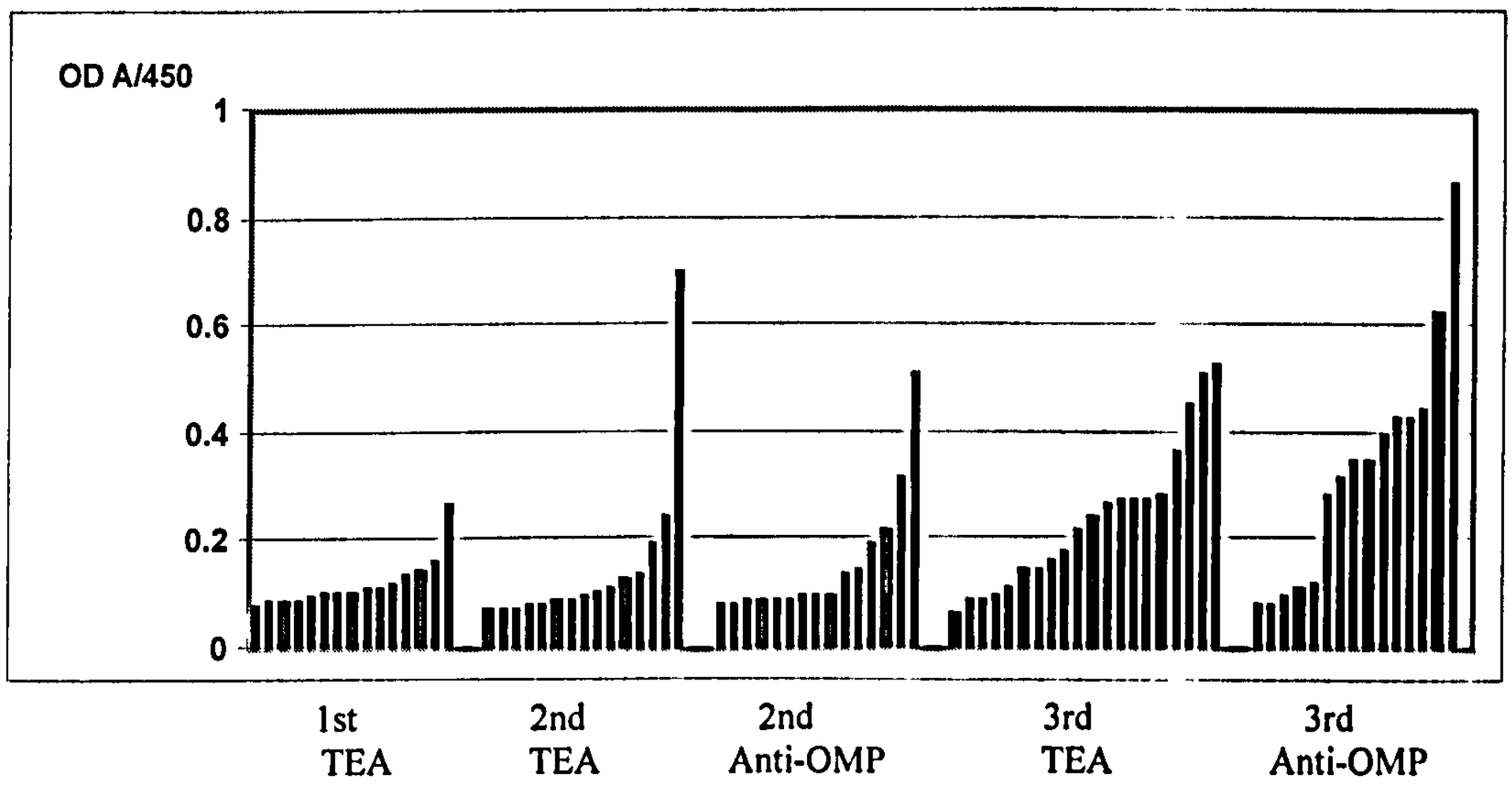


Figure 4.10 ELISA to compare 80 clones isolated from different rounds of selection. Fifteen clones which reacted best in initial anti-OMP ELISA were taken from each round of selection as indicated (20 clones for the third round of selection with TEA elution). Monoclonal phage ELISA was carried out for each clone on wells coated with OMPs and millk. The ELISA results (y axis) are corrected for values obtained on control wells coated with milk.

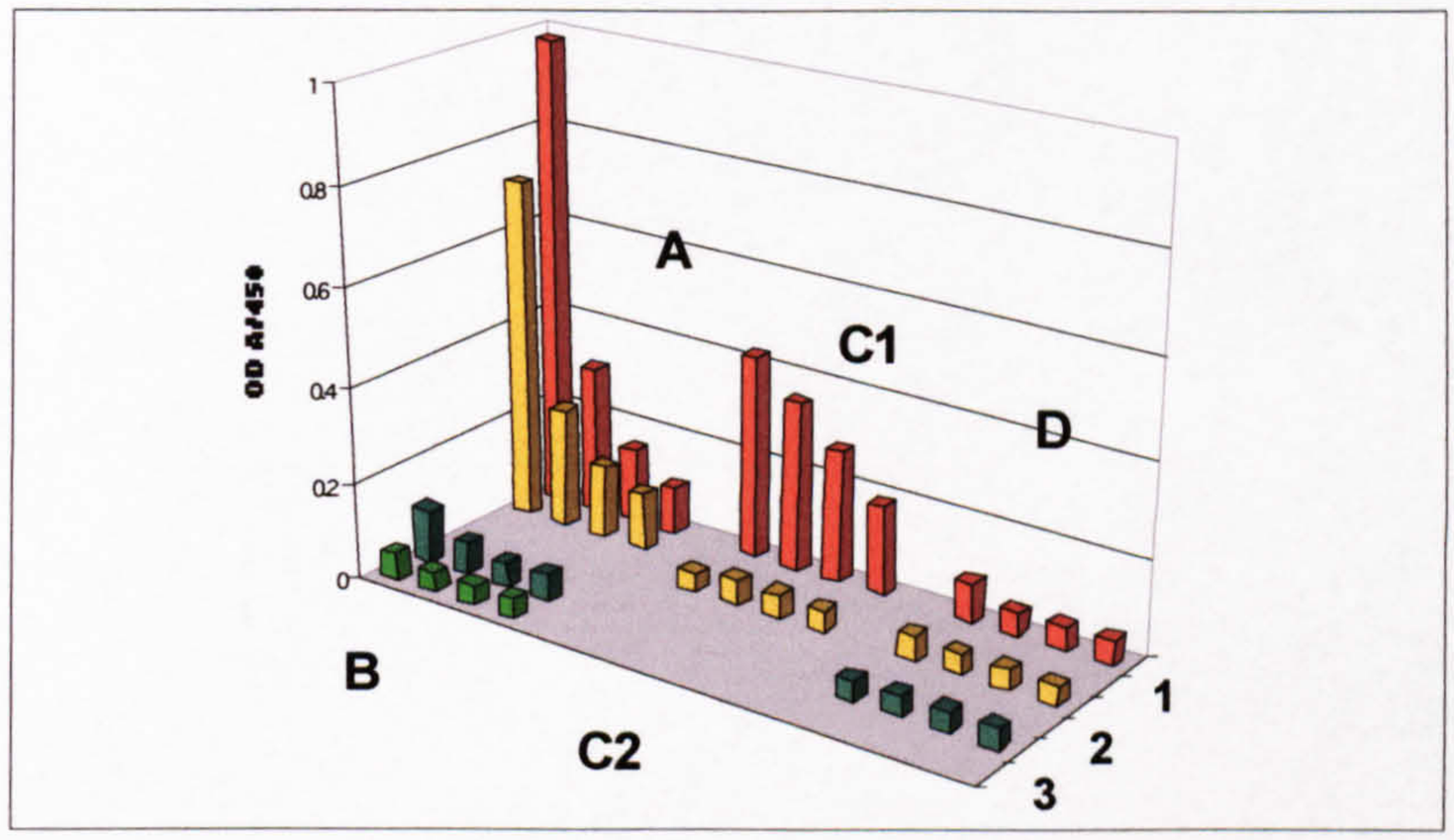


Figure 4.11 ELISA to confirm the binding of OMPs to ELISA plates.
Wells were coated with serial dilutions of OMPs (section A; two rows coated with dilution from left to right), PBS (section B; two rows), serial dilutions of an anti-OMP rabbit serum (section C; dilutions left to right) or BSA (section D). Rabbit anti-OMP serum was then added to sections A, B, and D1, followed by an anti-rabbit HRP conjugate (all sections except C2 and D3). The reactions were then developed with OPD substrate.

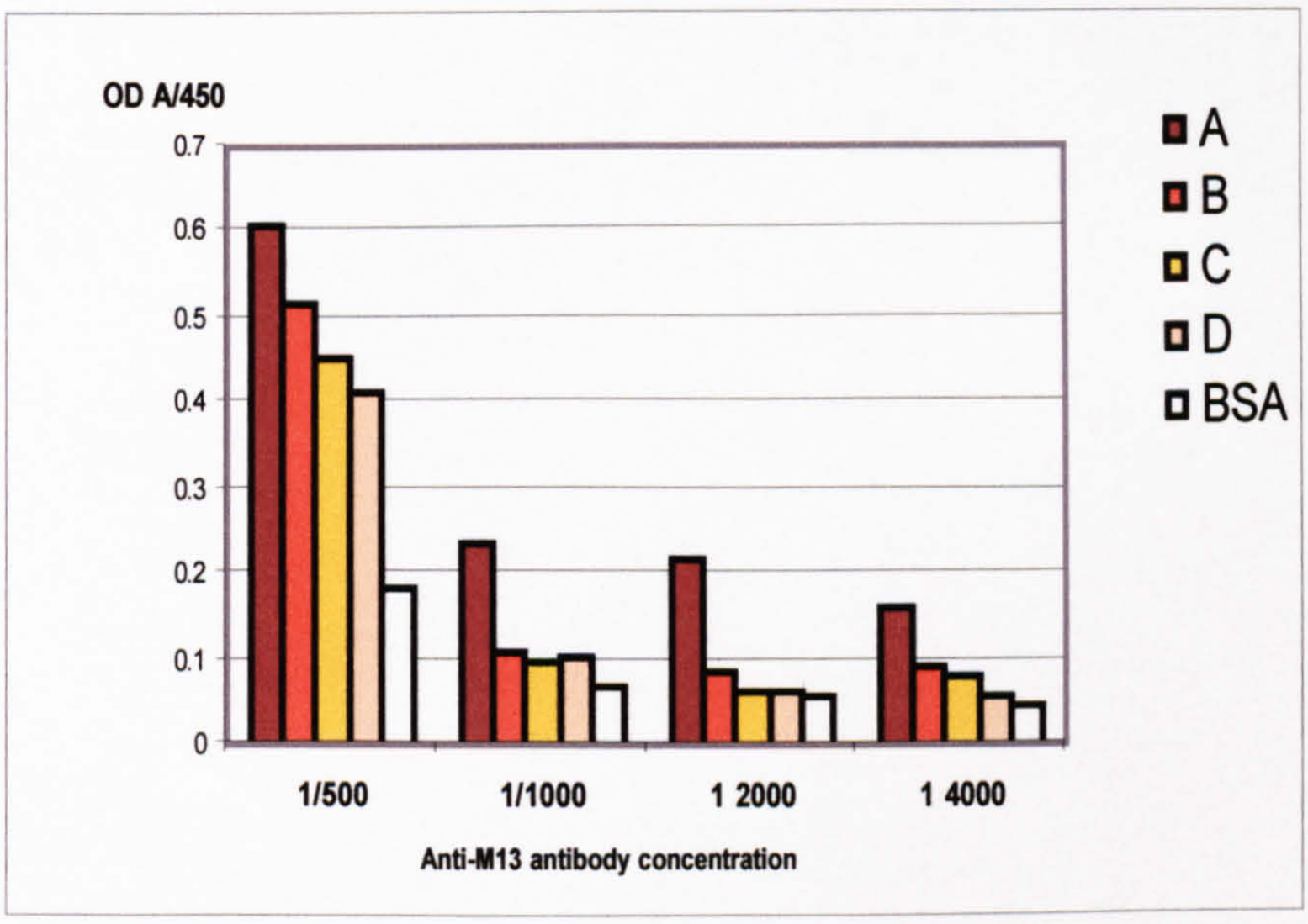


Figure 4.12 ELISA to optimise dilution of anti-M13 antibody for the detection of phage.
Wells were coated with 10-fold serial dilutions of helper phage and with BSA. Sections of the plate were then probed with different dilutions of the anti-M13 antibody (see x axis).
A: 2x10e9 phage
B: 2x10e8 phage
C: 2x10e7 phage
D: 2x10e6 phage

Comparison between BSA and milk

- Helper phage coated wells

In order to compare the relative values of BSA and milk blocking agents on microtitre plates or as components of the diluents, an ELISA plate coated with VCS-M13 helper phage was then blocked with BSA-PBS or MPBS, and these reagents used for dilution of the anti-M13 antibody. Results presented in Figure 4.13 showed that ELISA readings from reactions in which milk had been used were decreased markedly when compared to the use of BSA. This experiment also emphasised the importance of achieving appropriate dilution of the anti-M13 antibody. Note the marked fall in ELISA signal upon dilution of antibody from 1/500 (last-most column) to 1/1000 (next column to right).

-OMP coated wells

A further experiment was performed to study the effect of BSA and milk on the ELISA when OMPs instead of phage were coated to the microtitre plates. Phage antibodies prepared in different ways were then added with BSA-PBA and MPBS as diluents. The results again showed that milk had a negative effect on the assay particularly when used as blocking reagent (Figure 4.14). Different OD values were obtained with PEG-precipitated phage and phage antibody prepared from bacterial culture supernatant, probably reflecting the higher numbers of virus in the former sample. The assay also included phage which were reactive and unreactive with the OMPs. Since these viruses were both prepared by the same methods (from bacterial culture supernatants), their results in the assay are comparable and reaffirm the specificity of the resulting signals.

Comparison of immunoplates

Microtitre plates from two Nunc and IWAKI were compared in ELISA. Each plate was coated with OMPs and milk as a control negative control and phage from overnight culture supernatants of 12 positive clones were added. The successful binding of phage was then detected with an anti-M13-HRP conjugate. Results presented in Figure 4.15 showed the clear superiority of the Nunc plate: the final ODs obtained from the Nunc plate coated with OMPs were higher than from the other plate and the background values from milk coated wells were lower.

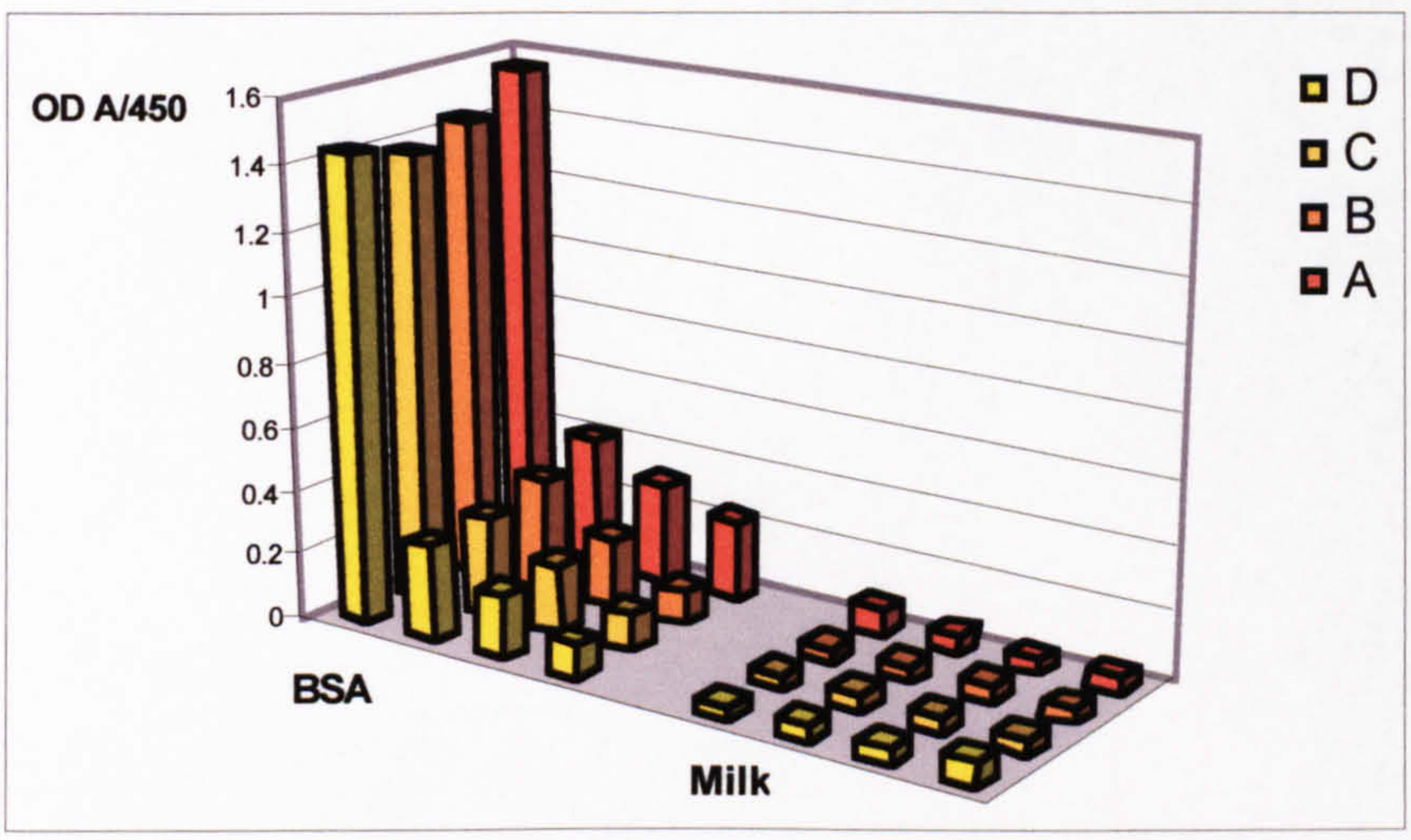


Figure 4.13 ELISA to compare BSA and milk as blocking agents for the detection of phage.

