Antibody phage-displayed libraries derived from chicken immunoglobulin genes: a source of highly specific diagnostic antibodies

By

Thamsanqa Emmanuel Chiliza

A dissertation submitted to the Faculty of Veterinary Science of University of Pretoria in partial fulfillment of the requirements for the degree of

Master of Science (Veterinary Science)
DECLARATION

I, Thamsanqa Emmanuel Chiliza, do hereby declare that this dissertation is my own work. It has been submitted to the University of Pretoria for the degree of Master of Science. It has not been submitted before for any degree at any other University.

_________________________
Thamsanqa Emmanuel Chiliza
ACKNOWLEDGEMENTS

I wish to acknowledge and express my sincere appreciation to the following people:

Prof Dion H. du Plessis my promoter, for his guidance, interest and encouragement during the project and writing of this dissertation.

Wouter van Wyngaardt, for his guidance and advice during this project and for providing the LDH “dedicated” IgY.

Cordelia Mashau, for her patience, guidance and time spent in training me in phage antibody selection method.

Dr Christine Vroon, for giving me the opportunity to work with her and for her valuable expertise in virus purification.

Dr Jeanni Fehrsen, for designing the Hend1 and Lstart1 primers.

Dr Marco Romito, for his expertise in animal care.

Prof Tony Musoke, Research and Technology Manager (OVI) for his constant motivation and support throughout this project.
Immunology Section staff at Ondersteypoort Veterinary Institute and fellow colleagues for their interest and support in this project.

Department of Science and Technology and Agricultural Research Council for funding of this project.

My family and friends for their support and encouragement.
TABLE OF CONTENTS

DECLARATION ..............................................................................................i

ACKNOWLEDGEMENTS .............................................................................ii

TABLE OF CONTENTS ...........................................................................iv

LIST OF FIGURES ......................................................................................viii

LIST OF TABLES ........................................................................................xiii

ABBREVIATIONS ......................................................................................xiv

ABSTRACT .................................................................................................xvii

CHAPTER 1: LITERATURE REVIEW .........................................................1

1.1 Antibody technology ..............................................................................1

   1.1.1 Hybridoma technology .................................................................1

   1.1.2 Phage display technology .............................................................3

       1.1.2.1 Polypeptide linker effect .......................................................8

       1.1.2.2 Types of phage antibody libraries .....................................9

1.2 Immunoglobulin genes ..........................................................................11

   1.2.1 The mouse immunoglobulin gene locus ....................................11

       1.2.1.1 Mouse B cell development ................................................12

   1.2.2 The chicken (*Gallus domesticus*) immunoglobulin gene locus ....14

       1.2.2.1 Chicken B cell development ................................................16

1.3 Disease targets ....................................................................................20

   1.3.1 Malaria ........................................................................................20

   1.3.2 Trypanosomiasis .........................................................................24
1.3.3 Malignant catarrhal fever .................................................26
1.4 Motivation and strategy..............................................................28
1.5 Aim, objectives and potential benefits........................................30
  1.5.1 Aim ..................................................................................30
  1.5.2 Objectives .........................................................................30
  1.5.3 Potential benefits .................................................................30

CHAPTER 2: MATERIALS AND METHODS .............................................31
  2.1 Antigens for immunisation ..........................................................31
  2.2 Immunisation of chickens ............................................................31
  2.3 IgY ELISA ..............................................................................32
  2.4 Western immunoblotting ..............................................................33
  2.5 Phagemid preparation .................................................................33
  2.6 Antibody library construction ....................................................34
    2.6.1 Preparation of RNA and synthesis of cDNA .......................34
    2.6.2 PCR amplification of VH and VL chain genes ...................35
    2.6.3 Assembly of scFv gene .......................................................36
    2.6.4 Electroporation and library size estimation .......................37
  2.7 Biopanning of antibody library against target antigens ................38
  2.8 Screening of phage pools ..........................................................40
    2.8.1 Polyclonal phage ELISA .................................................40
    2.8.2 Monoclonal phage ELISA ..............................................40
    2.8.3 Expression of soluble scFv antibody fragments ..................41
2.8.4 DNA sequencing of single scFv clones.................................41
2.8.5 Introduction of a flexible linker...........................................42
2.9 Immunoassays...........................................................................42
2.9.1 Sandwich ELISA.................................................................42
2.9.2 Competitive inhibition ELISA...............................................43

CHAPTER 3: RESULTS .........................................................................44
3.1 Antigens for immunisation .......................................................44
3.2 Immunisation of chickens and IgY ELISA ...............................45
3.3 Antibody library construction ..................................................47
3.4 Biopanning...............................................................................51
3.4.1 Selection of anti-LDH scFvs ..................................................52
3.4.2 Selection of anti-HRPII scFvs.................................................57
3.4.3 Introduction of a flexible linker..............................................64
3.5 Identification of pseudo $\psi$VL gene donors...............................66
3.6 Potential use of selected scFvs..................................................69
3.7 IgY immunoblotting of MCFV proteins.................................71
3.8 Other use of IgY.................................................................72

CHAPTER 4: DISCUSSION .................................................................74
4.1 Chicken immune response to antigens......................................74
4.2 Antibody library construction...............................................75
4.3 Antibody selection...............................................................77
4.4 Stability testing........................................................................79
4.5 Donor sequences......................................................................80
4.6 Introduction of a flexible linker..................................................81
4.7 Potential use of selected scFvs....................................................82
4.8 Conclusion.................................................................................82

CHAPTER 5: APPENDICES AND REFERENCES.................................84
5.1 Appendices..............................................................................84
  5.1.1 Appendix A: Buffers and stock solutions...............................84
  5.1.2 Appendix B: Media..............................................................87
5.2 References...............................................................................88
5.3 Publication..............................................................................104
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1:</td>
<td>Principle steps in the production of a hybridoma</td>
<td>2</td>
</tr>
<tr>
<td>Figure 1.2:</td>
<td>Diagram showing the structure of an antibody and single-chain variable fragment (scFv)</td>
<td>4</td>
</tr>
<tr>
<td>Figure 1.3:</td>
<td>Diagram showing the steps involved in constructing an antibody phage display library</td>
<td>5</td>
</tr>
<tr>
<td>Figure 1.4:</td>
<td>Diagram illustrating selection of phage particles expressing an antibody (scFv fragment) of interest by panning on an immobilised target</td>
<td>7</td>
</tr>
<tr>
<td>Figure 1.5:</td>
<td>ScFv gene constructs and encoded protein products, depending on a linker length (A) scFv monomer, (B) diabody and (C) triabody</td>
<td>9</td>
</tr>
<tr>
<td>Figure 1.6:</td>
<td>Diagram illustrating IgH VDJ (A) and Igκ VJ (B) rearrangement; 1, 2 and 3 are examples of possible V (D) J products for each case in mouse</td>
<td>13</td>
</tr>
<tr>
<td>Figure 1.7:</td>
<td>The chicken IgH chain locus organisation, the diagram is illustrating the process of Ig diversification by gene conversion</td>
<td>16</td>
</tr>
<tr>
<td>Figure 1.8:</td>
<td>Diagram illustrating AID involvement in the diversification of Ig genes by gene conversion</td>
<td>18</td>
</tr>
<tr>
<td>Figure 1.8:</td>
<td>The world map showing the distribution of malaria</td>
<td>21</td>
</tr>
<tr>
<td>Figure 1.9:</td>
<td>The principle of immunochromatographic RDT</td>
<td>23</td>
</tr>
<tr>
<td>Figure 2.1:</td>
<td>The structure and cloning sequence of the pHEN vector</td>
<td></td>
</tr>
</tbody>
</table>
used for phage display and expression of scFv antibodies

Figure 3.1: Picture A shows the sucrose density-gradient MCFV pellet and an electron microscope image in B shows MCFV particles (VP), viral envelope (VE) and nucleocapsid (NC)

Figure 3.2: Analysis of IgY (lane 1 and 2) by SDS-PAGE

Figure 3.3: Indirect ELISA analysis of antigen specific IgY titre in eggs collected from chicken “A” after a third booster immunisation

Figure 3.4: Indirect ELISA analysis of antigen specific IgY titre in eggs collected from chicken “B” after a third booster immunisation

Figure 3.5: Flow diagram showing the construction of two antibody phage displayed libraries

Figure 3.6: PCR amplicons lane 1, V\textsubscript{H}; lane 2 V\textsubscript{L}; and lane 3 the scFv gene assembled by pull-through PCR.

Figure 3.7: PCR amplicons obtained after colony PCR (“directly” linked library) using vector-specific primers that recognise insert flanking regions

Figure 3.8: PCR amplicons obtained after colony PCR (“normally” linked library) using vector specific primers that recognise insert flanking regions

Figure 3.9: Amino acid sequence alignment of randomly chosen clones from “directly” linked antibody library

Figure 3.10: Amino acid sequence alignment of randomly chosen clones from “normal” linked antibody library
Figure 3.11: Polyclonal phage ELISA showing an increase in ELISA absorbance values from round 2 to 4 indicating an enrichment of LDH, HRPII and VSG specific phage antibodies

Figure 3.12: Monoclonal phage ELISA (A) on LDH with clones randomly chosen from LDH selection 3

Figure 3.13: Soluble antibody fragment ELISA (A) corresponding to monoclonal phage clones shown in Fig. 3.12

Figure 3.14: Amino acid sequence alignment of anti-LDH scFvs LA2, LB10, LC9, LD3 and LE11 with the chicken germline Ig V_H and V_L

Figure 3.15(a): ELISA of selected anti-LDH scFvs showed specificity to LDH with no signal against ALDO and negative control (2% MP)

Figure 3.15(b): Stability test ELISA of anti-LDH scFvs that were stored for one month at RT, 4ºC and -20ºC

Figure 3.16: Monoclonal phage ELISA (A) on HRPII with clones randomly chosen from HRPII selection 3

Figure 3.17: Monoclonal phage ELISA (A) on HRPII with clones from HRPII selection 4

Figure 3.18: Soluble antibody fragment ELISA on HRPII (A) corresponding to monoclonal phage clones selected in round 3

Figure 3.19: Soluble antibody fragment ELISA on HRPII (A) corresponding to monoclonal phage clones selected in round 4
Figure 3.20: Amino acid sequence alignment of anti-HRPII scFvs HA2, HB5, HF11, HD2, HD6 and H4A5 with the chicken germline Ig V\text{H} and V\text{L} chain.

Figure 3.21: ELISA of selected scFvs on HRPII showed specificity to HRPII with no signal against immunising partner ALDO (A) and four scFvs showed stability following incubation at RT, 4\textdegree C and -20\textdegree C for one month (B).

Figure 3.22: Diagram illustrating conversion of “directly” linked V\text{H}-G-V\text{L} scFvs to flexible linked V\text{H}-(G_{4S})_{3}-V\text{L} scFvs using PCR and cloning into the pHEN displaying vector using Sfi1/Not1 restriction sites.

Figure 3.23: ELISA of newly constructed anti-LDH scFvs N-A2, N-B10, N-C9, N-D3 and N-E11.

Figure 3.24: Alignment of anti-LDH V\text{L} chain sequences to pseudo ψV\text{L} gene donors.