Wells were coated with phage as indicated in the key to the right of the Figure and blocked with either BSA or milk, as indicated. Dilutions of anti-M13 antibody were then made in BSA or milk consistent with the blocking agent, and applied to the wells to detect bound phage. Anti-M13 antibody was added in dilutions of 1/500, 1/1000, 1/2000 and 1/4000 from left to right in each row.

A: 2x10e12 phage
B: 2x10e11 phage
C: 2x10e10 phage
D: 2x10e9 phage

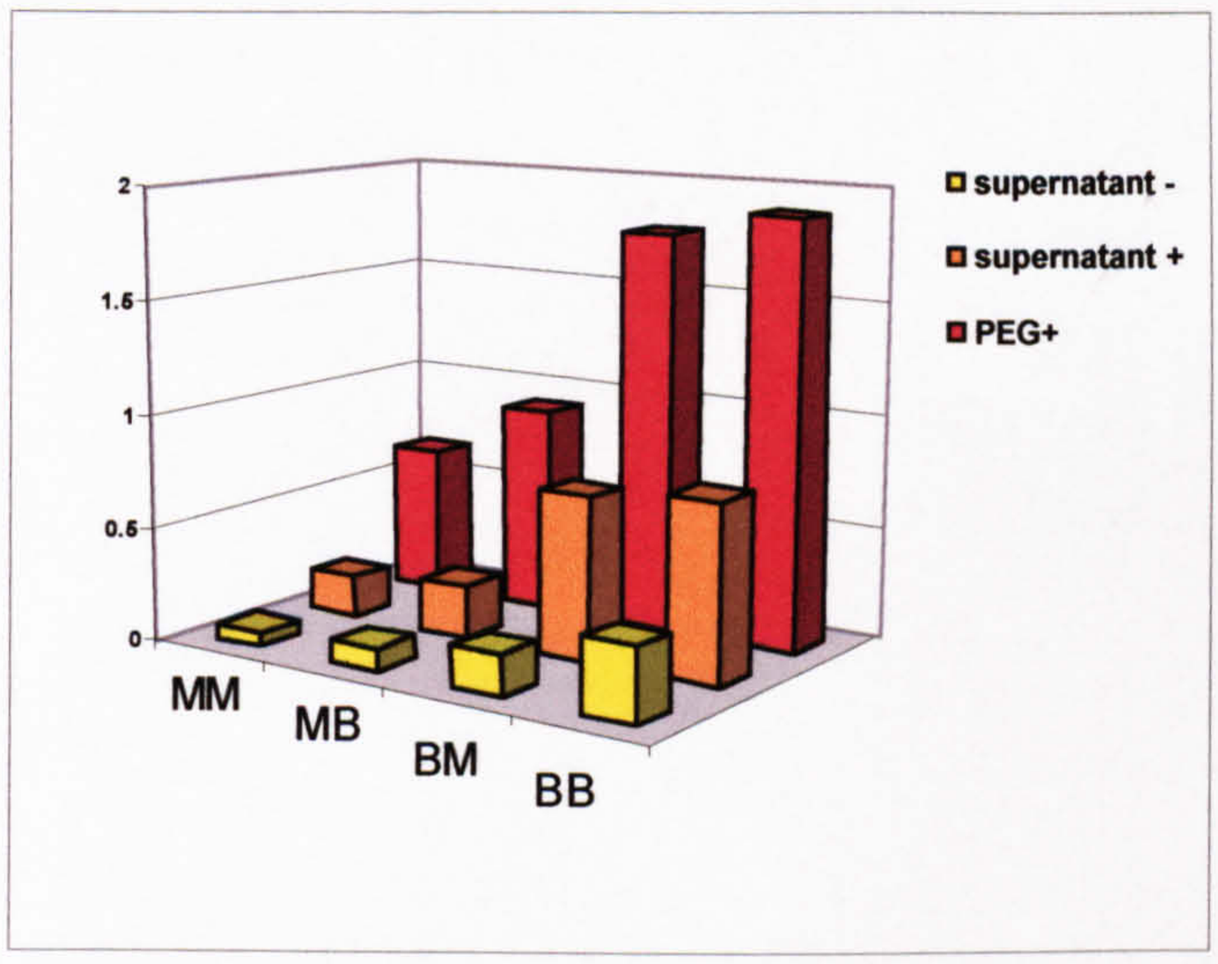


Figure 4.14 ELISA to compare BSA and milk as blocking and diluting agents for phage recognition of OMPs.

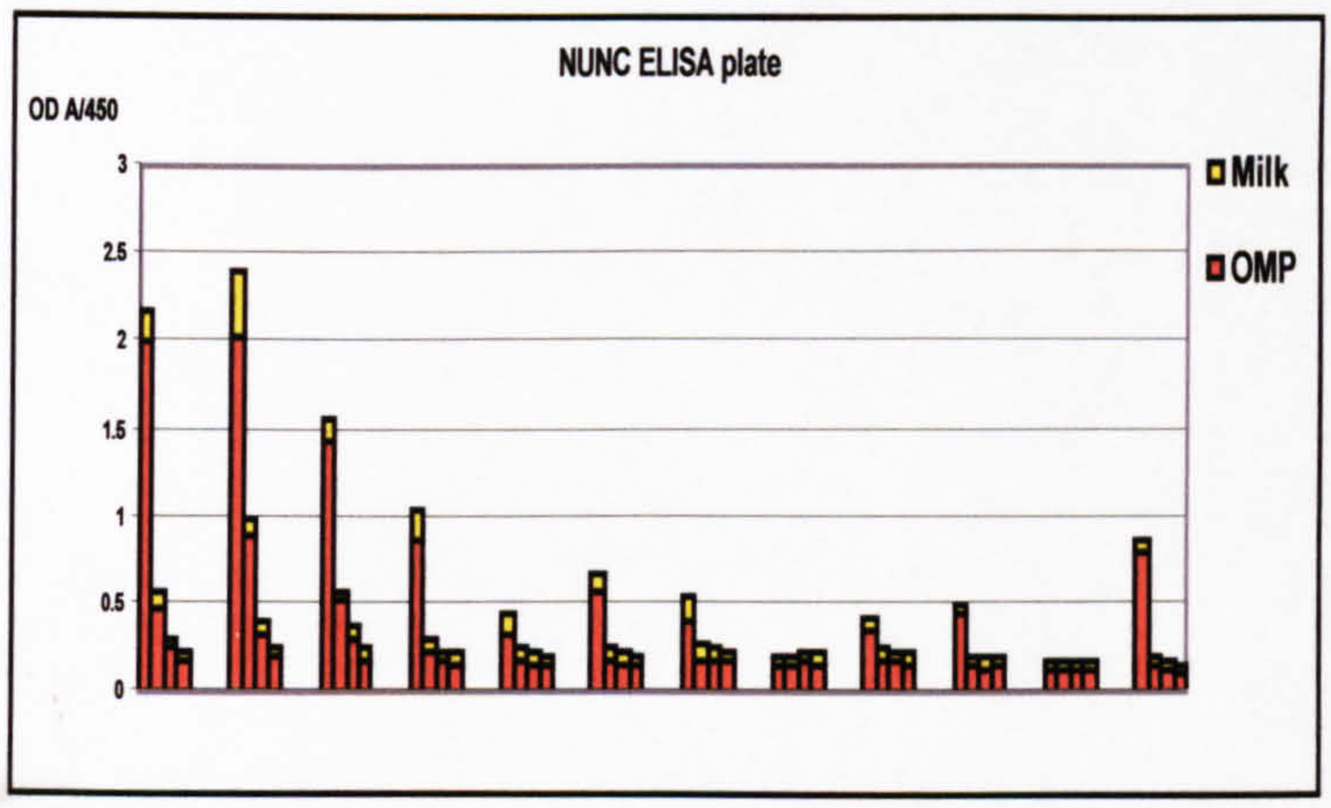
Wells were coated with OMPs and blocked with BSA or milk. Phage reactive with the OMPs ("+" in the key to right of the Figure) or negative control phage ("-" in the key) were added (either supernatants from infected bacteria or phage concentrated by PEG precipitation – see key) in diluents containing BSA or milk, and their binding detected with ant-M13 antibodies.

MM: milk was used for both blocking and dilution

MB: milk and BSA were used for blocking and dilution respectively

BM: BSA and milk were used for blocking and dilution respectively

BB: BSA was used in both steps.



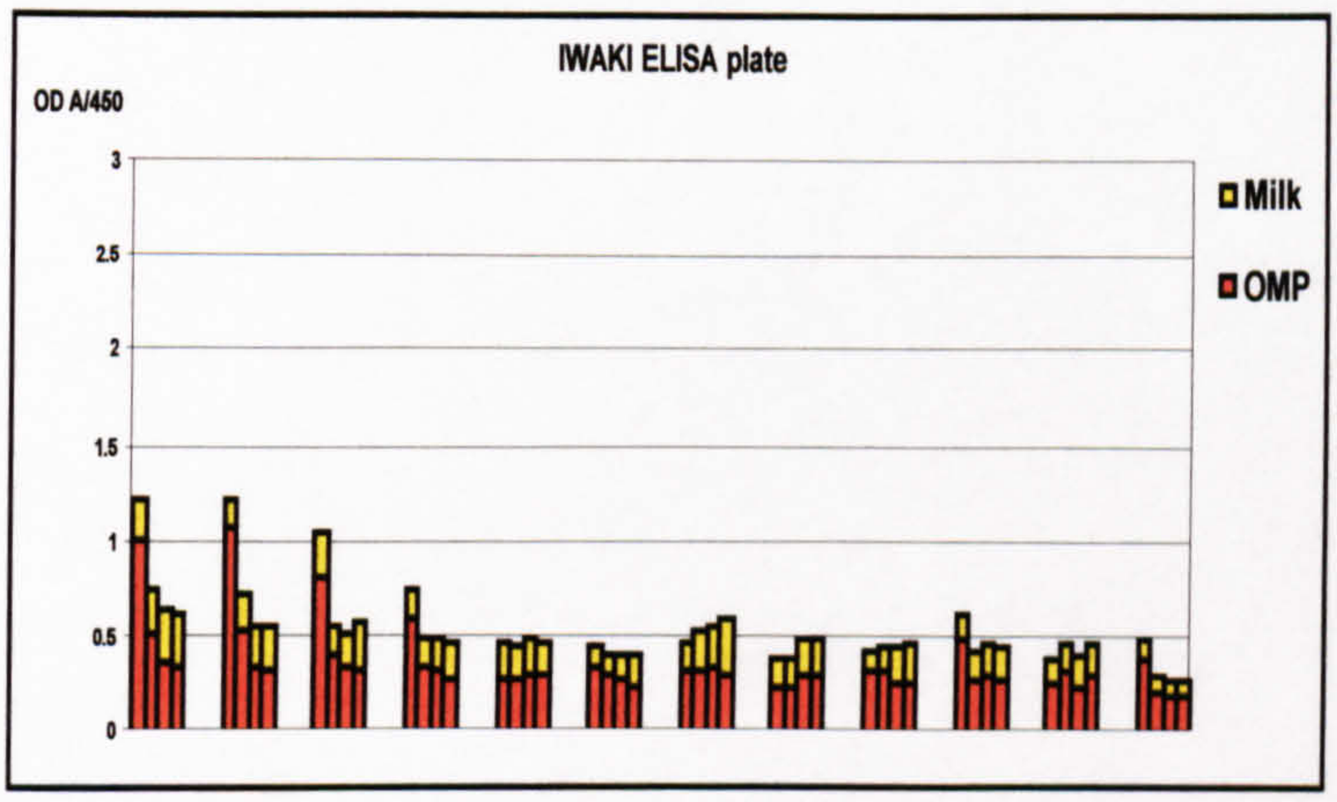


Figure 4.15 ELISA to compare NUNC and Iwaki microtitre plates.
Wells on each plate were coated with either OMPs or milk (see key to right of Figure), blocked, and 12 test phage (clustered bars across the Figure) added in serial dilutions (bars within each cluster). Binding of phage was detected with anti-M13 antibodies. The test phage were prepared from culture supernatants of clones taken from the third round of panning and eluted with anti-OMP serum. The lower section of each bar shows the ELISA reaction in OMP-coated wells. The upper (yellow) section shows the generally weaker reaction in wells coated with milk.

Optimal conditions for phage ELISA

Given the results of experiments described above the following condition were determined as optimal for phage ELISA to detect the OMPs:

- dilution of 1/500 of anti-M13 antibody
- using of ELISA plate from NUNC instead Iwaki
- using of 3% BSA-PBS instead 2% milk-PBS as blocking agent and diluent

4.3.6 Analytical PCR for the determination of scFv length

It has been well established from other studies that scFvs isolated by phage display can be reactive with the selecting target even if they are incomplete, possessing solely V_H or V_L inserts. To determine the frequency of full length inserts in clones selected from the library with OMPs, crude bacterial lysates were used in analytical PCR. Twenty-four clones selected from the last round of OMP selection and eluted with TEA were grown with 12 clones from the final round of selection and eluted with anti-OMP antiserum. PCR was performed on crude lysates using primers LMB3 and FOR_LinkSeq or LMB3 and pHEN-SEQ or Fdseq1. Primers LMB3 and FOR_LinkSeq amplify the heavy chain fragment, whereas primers LMB3 and pHEN-SEQ or Fdseq1 amplify the sequences of both light and heavy chains. By comparing the size of the resulting amplicons with that predicted for full-length scFvs, the phage antibodies could be characterised. Twelve clones from anti-OMP elution were ranked according to the strength of their reactivity in anti-OMP ELISA. Only 3 of the clones possessed full-length scFv inserts: these lay at the bottom of the ranking. Twenty four clones from TEA elution were analysed similarly. Eight possessed both light and heavy chains (Table 4.7). Although some of these phage reacted strongly with OMPs in ELISA, 5 are located at the end of the ranking. These results revealed that the clones with the strongest reactivity in ELISA with OMPs were those that lacked a heavy chain component of the scFv.

4.3.7 Western blotting

4.3.7.1 Western blotting using phage and anti-M13 antibodies

In order to determine the specificity of each clone selected from scFv library, OMPs were run on SDS-acrylamide gels and then blotted to nitrocellulose. Phage prepared by PEG precipitation or culture supernatants from *E. coli* infected with individual clones

Table 4.7 Inserts carried by clones from the third round of selection. A: clones eluted with anti-OMP serum. **B:** clones eluted with TEA. Amplicons of the sizes (bp) shown in the body of the table were recovered with primers LMB3 and the primers indicated of the top. Clones are ranked in order of decreasing reactivity in anti-OMP phage ELISA.