Figure 3.25: Alignment of anti-HRPII V\text{L} chain sequences to pseudo ψV\text{L} gene donors.

Figure 3.26: Sandwich ELISA using IgY as capturing antibody of LDH and scFv LC9 specific to LDH as a detection antibody.

Figure 3.27: Competitive inhibition ELISA using IgY at different concentrations to block LC9 binding to immobilised LDH.

Figure 3.28: Competitive inhibition ELISA using “cocktail” IgY at different concentrations to block the LC9 binding to
immobilised LDH

Figure 3.29: Western blot analysis of virus proteins of partially purified MCFV proteins immunoblotted with chicken IgY

Figure 3.30: ELISA of the new batch of HRPII and comparison to the old batch
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Nucleotide sequences of primers used</td>
<td>37</td>
</tr>
<tr>
<td>Table 2</td>
<td>The list of pseudo $\psi_{VL}$ genes that were involved during gene conversion events</td>
<td>66</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>AID</td>
<td>activation induced cytidine deaminase</td>
<td></td>
</tr>
<tr>
<td>ALDO</td>
<td>aldolase</td>
<td></td>
</tr>
<tr>
<td>$A_x$</td>
<td>absorbance at x nanometre</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
<td></td>
</tr>
<tr>
<td>bp</td>
<td>base-pair</td>
<td></td>
</tr>
<tr>
<td>$^\circ$C</td>
<td>degrees Celsius</td>
<td></td>
</tr>
<tr>
<td>CI-ELISA</td>
<td>competitive inhibition ELISA</td>
<td></td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
<td></td>
</tr>
<tr>
<td>CDR</td>
<td>complementarity determining region</td>
<td></td>
</tr>
<tr>
<td>dC</td>
<td>2’-deoxy-cytidine</td>
<td></td>
</tr>
<tr>
<td>dG</td>
<td>2’-deoxy-guanosine</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>dNTP</td>
<td>2’-deoxynucleoside-5’-triphosphate</td>
<td></td>
</tr>
<tr>
<td>dU</td>
<td>2’-deoxy-uracil</td>
<td></td>
</tr>
<tr>
<td>e.g.</td>
<td>for example</td>
<td></td>
</tr>
<tr>
<td>et al.</td>
<td>et alii (and others)</td>
<td></td>
</tr>
<tr>
<td>etc</td>
<td>continuing in the same way</td>
<td></td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
<td></td>
</tr>
<tr>
<td>Ff</td>
<td>filamentous phage</td>
<td></td>
</tr>
<tr>
<td>Fig.</td>
<td>figure</td>
<td></td>
</tr>
<tr>
<td>FW</td>
<td>frame-work</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>glycine</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>HAT</td>
<td>hypoxanthine-, aminopterin- and thymidine</td>
<td></td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
<td></td>
</tr>
<tr>
<td>HRPII</td>
<td>histidine-rich protein 2</td>
<td></td>
</tr>
<tr>
<td>i.e.</td>
<td>in other words</td>
<td></td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
<td></td>
</tr>
<tr>
<td>IgY</td>
<td>immunoglobulin class Y</td>
<td></td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
<td></td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
<td></td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
<td></td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
<td></td>
</tr>
<tr>
<td>MCFV</td>
<td>malignant catarrhal fever virus</td>
<td></td>
</tr>
<tr>
<td>mg</td>
<td>miligram</td>
<td></td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
<td></td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
<td></td>
</tr>
<tr>
<td>MP</td>
<td>milk powder</td>
<td></td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
<td></td>
</tr>
<tr>
<td>OD_x</td>
<td>optical density at x nm</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
<td></td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
<td></td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cells</td>
<td></td>
</tr>
<tr>
<td>RDT</td>
<td>rapid diagnostic tests</td>
<td></td>
</tr>
<tr>
<td>RSS</td>
<td>recombination signal sequence</td>
<td></td>
</tr>
</tbody>
</table>
RT - room temperature
scFv - single-chain variable fragment
SDS-PAGE - sodium dodecyl sulphate - polyacrylamide gel electrophoresis
s - seconds
S - serine
ssDNA - single-stranded DNA
μl - microlitre
μg - microgram
VAT - variable antigen
VH - variable heavy
VL - variable light
vol - volume
VSG - variant surface glycoprotein
WHO - world health organisation
wt - weight
xg - gravity
ABSTRACT

Antibody phage-displayed libraries derived from chicken immunoglobulin genes: a source of highly specific diagnostic antibodies

by

Thamsanqa Emmanuel Chiliza

Promoter : Prof DH du Plessis
Department : Department of Veterinary Tropical Diseases
Faculty of Veterinary Science, University of Pretoria
Degree : Master of Science

In meeting the high demand for monoclonal antibodies, the chicken immunoglobulin system was exploited to generate recombinant antibodies against multiple target antigens. Following simultaneous immunisation of two chickens with a mixture of *Plasmodium falciparum* recombinant lactate dehydrogenase (LDH), histidine rich protein II (HRPII) and aldolase (ALDO), recombinant trypanosome variable surface glycoprotein (VSG) and malignant catarrhal fever virus (MCFV) each chicken produced egg yolk antibodies (IgY) against four of the five antigens.
Using phage display technology, two single-chain variable fragment (scFv) antibody libraries, one with the immunoglobulin V\textsubscript{H} and V\textsubscript{L} chain regions joined by a single amino acid (G) and the other with a 15 amino acid flexible linker [(G\textsubscript{4}S)\textsubscript{3}] were constructed using pooled splenic RNA. The single amino acid-linked scFv repertoire was evaluated as a source of highly specific diagnostic antibodies by panning against each of the five different antigens. After two rounds of panning, polyclonal phage ELISA showed the presence of antigen-specific phage antibodies against three (LDH, HRPII and VSG) of the five antigens. Five different anti-LDH and six different anti-HRPII scFvs were identified by sequence analysis. Evidence of high levels of antigen-driven gene conversion events was found in the framework and complementary determining regions and the V\textsubscript{L} chain pseudogene donors were identified. Stability of the selected scFvs was determined by incubation at different times and at different temperatures. The specificity and potential use of an LDH-specific scFv as a diagnostic reagent was shown in sandwich and competitive inhibition ELISAs.
CHAPTER 1: LITERATURE REVIEW

1.1 Antibody technology

An antibody directed against a specific epitope (i.e. antigenic determinant) and produced by a single clone of B cells is called a monoclonal antibody (mAb). Monoclonal antibodies are widely used in the diagnosis of disease, biomedical research, identifying pathogens and locating potentially protective antigens for vaccine development. Since monoclonal antibodies usually recognise a single epitope with high specificity, they are particularly powerful diagnostic tools. There are two methods which are generally used to generate mAbs: (1) traditional hybridoma technology (Köhler and Milstein, 1975) and (2) antibody phage display technology (McCafferty et al., 1990).

1.1.1 Hybridoma technology

A process known as hybridoma technology has been widely used to generate mAbs since its introduction by Köhler and Milstein in 1975. This method is laborious, often inefficient and is usually used to generate murine antibodies. This means that when used therapeutically they are likely to be immunogenic in humans. Another limitation is the number of clones that can be accessed from a single host animal. The essentials of the method are that splenic B cells from an immunised mouse are fused with suitable myeloma (cancer) cells to form hybridomas which secrete antibodies of a single specificity. The hybridomas are cultured and selected for in hypoxanthine-, aminopterin- and thymidine-containing (HAT) medium. Unfused spleen and myeloma cells die and only the fused myeloma-spleen cell hybrids (hybridomas) survive (Köhler and Milstein, 1975). Hybridomas expressing mAbs are then distributed to 96 well plates for expansion and the supernatant fluid produced is screened by ELISA to identify antigen-specific
clones. The positive hybridomas are then re-screened in a second test. The clones from the second test may in turn be subjected to tertiary screening. Antigen-specific mAbs are then purified from a clone of hybridoma cells which produces a single specific antibody population. Appropriately characterised mAbs can be used in diagnostic assays or other applications. Fig. 1.1 shows the essential steps in hybridoma production.

Figure 1.1: Principle steps in the production of a hybridoma (from http://home.inje.ac.kr/~lecture/immunobiotech/ch9/antibodyeng.htm).
1.1.2 Phage display technology

The use of filamentous bacteriophages as peptide-displaying vectors was first introduced by George Smith in 1985 (Smith, 1985). This method physically links phenotype and genotype thereby allowing both selection and amplification of binding peptides. It was initially described as a method for mapping epitopes, but after the successful display of antibody fragments by McCafferty et al. (1990) interest in phage display increased tremendously since this technology has the potential to replace hybridomas in some cases.

The Ff filamentous phages are non-lytic, single stranded DNA (ssDNA) phages infecting susceptible F+ (male) *Escherichia coli* strains via their minor coat protein (pIII) which is located at the tip of the phage particle. This protein binds to the bacterial F+ sex pilus. The *pIII* gene of a typical filamentous phage encodes a polypeptide of 406 amino acid residues which is responsible for both infection of the host and normal morphogenesis of the phage particle (Smith, 1985). In phage display, exogenous peptide sequences can be cloned in one of several capsid proteins, namely the abovementioned pIII (predominantly), pIV, pVIII or pIX (Hoogenboom et al., 1991; 1998). The foreign peptide is expressed on the phage surface as a fusion protein and hence can specifically interact with cognate molecules (Smith, 1985; McCafferty et al., 1990; Hoogenboom et al., 1991; 1998). The phage particles expressing the protein or peptide of interest are selected by “panning” on an immobilised target as will be described later (Fig. 1.4).
To construct an antibody phage library, an mRNA is isolated from a secondary lymphoid organ such as the spleen. Genes coding for the variable regions of immunoglobulins are accessed by PCR, linked and introduced into a display vector (McCafferty et al., 1990). The variable heavy (VH) and variable light (VL) chain genes containing antigen-binding regions are usually linked via a synthetic flexible linker to construct genes that code for a single-chain variable fragment (scFv) (Fig. 1.2) repertoire which is cloned so as to form a fusion with pIII, the attachment protein (Fig. 1.3).

![Figure 1.2: Diagram showing the structure of an antibody and single-chain variable fragment (scFv) (from http://www.gene-therapeutics.com/.../Abb-scFv.gif).](http://www.gene-therapeutics.com/.../Abb-scFv.gif)

The amplified antibody variable regions are fused to the amino terminus of pIII while a signal sequence downstream of gene III directs the antibody fragments to the bacterial periplasm during morphogenesis of phage particles (Hoogenboom et al., 1998). Two types of vectors are generally used to construct phage display libraries, namely phage and phagemid vectors. Phage vectors are based entirely on the Ff filamentous phage genome.
while the phagemid genome consists of plasmid DNA and some phage intergenic regions (O’Connell et al., 2002). In a phage vector the expression of the antibody-coat protein fusion is under the control of a natural promoter and a strong transcriptional terminator (Hoogenboom et al., 1991). In the phagemid vector on the other hand, the lac Z promoter drives the antibody-pIII expression (Barbas III et al., 1991; Hoogenboom et al., 1991).

For the successful display of antibody fragments by phagemids, a leader sequence for secretion, a weaker promoter, a selection marker and both bacterial and viral replication origins are all required (Hoogenboom et al., 1998).

Figure 1.3: Diagram showing the steps involved in constructing an antibody phage display library: (A) amplification and assembly of scFv fragments by PCR, (B) cloning of scFv genes into vector DNA and (C) display of a functional scFv on the phage surface.
Packaging the DNA of a phagemid into a phage particle requires a helper phage to supply all the structural proteins required. The wild-type pIII from the helper competes with the antibody-pIII fusion for display on the phage surface. This means that most of the particles will probably not have antibody-pIII fusions (O’Connell et al., 2002). In fact, only about 10% of rescued phage particles are likely to display a single fusion particle on their surface (Hoogenboom et al., 1998). The rate of phagemid rescue differs for each member in the phage library due to the different effects exerted on the host E. coli by each scFv gene and/or its gene product. High levels of pIII fusion protein expression can inhibit infection with helper phage resulting in a loss of some of the library members due to failure in rescuing them (O’Connell et al., 2002). These potential drawbacks have, however, not prevented large antibody libraries from being made. Phagemid vectors are usually preferred over phages for antibody display due to higher transformation efficiencies and because of their monovalent display of the antibody fragment (Hoogenboom et al., 1998).

Phage libraries, on the other hand, display more copies of each scFv per particle and can as a consequence yield high levels of selection on antigen as compared to phagemid libraries. Many of the selected antibodies may, however, have a low intrinsic affinity for their antigens. This can be overcome by converting a multivalent phage display repertoire to a monovalent phagemid display library after a single round of selection if required. Monovalent display enables high-affinity antibodies to be more efficiently selected, especially against a less complex antigen while multivalent display enhances selection of rare antibodies (O’Connell et al., 2002). Although monovalent display virtually guarantees the selection of high affinity antibodies, multivalent display may be preferred
when a limited number of phage particles per clone are to be selected from a huge antibody library (Hoogenboom et al., 1998). Phage libraries have so far not been used as widely as phagemid repertoires for displaying antibody fragments.

Phage-display libraries are screened for scFv fragments that bind to a chosen antigen by a process called “panning” (Barbas III et al., 1991). The surface-immobilised target antigen is incubated with the phage antibody library and the unbound phage particles are washed away. Bound particles are then eluted, usually at high or low pH. The eluted phage particles are amplified by infecting *E. coli* TG1 cells. The panning cycle may be repeated, thus enriching the library with antigen-specific clones (Fig. 1.4).

Figure 1.4: Diagram illustrating selection of phage particles expressing an antibody (scFv fragment) of interest by panning on an immobilised target.
1.1.2.1 Polypeptide linker effect

In the design of scFv antibodies, the carboxy terminus of one V-domain is joined to the amino terminus of the other V-domain by a polypeptide linker. Usually the linker consists of a serine (Ser) residue which improves the hydrophobicity of the peptide linker to enable hydrogen bonding and a number of glycine (Gly) residues which gives the linker some flexibility (Argos, 1990). The use of flexible linkers allows the $V_H$ and $V_L$ domains to assemble in the natural Fv orientation (Plückthun and Pack, 1997; Hudson and Kortt, 1999; Todorovska et al., 2001). The linker length between $V_H$ and $V_L$ domains determines the flexibility, valency and size of the scFv. ScFvs are likely to be monomeric when the $V_H$ and $V_L$ domains are joined by a flexible polypeptide linker of 12 or more amino acid residues (15 residues are widely used), providing the minimal antigen binding site. By shortening the linker to less than 12 residues, the $V_H$ and $V_L$ domains cannot fold to return the natural Fv orientation, but instead two or more separate complementary scFv molecules associate to form bi-, tri- or tetrameric complexes. The complexes are referred to as multibodies i.e. multivalent antibodies. By shortening the linker to between zero and two residues, triabodies or tetrabodies can be formed by the association of three or four scFv molecules. A linker of three to ten residues can result in the association of two scFvs to form diabodies (Fig. 1.5). These multivalent antibodies are mono-specific and have higher avidity than the parent scFv (Hudson and Kortt, 1999; Atwell et al., 1999).

Multivalent antibodies can also be formed on the phage surface by association of soluble fragments with intact antibody-pIII fusion. These soluble fragments probably result from proteolysis of antibody-pIII fusion proteins in the periplasmic space of *E. coli* during phage particle morphogenesis (Andris-Widhopf et al., 2000).
1.1.2.2 Types of antibody phage libraries

There are two types of antibody phage libraries that can be constructed from animals namely: (i) "Naïve" antibody libraries: “Naïve” antibody libraries are constructed using mRNA isolated from B cells of a non-immunised animal (Nissim et al., 1994; Davies et al., 1995; van Wyngaardt et al., 2004). They contain the primary V-gene repertoire of the immune system and as a result can recognise a wide variety of antigens (Hoogenboom et al., 1998). The disadvantages associated with these libraries are the large size required to make for the successful selection of high affinity antibodies and the cost of constructing such repertoires. (ii) Immune antibody library: An immune antibody library is constructed from the mRNA of B cells of an immunised animal and therefore does not need to be so large to successfully yield high affinity antibodies. This is because the V-genes have undergone affinity-maturation and as a result, the library is highly bias toward the expression of antigen-specific V-genes. Compared to a “naïve” library, an immune phage antibody repertoire is thus enriched in affinity-matured antibodies specific for those antigens against which an immune response was elicited (Yamanaka et al., 1996;
Andris-Widhopf et al., 2000; Sapats et al., 2003; Finlay et al., 2005). The disadvantages of immune libraries include the time required for immunisation, the possibility of non-immunogenicity or toxicity of some antigens, and the fact that for each antigen a new library must be constructed. Antibodies against more than one immunogen can, however, be successfully isolated from the same library (Finlay et al., 2005). This finding is the basis of the present study.

For use in sensitive diagnosis, in vitro affinity-maturation of recombinant antibodies may sometimes be necessary. This is a two step process: firstly, the introduction of diversity in V-genes using a variety of methods including error-prone PCR and chain shuffling. The second step is the selection and screening of affinity matured antibodies from a new sub-library of variants (Hoogenboom et al., 1998). Since affinity maturation has already taken place in the animal, this process is less likely to be necessary in the case of recombinant antibodies from immune sources.
1.2 Immunoglobulin genes

When constructing antibody libraries, and in particular to understand the advantages of the avian immune system, it is important to know how different species generate antibody diversity. In this sub-chapter, the widely used laboratory animal, the mouse is compared to an alternative, namely the chicken (*Gallus domesticus*).

1.2.1 The mouse immunoglobulin gene locus

In the mouse the source of the primary Ig repertoire is a number of variable (V), diversity (D) and joining (J) gene segments (Tonegawa *et al.*, 1983). The Ig heavy (H) chain locus consists of approximately 140 V\(_H\) gene segments that can be grouped into 15 families. One-hundred-and-four of these segments are believed to be functional (de Bono *et al.*, 2004). There are also 13 active D\(_H\) (diversity) gene segments that can be grouped into four families and four functional J\(_H\) (joining) gene segments (reviewed by Schroeder, 2006). The Ig light (L) chain locus is divided into two sub-IgLs; the Ig\(_\kappa\) and Ig\(_\lambda\). The V\(_\kappa\) locus consist of 93 expressed V\(_\kappa\) gene segments that belong to 18 families. There are 5 members in the J\(_\kappa\) locus. One of these is non-functional and there is one constant \(\kappa\) (C\(_\kappa\)) element. The \(\lambda\) locus is made up of three V\(_\lambda\) gene segments and two C\(_\lambda\) segments each associated with a single J\(_\lambda\) gene segment (Lefranc, 2003).

Three types of framework (FW) 1 structure is found in the V\(_H\) gene segments. These can be used to group V\(_H\) families into clans, each with its own unique FW3 structure (Kirkham *et al.*, 1992). The FW1 of the V-domain links the V to the first C-domain and the FW2s encoded in V-region and FW4s in J-regions of both V\(_H\) and V\(_L\) chains constitute the hydrophobic core of the united V-domain (Schroeder, 2006). The antigen-
binding domains, complementarity-determining regions (CDRs) 1 and 2 are encoded by V-gene segments and CDR3s are generated during the V(D)J joining. The diversity of CDR3s thus depends on the multiplicity of VDJ combinations (Padlan, 1994; Xu and Davis, 2000) (see Fig. 1.6). The light chain CDR3 consists of VL and JL sequences only, thus L chain variability is encoded in the germline (Schroeder, 2006).

1.2.1.1 Mouse B cell development

The IgH chain locus undergoes rearrangement before the IgL locus (Burrows and Cooper 1997; Burrows et al., 2002). There are four stages involved in Ig gene assembly: (1) pro-B cell, (2) pre-B cell, (3) immature B cell, and (4) mature B cell stage (Schroeder, 2006). The DH-JH joining and in-frame VH-DJH rearrangement define the pro-B cell and pre-B cell stages, respectively. During late pre-B cell stage the L chain rearrangement is triggered by reactivation of the recombination activating genes (RAG) 1 and RAG2. The VK-Jk recombination occurs before the VL-Jk joining. The immature B cell stage is defined by surface expression of IgM which depends on the successful association of an in-frame L chain and an endogenous μ chain. The cells expressing IgM molecules on their surface are selected to undergo splicing of the Cμ-Cδ locus leading to surface co-expression of IgD marking the mature B cell stage (Burrows et al., 2002).

The matured B cells in the bone marrow migrate through the blood into the secondary lymphoid organs (spleen, lymph node and Peyer’s patches). In the presence of a cognate antigen in the lymphoid organ, the B cells undergo rapid proliferation in the lymph follicles. In the mouse, there are two types of genetic modification that follow productive V gene rearrangement; namely class-switch recombination and somatic hypermutation.
These two modifications are region-specific recombination and multiple single nucleotide substitutions on rearranged V-gene segments (Harris et al., 2002). The activated B cells are further diversified by activation induced cytidine deaminase (AID) -mediated somatic hypermutation of the V-domains and class switching which enables replacement of the constant (C) domain by means of switch sequence non-homologous recombination with a downstream C exon. B cell development takes only three weeks. By day 12 and days 16-17, pre-B cells and B cells expressing surface IgM are present in the foetal liver. This organ thus acts as a primary lymphoid organ for more than 7 days (Harris et al., 2002).

As will be seen, the way in which the mouse or human immune system generates diversity (Fig. 1.6) has practical implications when attempts are made to access this diversity.

Figure 1.6: Diagram illustrating IgH VDJ (A) and Igκ VJ (B) rearrangement; 1, 2 and 3 are examples of possible V (D) J products for each case in mouse.
1.2.2 The chicken (*Gallus domesticus*) immunoglobulin gene locus

In the chicken, Igs are divided into four classes, IgM, IgG, IgA and IgY (from egg yolk). As with the mouse, IgM is generated during the primary antibody response and expressed on the surface of B lymphocytes. IgG is mainly generated during the secondary antibody response while IgA is normally present in serum and secretions such as bile (reviewed by Ratcliffe, 2006). The egg yolk immunoglobulins (IgY) are transferred in the female bird from the serum to the egg yolk to confer passive immunity to the embryo and neonate (Jukes *et al*., 1934). Three Igs are homologous to mammalian IgM, IgG and IgA and have some similarities in their structure and function. Chickens, however, do not have homologues of mammalian IgD and IgE. IgY may be the ancestor of both IgG and IgE due to its ability to serve the same functions as IgG in the defence against systematic infections. Like IgE, it can mediate anaphylactic reactions. IgY has two antigen-binding sites, binds strongly to multivalent antigens and can neutralise pathogenic viruses (Warr *et al*., 1995).