A

clone	Fdseq1	PHEN-SEQ	FOR_LinkSeq	Insert carried
1	650	600	Small	VL
2	650	600	Small	VL
3	650 & 850	600 & 800	Small & 300	VL & incomplete VH
4	650	600	Small	VL
5	500	450	?	Incomplete VL
6	650	600	Small	VL
7	650 & 800	600 & 750	Small & 450	VL & incomplete VH
8	650	600	Small	VL
9	650	600	Small	VL
10	950	900	500	VL & VH
11	950	900	500	VL & VH
12	650 & 950	600 & 900	Small & 500	VL & VH

B

clone	Fdseq1	PHEN-SEQ	FOR_LinkSeq	Insert carried
1	650	600	Small	VL
2	650	600	Small	VL
3	950	900	500	VL & VH
4	650	600	Small	VL
5	450 & 700	400 & 650	250	?
6	650	600	Small	VL
7	650	600	Small	VL
8	650	600	Small	VL
9	650	600	Small	VL
10	650	600	Small	VL
11	650	600	Small	VL
12	650	600	Small	VL
13	650	600	Small	VL
14	650 & 950	600 & 900	500	VL & VH
15	650	600	Small	VL
16	650	600	Small	VL
17	650 & 950	600 & ?	500	VL & VH
18	650	600	Small	VL
19	650	600	Small	VL
20	650 & 950	600	500	VL & VH
21	950	600 & 900	Small & 500	VL & VH
22	650 & 950	900	500	VL & VH
23	650 & 950	600	Small & 500	VL & VH
24	950	600 & 900	Small & 500	VL & VH

were applied to the blot and their binding detected with anti-M13 reagents. This protocol failed to generate signals from any of the antigens on the blot. In consequence, a revised method using scFv-pIII fusion proteins was developed.

4.3.7.2 Western blotting using scFv and anti-pIII antibody

This method differs in several respects from conventional approaches to the expression of scFvs and their use as immunochemical reagents (Tesar *et al.*, 1995). In pHEN2, an amber codon lies between to coding sequence of the scFv and gIII. Conventionally, phagemids are used in suppressor strains of *E. coli* (*e.g.* TG1) for the propagation of phage bearing scFvs fused to pIII. The constructs are transferred to non-suppressor strains (*e.g.* HB2151) for expression of the scFv in 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 (Mersman *et al.*, 1998). Before applying this method to the identification of OMP targets by Western blotting, the system was tested in dot blotting and ELISA.

Dot blotting

E. coli carrying phagemid from two positive phage clones, were induced with 1 and 0.1 mM IPTG overnight. Bacterial extracts were prepared with Bugbuster and Tween 20 and the cell pellets from each extraction treated with alkaline SDS solution from a QIAprep plasmid kit. PEG-precipitated phage of each clone, the bacterial extracts, lysates and buffer containing just the detergents as negative controls were spotted to nitrocellulose. After blocking, anti-pIII antibodies were applied in a typical blotting protocol. The results (Figure 4.16) clearly showed that the anti-pIII antibody could recognise the scFv-pIII complex in the lysates. The strongest signals were obtained from the QIAprep lysates of pellets from detergent-extracted cells. No signals could be detected from the media and detergents used in preparation of the extracts ruling out the possibility that results were non-specific.

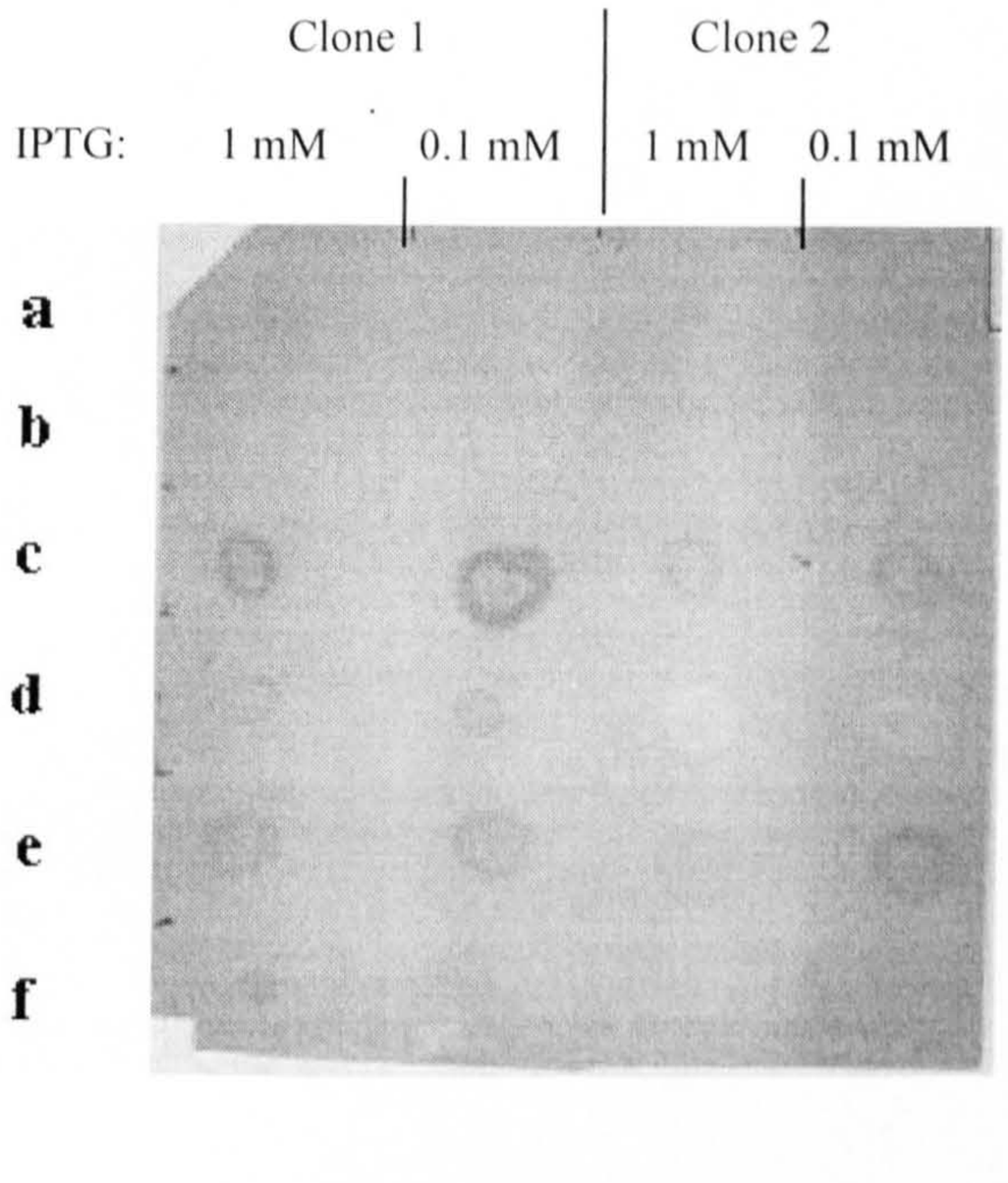


Figure 4.16 Dot blotting of bacterial lysates to test for scFv-pIII.
Two clones were chosen for analysis as shown at the top of the Figure. Cultures of each clone were induced with the indicated concentration of IPTG. Nitrocellulose was then spotted with extracts (specified below) and the presence of scFv-pIII fusion proteins detected with an anti-pIII monoclonal antibody.

a: Supernatants from overnight culture.
b: Tween 20 extracts.
c: Lysates from Tween 20-extracted cells.
d: Bugbuster extracts.
e: Lysates from Bugbuster-extracted cells.
f: Controls (4 spots, left to right) - PEG precipitated phage, bacterial culture medium, Bugbuster, Tween 20.

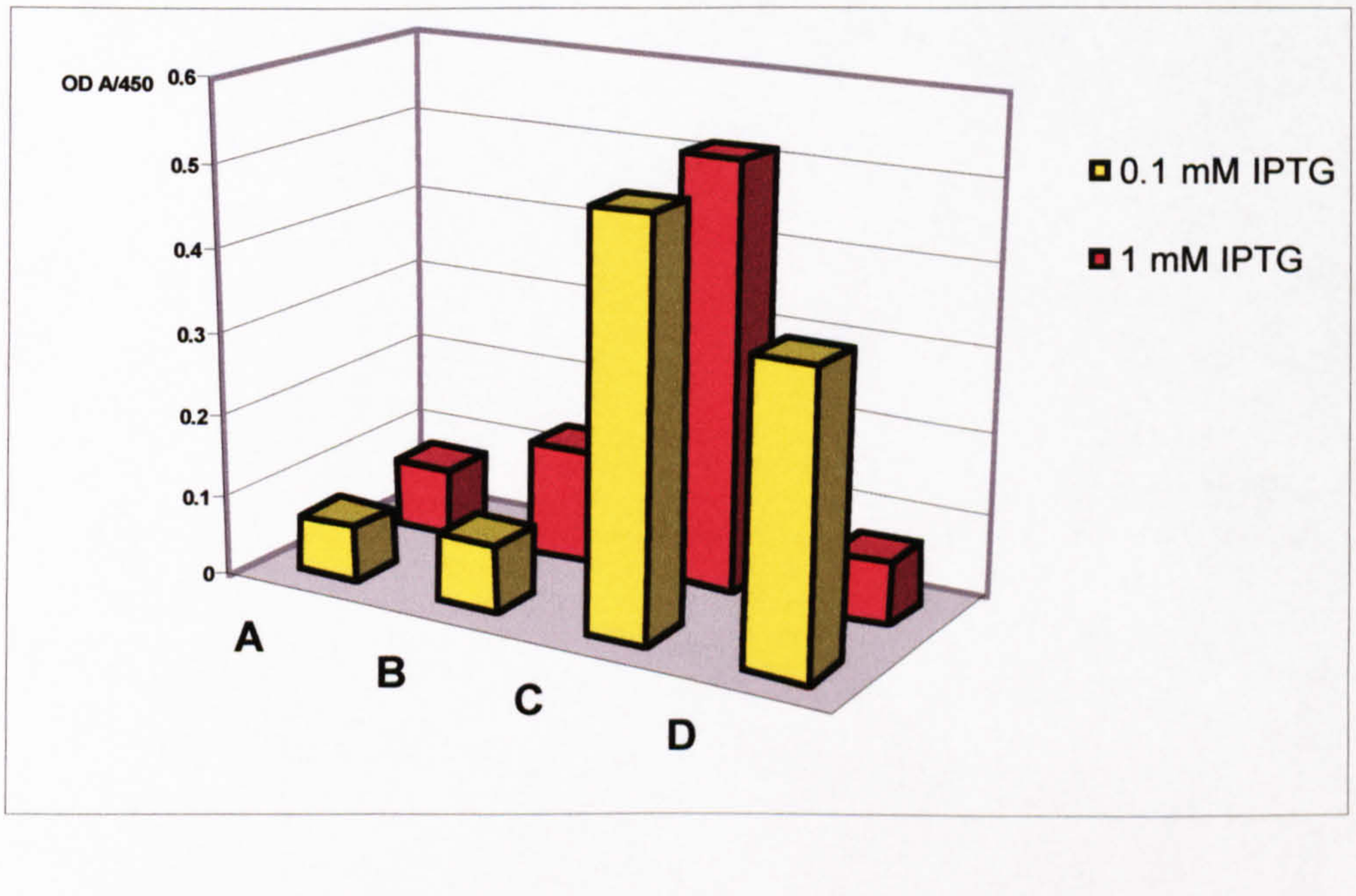


Figure 4.17 ELISA to assess the induction of scFv-pIII fusion proteins and their extraction with detergent for binding to OMPs.

Bacteria were induced to express a scFv-pIII fusion protein by culture in medium containing IPTG (see key to right of Figure). Detergent extracts were prepared from bacterial pellets and applied to ELISA wells coated with OMPs or milk. Binding of the fusion protein was detected with an anti-pIII monoclonal antibody. The bars show the difference in ELISA reactions between OMP and milk coated wells.

A: Tween 20 extract

B: QIAprep lysate of Tween 20 extracted bacteria

C: Bugbuster extract

D: QIAprep lysate of Bugbuster extracted bacteria

ELISA

For this experiment, a microtitre plate was coated with OMPs and the lysates prepared from one of the clones described above were used as a source of scFv-pIII protein. Again, the results (Figure 4.17) again showed that the scFv-pIII fusion was detectable by anti-pIII reagents. The experiment further demonstrated that the scFv fusion protein possessed specificity for components of the OMP preparation. In this experiment, the extract prepared with Bugbuster showed the highest signals.

Western blotting

Having confirmed that scFv-pIII fusion proteins could be prepared, that they could be detected specifically with anti-pIII reagents and that they recognised successfully components of the OMP preparation, the system was applied in Western blots. Samples of OMPs were run on SDS-polyacrylamide gels and the proteins were transferred to nitrocellulose membranes. scFv-pIII proteins were applied to the blot as Bugbuster extracts and their binding detected with anti-pIII antibodies and anti-mouse-HRP conjugates. Faint recognition could be detected using extracts from two scFv clones. By pooling extracts from 12 clones which individually gave little or no signal, stronger recognition of an OMP protein of about 40 kDa could be demonstrated (Figure 4.18).

4.3.8 Identification of the OMP component recognised by scFv-pIII fusion proteins

The band recognised in Western blots by the scFv-pIII proteins was carefully excised from an SDS-poylacrylamide gel and despatched to the University of Aberdeen for analysis by mass spectrometry. Once the quantity of protein used for tryptic digestion had be optimised, MALDI-TOF analysis clearly showed the protein to be PomA of *Mannheimia haemolytica* (molecular mass determined to be 40461 Da; 40% coverage of theoretical tryptic fragments with fragments from the sample).

4.3.9 Native acrylamide electrophoresis

Since phage and scFv-pIII fusion proteins tended to react weakly with OMPs on Western blots, it was postulated that the presence of SDS in gels might be denaturing epitopes recognised by the recombinant antibodies. Hence, native electrophoresis was chosen as an alternative. Electrophoresis was performed with acrylamide gels and running buffers without SDS. Samples of OMPs were either loaded directly to gels in a sample buffer lacking SDS and mercaptoethanol or mixed first with the detergents

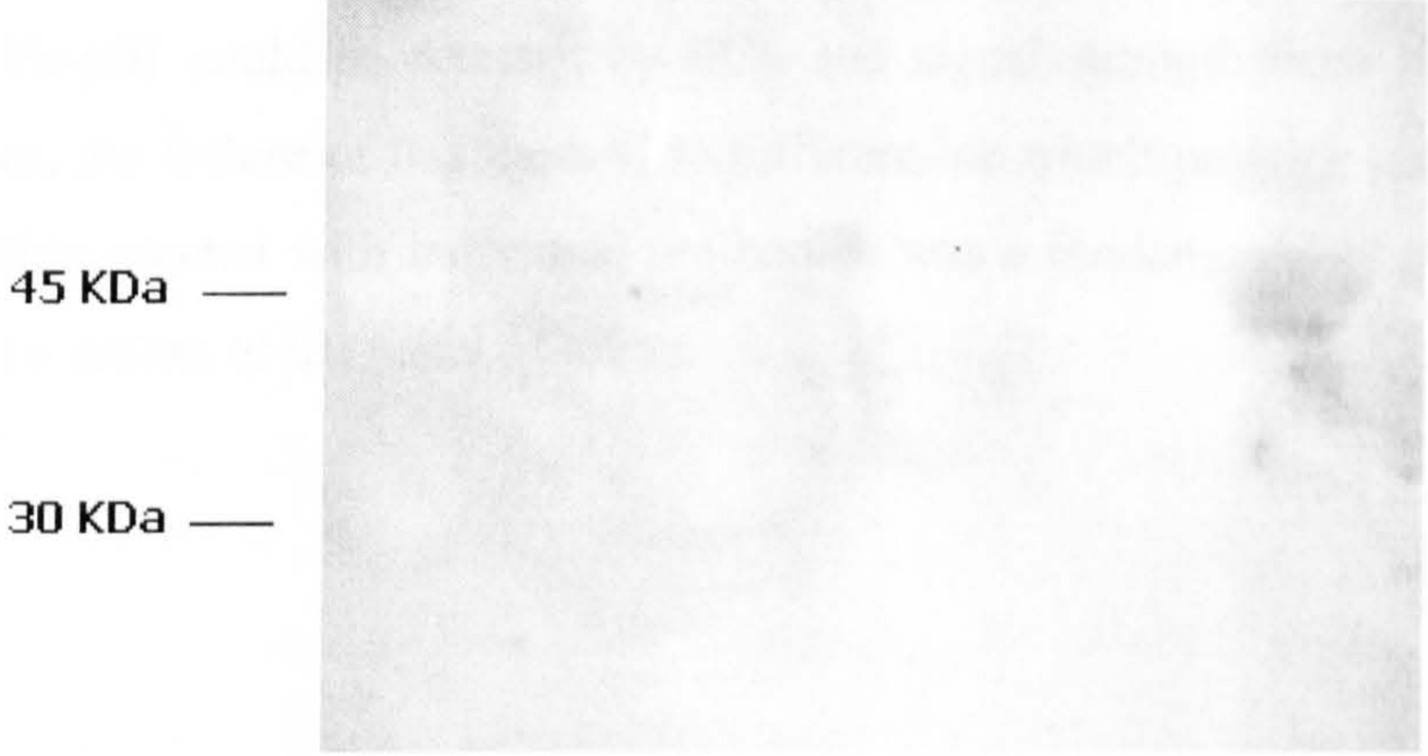


Figure 4.18 Western blotting of OMPs using a mixture of extracts from 12 scFv-pIII clones.