Unlike the mouse, the chicken IgH chain locus has only one unique functional V\textsubscript{H} and one J\textsubscript{H} gene segment. A total of 15 D\textsubscript{H} regions have been identified. All fifteen D\textsubscript{H} segments are functional and have very similar sequences. The D segments are flanked by V\textsubscript{H} and J\textsubscript{H} segments. The joining of D-J\textsubscript{H} and V-D\textsubscript{H} is homology-mediated based on a single base pair and 3 base pair overlaps in J\textsubscript{H} and V\textsubscript{H}, respectively (Reynaud *et al*., 1989; 1991). The chicken IgL locus (consist of only the V\textsubscript{\lambda}) also has only one unique functional V\textsubscript{L} and J\textsubscript{L} gene segment (Reynaud *et al*., 1985). Upstream of both functional V\textsubscript{H} and V\textsubscript{L} gene segments are a number of pseudogenes (ψ) (Reynaud *et al*., 1987; 1989). These pseudogenes (100ψV\textsubscript{H} and 25ψV\textsubscript{L}) contain the same gene sequences as the functional V\textsubscript{H}
and \( \psi \) VH and \( \psi \) VL gene respectively. Both \( \psi \) VH and \( \psi \) VL have either 5’ or 3’ truncations. This means that both lack the promoter and recombination signal sequences (RSSs) at their 5’ and 3’ ends. Functional RSSs are a prerequisite for gene rearrangement. They therefore cannot be expressed unless they donate their sequences to rearranged VDJH or VDJL genes (Reynaud et al., 1987; 1989). This process is called gene conversion. The chicken VH and VL chain diversity is mainly generated by multiple rounds of gene conversion involving the upstream pseudogenes. This mechanism, which is clearly distinct from that employed by mice and humans, generates the diversified avian antibody repertoire (Reynaud et al., 1987; McCormack et al., 1993).

Nine of 25 \( \psi \) VL segments are orientated in an inverted fashion (opposite polarity) to the functional VL gene, the majority in an alternating orientation (Reynaud et al., 1987). Due to homologous recombination among adjacent \( \psi \) VL segments, the alternating orientation has probably evolved to prevent pseudogenes from being lost due to homologous recombination. This also applies to the IgH \( \psi \) VH genes (McCormack et al., 1993). Since the VH gene locus is “rich” in pseudogenes, this contributes to generating a large heavy chain repertoire (Cohen and Givol, 1983; Reynaud et al., 1989). The VH pseudogenes have D segment sequences at the 3’ end. During recombination, the germline D element sequences act as an acceptor exploiting sequence homology to the D region-like sequences found in VH pseudogenes. Base pair modifications may also occur in the D segment during gene conversion (Reynaud et al., 1989; 1991). The addition of base pairs at the V-DH and D-JH adds to diversity at the junction yielding an increased variability of CDR3, which is encoded in the V-D-JH junction (McCormack et al., 1991). A single pseudogene can contribute more than once during an antibody response to a certain
antigen. As a result, a single V gene can be converted into different end products (Cohen and Givol, 1983). The germ-line element contribution to the chicken IgH and IgL chain diversification is limited to one V_L, J_L and one V_H, J_H functional gene (Parvari et al., 1988; 1990). As with the V_H chain, gene conversion of rearranged functional V_L gene with non-functional pseudo-V_L genes also creates diversity in the pre-immune repertoire of the chicken L chain with 25 pseudogenes acting as sequence donors (Reynaud et al., 1987). The gene conversion mechanism is shown schematically in Fig. 1.7 and described in more details in the next section (1.2.2.1).

![Figure 1.7: The chicken IgH chain locus organisation, the diagram is illustrating the process of Ig diversification by gene conversion. Essentially the same process introduces diversity into the IgL chain locus (adapted from Michael et al., 1998).](image)

1.2.2.1 Chicken B cell development

In birds, the bursa of Fabricius develops during embryonic life. This organ is the site of B cell development. The embryonic bursa is colonised by dividing stem cells at day 8-12. Lymphoid stem cells colonise the bursa by the eighth day of embryogenesis and by day
the cells start to express surface immunoglobulin (sIg). Prior to hatching more than 95 percent of the stem cells express sIg (Thompson and Neiman, 1987). The colonisation of the embryonic bursa is followed by rapid B cell expansion in the bursal follicles (Reynaud et al., 1985). Gene rearrangement occurs once during early embryonic development and it in turn triggers gene conversion. The productive rearranged cell-surface expressing sIg genes are selected before the onset of gene conversion to undergo clonal expansion. Non-productive rearranged Ig genes are eliminated (Reynaud et al., 1987; 1991; Paramithiotis and Ratcliffe, 1993). The Ig gene rearrangements generate sIg receptors with specificity which is mostly determined by the germline (V_H and V_L) encoded genes. The sIg receptor is thought to transduce signals to the bursal cells which then produce receptor specific chemokines that attract the sIg receptor-expressing cells into the bursal follicle (reviewed by Ratcliffe, 2006).

It has been suggested that affinity maturation by gene conversion is antigen driven. The B cells migrate into the follicular cortex after encountering their cognate antigen. In the follicular cortex, rapid cell division induces gene conversion in the presence of locally expressed activation induced cytidine deaminase (AID) which is responsible for the diversification of Ig genes. Therefore gene conversion is AID-dependent (Arakawa et al., 2002; Harris et al., 2002; Withers et al., 2005). The germline V gene and pseudogenes sequence homology promotes genetic exchange during gene conversion (Reynaud et al., 1987). During the Ig locus transcription process, a transcription “bubble” is generated which renders the DNA single stranded (ssDNA). AID recognises and acts on ssDNA in the “bubble” by directly deaminating dC to dU yielding the so called initiating lesion. It then nicks the DNA strand. The nicked DNA is homologously inserted into the target
upstream pseudogene sequence. DNA polymerase then accurately replicates the pseudogene sequence and when elongation is completed, the elongated strand re-associates with the functional V gene introducing sequences encoded by the pseudogene (Fig. 1.8). The base pair mismatch is repaired by gene conversion using the replicated strand as the template to repair the dU:dG mismatches in the non-replicated DNA strand (Ratcliffe, 2006).

Figure 1.8: Diagram illustrating AID involvement in the diversification of Ig genes by gene conversion (adapted from Arakawa et al., 2004).
The diverse B cell repertoire confers specificity for the target antigen, but this repertoire is not restricted only to encountered antigens. The presence of gut-derived materials (antigens, bacterial mitogens or superantigens) and expression of sIg receptor is required for the bursal B cell development after hatching. It has been proposed that these later stages are triggered by individual gut-derived antigen-antibody interactions (Ratcliffe, 2006).

As with mammals, somatic hypermutations also contribute to affinity-maturation of the immune response. Somatic hypermutations or gene conversion events increase with time from pre- to post-hatch. Up to seven of these events may occur (Reynaud et al., 1987). Pre-immune repertoire generation in the bursa ends 21 days after hatching and somatic point mutations take-over to further diversify the V genes, resulting in antibodies with matured affinity. High affinity antibodies are predominantly produced during the late immune response (Parvari et al., 1990). The bursa continuously matures after hatching while B cells rapidly proliferate in each bursal follicle and the cells migrate to the secondary lymphoid organs (eg. spleen) for further specific development. When the chicken is between the ages of 8 and 12 weeks the bursa reaches complete maturity which results in involution. Twenty-four months after hatching, only a sclerotic remnant of the original organ is present (Thompson and Neiman, 1987).
1.3 Disease targets

For the development of mAb-based diagnostic immunoassays, it is crucial to identify suitable target antigens. For field use, tests based on immunochromatographic principles have the potential to replace existing techniques. The present study focuses on recombinant antibodies that can potentially be used in such immunoassays. Several target antigens which had been previously identified were of interest to this study.

1.3.1 Malaria

As early as 1880, the causative agent of malaria was identified as a single-celled parasite called *Plasmodium*. There are four *Plasmodium* species which cause human malaria. These are *P. vivax*, *P. ovale*, *P. malaria*, and *P. falciparum*. The parasite is fatal in less than 1% of cases. The parasites are transmitted to human hosts from the infected female *Anopheles* mosquito during blood meals. The disease occurs worldwide, mostly in tropical and sub-tropical regions. More than 100 countries around the world are affected with sub-Saharan Africa being the most affected region (see Fig. 1.8). At least one million people die from malaria every year and most of these deaths occur in sub-Saharan Africa (World Health Organisation [WHO]).

The human malaria parasite life cycle consists of two separate cycles, the asexual and sexual. The asexual life cycle takes place in the human host’s erythrocytes (red blood cells) while the sexual life cycle occurs in the mosquito gut. Within 48 hours of red blood cells (RBC) being infected, the parasite develops into trophozoites and divides asexually producing schizonts. The accumulation of schizonts forces the RBC to burst and release daughter merozoites which reinitiate the cycle by invading more RBC. Death results from
anaemia and obstruction of blood flow by clogging of capillaries in vital organs such as the brain (cerebral malaria) (WHO).

Figure 1.9: The world map showing the distribution of malaria (from WHO).

The most widely used method for malaria diagnosis is microscopic blood-smear examination. There are limitations associated with this method including the time (up to 60 minutes) required to prepare the blood film. A skilled microscopist is required to interpret results, particularly at low levels of parasitaemia. This method may be impractical when large numbers of blood samples need to be accurately examined, more especially in developing countries where expertise may be scarce (Snounou et al., 1993; Srinivasan et al., 2000; Moody, 2002). In an attempt to overcome challenges in malaria diagnosis Snounou and colleagues (1993) developed a PCR based on species-specific
sequences of the small subunit ribosomal RNA genes (ssrRNA) to detect all four human malaria parasites. Although PCR can be more sensitive than routine microscopic analysis (Snounou et al., 1993), it does not meet the recommended criteria of a rapid diagnostic method since it usually takes more than an hour to obtain results (Moody, 2002). The World Health Organisation has identified a need for easy to perform, rapid, sensitive and cost-effective malaria diagnostic tests. Antibody-based malaria rapid diagnostic tests (RDT) appear to meet the WHO recommendations (Moody, 2002). These test kits are based on the detection of parasite-specific antigens using mAbs immunochromatographic capture procedures.

Immunochromatographic technology is based on two phases; the mobile and stationary phase. In the mobile phase the antigen-specific mAb captures the target antigen in the patient’s blood and migrates across the nitrocellulose membrane surface. There are usually three different mAbs in the stationary phase. The first mAb to be encountered by the migrating antigen-antibody complex is usually specific to \textit{P. falciparum}. The second mAb is pan-specific and recognises a highly conserved antigen among the four malaria parasites (\textit{P. vivax}, \textit{P. ovale}, \textit{P. malarae} and \textit{P. falciparum}). The third mAb serves as a control recognising the migrating mAb whether antigen bound or non-bound (see Fig. 1.9) (Moody, 2002).

Several malaria parasite antigens have been identified as potential candidates for the development of either malaria RDT and/or anti-malarial drugs. These are the histidine-rich protein II (HRPII), lactate dehydrogenase (pLDH), and \textit{Plasmodium} aldolase (ALDO). The suitability of these antigens for malaria diagnosis is based on the fact that
they are produced by the malaria parasites during their asexual life cycle in the human
and are unique to the parasite (Leech et al., 1984; Howard et al., 1986; Rock et al., 1987;
Knapp et al., 1990; Döbeli et al., 1990; Meier et al., 1992).

Figure 1.10: The principle of immunochromatographic RDT (from Moody, 2002). The
antigen-specific mAb is mixed with patient’s blood sample and allowed to migrate across
the stationary phase. The positive tests (A) *P. falciparum* and (B) one of the four malaria
parasites infection. In the negative test only the control gives a signal confirming the
migration of antigen-specific antibody across the immobile phase.
Three proteins rich in histidine residues (HRPI, HRPII, and HRPIII) are produced by *P. falciparum*, but HRPII was shown to be unique and dominant in this parasite. HRPII is synthesised by the intracellular parasite and expressed on the surface membrane of the infected RBC (IRBC) during its asexual life cycle (Leech *et al.*, 1984; Howard *et al.*, 1986; Rock *et al.*, 1987). During the erythrocytic life cycle the parasite requires energy in the form of ATP for the synthesis of RNA, DNA and proteins to ensure its survival. The only source of the required energy is the glycolytic pathway. Two well-characterised enzymes LDH and ALDO play a key role in the glycolytic pathway and have also been identified as target antigens for the development of enzyme-based immunoassays (Knapp *et al.*, 1990; Döbeli *et al.*, 1990; Meier *et al.*, 1992; Gomez *et al.*, 1997).

### 1.3.2 Trypanosomiasis

Trypanosomiasis is a disease of human and domestic livestock. It is caused by blood parasites called African trypanosomes. Three species (*Trypanosoma brucei, T. congoense* and *T. vivax*) are of economic importance to the agriculture sector. In humans, *Trypanosoma brucei, T. b. gambiense* and *T. b. rhodesiense* cause sleeping sickness. *T. congoense* and *T. vivax* affect livestock causing a disease known as Nagana. Trypanosomes are transmitted to mammalian host by tsetse flies (*Glossina* sp.) and reside in the bloodstream (Donelson, 2003).

Microscopic blood-smear examination is commonly used to diagnose trypanosomiasis, but cyclical parasitaemic fluctuation can limit the usefulness of this method (Kashiwazaki *et al.*, 1994). Other diagnostic approaches include PCR and the detection of parasite antibodies and antigens (Masake and Nantulya, 1991; Masiga *et al.*, 1992; Singh
et al., 1995; Eisler et al., 1998; Masake et al., 1997; 2002). Trypanosome-specific antibodies in the blood may indicate past infections. On the other hand, current infections can be identified by detecting circulating parasite antigens and diagnostic immunoassays based on immunochromatographic principles that have been developed to detect species-specific antigens (Kashiwazaki et al., 1994).

In the bloodstream, the trypanosome surface is coated with approximately 10 million identical copies of the variant surface glycoprotein (VSG). Several hundred different VSG genes are encoded in the parasite genome and expressed one at a time (antigenic variation), thus preventing immune recognition of the parasite (Mehlert et al., 1998). VSG is made up of an N-terminal domain and a C-terminal domain and is anchored to the parasite surface via covalent attachment of the C-terminal residues (Carrington and Boothroyd, 1996). VSG determines the variable antigen type (VAT) of the individual trypanosome which elicits a specific immune response. VATs are predominantly expressed at early stages of infection and specific antibodies are found in the blood within 6 to 32 days after infection. The majority, if not all different strains of trypanosomes express VAT, thus making it a good candidate for development of diagnostic tests for detection of anti-VAT antibodies (Verloo et al., 2000; 2001; Urakawa et al., 2001; Lejon et al., 2005). The potential of VSG (VATs) as an antigen for detection of specific antibodies has led to cloning and expression of recombinant VSG for use in diagnosis (Urakawa et al., 2001). Other potential candidates such as the single conserved N-linked carbohydrate (GlcNAc$_2$-Man$_{5,9}$) moiety has been found in C-terminus of all VSGs studied so far (Mehlert et al., 1998). This carbohydrate appears to play a key role in the process of binding and uptake of host macromolecules required for survival and
growth. It therefore, offers a great advantage over the expression of multiple specific proteins. The GlcNAc$_2$-Man$_{5,9}$ moiety is found exposed at the flagellar pocket (Magez et al., 2001), and also makes a good target for diagnostic immunoassays (Magez et al., 2001; Stijlemans et al., 2004).

1.3.3 Malignant catarrhal fever

Malignant catarrhal fever (MCF) is generally a fatal disease syndrome of domestic and wild ruminants as well as pigs (Løken et al., 1998; Li et al., 2005). MCF in cattle is caused by two related viruses; wildebeest-associated alcelaphine herpesvirus-1 (AlHV-1) (Bridgen et al., 1989) and sheep-associated infection caused by ovine herpesvirus-2 (OvHV-2) (Dunowska et al., 2001). MCF viruses belong to the Rhadinovirus genus of the Gammaherpesvirinae subfamily of the Herpesviridae. Similarities between AlHV-1 and OvHV-2 have been shown at the genomic level (Ensser et al., 1997; Dunowska et al., 2001). Recently, a new member of MCF virus associated with goats has been described and designated caprine herpesvirus-2 (CpHV-2) (Li et al., 2001).

The clinical signs of MCF include sudden fever, discharge from eyes and nose with matting of facial hair, inflammation toward mucosal surfaces and blood vessels, body temperature of 40.6 to 42°C and death, resulting in significant economic losses in cattle and other susceptible domestic ruminants (Pierson et al., 1973; Bridgen et al., 1989; Løken et al., 1998). In the past, viral antigen was detected in both living and dead infected cells (Patel and Edington 1980) and other serological tests such as virus neutralisation, complement fixation, indirect immunofluorescence, direct-binding ELISA and competitive-inhibition ELISA (CI-ELISA) are also used for MCF diagnosis. The
identification of MCF affected animals has also been based on the detecting of antibodies to AlHV-1. AlHV-1 infected cells were used as antigens in ELISA or indirect immunoflorescence (Rossiter, 1981, Løken et al., 1998). The sensitivity and reliability of results obtained using this approached remained questionable due to the fact that MCF viruses share common antigens with other herpesviruses. Some animals may not develop detectable levels of antibody prior to death which may result in false results; therefore, the presence of antibody alone may not be reliable for diagnosis of MCF infection (Washington State University website notes). Currently PCR is used for diagnosis of MCF from tissue samples collected from the infected animal using a virus specific primer set (Wiyono et al., 1994; Li et al., 2001). Histopathology remains the most recognised diagnostic procedure (Müller-Doblies et al., 1998).

At least 11 MCF virus proteins have been reported to elicit immune response in animals suffering from MCF (Adams and Hutt-Fletcher, 1990; Li et al., 1995a). Among the conserved glycosylated proteins found in AlHV-1 and OvHV-2 (Dunowska et al., 2001), a potential candidate (130 kDa protein) for the development of diagnostic immunoassays specific to MCF viruses only, had been shown to exist. A monoclonal antibody against this conserved glycosylated protein epitope among MCF viruses has been used to develop specific, rapid and economical CI-ELISA for detection of MCF virus antibodies in sheep and other ruminants (Li et al., 1994; 1995b). The present study aims at selecting recombinant antibodies that may be used in future for the development of such immunoassays. Such monoclonal antibodies can potentially replace the monoclonal antibody used in the CI-ELISA of Li et al. (1994).
1.4 Motivation and strategy

Chickens pass their immunity to the developing embryo by relocating maternal antibodies from the serum to the egg-yolk and neonates (Jukes et al., 1934; Patterson et al., 1962). Immunising laying chickens therefore enables antibody titres to be determined by simply collecting the eggs and isolating antibodies (IgY) from the yolk. Other advantages of using the chicken as an immunisation host include the fact that a relatively small quantity of antigen is required to trigger an immune response and the relative tolerance of the bird to complete Freund’s adjuvant (Gassmann et al., 1990; Tini et al., 2002). The chicken is nowadays therefore often used to produce antibodies for diagnostic and immunotherapeutic applications. Of relevance to this project is the fact that combinatorial antibody library can be constructed from immunised chickens (Davies et al., 1995; Yamanaka et al., 1996; Andris-Widhopf et al., 2000; Sapats et al., 2003; Finlay et al., 2005) and used to select antigen-specific clones.

As mentioned earlier, the mouse IgH locus consists of 15 V_H gene families which are grouped into clans on basis of their FW1 sequence composition. While the IgL locus is divided into two Igk and Igλ which contain 18 V_k and 3 V_λ family members, respectively. To construct an antibody library using mouse genes, the IgH and IgL gene repertoires are amplified using a mixture of 23 IgH specific primers (19 sense primers {V_H 5’} and 4 reverse primers {V_H 3’}) and 22 IgL specific primers (18 sense primers {17 V_k 5’ and one V_k 5’} and four reverse primers {three V_k3’ and one V_k3’ specific}) (Andris-Windpof et al., 2001; Burmester and Plückthun, 2001). In contrast, the technical advantage of using chicken genes in the construction of antibody libraries is that only one set of primers for each V_H and V_L gene segment is required to successfully access the
whole immunoglobulin repertoire (Davies et al., 1995; Yamanaka et al., 1996; Andris-Widhopf et al., 2000; Sapats et al., 2003; van Wyngaardt et al., 2004; Finlay et al., 2005). This is because the 3’ region of FW 4 in JL or JH is highly conserved and only one functional VH and VL gene segment exists (Reynaud et al., 1987; 1989; Thompson and Neiman, 1987) (see Fig. 1.6 and 1.7). Once an antibody-phage library is constructed, it can be used as a source of antigen-specific antibody clones without further use of animals.

This project was aimed at determining whether high-affinity chicken scFvs directed against five different diagnostic targets can be derived from a single immune bird. Accordingly, two chickens were immunised with a mixture of LDH, HRPII, ALDO, VSG and MCF virus. Its splenocytes were used as a source of mRNA from which filamentous phage libraries displaying single-chain Fv antibody fragments was constructed. The library was screened for binders which can potentially be incorporated into a variety of immunoassay formats. The approach described here yielded useful affinity-matured binders and provided a focussed alternative to large "universal" repertoires (Nissim et al., 1994; Davies et al., 1995; van Wyngaardt et al., 2004) as a source of useful antibody fragments.
1.5  Aim, objectives and potential benefits

1.5.1  Aim

To establish whether phage display libraries can be constructed using splenic mRNA from a chicken immunised with five different proteinaceous antigens, namely *Plasmodium falciparum* lactate dehydrogenase, histidine-rich protein II and aldolase; *Trypanosoma sp.* variant surface glycoprotein and lastly, malignant catarrhal fever virus.