OMPs were separated by SDS-PAGE and blotted to nitrocellulose. The scFv-pIII mixture was applied and binding to the blot detected with an anti-pIII monoclonal antibody.

a: Signals from a protein of about 40 kDa.

M: Rainbow protein marker (Amersham Pharmacia)

The migration of markers of 45 kDa and 30 kDa is indicated.

CHAPS, Tween 20 or sodium deoxycholate. In the absence of detergent or when CHAPS was used, protein staining revealed very little migration of material into the gel. This might indicate that the complexity of the OMP preparation was too high in molecular weight to enter the resolving gel, or that under the electrophoresis conditions employed, the proteins lacked sufficient charge to drive their migration. With Tween 20 or sodium deoxycholate, the OMP sample did enter the gel slightly as shown by staining with Coomassie Blue, but there was no effective resolution of the components present. However, when these samples were transferred to nitrocellulose by electroblotting, binding of scFv-pIII could be detected by ECL and signal strength from probing was good. However, the failure of this method to differentiate which proteins present in the OMP preparation reacted with individual antibodies was a fundamental obstacle to the further characterisation of the panel of scFvs.

4.4 Discussion

Phage display has enabled the generation of large repertoires of human antibodies and the rapid isolation of antibodies against numerous targets. If sufficiently large and diverse, naïve or synthetic libraries can be used to generate antibodies to any antigen of interest providing that a suitable screening method can be devised (O'Brien and Aitken, 2002)). The potential uses of these antibodies for the detection and treatment of infectious diseases are only just being unlocked.

Overall, screening the Griffin.1 library of human scFv antibodies against the OMPs of *Mannheimia haemolytica* was successful. Phage which reacted with components of the OMP preparation were readily isolated and once ELISA methods had been optimised, the interaction of individual scFvs with the OMPs could be measured. While some obstacles were encountered in defining which bacterial proteins in the preparation were recognised by individual recombinant antibodies, in one instance unambiguous identification was possible.

A well-established protocol was used for the isolation of OMPs from *Pasterella haemolytica* (Davies *et al.,* 1991) which exploits the differential solubility of the cytoplasmic and outer membranes of Gram negative bacteria in Sarkosyl. The protein composition of isolated OMPs from *Mannheimia haemolytica* was analysed by SDS-PAGE. Three major bands, one of 31 kDa and two closely migrating proteins around 40 kDa could be observed along with numerous less abundant proteins. The overall appearance of these gels was similar to those described by others (Morton *et al.*, 1996; Brennan *et al.*, 1997) using bacteria of the A biotype. Estimates of the molecular masses of the major OMPs vary slightly in these papers: Morton *et al.*, (1996) reported the molecular weights to be 30.5 and 43 kDa using gels of 11-12 % acrylamide; Brennan *et al.*, (1997) reached similar conclusions (31 and 42 kDa on 12 % gels) as did Iovane *et al.* (1998) (28, 31 and 40 kDa). The differences may be due to the differing co-migration of OMPs in gels containing different concentrations of acrylamide as Mahasreshti *et al.*, (1997) reported that the 30 kDa band observed on 12% gels separated into two proteins of 32 and 35 KDa on 18% gels. They also reported that the major protein of the outer membrane, PomA, appeared in two forms of 35 kDa and 40 kDa when samples were incubated at 37 °C but that it was heat modifiable; incubation for 10 min at 100 °C resulted in a single band of 40 kDa.

The Griffin.1 library was screened against OMPs which had been immobilised on immunotubes. The bound phage antibodies were eluted with TEA or an anti-OMP

antiserum. As reported by other investigators (O'Brien and Aitken, 2002), phage ELISA indicated increasing strength of reactivity towards the OMPs in this assay, round-on-round, perhaps reflecting the selection of scFvs of increasing affinity as the concentration of target at the selecting surface was progressively reduced. Valuable as phage ELISA was in these experiments, it would be unwise to draw firm conclusions from the absolute values obtained at the end of each assay. One observation made during the course of these experiments was that an individual clone could vary in its reactivity towards the OMPs between different assays, perhaps reflecting different phage titres obtained from one experiment to the next, variable proteolytic breakdown of scFv displayed at the phage surface or inconsistencies in the binding of the target OMP component to microtitre plates. It is therefore the pattern of results from these experiments which is significant rather than their absolute values.

Analytical PCR revealed that antibodies which reacted most strongly with OMPs typically possessed only a V_L domain. de Bruin *et al.*, (1999) reported similar observations when they screened the Griffin.1 library for antibodies against two target proteins. They noted that all phage antibodies recovered from the final panning performed poorly in ELISA and Western blot analysis. PCR and sequencing revealed that only 1 from 100 phage antibodies carried a complete scFv insert, the majority lacking the V_H component. de Bruin *et al.* showed that increasing the stringency of elution from the protocol recommended by the team that constructed the Griffin.1 library resulted in selection of phage antibodies with full length inserts.

Despite the strong ELISA signals obtained from phage isolated in our study, attempts to define their specificity using Western blotting only resulted in faint reactions. Signal strength was improved when a mixture of antibodies was used. This may be because the pooled scFvs act rather like a polyclonal serum, binding to different epitopes on the same target protein. Western blotting is routinely used with recombinant antibodies selected by phage display and nitrocellulose or polyvinylidene fluoride membranes have been used to immobilise antigens for panning (Liu and Marks, 2000; Nakamura *et al.*, 2001; Foy *et al.*, 2002). There is therefore no reason in principle for the method to yield low signals. It may be that the common lack of a V_H insert in ELISA positive phage impaired scFv affinity for the target. Alternatively, the use of SDS and mercaptoethanol in preparing samples for electrophoresis might have denatured target epitopes sufficiently to reduce the binding of scFvs to blots. As shown in this study, native gel electrophoresis has some potential to overcome these

drawbacks but the technique as implemented lacks the resolution required to separate the different proteins from the OMP preparation. For future work with the panel of anti-OMP scFvs, immunoprecipitation would avoid these problems but the yield of target protein tends to be low. If the OMPs could be labelled *in vitro* prior to interaction with scFvs, this would improve the sensitivity of the technique significantly. For example, if labelled with biotin, the precipitated protein could be blotted and then detected by reaction with streptavidin-HRP conjugates. At present, the diversity of the panel of anti-OMP scFvs isolated by phage display remains uncertain. While this could be assessed through identification of target proteins as suggested above, there remains the possibility that multiple clones recognise distinct epitopes on the same OMP. Competition ELISA might be one way to tackle this, perhaps testing soluble scFvs against scFv-pIII fusion proteins to avoid the need to label one component of the competing antibody mixture. The other would be to assess through DNA sequencing the diversity of clones reactive with a common OMP. As time for this phase of the project was limited, little sequence data was gathered from the anti-OMP scFv clones isolated by phage display.

Comparison between phage antibodies selected through the two different methods of elution - release with TEA and competitive elution with anti-OMP antibody - did not reveal marked differences. In both groups, the reactivity of individual clones increased progressively during the rounds of selections, ELISA signals from clones at the same round of selection under each protocol did not differ, and in both selection methods, the clones with the highest ELISA signal carried only the VL chain. Finally, clones from each selection method failed to recognise OMPs on Western blots when used individually.

One protein band from the OMP preparation was detected by some scFvs or by mixtures of them. Analysis using mass spectrometry revealed this to be PomA of *Mannheimia haemolytica*. The amino-terminal sequence of this protein is homologous to OmpA from *Escherichia coli* and related proteins from other gram negative bacteria such as *Haemophilus influenzae* and *Salmonella typhimurium* (Mahasreshti *et al.*, 1997). As a porin, OmpA is important in maintaining the integrity of the *E. coli* outer membrane (Sonntag *et al.*, 1978) and by virtue of its surface location, it stimulates a strong antibody response (Puohiniemi *et al.*, 1990). In Western blotting, PomA of *Pasteurella haemolytica* is recognised by antibodies raised against OmpA from *Escherichia coli* (Mahasreshti *et al.*, 1997) and it must also be located at the bacterial

surface since it is accessible to treatment with chymotrypsin. Antibodies against OmpA and several other members of this family of proteins are bactericidal, opsonic or protective (Ichihara *et al.*, 1981; Murphy *et al.*, 1986; Mathews and Gilleland, 1987; Hughes *et al.*, 1992). Vaccination with *Pasteurella haemolytica* A1 OMPs confers protection against pneumonic pasteurellosis (Mosier *et al.*, 1989; Morton *et al.*, 1995; Srinand *et al.*, 1995) and cattle vaccinated with live *Pasteurella haemolytica* developed a significant increase in serum antibodies to partially purified PomA (Mahasreshti *et al.*, 1997). Hence, PomA of *Mannheimia haemolytica* has potential as a vaccine target. It would be interesting to see if the scFvs isolated against this target could mediate bacteriocidal activity when conjugated to a heavy chain constant domain (Wang *et al.*, 1999). This might offer a novel therapeutic against pasteurellosis.

As regards the generation of antibodies against components of the bacterial surface, a further feature of these results is significant. No attempt was made to quantify levels of lipopolysaccharide (LPS) in the OMP preparations but its presence is inevitable. Preliminary experiments attempted to determine if scFvs against LPS were commonly isolated from the Griffin.1 library. OMPs prepared with Sarkosyl were treated with Proteinase K in an attempt to digest the proteins present. This had a marginal effect on the reaction of a selection of phage but SDS-PAGE indicated that traces of the major protein components were still present. In contrast, the reaction of phage with OMPs prepared with the detergent n-dodecyl β-D-maltoside (DDM) was significantly reduced after protease digestion, but insufficient material remained for analysis by SDS-PAGE. The results (not shown) were therefore inconclusive. This issue could be investigated more thoroughly by treatment of larger quantities of OMPs with protease before assay in monoclonal phage ELISA or by coating microtitre plates with purified LPS.

With the rapid advance of genomic projects for major bacterial pathogens, the approach described in this chapter has significant potential for the identification of bacterial proteins with therapeutic or vaccine potential. In one recent investigation, Pizza *et al.* (2000) analysed the genomic sequence of a virulent serogroup B strain of *Neisseria meningitidis* for vaccine candidates. A total of 350 candidate antigens were expressed in *Escherichia coli*, were purified and sera raised against each protein by immunising mice. The sera were then tested for bactericidal activity. Given the success with which scFvs against OMPs were isolated in this project, application of

phage display in this context would seem attractive. Selection of phage can be done in the wells of a microtitre plate (O'Brien and Aitken, 2002), hence antibodies against 350 proteins could be isolated on only 4 ELISA plates. For biological evaluation the Fc portion of an antibody would be required for complement activation. Fab or scFv antibodies selected from phage display libraries could either be fused to Fc domains by recloning or with appropriate immunochemical reagents, a second full-length antibody directed against a tag (*e.g.* pIII as used in this chapter with scFv-pIII fusion proteins for dot blots, ELISA and Western blotting) could be used to activate complement.

This chapter thus fulfils the aims set earlier in the thesis: to use phage display technology to isolate recombinant antibodies against the outer membrane proteins of *Mannheimia haemolytica*. The panel of scFvs generated need to be characterised further, but the reactivity of clones towards PomA has demonstrated that this approach offers significant potential as a tool for the investigation of bacterial infection.

Chapter 5

Conclusion

5 Conclusion

The scope of this study has been broad. It has examined the basic immunology of the bovine antibody system and it has brought new techniques of molecular biology to the applied immunology of this species. It both areas, the project has attempted to help close the gap between present understanding of the biology of humans and laboratory animals, and that of cattle, a group of animals of huge importance to industrial and rural economies across the globe.

At the start of the project, it was well known that the bovine antibody system showed at least one unique feature. Its use of a single gene family in the formation of the heavy chain repertoire is in marked contrast to humans or mice (Cook and Tomlinson, 1995) but is a feature shared with other veterinary species (*e.g.* sheep (Dufour *et al.*, 1996), chickens (Reynaud *et al.*, 1994) and pigs (Sun *et al.*, 1994). Rather, it is the length of CDR3H that sets bovine antibodies apart (Berens *et al.*, 1997; Saini *et al.*, 1999). In seeking to characterise the bovine J_H locus, it was imagined that strong similarities would emerge with the equivalent region of the ovine heavy chain locus *i.e.* that 6 segments would be present but that 2 would account without ambiguity for the sequences of FR4H observed in bovine Ig cDNA. Useful as this data would be, it was envisaged that its main value would simply be to provide a fixed point of reference from which to isolate and characterise bovine D segments and thereby explain why CDR3H is atypically long.

The result of these studies – that the J_H segment which undergoes rearrangement does not appear to originate from the main J_H locus – has instead revealed another unique aspect of the bovine Ig system and set another set of puzzles for future investigations: where does the sequence observed in rearranged B-cells come from; when is it accessed during B cell development; what process brings it into the coding sequence for bovine Igs. Clearly the donor sequence lies outwith the 3200 bp which has been sequenced in this study; whether it lies on the same chromosome as the heavy chain locus (chromosome 21) or elsewhere in the genome remains unknown. In either case, this appears to be another highly unusual property of the immunology of cattle as a group of vertebrates. The availability of BACs for characterising significant parts of the Ig locus and other regions of the genome should assist the location of these sequences and elucidation of how they are introduced (gene conversion or perhaps trans-chromosomal rearrangement).

As originally planned, the characterisation of the bovine J_H locus has provided a fixed point of reference for the isolation of D segments. These studies have already been initiated by others in the laboratory. Three potential suggestions have been made regarding the length of bovine CDR3H (Aitken *et al.*, 1999): that D segments are unusually long; that D-D fusions emerge frequently from the process of rearrangement; that terminal transferase introduces significant lengths of non-templated sequence during rearrangement. Since the DQ52 segment described in this thesis was found to be longer than equivalent segments from other species, there may already be an indication that bovine D segments will emerge as unusually long.

A further puzzle surrounding the formation of antibodies in cattle is the process which introduces diversity, post rearrangement. Leading candidates for this include gene conversion (Parng *et al.*, 1996) and somatic hypermutation (Berens *et al.*, 1997). Distinguishing between these processes in the Ig reading frame is difficult. However, it might be predicted that gene conversion would favour the introduction of substitutions within the protein coding sequence, whereas somatic hypermutation (at least in mice) targets the transcriptional unit (Wagner and Neuberger, 1996). The work described in this thesis successfully recovered sequence from regions downstream of the rearranged J_H segment using genomic DNA from B cells. During the process of diversification, perhaps random substitutions would appear here if somatic hypermutation was active whereas little substitution would occur is gene conversion was the driving force for diversification.

Attempts to construct a bovine Fab library were time-consuming and ultimately unsuccessful but the selection of scFvs against *Mannheimia haemolytica* from the Griffin.1 library showed the potential application of phage antibody technique investigations of infectious diseases. Clearly, the weak signals obtained in Western blotting pose a challenge for the future but ways in which this could be tackled have been suggested. A wider question concerns the use to which these reagents could be put. There is little doubt that scFvs selected from phage antibody libraries could be applied to the diagnosis of livestock infections. To take one aspect, the ability to express these monoclonal Igs in bacteria offers significant opportunities for cost-saving. Perhaps this could be utilised in designing assays to be run on site by personnel with little specialist training (*e.g.* farm staff). This might enable rapid

isolation of animals showing signs of infection, selection of animals in good health for transport to market, or monitoring of animals for selected pathogens before introduction to a herd. There is some precedent for this in the steady rise of patient-run assays in human medicine (*e.g.* pregnancy tests and assays for blood sugar levels).