1.5.2  Objectives

To stimulate splenic B cell proliferation by the simultaneous immunisation of a chicken with five different antigens; isolate spleens, prepare mRNA and convert mRNA into cDNA; construct a phage library displaying affinity-matured chicken scFvs; and to select antibody clones with sufficiently high specificity for future use in diagnostic methods.

1.5.3  Potential benefits

Once an antibody-phage library is constructed, it can be used as a potential source of antigen-specific antibody clones by panning against any one of the chosen immunogens. The resulting antibodies can also be used in research and for the diagnosis of human or animal diseases. This study will contribute to the development and application of recombinant antibody technology in the field of veterinary science.
CHAPTER 2: MATERIALS AND METHODS

2.1 Antigens for immunisation

Recombinant lactate dehydrogenase (LDH), aldolase (ALDO) and histidine-rich protein II (HRPII) of *Plasmodium falciparum*; and recombinant variant surface glycoprotein (VSG) of *Trypanosoma* spp. were a gift from Vision Biotech Pty (Western Cape). Malignant catarrhal fever virus (MCFV) was cultured and purified using a modified method of Schloer and Breese (1982). WC11 virus strain (cell culture passage strain of AlHV-1) was propagated on foetal bovine cells at the 8th passage level in BioWhittaker’s minimum essential medium eagle (EMEM) supplemented with 7% serum. When an estimated 70 to 90% of the cells exhibited cytopathic effects, the supernatant fluid was harvested and centrifuged at 4 500 xg (Sorvall SLA 3000 rotor, Beckman) for 30 min. Virus particles were pelleted from the supernatant by ultracentrifugation through a 7 ml cushion of 35% sucrose (wt/wt) in Beckman SW 28 rotor (120 000 xg, 90 min at 4°C), and resuspended in phosphate buffer saline pH 7.4 (PBS). Purified virus was obtained following equilibrium density-gradient ultracentrifugation in a 20-40% sucrose gradient at 70 000 xg at 4°C in a Beckman SW 28 rotor for 40 min. The pellet was resuspended in PBS and centrifuged at 120 000 xg in a Beckman SW 28 rotor at 4°C for 90 min, resuspended in PBS and examined by electron microscopy.

2.2 Immunisation of chickens

Ethical approval to use two chickens in this study was granted by the Onderstepoort Veterinary Institute Animal Ethics committee. Two specific pathogen-free adult laying hens were immunised by intra-muscular inoculation of a mixture of LDH, HRPII, ALDO, VSG and MCFV antigens (50-100 µg of each in 500 µl of PBS), emulsified with an equal
volume of Montanide ISA 70 adjuvant (Seppic, France). At three week intervals the chickens received the first, second and third booster immunisations. Two weeks after the third booster, a fourth inoculation with the same mixture of antigens but this time emulsified in Freund’s incomplete adjuvant (Sigma) using the method described by Vulliet (1996) was performed. After the third booster, the eggs were collected and the egg yolk antibodies (IgY) were prepared using the polyethylene glycol (PEG) precipitation method of Polson (1990) and analysed by SDS-PAGE using 10% polyacrylamide gels. The gel was stained with Coomassie Brilliant Blue and the samples were incubated at 100°C for 5 min prior to loading on the electrophoretic gel. The bird’s immune response to the target antigens was confirmed in ELISA using IgY prior to the collection of spleens. Twenty-one days after the fourth booster, chickens were euthanased by administering 1 ml/kg of pento-barbitone intraperitoneally. The spleens were removed and used for RNA extraction (see 2.4).

2.3 IgY ELISA

Wells of a Nunc Polysorp ELISA plate were coated overnight at 4°C either with target antigen (10 µg/ml) or with 2% milk powder in PBS as a negative control. The following day non-specific binding was blocked with 300 µl/well of 2% MP at 37°C for 1 h. Plates were washed three times (3x) with PBS containing 0.05% Tween, then 3x with PBS. Egg-yolk IgY was diluted in PBS containing 0.5% Tween to 100 µg/ml and 50 µl was added to each well. After 1 h incubation at 37°C, plates were washed as described above and IgY was detected with 1:10 000 dilution of horseradish peroxidase-conjugated goat anti-chicken IgG (seroTec, USA) in PBS containing 2% MP and incubated at 37°C for 1 h. Wells were washed as described above and colour was developed by adding 50 µl
(1mg/ml) of the enzyme substrate O-phenylenediamine dihydrochloride (Sigma, Germany) and 10 µl of hydrogen peroxide. The absorbance was measured at 492 nm using BDSL Immunoskan MS (Labsystems) after stopping the reaction with 50 µl of 1M H₂SO₄.

2.4 Western immunoblotting

Western immunoblotting was performed by Dr. Christine Vroon (OVI-Biotechnology Division). Partially purified MCFV and uninfected foetal bovine testes (FBT) cells were separated in SDS-PAGE using a 10% polyacrylamide gel and the proteins were transferred to an Immobilon-P Transfer membrane (Millipore). The membrane was treated with blocking buffer (PBS containing 5% skim milk and 0.05% Tween) for 1 h and exposed to 1/100 dilution of IgY in blocking buffer for 1 h and washed four times for eight minutes with PBS-0.05% Tween. The membrane was exposed to a 1000 times diluted horseradish peroxidase-conjugated anti-chicken IgG in blocking buffer for 1 hr and bound anti-chicken antibodies were detected with substrate solution (10 ml PBS, 6 mg of 3,3’-diaminobenzidine and 10 µl hydrogen peroxide).

2.5 Phagemid preparation

The phagemid vector, pHEN1 was used for phage display and expression as soluble fragments of scFv antibodies. The vector is designed to allow cloning of inserts using SfiI/NotI restriction sites on gene III (Hoogenboom et al.; 1991) (see Fig 2.1). Vector DNA was isolated from an overnight culture of E. coli TG1 bacterial stock cells using a Plasmid Midi Prep kit (Qiagen) and digested with SfiI and NotI restriction endonucleases as described by van Wyngaardt et al. (2004). The digested vector was first purified with a
PCR purification kit (Qiagen). It was then purified by electrophoresis in 1% agarose gel and extracted using a QIAquick gel extraction kit (Qiagen). The concentration of DNA was determined by measuring absorbance at 260 nm ($A_{260} = 50$ ng/µl).

Figure 2.1: The structure and cloning sequence of the pHEN vector used for phage display and expression of scFv antibodies (from Hoogenboom et al., 1991).

2.6 Antibody library construction

2.6.1 Preparation of RNA and synthesis of cDNA

Total spleen RNA was prepared as described by Andris-Widhopf et al. (2001). Chicken spleens were minced and homogenised through a fine stainless steel mesh in 30 ml TRI
reagent (Sigma). The homogenate was centrifuged for 10 min at 2500 xg and the supernatant fluid was transferred to a clean COREX (15 ml) glass tube, mixed with 3 ml of 1-bromo-3-chloropropane (BCP) (Sigma) and incubated at room temperature (RT) for 15 min, then centrifuged at 10 000 xg for 15 min at 4°C. The resulting clear aqueous phase was transferred to a clean tube and 15 ml of isopropanol was added, mixed and incubated at RT for 10 min. The mixture was centrifuged at 10 000 xg for 30 min at 4°C to pellet the RNA. The RNA pellet was washed with 75% ethanol and centrifuged at 10 000 xg for 30 min at 4°C. The total RNA pellet was air-dried and resuspended in 500 µl of nuclease-free water (stored at -80°C). The nucleic acid concentration was determined by measuring absorbance at 260 nm ($A_{260} = 40$ ng/µl). A total amount of 6 mg was obtained from both chickens. Complementary DNA (cDNA) was synthesized from approximately 25 µg of total RNA using a TaKaRa RNA PCR Kit (AMV) Ver. 3.0 following the manufacture’s instructions. Fifty individual and identical cDNA synthesis reaction mixtures were prepared each consisting of 5 mM MgCl₂; 1x RT buffer; 1mM dNTPs; RNase inhibitor (1 unit/µl); reverse transcriptase (0.25 unit/µl); 0.125 µM oligo dT-adapter primer and 500 ng of total RNA. Nuclease free-water was added to make a final volume of 10 µl for each reaction. The cDNA was synthesised by incubating for 10 min at 30°C, 60 min at 42°C, 5 min at 95°C and 5 min at 5°C.

### 2.6.2 PCR amplification of V\textsubscript{H} and V\textsubscript{L} chain genes

The VHF1/VHR1 and VLF1/VLR1 sets of primers (see Table 1) were used for PCR amplification of V\textsubscript{H} and V\textsubscript{L} gene segments in two separate reactions. PCR was performed using TaKaRa RNA PCR Kit (AMV) Ver. 3.0 in a total volume of 50 µl in which 10 µl of cDNA synthesis reaction was mixed with 40 µl reaction mixture consists of 1x PCR
buffer, 20 pmol of forward and reverse primers, 1.25 units of TaKaRa Ex Taq HS and distilled water. The PCR conditions consisted of 30 cycles of 1 min at 94ºC, 1 min at 60ºC, 1 min at 72ºC, followed by a final extension of 5 min at 72ºC. This was performed on a PERKIN ELMER GeneAmp PCR system 2 400 thermocycler. A total of 25 separate PCR’s were performed for each V_H and V_L gene amplification. The products were pooled; purified using a QIAquick PCR purification kit (Qiagen) and then further by gel electrophoresis in a 2% agarose gel containing 10 µg/ml of crystal violet to make the DNA visible (van Wyngaardt et al., 2004). DNA fragments of 390 and 350 base pairs (bp) respectively (Davies et al., 1995) were extracted using a QIAquick gel extraction kit (Qiagen), and stored at -20ºC in elution buffer (EB).

### 2.6.3 Assembly of scFv gene

The Hend1/Lstart1 and NewlVarH/NewlVarL set of primers (see Table 1) were used to introduce overlapping sequences which enabled the scFv gene fragments to be assembled by overlap extension PCR (Horton et al., 1989; Huston et al., 1988). The Hend1/Lstart1 primers encode a linker consisting of a single amino acid (G) residue. While the NewlVarH/NewlVarL primers encode a 15 amino acid linker sequence (G₄S)₃. The overlap extension PCR consisted of preheating for 2 min at 94ºC followed by the first 15 cycles of 1 min at 94ºC; 1 min at 60ºC; 2 min at 72ºC and a final extension of 5 min at 72ºC. The resulting product was diluted 25x in a 100 µl pull-through PCR reaction mixture made-up of 20 pmol of VHF2 and VLR1 primers. The VHF2 and VLR1 primers introduced the SfiI and NotI restriction sites at the both ends of scFv gene fragment respectively (Sapats et al., 2003; van Wyngaardt et al., 2004). The assembled scFv fragments (~750 bp) were purified in 1.2% agarose gel as previously described and
digested with SfiI and NotI restriction endonucleases under appropriate conditions to yield an insert with sticky-ends. The digested insert DNA was purified using a Qiagen PCR purification kit and stored at -20°C until used.