Recombinant antibodies are having an increasing impact in human therapy; it is questionable to what extent the economics of modern livestock farming could sustain this area of application, and how delivery to large numbers of animals would actually be carried out. In selected, high-value animals (*e.g.* horses and companion species), there may be the opportunity to apply the techniques described in this thesis to the development of protein therapeutics against infectious diseases. The exposure of PomA at the surface of *Mannheimia haemolytica* and the availability of a panel of recombinant monoclonal antibodies offers the opportunity to test these ideas. The work would entail linkage of the scFvs to constant region domains able to fix complement and expression of the constructs in mammalian cells owing to their size and requirement for glycosylation. Depending upon yield, assays of bactericidal activity *in vitro* might progress to limited testing against established or challenge infections of *M. haemolytica in vivo.* One value of this kind of study aside from proof of principle would be that more informed costing could be made for production of a protein therapeutic against bacterial diseases of livestock animals. It would be interesting to also investigate the delivery of recombinant antibodies *via* DNA immunisation methods (Lorenzen *et al.*, 2000) or attenuated bacterial species able to colonise the tissue to be protected (Beninati *et al.*, 2000).

One area where phage antibodies are certain to have an impact is in basic studies of the pathogenesis of bacterial infection. Reference has already been made to this topic earlier in the thesis but it is worth repeating that as genome projects provide increasing volumes of data, the need to produce antibodies against proteins implicated in the pathogenic process will similarly escalate. There is an obvious value for these reagents in evaluating the vaccine potential of their targets. More speculatively, the use of antibody arrays (de Wildt *et al.*, 2000; Holt *et al.*, 2000) to monitor the expression of proteins (*c.f.* transcriptional analysis) would offer the chance to observe expression at the level of the proteome or its constituent subsets (*e.g.* surface-located proteins, proteins induced exclusively *in vivo*).

Appendices

Appendix 1:

Sequence from the bovine J_H locus. Sequences were assembled from 5 individual sources indicated with different colours (see below), the numbers in brackets indicating repeated sequencing runs from the same clone. From these, a consensus was derived (indicated by "Cattle" at the left of the Figure). Data are compared with the sheep J_H locus (Dufour and Nau, 1997) using bars to indicate differences. In the body of the Figure, dashes indicate identity with the consensus, differences are shown with the nucleotide identified at that position. Arrows indicate primer binding sites.

Black : genomic DNA from liver

Red: genomic DNA recovered from palate tissue

Blue: peripheral blood genomic DNA

Purple: a lambda clone isolated from a library of bovine genomic DNA

Green: clone 15, a lambda clone carrying the bovine J_H locus (Knight *et al.*, 1988)

```
Cattle                                          AATTCC  CCGGATCTGT  GGGCAGGGTT
C15.RR2                                    >    ------  ----------  ----------

Cattle     CCCAAGGGGG  TCGGTGGCCG  ACCCCCCTGG  CCCTTGCTCG  CCTGCGGCCA  GCCCTAAGGG
C15.OJ1R<  -----A---G  ---------N  ---C--T---  -CC---T-.-  -T--.-----  ---C-----T
C15.RR2    -----G---N  ---------G  ---N--C---  -NN---N-C-  -C--C-----  ---N-----G

Cattle      TTTTGGCCAA  GCCAGAAACC  ACAGTGGAAC  TCGGTGGGGC  CACAAAAATT  GACAACTTT.
C15.OJ1R    ----------  G----A-N--  N-----.--T  -G--------  -A--A-----  -----T---T
C15.RR2     ----------  N----N-A--  A-----G--C  -C--------  -N--N-----  -----C---.

Cattle      ACAAAAACCC  CTGAGCAAAG  TGGCCCCAGG  GAAGCCGGGG  GGGGTCTCAC  TTTGAGGGTT
C15.OJ1R    ----------  T-A-C--N--  ----------  .---------  -----C-C--  ---N------
C15.RR2     ----------  N-G-G--N--  ----------  G---------  -----T-N--  ---G------

Cattle      CCCTGTCAGC  GCACACGGGG  TCAGGTCAGC  CAGGAGCCTG  AAGGCACTGG  AAAAGAGCCA
C15.OJ1R    --------G-  ----------  -C------G-  ---N-G-N-N  ------N---  ----N-N--N
C15.RR2     --------C-  ----------  -N------N-  ---N-N-C-G  ------C---  ----A-A--N

Cattle      CCTGGGCTGA  CGGTGAAGGG  GCCTCCCAGG  CGGGCACGGA  GCCAGGCCCA  GCCCAGGCAA
C15.OJ1R    -N-G--C---  ----N-----  --------G-  -----A----  ----------  ----------
C15.J10R(1)                                     < A-        ----------  ----------
C15.J10R(2)                                                 < -----     ----------
C15.RR2     -C-N--N---  ----G-----  --------N-  -----N----  ----------  N---------

Cattle      GGGGCCAGGT  CCCCAAGGCC  TGGGGTGCAG  GGAGTCCA.G  GGAATTGGGG  GTTGAGGTGG
C15.OJ1R    ----------  ----------  ----------  --------.-  ----------  ----------
C15.J10R(1) ----------  ---------N  ----------  --------.-  ----------  ---N------
C15.J10R(2) .---------  ---------.  -T--------  -------.-.-  ----------  ---------.
C15.RR2     -------N--  ----------  -N--------  --------A-  ----------  ----------
C15.J8R(1)                                                  <  ----     ---N-----A

Cattle      AAAGCCTCGGG  AGGGGCAGGG  AGCATGTGAC  CTCAT.GGGA  ACAAAGCAGA  TCAGGAGAC
C15.J8R(1)  -N-N------.  -A-------A  -----N-TC-  -----.---.  ----------  -----....
C15.OJ1R    -----------  ----------  ----------  -----.----  ----------  ---------
C15.J10R(1) -N---------  ----------  ----------  -----.----  ----------  ---------
C15.J10R(2) -----------  -.--------  ----------  -----.----  ----------  ---------
C15.RR2     ---N---NT--  -------A--  -----T-T--  --NN-T----  -----A--N-  ---------

Cattle      AGCAG.A.CACT.    CAAATCCCAG  ACCAGGGGCC  TGAGGCAGAC  AGGTGCT
C15.J8R(1)  A-----C-G-C--.  ----------  ---------.  ----.-----  -------
C15.OJ1R    .-----.-.----.  ----------  ----------  ----------  -------
C15.J10R(1) .-----.-.----.  ----------  ----------  -N--------  -------
C15.J10R(2) .-----.-.----.  ----------  ----------  ----.-----  -------
C15.RR2     .---NN.-.----T  N----T---A  -------N-T  NA-N-A-A-A  --T-.--

Sheep1      TCGAGGGTGG  GGGGCAAGCG  TCTTCAGGAA  GACAGACTTA  CAGCTCCCGG  GGGCTTCCTG
            |||   |               |                       |      ||||||  |||||
Cattle      TGTCGGTTGG  GGGGCAAGTG  TCTTCAGGAA  GACAGACTTG  CAGC......  .....TCCTG
C15.J8R(1)  ----------  --------T-  -N-----N--  ---------G  ----......  .....-----
C15.OJ1R    ----------  --------T-  -N--------  ---------G  ----......  .....-----
C15.J10R(1) ----------  --------T-  -C--------  ---------G  ----......  .....-----
C15.J10R(2) ----------  --------T-  -C--------  ---------G  ----......  .....-----
C15.RR2     -N-T------  ------  >

Sheep 61    GGGGCTTCCT  GGGGACCCTG  AGTGGGTTTC  CATGCCCCCC  AGCACAGGGC  CAGCCCACTG
                  |                 |           |   |
Cattle      GGGGCTCCCT  GGGGACCCTG  GGTGGGTTTC  CGTGCCTCCC  AGCACAGGGC  CAGCCCACTG
C15.J8R(1)  ------C---  ----------  G---------  -G---T---.  ----------  ----------
C15.J8R(2)                                                 <  ---A------  A------T--
C15.OJ1R    ------C---  ----------  G---------   <
C15.J10R(1) ------C---  ----------  G---------  -G---T---.  ----------  ----------
C15.J10R(2) ------C---  ----------  G---------  -G---T---.  ----------  ----------
```

Ovine JH1R

Ovine JH1

Sheep 121	TGACTATGCT	GACTTCCATC	TCTGGGACCA	GGGT.GCCCTG	GTCACCGTCT	.CCTCAGGAGA
Cattle	TGACTATGCA	GACTTCCATC	TCTGGAGCCA	GGCT.GCCCTG	GGCTGCCCTG	.CCTCAGGTGA
2-4.OJ1(2)		> ---	-----AG---	--C-.------	-G-TG-CCTG	.-------T--
2-4.OJ1(3)		> ---	-----AG---	--C-.------	-G-TG-CCTG	.-------T--
2-4.OJ1(1)		> ---	----NAN---	--C-.------	-G-TG-CCTG	.-------T--
L.JH2		>				.CC-----T--
L.OJ1(1)		> ---	-----AG---	----.------	-G--------	.-------T--
L.OJ1(2)		> ---	-----AG---	A-C-T------	NG--------	.N----N-T--
L.J8R(2)	<--N----T-	----T-----	-T--AAG---	--T-.---T--	-G--------	.-------T--
C15.J8R(3)			< -	--T-.N--T--	-G-N-G----	.---N---TN-
C15.J8R(1)	---------A	----------	-----AG---	--C-.------	-G--------	.-------T--
C15.J8R(2)	---T---CAA	ACT-------	-T--AAG---	--T-.---T--	-G--------	.-------.--
C15.J10R(1)	---------A	----------	-----AG---	--C-.------	-G--------	C-------T--
C15.J10R(2)	---------A	----------	-----AG---	--C-.-.----	-G--------	.-------T--

Sheep 181	ATCTCGTGTC	CAGTGACCAA	ATCAGGGGAC	AATGGGACTT	GGGTGGACTG	GGGATGGGAG
Cattle	ATCTGGTGTC	TAGTGTCCAA	GTCAGGGGAC	AGCAGGACTT	GGGTGGACTT	GGGATGGGAG
2-4.OJ1(2)	----G-----	T----T----	G---------	-GCA------	---------T	----------
2-4.OJ1(3)	----G-----	T----T----	G---------	-GCA------	---------T	----------
2-4.OJ1(1)	----G-----	T----T----	G---------	-GCA------	---------T	----------
L.JH2	----G-----	T----T----	G---------	-GCA------	---------T	----------
L.OJ1(1)	----G-----	T----T----	G---------	-GCCA-----T	---------T	----------
L.OJ1(2)	----G-----	T----T----	G---A-----	-GCAC-----	---------T	-N-----N--
L.J8R(2)	--T-G----T	T----T----	G------.--	-GCA------	---------T	------A--
C15.J8R(3)	--T-G-----	N----T----	G---------	-GCA------	-----A---T	----------
C15.J8R(1)	----G-----	T----T----	G---------	-GCA------	---------T	----------
C15.J8R(2)	--T-G--T-T	N----T----	G------.--	-GCA---N--	---------T	--N----N--
C15.J10R(1)	----G-----	T----T----	G---------	------ <		
C15.J10R(2)	----G----N	T---- <				

JH10R

Sheep 241	TCAGGGCCAG	CAAGGAGGCA	GTG.TGTGTTT	TTGTAGAAAA	GAAGCAGCAG	AATAGAAGCT
Cattle	TCAGGGCCAG	CAAGGAGGTA	GGG.TGTGTTT	TTGTGGAAAA	GAATTAACAG	AAGAGAAGCC
2-4.OJ1(2)	----------	--------T-	-G-.-------	----G-----	---TT-A---	--G------C
2-4.OJ1(3)	----------	--------T-	-G-.-------	----G-----	---TT-A---	--G------C
2-4.OJ1(1)	----------	--------T-	-G-.-------	----G-----	---TT-A---	--G------C
L.JH2	----------	--------T-	-G-.--N----	----G-----	-----A---	--G------C
L.OJ1(1)	---------A	--------T-	AG-G-------	-- >		
L.OJ1(2)	---CN----A	---N----T-	TG-G-------	--T-G-----	---NCCN--A	--G------>
L.J8R(2)	-----.----	----N---T-	-G-.-------	----G-----	---GC-A---	--G------C
C15.J8R(3)	---------C	--------T-	-G-.-------	----G-----	N-----A--N	--N------C
C15.J8R(1)	--G-------	----N---T-	-G-.-------	----G-----	N-----A---	--G------C
C15.J8R(2)	--G-------	----A---T-	-G-.-------	----G-----	N-----A---	--N------C