Table 1: Nucleotide sequences of primers used.

| Primer      | Nucleotide sequence (5’-3’)
|-------------|-----------------------------
| a VHF1      | CTGATGGCGCCGGTGACGTTGGAC    |
| b VHF2      | GTCTCCTCGCAACTGCGGCCCAGCCGCCGCTGATGGCGCCGGTGACG |
| a VHR1      | CCGCCTCGGAGGAGACGATGACTTCG |
| a VLF1      | GACTCAGCCGTCTCGGTGTCAG      |
| b VLR1      | TGATGGTGCGGCAGCGGCATTGGGTG |
| c Hend1     | CCGGAGGAGACGATGACTTCGG      |
| c Lstart1   | GCGCTGACTCAGCCGTCTCGG      |
| b NewlVarH  | CCGCAGAGCCACCTCACTGAAACCCTCACTCCACCGCTCCACCGGAGGAGACGATGACTTCGG |
| b NewlVarL  | TCAGGTGGAGGTGCTCGGAGGCAGGCTCAGGATCGACTCAGCCGTCCTCGG |
| b OP52F     | CCCTCATAGTTAGCGTAACG |
| b M13R      | CAGGAAACAGCTATGAC |

All primers were synthesised by Invitrogen, a derived from Yamanaka et al., 1996 and Andries-Widhopf et al., 2000; b as published by van Wyngaardt et al., 2004; c designed by Jeanni Fehrse unpublished.
2.6.4 Electroporation and library size estimation

Ligation of SfiI/NotI-digested scFv inserts (1.6 µg) and vector DNA (3.2 µg) was carried out using a rapid T4 DNA ligation kit (Roche), using a 2:1 molar ratio of vector:insert following the manufacturer’s instructions. The ligated scFv phagemid library was desalted using the method of Atrazhev and Elliot (1996). The DNA was then electroporated (Gene-pulser; Biorad) into electrocompetent *E. coli* TG1 cells (Stratagene). Five electroporations for each library were performed at 2.5 kV, 25 µF, and 200 Ω. Serial dilutions of transformed cells were made to determine the library size. The cells were cultured on TYE agar plates containing 100 µg/ml ampicillin and 2% glucose (A/G). The remaining bacterial culture was plated out onto 15 TYE (A/G) agar plates (150 mm) and incubated overnight at 30ºC. The resulting lawns of bacterial cells were scraped into 2x TY medium. A 15% glycerol stock of the bacteria was prepared and stored at -70ºC. The library was re-amplified by diluting 1ml stock in 2x TY (A/G) to give OD$_{600}$ reading of <0.1 and grown to OD$_{600}$ 1. Cells were pelleted and a sub-stock of cells in 15% glycerol was prepared and stored at -70ºC. The phage antibody stock was prepared by diluting 1 ml of the sub-stock to OD$_{600}$ 0.05 in 2 litre of 2x TY (A/G), grown at 37ºC with shaking to an OD$_{600}$ of 0.5 and infected with M13-K07 helper phage at a ratio of phages: bacteria of 20:1. It was then incubated for 30 min at 37ºC in a water-bath, a further 30 min at 37ºC with shaking at 100 rpm. The cells were pelleted and resuspended in 2xTY containing 50 µg/ml ampicillin, 25 µg/ml kanamycin and shaken at 270 rpm overnight at 30ºC. Phage particles from supernatant of an overnight culture were precipitated with 1:5 volumes of 20% PEG/2.5 M NaCl and resuspended in PBS. A 15% glycerol stock of antibody-phage particles was prepared and stored at -70ºC. Twenty clones were randomly chosen from titration plates. Each clone was tested for scFv insert
by colony PCR using the OP52/M13 primer set (Table 1) that flanks the cloning site of the vector (94°C for 2 min, then 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and final extension at 72°C for 4 min).

2.7 Biopanning of antibody libraries against target antigens

Maxisorp Immunotubes (Nunc, Denmark) were coated overnight at 4°C with 10 µg/ml of the target antigen in 2 ml PBS and the following day blocked for 1 h at 37°C by adding PBS containing 2% milk powder (MP) (wt/vol). Tubes were washed three times with PBS-0.1% Tween and 3 times with PBS. Panning was carried out by diluting 1 ml of the PEG-precipitated phage stock with an equal amount of PBS-4% MP/0.2% Tween and then added to the immunotube. The tube was rotated for 30 min at room temperature, and then allowed to stand for 1 h. The tube was then washed 20 times with PBS-0.1% Tween (PBS-T) and 20 times with PBS. Bound phage particles were eluted by adding 1 ml of 100 mM triethylamine (pH 12) and incubated at RT for 10 min with slow horizontal rotation. The triethylamine was neutralised by adding 500 µl of Tris-HCl pH 7.4. One ml of the eluted phage was used to infect 5 ml of freshly grown *E. coli* TG1 cells (OD₆₀₀ 0.5) at 37°C. The culture volume was increased to 10 ml by adding 4 ml of 2x TY. It was then incubated at 37°C in a water bath for 30 min to allow infection. The phage infected cells were pelleted, resuspended in 1 ml of 2x TY, plated out on three TYE (A/G) agar plates (150 mm) and incubated overnight at 30°C. The output phage titre was determined by plating 100 µl of infected *E. coli* TG1 cells with serial dilutions of phage on TYE (A/G) agar and incubating overnight at 30°C. The resulting lawn of cells was scraped off and a glycerol stock was prepared. For phage rescue, bacterial stock harbouring the phagemid was diluted to an OD₆₀₀ of 0.05, grown to an OD₆₀₀ of 0.5 in 25 ml 2x TY (A/G) at 37°C
(shaking). Five ml of the culture was infected with M13KO7 helper phage at a phage:bacterium ratio of 20:1 and incubated at 37°C for 30 min in a water-bath to allow infection. The cells were then pelleted by centrifugation and resuspended in 25 ml of 2x TY containing 100 µg/ml ampicillin and 25 µg/ml kanamycin (A/K), and subsequently incubated at 30°C overnight with shaking. Bacteria in overnight culture were pelleted and phage particles were collected from the supernatant by 20% PEG/2.5 M NaCl precipitation on ice for 1 h. The phages were pelleted by centrifugation and resuspended in 1 ml PBS. Half of precipitated phage particles were used for the next round of panning.

A total of four rounds of panning were performed.

2.8 Screening of phage pools

2.8.1 Polyclonal phage ELISA

Pooled phage inputs for each round of panning were tested for antigen-specific binding by indirect ELISA. Wells of a Nunc Polysorp ELISA plate were coated overnight at 4°C either with target antigen dissolved in PBS at 10 µg/ml concentration or 2% milk powder as a negative control. The following day coating solution was removed and non-specific binding was blocked with 300 µl/well of 2% MP at 37°C for 1 h. Plates were washed three times with PBS-T, then 3x with PBS. The input phage preparations were diluted 200x in PBS-2% MP/ 0.1% Tween (PBS-MP/T) and 50 µl of the dilution was added to each well. After 1 h incubation at 37°C, plates were washed as described above and bound phage particles were detected with a 1:1 000 dilution of mouse anti-M13 monoclonal antibody (Progen, Germany) in PBS-MP/T and incubated at 37°C for 1 h. The plate was washed as previously described and probed with horseradish peroxidase-conjugated polyclonal rabbit anti-mouse immunoglobulin (DakoCytomation, Denmark)
in PBS-MP/T, incubated at 37°C for 1 h and after washing with PBS-T, the wells were developed with 50 µl (1 mg/ml) of enzyme substrate O-phenylenediamine dihydrochloride (Sigma, Germany). The absorbance was measured at 492 nm after stopping the reaction with 50 µl of 1M H₂SO₄.

2.8.2 Monoclonal phage ELISA

Individual colonies were randomly chosen from the output of the third or fourth round of panning and inoculated into a 96-well flat-bottom culture plate (Costar, USA) containing 100 µl of 2x TY (A/G). Plates were incubated overnight at 30°C with shaking at 220 rpm. A 96-well replica plater (Sigma, Germany) was used to transfer inoculum from the overnight culture (master) plate to a fresh 96-well culture plate containing 150 µl of 2x TY (A/G) per well. This new plate was then incubated for 2.5 h at 37°C with shaking, then 50 µl of 2x TY (A/G) containing 2x10⁹ pfu M13KO7 helper phage was added to each well to rescue the phage particles and further incubated at 37°C for 30 min without shaking; then centrifuged at 600 xg for 10 min. The supernatant was replaced with 2x TY (A/K) and the plate was incubated overnight at 30°C with shaking at 220 rpm. The supernatant liquid containing the phage particles was collected and used for ELISA as previously described (van Wyngaardt et al., 2004). The bacterial stock was prepared by adding glycerol to the master plate and stored at -70°C.

2.8.3 Expression of soluble scFv antibody fragments

Monoclonal soluble scFv fragments were expressed in *E. coli* TG1 cells. Positive phagemid clones identified by ELISA were tested for their ability to bind as soluble antibody fragments. Inocula were transferred from the master plate using a replica plater
to a 96-well culture plate containing 100 µl of 2x TY supplemented with 100 µg/ml ampicillin and 0.1% glucose per well and grown at 37°C for 2-3 h with shaking. To induce expression of soluble scFv protein antibodies, 50 µl of 2x TY containing 100 µg/ml ampicillin and 3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to each well. ScFv induction was overnight at 30°C with shaking. Cells were pelleted by centrifugation and supernatant fluid containing soluble antibody fragments was used in ELISA as described above except that PBS containing 0.05% Tween was used to wash the ELISA plate (van Wyngaardt et al., 2004).

2.8.4 DNA sequencing of single scFv clones

Single stranded phagemid DNA was isolated from an overnight culture of bacterial cells harbouring antigen binding scFv antibodies. The M13 and OP52 primers (Table 1) were used for sequencing and the DNA sequence was analysed using BioEdit Sequence Alignment Editor and protein sequences were deduced using Gene Runner program and CLC Free Workbench 3.2.2 program was used to align the sequences.

2.8.5 Introduction of a flexible linker

The VH and VL gene fragments were amplified from phage DNA using the NewlVarH/VHF2 and NewlVarL/VLR1 primer sets that encode for a flexible linker; the PCR products were pooled and joined by SOE-joining as described by Huston and colleagues (1988). Restriction enzyme sites (SfiI/NotI) were introduced as described above and the modified scFv gene was cloned back into the pHEN display vector and transformed into chemically competent TG1 cells by heat-shock at 42°C for 45 s.
Chemically competent *E. coli* TG1 cells were prepared using a one-step method as described by Chung and colleagues (1989).

2.9 Immunoassays

2.9.1 Sandwich ELISA

A Nunc Polysorp plate was coated with 50 μl of LDH-specific IgY (100μg/ml) in PBS (pH 7.4) and 2% MP as a negative control at 4°C overnight. Three times washing was performed with PBS-0.05% Tween between each step. Non-specific binding was blocked with 2% MP for 1 h at 37°C. The plate was washed and 10 μg/ml of LDH in PBS containing 0.05% Tween 20 was added to each well and incubated at 37°C for 1h. Then a 1:1 dilution of scFv LC9 containing supernatant with 4% MP was added and incubated at 37°C for 1 h. The scFv binding was detected as described in section 2.8.3.