Sheep 301	ACGCTGCTGG	GACTCGGGTC	TCTGGGGCCA	GCGCACCCCG	GTCACCGTGT	CCTTGGGCAG
Cattle	ATGCTGCTGG	GACTTGGATC	TCTGGGGCCA	GCGCACCCCG	GTCACCGTGT	CCTTGGGGAG
2-4.OJ1(2)	-T--------	----T--A--	----------	----------	----T-----	-------G--
2-4.OJ1(3)	-T--------	----T--A--	----------	----------	----T-----	-------G--
2-4.OJ1(1)	-T--------	----T--A--	----------	----------	-N--T-----	-------G--
L.JH2	-T--------	----T--A--	----------	----------	----------	-------G--
L.J8R(2)	-T--------	----TA-A--	----------	----------	----------	-------G--
C15.J8R(3)	-T--------	----T--A--	----------	----------	----------	-------G--
C15.J8R(1)	-T--------	----T--A--	-N--------	----------	----------	-------G--
C15.J8R(2)	-T----N---	----T--A--	-N--------	----------	----------	-------G--

```
Sheep 361     TGTCTCCCTT   GCAGTGCTGC   CTGGGCCTCG   GGCAGTTCCC   TGGG.TCTGTG   CAG.CCCACCA
                                ||      AA  .......  ...               |  |        |      |
Cattle        TGTCTCCCTT   GCAGCACTGC   CTGGGCCTCG   GGCAGTTCCC   TGGGGTCCGTG   CAGCCCCACCG
2-4.OJ1(2)    ----------   ----CA----   ----------   ----------   ----G--C---   ---C------G
2-4.OJ1(3)    ----------   ----CA----   ----------   -------N--   ----G-NC---   ---C------G
2-4.OJ1(1)    ----------T ---NCA----    --------T-   -------N--   ----G--C--N   --NC-- >
L.JH2         ----------   ----CA----   AA--......   ...-------   ----G--C---   ---C------G
L.J8R(2)      ----------   ----CA----   AA--......   ...-------   ----G--C---   ---C------G
C15.J8R(3)    ---N------   ----CA----   ----------   ----------   ----G--C---   ---C------G
C15.J8R(1)    C---------   ----CA----   ----------   ----------   ----G--C---   ---C------G
C15.J8R(2)    C---------   ----CA----   ----------   ----------   ----G------   ---C------G


Sheep 421     TGCCCGGAGC   AAGGCAGGGT   CCT.GGTCTAG   TGGGGTCCTG   CCCTTGGATC   TGCCAGAGCC
                   |                           | |   |      ||                   |
Cattle        TGCCCTGAGC   AAGGCAGGGT   CCT.GGTCCAA   CGGGGTCGCG   CCCTTGGATC   TGCCGGAGCC
2-4.OJ1(2)    -----T----   ----------   ---.----C-A   C------GC-   ----------   ----G-----
2-4.OJ1(3)    -----T----   ----------   ---.----C-A   C------GC-   ----------   -T--G-----
L.JH2         -----T----   ----------   ---.----C-A   C------GC-   ----------   ----G-----
L.J8R(1)           < ---   ----------   ---T----C-A   C------GC-   ----------   ----G-----
L.J8R(2)      -----T----   ----------   ---.----C-A   C------GC-   ----------   ----G-----
C15.J8R(3)    -----T----   ----------   ---.----C-A   C------GC-   ----------   ----G-----
C15.J8R(1)    -----T----   ----------   ---.----CNA   C----N-GC-   ----------   ----G-----
C15.J8R(2)    -----T----   ----------   ---.----C-A   C------GC-   ----------   ----G-----

                                                                       JH8R
                                                           <------------------------
                                                                          JH8
                                                                        ------------>
Sheep 481     CCTGGAGGTG   AGGCAGCTGA.  GCCTGAGAGG   AGGACAGGAG   CTG.CT.GGGTG   GGAGGAAACG
                     | |     |       |          |                  |      |              |
Cattle        CCTGGAGATA   AGCCAGCTGAG  GCCTGAGGGG   AGGACAGGAG   CTGACT.GGGCG   GGAGGAAAGG
2-4.OJ1(2)    -------A-A   --C-------.  -------G--   ----------   ---A--.---C-   --------G-
2-4.OJ1(3)    -------A-A   --C-----T-G  -------G--   ----------   ---A--T---CC   --------G-
L.JH2         -------A-G   --C-------G  -------G--   ----------   ---A--.---C-   ----- >
L.J8R(1)      ----A--A-G   --C-------G  -------G--   ---------         <
L.J8R(2)      -------A-G   --C-------G  -------G--   ---------         <
C15.J8R(3)    -------A-A   .-C-------G  --N.--NG        <
C15.J8R(1)    -------A-G   --C-.-----G  -------G--   ---------   <
C15.J8R(2)    -------A-G   --C-------G  -------G--   ---------   <


Sheep 541     TGG.CAGAGCC CGACGGGTCA   GGGTCTGTGT    GGTGA.GGGC.A   GGGGAGGCCC   CTGGGCCTGA
                 |      | |     |              |     |                                |   |   |
Cattle        TGGGCAGAGC. TGACGGGGCA   GGGTCTGTGC.   AGTGA.GGGC.A   GGGGAGGCCC   CCGGGACTGG
2-4.OJ1(2)    ---G--A---. T---C--G--   ---------CA   AN---.----A-   ---A--N---   -C---A---G
2-4.OJ1(3)    ---G--G---. T---N-NG--   N-----N-NC.   A----G----.-   ---N------   -C-N.A---G
2-4.JH8             >          --G--   ---C-----C.   A----.----.-   ----------   -C---A---G
L.JH8               >                   --------C.   A----.----.-   ----------   -C---A---G
Pal.JH8             >     T---T--G--   G--------C.   A----.---T.N   ----------   -C---A---G
C15.JH8             >             --   ---------C    A----.----.A   ----------   -C---A---G
BG.JH8              >       ------G--  ---------C    A----.----.-   ----------   -C---A---G


Sheep 601     CCTCCCAAGG   GTTCATGTCT   TGGGGACAGC    CGGGACGGCG   TCCTTCATCA   TTGCTTTTGA
                    |                        |                 |         |   |      |
Cattle        CCTCCCCAGG   GTTCATGTCT   TGGGGGCAGC.   CGGGACTGCG   TCCCTCAGCA   ATGCTTTTGA
2-4.OJ1(2)    ---N--C---   ---N--  >
2-4.OJ1(3)    ------C---   T--N--N-N-   ----- >
2-4.JH8       ------C---   ----------   -----G----.   ------T---   ---C---G--   A---------
L.JH8         ------C---   ----------   -----G----.   ------T---   ---C---G--   A---------
Pal.JH8       ------C---   ----------   -----G----.   ------T---   ---C---G--   A---------
C15.JH8       ------C---   ----------   -----G----C   ------T---   ---C---G--   A--------G
BG.JH8        ------C---   ----------   -----G----.   ------T---   ---C---G--   A---------
```

```
Sheep 661    CTCCTGGGGC  CAGCGCGCCC  CGGTCACAGT  CTCCTCAGGT  GAGACGGCCC  TCTGCCCGCT
                         |           ||  | | |                       |
Cattle       CTCCTGGGGC  CAGCGCACCC  CCATCTCCGC  CTCCTCAGGT  GAGACGGCTC  TCTGCCCGCT
2-4.JH8      ----------  ------A---  -CA--T-C-C  ----------  --------T-  ----------
L.JH8        ----------  ------A---  -CA--T-C-T  ----------  --N-----T-  -N--------
Pal.JH8      ----------  ------A---  -CA--T-C-T  ----------  -------NT-  -N--------
C15.JH8      ----------  ------A---  -CA--T-CAT  ----------  --------T-  ----------
BG.JH8       ----------  ------A---  -CA--T-CAT  ----------  --------T-  ----------

Sheep 721    CGGTCCTGGG  CTGGGAAAGA  AGTCTCCAGA  GGCCCCTCGG  TCTGTGGCAG  ACCCTCTGTG
              |           |   |   A  |         |           |           |         |
Cattle       CCGTCCTGGG  CCGGGGAAGG  CGTCTCCAGG  GGCCCCTTGG  TCTGGGGCAG  ACCCTCCGTG
2-4.JH8      -C--------  -C---G---G  C--------G  -------T--  ----G-----  ------C---
L.JH8        -C--------  -C---G--NA  C--------G  -------T--  ----G----N  ------C---
Pal.JH8      -C--------  -C---G---A  C--------G  -------T--  ----G-----  ------C---
C15.JH8      -C--------  -C---G---G  C--------G  -------T--  ----G----A  ------C---
BG.JH8       -C--------  -C---G---G  C--------G  -------T--  ----G-----  ------C---

Sheep 781    GCCCCTGGGG  GATCTGCCTC  TGAGGCCGTT  TGCCTCTCTG  CCCTGTTGA.G  GTGGTGCCTC
              |           |                                              |A
Cattle       GTCCCTGGGG  GATCTGCGTC  TGAGGCCGTT  TGCCTCTCTG  CCCTGTTGA.G  GTGGCGCCTC
2-4.JH8      -T--------  -------G--  ----------  ----------  ---------A-  ----CA----
2-4.JH9                                                  <--  -------N-A-  ----CA-N--
L.JH8        -T--------  -------G-N  ----------  -------N--  ---------.-  -N--C-----
Pal.JH8      -T--------  -------G--  ----------  ----------  ---------.-  ----C-----
Pal.JH9                                                                   <--N--
C15.JH8      -T--------  -------G--  ----------  ----------  ---------.-  -N--C-----
BG.JH8       -T--------  -------G--  ----------  ----------  ---------.-  ----C-----
BG.JH6                                                   <   ----A-.-  ----C-----

Sheep 841    TGCCTGTGGA  ATGTGGCCAG  GCAGA..GTGGG  CTCTGTGGAC    CTGGGGGCCT  GGCTTTGCAT
                         | |                                    ||                  ||
Cattle       TGCCTGTGGA  ACGCGGCCAG  GCAGA..GTGGG  CTCTGTG..C  CTGGGGGCCT  GGCTTTGTGT
2-4.JH8      ----------  -C-C------  -----..-----  -------..-  ----------  -------TG-
2-4.JH9      ---N----A-  -C-C-C----  C--A-A.-----  -------..-  ---N------  -CT----TG-
L.JH8        ----------  -C-C------  ---N-..-----  -----N-..-  ----------  -------TG-
Pal.JH8      ----------  -C-C------  -----..-----  -------..-  ----------  -------TG-
Pal.JH9      ---N------  -C-C-----A  --CAANA-----  N------..-  T---------  -------TG-
C15.JH8      -N--------  -C-C.----A  ---AA..N----  ----N--..C  ----------  ------TTG-
BG.JH8       ----------  -C-C------  ---G-..-----  ----.---..-  ----------  -------TG-
BG.JH6       ---T------  -C-C----CA  --CANA.-----  --T-T---..-  ----------  -------TG-

Sheep 901    GGGAGCAGA.G  GCCGCCAAGC  CTTGGGTTTT  TGCAC.AGC.CC  TAACGGGGCC  CATGGCACTG
                  |          |   |          |           |   |
Cattle       GGGGGCAGA.G  GACGCCAGGC  CTTGGGGTTT  TGCAC.AGCCCC  TAGCGGGGCC  CATGGCACTG
2-4.JH8      ---G-----.-  -A-----G--  ------G---  -----A---C--  --G-------  ----------
2-4.JH9      ---C.--N-.-  -A-----GC-  N-----G---  -----.---C--  --G-------  ----------
L.JH8        ---G---N-.-  -A-----G--  ------G---  -N---.---C--  --N-------  ----------
Pal.JH8      ---G-----.-  -A-----G--  ------G---  -----.---C--  --G-------  ----------
Pal.JH9      ---G.----A-  AA-N---GC-  ------G---  -----.---C--  --G-------  ----------
C15.JH8      ---G---A-.A  -A-C---C--  ---T--GG--  -.---.---C--  --A------.  -----N----
C15.J6(1)                                    <   -----N---  -----.---C--  .NAG------  ----------
BG.JH8       ---G-----.-  -A-----G--  ------G---  -----.---C--  --G-------  ----------
BG.JH6       ---.-----.-  -A-----G--  ------G---  -----.---C--  --G-------  ----------

Sheep 961    TGACTATATC  GACTACTGGG  GCCCAG.GACT  CCTGGTCACC  GTCTCCTCAG  GTGA.GCCCTC
                      |     |                 |     ||||                        |
Cattle       TGACTAT.TC  GACAACTGGG  GCCCAG.GAAT  CCAAAACACC  GTCTCCTCAG  GTGA.GTCCTC
2-4.JH8      -------.--  ---A------  ------.--AC  --AAAA----  ----------  ----A-T---N
2-4.JH9      -------.--  ---AN-----  ------.--AC  --AAAA----  ----------  ----.-T----
L.JH8        ---N---.--  N--A------  ----CAN--A-  --AAAA----  --T------N  -N--.-T---N>
Pal.JH8      -------.--  ---A------  ------.--A-  --AAAA----  ----------  ----.-T----
Pal.JH9      -------.--  ---A------  ------.--A-  --AAAA----  ----------  ----.-T----
C15.JH8      -------  >
C15.JH6(1)   ---T---.--  ---A-T----  ------.N-A-  --AAAA-N-G  ----------  --N-.-T-N--
BG.JH8       -------.--  ---A------  ------.--A-  --AAAA----  -------N--  ----.-T---N
BG.JH6       -------.--  ---A------  ------.--A-  --AAAA----  ----------  ----.-T----
```

```
Sheep 1021   ACC.AGCCT.TC .TCTCCTCACT  CT..CT.CTGAGT  TTTGGTGCAC .TTTGGGGGAA ATCGAGGGTG
               |      |                    ||    | |                      |
Cattle       AAC.AGCCC.TC .TCTCCTCACT  CTGTCT.CAGGGT  TTTGGTGCAC .TTTGGGGAAA ATCGAGGGTG
2-4.JH8      -A-A----C.-- .----------  --GT--.-A-G--  ---------- .-------A-- ----------
2-4.JH9      -A-.----C.-- .----------  --GT--.-A-G--  ---------- .-------A-- ----------
Pal.JH8      -A-A----C.-N .-N--------  --GT--T-A-G--  ---------- .-------A-- ----------
Pal.JH9      -A-.----C.-- T-T--------  --GTN-.-A-G--  ---------- T-------A-- ---N------
C15.J6(1)    -A-.----C.-N .--------T-  T-GTT-.NA-G--  ---------- .------NA-- ----------
BG.JH8       -A-A----CC-- .----------  TN-GTT-NAANG--  ----------   ---T---G-- -----A----
BG.JH6       -A-.----C.--   ----------  --GT--.-A-G--  ----------   -------A-- ----------
```

```
Sheep 1081   TCGGGTCTAG  AGGGCCTGGG  ACGGCCAGGG  GTCTGAGACG  GGGAAGGCCC  AGGGGCCCAG
                                       |   | |     |         |   |  | |
Cattle       TCGGGTCTAG  AGGGCCTGGG  GCGG.CTGGG  ATCTGAGACA  CGGAGGACCC  AGGGGCCCAG
2-4.JH8      ----------  ----------  G---.-T---  A--------A  C---G-A---  -A-------N
2-4.JH9      ----------  ----------  .---.-T---  A--------A  C---G-A---  ----------
Pal.JH8      ----------  ----------  G---.-T---  A--------A  C---G-A---  -N--------
Pal.JH9      ---------N  ----------  G---.-T---  A-N------A  C---G-A---  ----------
C15.JH6(1)   -----.---N  ----------  .---.TT---  A-N-N----A  C---G-A---  ----------
BG.JH8       ----------  -A--------  G---      >
BG.JH6       ----------  ----------  G---.-T---  A--------A  C---G-A---  ----------
```

```
Sheep 1141   GCTTACAGCA  GCAAGGAGCA  GAGGCTCCAG  GCACCCTCTC  CTCCTGGGCT  CTGTAGCCAG
                          |
 Cattle      GCTTACAGCA  GCGAGGAGCA  GAGGCTCCAG  GCACCCTCTC  CTCCTGGGCT  CTGTAGCCAG
2-4.JH8      ----------  N-C-N-----  --N---T--N  -------T-T  --T--.----  -----A---N
2-4.JH9      ----------  --G-------  ------T---  ----------  ----------  ----------
Pal.JH8      ------- >
Pal.JH9      ----------  --G-------  ----------  ----------  ----------  ----------
C15J6(1)     ----------  --G-------  ----------  --------C-  ----------  ----------
BG.JH6       ----------  --G-------  ----------  ----------  ----------  ----------
```

```
Sheep 1201   GGCCTTCTCT  GAGGGCCTCA  GCCACCCTTG  GGCTCTGGAC  TCCCAAGGTC  CCAGCTGTGC
               |          |                                              |||
Cattle       GGCTTTCTCT  GGGGGCCTCA  GCCACCCTTG  GGCTCTGGAC  TCCCAAGGTC  CCAGCTG...
2-4.JH8      --NT----N-  TG---N--T-  AN--------  -CTNT-----  -T--------  --- >
2-4.JH9      ---T----C-  -G--------  ----------  ----------  ----------  -------...
Pal.JH9      ---T----N-  -G--------  ----------  ----------  ----------  -------...
C15JH6(2)                      <--   N--NGGN---  --NN-N----  N---------  ---NT--...
C15J6(1)     ---T------  -G--------  ----------  ----------  ----------  -------...
BG.JH6       ---T----N-  -G--------  ----------  ----------  ----------  -------...
```

```
Sheep 1261   TGGCCTCGTG  AGGCCACATG  TGAGGTAGTC  CGGAGGCCTC  AGCCGGCCAC  GAGCTCGTGC
             ||||||||||  ||||||||        |                               |
Cattle       ..........  ........TG  TGAGGTGGTC  CGGAGGCCTC  AGCCGGCCAC  GAGCTCTTGC
2-4.JH9      ..........  ........--  -?----G---  ----------  ----------  ------T---
Pal.JH9      ..........  ........--  -G----G---  ----------  ----------  ------T---
C15JH6(2)    ..........  ........--  -A----G---  --N-------  ----------  --N---T---
C15J6(1)     ..........  ........--  -G----G---  ----------  ----------  ------T---
BG.JH6       ..........  ........--  -G----G---  ----------  ----------  ------T---
```

```
                                    JH7
                                    ---------->
Sheep 1321   TTGGGGTCCC  AGCATCACTG  TCACAGTGTA  ACGACTGGCT  CAAGCACTGG  GGCCAGGGAC
             |        |            |           |                          |    | |
Cattle       CTGGGGTCAC  AGCATCATTG  TCACTGTGTA  ACAACTGGCT  CAAGCACTGG  GGTCAGGAAG
2-4.JH9      C-------A-  -------T--  ----T-----  --A-------  ----------  --T----A-G
Pal.JH9      C-------A-  -------T--  ----T-----  --A-------  ----------  --T----A-G
C15JH6(2)    C-------A-  -------T--  ----T-----  --A------N  N-NN-C---N  --C--  <
C15J6(1)     C-------A-  G------T--  ----T-----  --A-------  -G----  <
BG.JH6       C-------A-  -------T--  ----T-----  --A-------  ----------  --T----A-G
```

JH6

```
Sheep 1381   CCCGA.CGCTG  TCTGCTCAGC  TGAGCCCTCC  CCACCCCACT  TC.ACTGCACC  TGGGGAGGCC
                | ||  |                         |                        |
Cattle       CCTGGGCACTG  TCTGCTCAGC  TGAGCCCTCA  CCACCCCACT  CC.ACTGCACC  TGGGGAGACC
2-4.JH9      --T-GG-A---  ----------  ---------A  ----------  C-.--C-----  -------A--
2-4.J4(2)                             < ------T-  C-.--------  ----A--A--
L.JH4                                             <  ------   -------A--
Pal.JH9      --T-GG-A---  ----------  ---------A  ----------  C-.--------  -------A--
C15.JH4                  <   ------A  --------A-  TCC-T-----T  ----A--A-T
BG.JH6       --T-GG-A---  ----        <
```

```
Sheep 1441   TGGGGTGTCA  GAGATCCAGG  GGCATTCTGG  AGGTCAAGAA  AGGGAGCTGG  GGAGAGGGT
                | |                      |
Cattle       TGGAGCGTCA  GAGATCCAGA  GGCATTCTGG  AGGTCAAGAA  AGGGAGCTGG  GGAGAGGGT
2-4.JH9      ---A-C----  ---------A  ----------  ----------  ----------  ---------
2-4.J4(2)    ---A-C----  ---------A  ---------N  --------N-  ---A------  ---A-----
L.JH4        ---A-C----  ---------A  ---------A  ----------  ---A------  ---------
Pal.JH9      ---A-C----  ---------A  ----------  ----------  ----------  ---------
C15.JH4      --AA-C----  N-------NA  ------N--A  -------N--  ---A------  -A-N-----
```

```
Sheep        ..........  ...........  .......
             ||||||||||  ||| |||||||  |||||||
Cattle       TCTGGTAAAC  AGGC.AGAGCC  AGACCTC
2-4.JH9      ----------  ----.------  -------
2-4.J4(2)    ----------  ----.------  -------
L.JH4        -T--------  --CAN--..--  -------
Pal.JH9      ----------  ----.------  -------
C15.JH4      -N--------  ----A-N-CGG  -------
```

```
Sheep 1500   CC.CGCCCCA.AG  GACACTGCAA  TGTGGGTATG  AGGCGG.CTCC  TCTGG.CGGGT  CTGGCTGTCT
                |                 || |          ||                 ||   |           |
Cattle       CC.TGCCCCA.AG  GACACCACGA  TGTGGGTACA  AGGCGG.CTCC  TTCGG.TGGGT  CTGGCTGCCT
2-4.J4(2)    -N.T------.--  N----CA-G-  --------CA  ------G----  -T---GT----  -------C--
2-4.F(3)     --TT------G--  -----CA-G-A  --------CA  ------.---N  -TA--.T----  -N-----C--
2-4.JH9      --.T------.--  -----CA-G-  -------GCA  ------.----  -T---.T-.--  -------CA-<
L.JH4        --.T------.--  -----CG-G-  --------CA  --N---.----  -T---.T----  -------C--
Pal.JH9      --.T------.--  -----CG-N-  N-------CA  ------.----  -T---.T----  -------C--
C15.JH4      -N.T------.--  -----CA-G-  --------CA  --.---.----  -TC--.T----  -------C--
```

JH9

```
Sheep 1561   GAC.TTGAGCA  GGACCGGGGG  CTTCCGTCGC  TGTCTGGGGC  AGGTG.GCTGC  TCAAGGCTGG
                          |           |           |  |           ||           |
Cattle       CAC.TTGAGCA  GGACCAGGGG  CTTCCGTCAC  TTTCTGGGGC  AGGCA.GCTGC  TCGAGGCTGG
2-4.J4(2)    ---.-------  -----A----  --------A-  -?--------  ---CA.-----  --G-------
2-4.F(3)     ---N-------  -A---A----  --------A-  -T------.-  ---CAA-----  --G-------
L.JH4        ---.-------  -----G----  --------A-  -T--------  ---CA.-----  --G-------
Pal.JH9      ---.-    <
C15.JH4      ---.-------  -----A----  --------A-  -T--------  ---CA.-----  --G-------
```

```
Sheep 1621   ACTTAGGTGT  CTGTGGGTCA  CGGTCAGCTG  GTCCAGGCAG  GCACTGGTCT  GGCCTCTGGG
                    |           |   |    |  ||   |    |        |                 |
Cattle       ACTTAGGAGT  CTGTGGTTCA  TGGTCCGCCA  GCCCAGCCAG  GCAGTGGTCT  GGCCTCTGTG
2-4.J4(2)    -------A--  ------T---  T----C--CA  -C----C---  ---G------  --------T-
2-4.F(3)     -------A--  ------T---  T----C--CA  -C----C---  ---G------  --------T-
L.JH4        -------A--  ------T---  T----C--CA  -C----C---  ---G------  --------T-
C15.JH4      -------A--  ------T---  T----C--CA  -C----C---  ---G------  --------T-
```

```
Sheep 1681   GGCCAAAATG  GGACA.TAGTG  TCTCTGGCAC  .AGTCAGGTGG  GGCGGGGCCG  GCAGAGGGCC
                    |                                            |      |          |
Cattle       GGCCAGAATG  GGACA.TAGTG  TCTCTGGCAC  .AGTCAGGTGG  GGTGGGGCCA  GCAGAGGGTC
2-4.J4(2)    -----G----  -----.-----  ----------  .----------  --T------A  --------T-
2-4.F(3)     -----G----  -----.-----  ----------  A----------  --T------A  --------T-
2-4.F(1)         <--N-   -----AN----  -N----N---  A---N---NN-  --T---N--A  --------TA
L.JH4        -----G----  -----.-----  ---N------  .----------  --T------A  --------T-
C15.JH4      -----G----  -----.-----  ----------  .----------  --T------A  --------T-

Sheep 1741   ACAGGCAAGC  GACTTTGACC  AGCGGCTTCC  CTGTGGTGCC  TGGAGATGGG  GTGGGGGCCC
                 | |                     ||  |          |
Cattle       ACTGACAAGC  GACTTTGACC  AATGGTTTCC  CTGTGGCGCC  TGGAGATGGG  GTGGGGGCCC
2-4.J4(2)    --T-A-----  ----------  -AT--T----  ------C---  ----------  ----------
2-4.F(3)     --T-A-----  ----------  -AT--T----  ------C---  --A-------  ----------
2-4.F(1)     --T-A---A-  ----------  -AT--T----  ----N-C---  --N----A--  --N-------
L.JH4        --T-A-----  ----------  -AT--T----  ------C---  ----------  ----------
C15.JH4      --T-A-----  ----------  -AT--T----  ------C---  ----------  ----------

Sheep 1801   AGGTGCCTCG  AGCCTTGCCA  GGCTCCCGA.G  GTTTTTGTTG  GGCGAGGCTG  GAGATAATCG
                  |           | |                                              | |
Cattle       AGGCGCCTCG  AGCCTCGTCA  GGCTCCCGA.G  GTTTTTGTTG  GGCGAGGCTG  GAGATA.TCA
2-4.J4(2)    ---C------  -----C-T--  ---------.-  ----------  ----------  ------.--A
2-4.F(3)     ---C------  -----C-T--  ---------.-  ----------  ----------  ------.--A
2-4.F(1)     --.C------  -----C-T--  ---------A-  ----------  ----------  ------.--A
L.JH4        ---C------  -----C-T--  ---------.-  ----------  ----------  ------.--A
C15.JH4      ---C------  -----C-T--  ---------.-  ----------  ----------  ------.--A

                                                                    JH2A
                                                                    ---->
Sheep 1861   CCACTGTGAT  TACTACGGTG  TAGATGTCTG  GGGCCGAGGA  CTCCTGGTCA  CCGTCTCCTC
                     |        |  |        | |                      |     ||
Cattle       CCACTGTGTT  TACTATGGTA  TAGACGCCTG  GGGCCGAGGG  CTCAGGGTCA  CCGTCTCCTC
2-4.J4(2)    --------T-  -----T---A  ----C-C---  ---------G  ---AG-----  ----------
2-4.F(3)     --------T-  -----T---A  ----C-C---  ---------G  ---AG-----  ----------
2-4.F(1)     --------T-  -----T---A  ----C-C---  ---------G  ---AG-----  ----------
2-4.JH2A                                                     >      --  -N--T-----
L.JH4        --------T-  -----T---A  --N-C-C---  ---------G  ---AG-----  ----------
C15.JH4      --------T-  -----T---A  ----C-C---  ---------G  ---AG-----  ----------
C15.JH2A                                             >       --CT-----  ----------

             ---->                                                                 <
Sheep 1921   AGGTAAGAGC  GGCC.ATACAG  AGCCTTTGCT  TTCTCTCCTA  TTCGTGGGAT  TTTTCTGAGC
                         |   |  |     |              |   | |        |
Cattle       AGGTAAGAGC  AGCCCATCCAG  GGCCTTTGCT  TTATCTCAT.  TTCGTGCGAT  TTTTCTGAGC
2-4.J4(2)    ----------  A---C--C---  G---------  --A----A--  ------CA--  ---------<
2-4.F(3)     ----------  A---C--C---  G---------  --A----A-.  ------CA--  ---------<
2-4.F(1)     ----------  A---C--C---  G----NGGGG  GNA----A-.  ------CA--  -------NN<
2-4.JH2A     --------N-  GN--C--C---  G---------  --N----A-.  ---CNNC---  ----N-----
L.JH4        --------N-  A---C--C---  G---------  --A----A-.  ------C---  ----NT---<
C15.JH4      ----------  A---C--C---  G---------  --A----A-.  ------CA--  ---------
C15.JH2A     ----------  A---C--C---  G---------  --A----A-.  ------CA--  ---------<

                                  JH4
                                  <----------------------------------------
Sheep 1981   ATCACTG....GTC  CTCTGATGTG  TCCTTGTCCC  CTCCTCCCCG  GGGGGACTGG  GCAGACTGGC
                    ||||                                                |
Cattle       ATCACTGTCTGGTC  CTCTGATGTG  TCCTTGTCCC  CTCCTCCCCG  GGGGAACTGG  GCAGACTGGC
2-4.JH2A     ---N----------  ---N------  ----------  ----------  ----A-----  ----------
C15.JH2A     --------------  ----------  ----------  ----------  ----A-----  ----------

Sheep 2041   CAGGAGGG.AC  CAGCTGCCCT  ATGCATTTCA  GAGTCTCT.AT  CTTCTGATAG  CTTTAAAAAA
                     |                                    |    |   |   ||
Cattle       CAGGAGGGGAC  CAGCTGCCCT  ATGCATTTCA  GAGTCTCTTAT  TTTCCGATGC  CTTTAAAAAA
2-4.JH2A     --------G--  ----------  ----------  --------T--  T---C---GC  ----------
2-4.EμR                                                        <           ----------
C15.JH2A     --------G--  -G--------  ----------  --------T--  T---C---GC  ----------
```

```
Sheep 2101   CCAGAATCTT  GCTGGCATTC  AGAGGGGGCT  TGGGCAGGAA  GGGCCACCAG  TGGG.GGAGTC
             |                          |           |          |
Cattle       TCAGAATCTT  GCTGGCATTC  AGAAGGGGCT  TGGGCGGGAA  TGGCCACCAG  TGGG.GGAGTC
2-4.JH2A     T-------T-  ----------  -G-A------  -----G----  T---------  ----.------
2-4.EμR      T-------C-  ----------  -A-A------  -----G----  T---------  ----G------
C15.JH2A     T-------T-  ----------  -G-A------  -----G----  T---------  ----.------
```

```
Sheep 2161   CCAGGCCTCC  CTTGGCAGCA  G.........  ..........  ...GGCAACTTG  CTGTGGTCCT
               | |                   |||||||||  ||||||||||  |||             ||
Cattle       CCAGGCCCCT  CTTGGCAGCA  GGGCAGCTTG  GGATGCGGTG  GAAGGCAACTTG  CTGCAGTCCT
2-4.JH2A     -------C-T  ----------  -GGCAGCTTG  GGATGCGGTG  GAA---------  ---CA-----
2-4.E_R      -------C-T  ----------  -GGCAGCTTG  GGATGCGGTG  GAA---------  ---CA-----
C15.JH2A     -------C-T  ----------  -GGCAGCTTG  GGATGCGGTG  GAA---------  ---CA-----
```

```
Sheep 2201   AGCATCTGCG  GAGGAGCGTG

Cattle       AGCATCTGCG  GAGGAGCGTG
2-4.JH2A     ----------  ----------
2-4.EμR      -------N--  ----------
C15.JH2A     -------G--  ----------
```

```
Sheep 2221   TCTGGATAAC  TTAGGGCCTC  AGGAGCGCCG  CCCGCAGTGG  G.GCAGAGAAG  GCCCTCCTCG
                       |               |   |       ||  |    |         |       |
Cattle       TCTGGATAA.  TTAGGGCCTC  AGGAACGCTG  CCCGTGGCGG  GCGCAGAGAAC  GCCCCCCTCG
2-4.JH2A     -N-------.  ----------  ----A---T-  ----TG-C--  -C--------C  ----C-----
2-4.EμR      ---------.  ----------  ----A---T-  ----TG-C--  -C--------C  ----C-----
C15.JH2A     -C-------.  ----------  ----A---T-  ----TG-C--  -C---N-N--C  ----C-----
```

```
Sheep 2281   GGTGAGGTTG  GCTCTGCACT  AGACTGTGTT  TAAAATTCTT  TATTGGGCAG  GAAGAGAATT
             |        ||   |                                          |
Cattle       CGTGAGGTGT  GTTCTGCACT  AGACTGTGTT  TAAAATTCTT  TATTGGGTAG  GAAGAGAATT
2-4.JH2A     C-------GT  -T--------  -N--------  ----------  -------T--  ----------
2-4.EμR      C-------GT  -T--------  ----------  ----------  -------T--  ----------
C15.JH2A     --------GT  -T--------  -N--------  ----------  -------T--  ---N-N----
```

```
Sheep 2341   GTCTAGGTGA  GGAC.GACACG  CAGTGTCCCG  ACCGCGGCAA  GAGAGGGGAG  GCTGGGGAGG
                             |    |               |  |   |                  | |
Cattle       GTCTAGGTGA  GGACGGACATG  CAGTGTCCCG  ATCGTGGCGA  GAGAGGGGAG  CCCGGGGAGG
2-4.EμR      ----------  ----G----T-  ----------  -T--T---G-  ----------  C-C-------
2-4.JH2A     -------N--  -- >
C15.JH2A     -------T--  ----C----CC  --A-GT----  -T-AT---G-  N-N-A-----  C-T-------
```

```
Sheep 2401   TGACGGGCGC  TGGGCTTTGT  GAGGCCACTG  TAAGAGAAAG  AAAAGCTGTT  CGCCAGAGGA
                                        | |         ||      |  |
Cattle       TGACGGGCGC  TGGGCTTTGT  GAGGCCAGTC  TAAGAGA..G  AAAGGCCGTT  CGCCAGAGGA
2-4.EμR      ----------  ----------  -------G-C  -------..-  ---G--C---  ----------
C15.JH2A     ---T------  -.A-------  --N-.-NC-C  ---N-N-AA.  N--A--T---  ..G--A-A--
```

```
Sheep 2461   GGTGTGCTTG  CGAATACCAA  GACAGGGCAT  CTTCAAAGCG  ACTCCTGATA  GTCTGGAAAA
                         |                       |||         |           |
Cattle       GGTGTGCTTG  CGAACACCAA  GACAGGGCAT  CTCTGAAGCG  ATTCCTGATA  GTCTGAAAAA
2-4.EμR      ----------  ----C-----  ----------  --CTG-----  -T--------  -----A----
C15.JH2A     ----------  ----T    >
```

```
Sheep 2521   TTGAAACTTT  AAAAAGAGAG  ATGTTTAAAG  TATTTTAAAT  TTTTATCATT  TAATTAACAA
                                 ||  |
Cattle       TTGAAACTTT  AAAAA..GAA  ATGTTTAAAG  TATTTTAAAT  TTTTATCATT  TAATTAACAA
2-4.EμR      ----------  -----..--A  ----------  ----------  ----------  ----------

Sheep 2581   CTGCGAATCA  TGGCTTTGGA  GAGTTGAGTA  AG.....AGT  TTGGCTGAAA  AGTACTAACT
              |  |    |                       ||   |||||        |
Cattle       CCGCAAATCG  TGGCTTTGGA  GAGTTGAGAC  AGGTACAAGT  TTGGCCGAAA  AGTACTAACT
2-4.EμR      -C--A----G  ----------  ----------  ----------  ----------  ----------

Sheep 2636   AGGTTCCATC  GGCCCTCGGC  CCCAATTCAG  GGCTGTTTTG  AGAATAATAA  ATTCAGCTTA

Cattle       AGGTTCCATC  GGCCCTCGGC  CCCAATTCAG  GGCTGTTTTG  AGAATAATAA  ATTCAGCTTA
2-4.EμR      ----------  ----------  ----------  ----------  ----------  ----------

Sheep 2696   TTTTTTTAAT  GTAATTGGTG  GTGCCGAGTT  AGTCAAGATG  GCCACGGGCC  AGACTGACCA
                                                             |  |
Cattle       TTTTTTTAAT  GTAATTGGTG  GTGCCGAGTT  AGTCAAGATG  GCCACGGGCA  GGACTGACCA
2-4.EμR      ----------  ----------  ----------  ----------  ---------A  G---------

Sheep  2756 CCTGCAGCA    2764

Cattle       CCTGCAGCA
2-4.EμR      --------- <
```

Appendix 2: General Methods

Plasmid preparation using QIAGEN reagents

Samples of 3-5 ml from an overnight bacterial culture were spun in a microfuge to collect the cells. Each pellet was resuspended in 250 µl of the manufacturer's resuspension solution and was then mixed with 250 µl of lysis buffer. Once the mixture had visibly clarified, 350 µl of neutralisation buffer was added to precipitate the proteins and genomic DNA. The mixture was centrifuged at high speed and the supernatant carefully recovered. This was poured into a spin-column preloaded by the manufacturer with ion-exchange resin to absorb the DNA. The lysate sample was spun through using a microfuge and after washing with solution containing 80% ethanol, and further centrifugation to remove traces of wash solution, the DNA was eluted by adding TE or distilled water. The recovered DNA was collected by transferring the spin-column to a fresh microfuge tube and recentrifuging.

Typical digestion of DNA with restriction endonuclease

Four µl of DNA was digested in a reaction of 10 µl containing 1 µl of each restriction enzyme, a 10x reaction buffer selected to optimise reaction efficiency (the composition of restriction buffers used in this project is indicated in appendix section), and sterile distilled water. The reaction was incubated a temperature appropriate for the enzymes being used for 1.5-2 hrs.

Agarose gel electrophoresis

General purpose agarose (electrophoresis grade) was dissolved in TAE buffer to a concentration appropriate for resolution of DNA fragments of interest (Sambrook and Russell, 2001) using a microwave oven. Ethidium bromide was added from a stock of 10 mg/ml in water to achieve a final concentration 1 µg/ml. After cooling, the gel was poured to an electrophoresis tray containing a comb to form sample wells and allowed to set at room temperature. It was then transferred to the electrophoresis tank and samples were loaded in sample buffer. Powerpacks were set to apply a voltage equal to 8-10 volts per centimetre of gel.

Extraction of DNA from gel slices using QIAEXII resin

The DNA fragment of interest was excised from an agarose gel with a sharp blade and transferred to a sterile microfuge tube. After adding three volumes of dispersing buffer supplied by Qiagen, the manufacturer of the kit, and a volume of QIAEXII resin appropriate to the quantity of DNA to be recovered, the sample was incubated at 50 °C for 10 min, vortexing every 2 min to keep the resin in suspension. This dispersed the agarose gel, releasing the DNA to the solution. The sample was then centrifuged at high speed in a microfuge for 30 seconds and the supernatant was removed. The pellet of resin was washed using first dispersing buffer and then buffer containing 80% ethanol. To do this, buffer was added to the tube, the sample was vortexed to resuspend the resin, the tube was centrifuged for 30 seconds and finally the supernatant was discarded. The pellet of resin was air-dried for about 30 min and the DNA was recovered by adding at least 20 µl of distilled water. By spinning in a microfuge, the supernatant containing the eluted DNA was isolated for further manipulation. This method was also occasionally used to extract DNA from solutions (*eg* restriction digests).

Extraction of DNA from gel slices using QIAQuick® Gel extraction kit. (QIAGEN)

DNA of the desired size was excised from the gel and dispersed in the manufacturer's buffer at 50 °C. The solution was then centrifuged through a QIAQuick membrane, washed with buffer containing 80% ethanol. DNA was eluted in 50 µl of water.

DNA precipitation with ethanol

One tenth volume of 3 M sodium acetate (pH 5.2 or 7) and 2.5 volumes of cold absolute ethanol were added to the sample. The mixture was incubate at – 20 °C for 30 min and then was spun at high speed (4 °C) for 10-15 min. The supernatant was taken out and the pellet dried by air. An appropriate volume of TE or distilled water was added to dissolve the pellet and recover the DNA.

Preparation of electrocompetent cells

The desired strain of *E. coli* was streaked onto a TYE plate and was incubated at 37 °C. The following day a single colony was picked into 30 ml of 2xTY broth and was incubated overnight at 37 °C. Next day, one litre of 2xTY was inoculated with 10 ml of this culture and was incubated at 37 °C with thorough shaking. The OD of the culture was monitored at 550 nm and the culture was transferred on ice when it reached a value of between 0.3 and 0.5. Cells were harvested by centrifugation at 6000 rpm, the supernatant was discarded and the pellet was gently resuspended in 1/5 volume sterile 10% glycerol, 90% distilled water. Harvesting was repeated 3 times with successively smaller volumes of cold 10% glycerol until the volume of the resuspended pellet was twice that of the packed cells. Working in a cold room at 4 °C, the suspension was divided into 200 µl aliquots in microfuge tubes. These were finally snap-frozen with liquid nitrogen and stored at -80 °C. All of the above operations were done on ice or at 4 °C in order to generate cells of high competence.

Preparation of media for white-blue screening

Forty µl of 4% (w/v) X-gal in dimethylformamide and 40 µl of 25 mM IPTG were spread onto LB plates containing antibiotics required for selection. The plates were incubated at 37 °C or room temperature for at least 30 min to let the substances diffuse into the agar. An appropriate volume of the bacterial transformation reaction was spread onto each plate and the plate was incubated at 37°C overnight. The following day, blue (non-recombinant) and white (recombinant) colonies were visible for further analysis.

Storing bacterial cultures at -80 °C

Cultures of bacteria grown on agar plates were scraped and resuspended in 0.5-1 ml of LB and after adding 0.5 volumes of sterile 50% glycerol in water, the suspensions were transferred to -80 °C in polypropylene containers suitable for low temperature storage.

Preparation of bacterial lysates as templates for PCR

A single bacterial colony was inoculated to an eppendorf tube containing 250 µl of LB with an appropriate antibiotic and the culture was incubated at an appropriate

temperature overnight. The following day, the tube was centrifuged to pellet the cells and the supernatant was discarded. The pellet was resuspended in 30 µl of distilled water and the tube was heated in a boiling water bath for 10-15 min to lyse the bacteria. The tube then was centrifuged to pellet the debris. Eight µl of supernatant was used as template for PCR reactions of 25 µl. After cycling, 7-8 µl of the reaction was run on an agarose gel.

PCR machines

Unless otherwise indicated, all PCR reactions were run on Perkin Elmer GeneAmp 9700 and 2400 instruments using cycling conditions specified in the relevant section of the thesis.

Western blotting

After electrophoresis, the SDS gel was equilibrated in cold transfer buffer for at least 30 min. The blotting membrane (typically Hybond C, Amersham), fibre pads and filter paper were also soaked for 10-15 min. The gel holder was submerged in transfer buffer and a sandwich comprising the gel, the blotting membrane, and filter papers, top and bottom, was packed between fibre pads and assembled into the holder. Each layer was kept moist, avoiding the entry of air bubbles. The cassette was transferred to the blotting tank, ensuring that the blotting membrane was nearest the positive terminal. Blotting was performed at 100 volts for 1 hr or 30 volts, 40 milliamps overnight. The membrane then was blocked using 50 ml of 3% skimmed milk in Tris-NaCl (TBS) (pH 7.4). This was done with constant agitation on a shaking table for 1 hr or overnight at room temperature. The membrane was transferred to 3% skimmed milk in TBS containing an appropriate dilution of the primary antibody and was incubated with shaking for 2-3 hrs at 37 °C or 8 hrs at room temperature. Unbound antibodies were removed by washing with 40 ml aliquots of TBS. Four washes, each of 5 min were routinely used. The secondary antibody-enzyme conjugate was added, typically at a dilution of 1/1000 in 3% skimmed milk TBS for incubation at 37 °C for 1 hr. Washing was done as described. The membrane was then transferred to the developer solution, allowing the bands to develop. Once the image was clear, the membrane was washed with distilled water and dried on filter paper.

Appendix 3: Chemicals

Acetic acid (Glacial): BDH Laboratory Supplies, England. # 10001 CU

30% Acrylamide-Bis solution (19:1): BIO-RAD Laboratories, USA. # 161-0154

Agarose: Roche Diagnostic GmbH, Germany # 1 388 991

Ammonium persulfate (APS): SIGMA-Aldrich Chemical GmbH, Germany # A-3670

Blood agar: Oxoid Ltd., England. # CM271

Bovine serum albumin (BSA): Fraction V, SIGMA-Aldrich Chemical GmbH, Germany # A-2153

Brain heart infusion: Oxoid Ltd., England. # CM225

Coomassie Brilliant Blue: SIGMA Chemical, USA. # B-0149

Bromophenol blue: SIGMA Chemical, USA. # B-5525

4-chloro-1-naphthol: SIGMA-Aldrich Chemical GmbH, Germany # C-8890

Chloroform: Fisher Scientific, UK. # C/4920/17

Citric acid: Fisher Scientific, UK. # C/0200/53

dNTPs: Promega, # U 1201, 1211, 1221, 1231

Diaminoethane tetra-acetic acid, disodium salt (EDTA): Fisher Scientific, UK. # D/0700/53

di-Sodium hydrogen orthophosphate (anhydrous): BDH Laboratory Supplies, England. # 1024944 C

di-Sodium hydrogen orthophosphate (dihydrate): Fisher Scientific, UK. # S/4450/53

Dithiothreitol (DTT): Melford Laboratories Ltd, UK. # 16286

Glucose: BDH Laboratory Supplies, England. # 10117

Glycerol: SIGMA-Aldrich, Riedel-deHaen, Germany, # 15523

Glycine: Fisher Scientific, UK. # G/0800/60

Hydrogen peroxide, 30% solution: Sigma Chemical, USA # H-1009

Isoamyl alcohol: Fisher Scientific, UK. # A/7080/08

Isopropanol: BDH Laboratory Supplies, England. #10224BQ

Isopropyl-B-D- thiogalactopyranoside (IPTG): Melford Laboratories Ltd, UK. # 16327

Potassium chloride : BDH Laboratory Supplies, England. # 101984L

di-Potassium hydrogen orthophosphate: BDH Laboratory Supplies, England. # 104363A

Sodium chloride: SIGMA-Aldrich, Riedel-deHaen, Germany. # 31434

N-Lauroyl sarcosine, sodium salt (Sarkosyl): SIGMA-Aldrich Chemical GmbH, Germany. # L-5125

Lysozyme: Promega.

Methanol: BDH Laboratory Supplies, England. # 10158 BG

Micro agar: Duchefa, Netherlands # M 1002

O- phenylenediamine tablets, 15 mg/tablet: SIGMA-Aldrich Chemical GmbH, Germany # 4664 SIGMA

Phosphate buffered saline tablets: SIGMA-Aldrich Chemical GmbH, Germany # P-4417

Rainbow molecular weight markers: Amersham Pharmacia Biotech, England. # RPN 756

Skimmed milk: Marvel. Premier Brands. UK.

Sodium bicarbonate: SIGMA-Aldrich Chemical GmbH, Germany # S-8875

Sodium carbonate (anhydrous): SIGMA-Aldrich Chemical GmbH, Germany # S-1641

Sodium di-hydrogen orthphosphate: BDH Laboratory Supplies, England. # 301324Q

Sodium hydroxide: Fisher Scientific, UK # S/4920/53

Tri-sodium citrate: Fisher Scientific, UK # S/3320/53

Sodium Dodecyl Sulphate: Fisher Scientific, UK. # S/P530/53

Sucrose: Fisher Scientific, UK # S/8600/63

Sulphuric acid (1.84): Fisher Scientific, UK. # S/9230/PB/7

TAE buffer: Anachem Ltd.

TEMED: SIGMA-Aldrich Chemical GmbH, Germany # T-9281

Tris-HCl: TRIZMA: SIGMA-Aldrich Chemical GmbH, Germany # T-3253

Tris:Trizma base: SIGMA-Aldrich Chemical GmbH, Germany # T-1503

Tryptone: Oxoid Ltd., England. # L42

Tween 20: SIGMA-Aldrich Chemical GmbH, Germany # P-1379

Yeast extract: Oxoid Ltd., England. # L21

Yeast extract: Dachefa, Netherlands # T-1333

Immunotubes: Nagle Nunc International, Denmark

Hybond-C Super: Nitrocellulose transfer membrane for proteins. Amersham Pharmacia Biotech, England.

Hybond-N+: Nylon transfer membrane for DNA: Amersham Pharmacia Biotech, England.

Hybond-P: PVDF transfer membrane for protein. Amersham Pharmacia Biotech, England.

Antibodies

HRP anti-rabbit antibody: SIGMA-Aldrich Chemical GmbH, Germany

Mouse anti-pIII: Mo Bi Tec. GmbH, Germany. # PSKAN3

HRP anti-M13: Amersham Pharmacia Biotech, England. # 27-9421-01

HRP anti-mouse: SIGMA-Aldrich Chemical GmbH, Germany # A-9309

Media

Luria broth:

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Distilled water	up to 1 litre

2xTY

Tryptone	16 g
Yeast extract	10 g
NaCl	5 g
Distilled water	up to 1 litre

TYE

Tryptone	10 g
Yeast extract	5 g
NaCl	8 g
Agar	15 g
Distilled water	up to 1 litre

BHI

Brain Heart Infusion	37 g
Distilled water	up to 1 litre

SOC

Tryptone	20 g
Yeast extract	5 g
NaCl	0.5 g
Distilled water	up to 1 litre

The medium is autoclaved and 10 ml of 1 M $MgCl_2$ and 10 ml of 1 M of $MgSO_4$ is added prior to use.

Buffers

Citrate buffer: consists of 53 % of 0.1 M trisodium citrate and 47 % of 0.1 M citric acid monohydrate

HRP Substrate solution: 1 OPD tablet (15 mg) and 6 µl of 30% hydrogen peroxide in 15 ml citrate buffer

DNA sample buffer (agarose):

Bromophenol blue	0.25%
Xylene cyanol	0.25%
Sucrose	40 %

SDS-Polyacrylamide electrophoresis

Stacking gel 4%

30% Bis-acrylamide	487.5 µl
0.5 M Tris-HCl (pH 6.8)	1.25 ml
10% SDS	50 µl
Distilled water	3.26 ml
TEMED	5 µl
10% APS	2.5 µl

Resolving gel	12%	10%
30% Bis-acrylamide	4 ml	3.3 ml
1.5 M Tris-HCl (pH 8.8)	2.5 ml	2.5 ml
10% SDS	0.1 ml	0.1 ml
Distilled water	3.3 ml	4 ml
TEMED	5 µl	5 µl
10% APS	0.1 ml	0.1 ml

5x Running gel buffer

Tris base	9 g
Glycine	43.2 g
SDS	3 g
Distilled water	up to 100 ml

Destain:

Methanol	450 ml
Distilled water	450 ml
Glacial acetic acid	100 ml

Gel stain

Methanol	450 ml
Glacial acetic acid	100 ml
Brilliant Coomassie Blue	0.125 g
Distilled water	450 ml

Western blotting

Western blotting transfer buffer

Tris-base	3.03 g
Glycine	14.4 g
Methanol	200 ml
Distilled water	up to 1 litre

pH 8.1-8.4 **(pH not adjucted)**

Tris-NaCl (TBS)

Tris-base	1.21 g
NaCl	8.7 g
Concentrated HCl	0.8 ml
Adjust the pH to 7.4	

Developer for Horse Radish Peroxidase (should be prepared fresh: maximum 30 min prior to use)

4-chloro-1-naphthol	30 mg
Methanol	10 ml
Tris-NaCl pH 7.4	50 ml
30% H_2O_2	30 µl

Restriction enzyme buffers: (x 10)

React buffer 2 for ***Bst*EII, *Sst*I and *Eco*RV** (Life Technologies):

500 mM	Tris-HCl (pH 8)
100 mM	$MgCl_2$
500 mM	NaCl

React buffer 5 for ***Rsr*II** (Life Technologies):

100 mM	Tris-HCl (pH 8.2)
80 mM	$MgCl_2$

React buffer 4 for ***Rsr*II** (New England Biolabs):

500 mM	potassium acetate
200 mM	Tris acetate
100 mM	Magnesium acetate
10 mM	DTT
pH 7.9	

React buffer 4 for ***Spe*I** (Life Technologies):

200 mM	Tris-HCl (pH 7.4)
50 mM	$MgCl_2$

React buffer B for ***Spe*I** (Promega):

60 mM	Tris-HCl (pH 7.5)
500 mM	NaCl
60 mM	$MgCl_2$
10 mM	DTT

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