2.9.2 Competitive inhibition ELISA

The ELISA plate was coated with LDH as described section 2.7. LDH-specific IgY (provided by Wouter van Wyngaardt) was added at 100 μg/ml and 1 mg/ml and “cocktail” IgY (used in section 2.3) was added at 100 μg/ml and 2 mg/ml concentration in PBS containing 0.5% Tween. The scFv LC9 was added and detected as described above.
CHAPTER 3: RESULTS

3.1 Antigens for immunisation

Of the antigens used in this study, all except MCF virus were recombinant proteins obtained in purified form by sucrose-gradient ultracentrifugation (Fig. 3.1 A). The virus was examined by electron microscopy an integument zone between the viral envelope (VE) and the nucleocapsid (NC) was observed with the capsids being arranged to form ring-like or hexagonal shapes as described by Todd and Storz (1983) (Fig. 3.1 B).

![Figure 3.1: Picture A shows the sucrose density-gradient MCF virus pellet and an electron microscope image in B shows MCFV particles (VP), viral envelope (VE) and nucleocapsid (NC).](image)

3.2 Immunisation of chickens and IgY ELISA

The egg yolk antibody (IgY) titre of laying hens should in theory, directly correlate with B-cell proliferation in the spleen. This organ therefore is expected to have a high concentration of immunoglobulin mRNA. In order to monitor the two chickens’ immune
response to the different target antigens, IgY was purified from eggs collected after the third booster immunisation and analysed by SDS-PAGE (Fig. 3.2). Chicken “A” had antibodies against ALDO, HRPII, VSG and MCFV (Fig. 3.3) while chicken “B” had antibodies against LDH, ALDO, VSG and MCFV antigen (Fig. 3.4) as shown by indirect ELISA (triplicate reading averages). The foetal bovine cells were also included in the ELISA test to measure possible background as the MCFV was cultivated in these cells. After the fourth booster immunisation, spleens were harvested from both chickens and the total RNA was isolated.

![Figure 3.2: Analysis of IgY (lane 1 and 2) by SDS-PAGE. Lane M is the protein molecular mass marker IV (peQLab). Bands at approximately 68 kDa and 32 kDa represent the immunoglobulin’s heavy and light chain respectively.](image)
Figure 3.3: Indirect ELISA analysis of antigen specific IgY titre in eggs collected from chicken “A” after a third booster immunisation.

Figure 3.4: Indirect ELISA analysis of antigen specific IgY titre in eggs collected from chicken “B” after a third booster immunisation.
3.3 Antibody library construction

Two phage-displayed scFv antibody libraries, one with the immunoglobulin VH and VL regions joined by a single amino acid (G) (the “directly” linked library) and the other with a normal 15 amino acid flexible linker [(G4S)3] were constructed using RNA isolated from the spleens of two immunised chickens (Fig 3.5). In the case of both libraries, cDNA was synthesised using oligo-dT primers from both chickens and then pooled and directly used as a template for PCR amplification of the V\textsubscript{H} and V\textsubscript{L} chain gene fragments. The gene fragments from both chickens were pooled in one reaction tube together with primers that contain overlapping linker sequences which allow the V\textsubscript{H} and V\textsubscript{L} gene segments to be assembled by overlap extension to form single-chain variable region fragments (scFvs) (Fig. 3.6). The resulting scFv genes were inserted into the phagemid pHEN. After electroporation of the gene repertoire into electro-competent \textit{E. coli} TG1 cells, the resulting libraries had calculated sizes of 6.7 x 10\textsuperscript{7} for the single amino acid (“directly” linked) and 1.7 x 10\textsuperscript{7} transformants for the flexibly linked repertoire (Fig. 3.5). Colony PCR of 20 randomly chosen clones showed that between 90 and 100% could be expected to contain the scFv DNA insert of ~ 750 bp (Fig. 3.7 and 3.8). Vector self-ligation background was estimated to be \( \leq 10\% \) of the library.

Library diversity. DNA from ten randomly chosen clones from each of the libraries was sequenced. Their amino acid sequences were deduced and three complementary-determining regions (CDRs) and four framework (FW) regions were identified in each of the heavy (V\textsubscript{H}) and light (V\textsubscript{L}) chain fragments. A single amino acid (G) linker (“directly” linked library) or a 15 amino acid [(G4S)3] “normal” linker was also present. Following
alignment with each other, significant diversity in the V_H and V_L chain was observed especially in the CDRs. Variability was also noted in the framework regions. No two clones had identical V_H or V_L fragments. The aligned amino acid sequences of five clones from each library are shown in Fig. 3.9 and 3.10.

Figure 3.5: Flow diagram showing the construction of two antibody phage displayed libraries: (1) reverse transcription-PCR of RNA to cDNA, (2) amplification of V_H and V_L gene segments, (3-4) assembly of scFv gene by extension PCR, (5) pull-through PCR to introduce restriction sites at the ends and (6) cloning scFv gene in pHEN vector DNA.
Figure 3.6: PCR amplicons lane 1, V_H; lane 2 V_L; and lane 3 the scFv gene assembled by pull-through PCR. M is a 100 bp ladder DNA molecular mass marker.

Figure 3.7: PCR amplicons obtained after colony PCR (“directly” linked library) using vector-specific primers that recognise insert flanking regions. M is a 100 bp ladder DNA molecular mass marker. Lanes 1-20 show amplicons from randomly picked colonies and lane 21 is the negative control (pHEN DNA).
Figure 3.8: PCR amplicons obtained after colony PCR ("normally" linked library) using vector specific primers that recognise insert flanking regions. M is a 100 bp ladder DNA molecular mass marker. Lanes 1-20 show amplicons from randomly picked colonies and lane 21 is the negative control (pHEN DNA).

Figure 3.9: Amino acid sequence alignment of randomly chosen clones from "directly" linked antibody library. A single amino acid linker (G) separates the V$_H$ and V$_L$ chains.
Figure 3.10: Amino acid sequence alignment of randomly chosen clones from “normal” linked antibody library. A normal 15 amino acid linker [(G₄S)₃] joins the V_H and V_L chains.

### 3.4 Biopanning

Since this was the first study in which a chicken antibody library was constructed by linking of heavy and light chain with a single amino acid (G) residue, the normally linked library was kept as an alternative in case panning failed with the “directly” linked library. Therefore antigen-specific antibodies were selected from the “directly” linked antibody library (6.7 x 10⁷ clones). Four rounds of panning against each individual antigen were performed. Polyclonal phage ELISA indicated an enrichment of anti-LDH, -HRPII and -VSG phage antibodies with each round of panning. All the antigens except ALDO and MCFV showed ELISA signals after only the second round (Fig. 3.11).
Figure 3.11: Polyclonal phage ELISA showing an increase in ELISA absorbance values from round 2 to 4 indicating an enrichment of LDH-, HRPII- and VSG-specific phage antibodies. No apparent enrichment occurred with ALDO and MCFV.

3.4.1 Selection of anti-LDH scFvs

Ninety individual clones resulting from the round 3 panning on LDH were cultured in a 96-well culture plate and screened for anti-LDH activity in a monoclonal phage ELISA. Of the 90 phage clones, 21 reacted with LDH (Fig. 3.12). After IPTG induction they were then all tested in soluble antibody fragment form using the supernatant directly in ELISA. Of 21 positive phage clones, only 12 reacted as scFvs (Fig. 3.13).
Figure 3.12: Monoclonal phage ELISA (A) on LDH with clones randomly chosen from LDH selection 3. The negative control 2% MP is shown in B.
Figure 3.13: Soluble antibody fragment ELISA (A) corresponding to monoclonal phage clones shown in Fig. 3.12. The negative control 2% MP is shown in B.
Sequence analysis. The DNA coding for ten of the scFv binders was sequenced. Their amino acid sequences were deduced and the CDRs and FW regions were identified in each of the V_H and V_L chain fragments. Following alignment of the sequences with each other, five unique clones were identified. They were designated LA2, LB10, LC9, LD3 and LE11. Although there were differences in the V_H and V_L chain CDRs, similarities were also noted between particular clones with some having identical V_H fragments. Clone LA2 and LD3 had identical V_H and V_L chain fragments with a major difference found in the FW region 1 of the V_L chain. Two clones, LB10 and LE11, expressed the same V_H chain but with two different V_L chains.

Figure 3.14: Amino acid sequence alignment of anti- LDH scFvs LA2, LB10, LC9, LD3 and LE11 with the chicken germline Ig V_H and V_L. Diversity was found predominantly in the CDR regions.
In comparison with the germline sequence (Reynaud et al., 1983; 1989), up to seven additional amino acids were found in VHCDR3 of all identified fragments indicating that the gene conversion process had taken place (McCormack et al., 1993). Evidence of extensive somatic hypermutation was also found in the FW regions. This is shown by amino acid substitutions in the FW regions (Fig. 3.14).

**ELISA.** The identified clones were further tested in ELISA against both LDH and ALDO to further establish their specificity. All bound to LDH but not ALDO (Fig. 3.15a). The stability and hence potential usefulness of these fragments was then tested by incubating aliquots (supplemented with 2% sucrose) at room temperature (RT), 4ºC and -20ºC; and testing for binding in ELISA after one month. ScFv A2 and C9 were stable under all the tested conditions while others had partially or totally lost their binding ability depending on incubation temperature (Fig. 3.15b).

Figure 3.15(a): ELISA of selected anti-LDH scFvs showed specificity to LDH with no signal against ALDO and the negative control (2% MP).
Figure 3.15(b): Stability test ELISA of anti-LDH scFvs that were stored for one month at RT, 4°C and -20°C. LA2 and LC9 were shown to be stable compared to other clones.

3.4.2 Selection of anti-HRPII scFvs

Ninety fusion phage clones from selection rounds 3 and 4 of the HRPII panning experiment were cultured in separate 96-well culture plates and tested for their binding to the target antigen in monoclonal phage ELISA. Of the 90 clones, 63 from selection round 3 and 59 from selection round 4 reacted with the antigen (Fig. 3.16 and 3.17). They were then all tested in the soluble antibody fragment form. All the positive phage clones from round 3 and round 4 also reacted as scFvs (Fig. 3.18 and 3.19).
Figure 3.16: Monoclonal phage ELISA (A) on HRPII with clones randomly chosen from HRPII selection 3. The negative control 2% MP is shown in B.
Figure 3.17: Monoclonal phage ELISA (A) on HRPII with clones from HRPII selection. The negative control 2% MP is shown in B.
Figure 3.18: Soluble antibody fragment ELISA on HRPII (A) corresponding to monoclonal phage clones selected in round 3. The negative control 2% MP is shown in B.
Figure 3.19: Soluble antibody fragment ELISA on HRPII (A) corresponding to monoclonal phage clones selected in round 4. The negative control 2% MP is shown in B.
Sequence analysis. DNA coding for 10 of the round 3 and the same number of round 4 scFv binders which had been randomly selected were sequenced. The amino acid sequences were deduced and the three CDRs and four FW regions were identified in each of the V<sub>H</sub> and V<sub>L</sub> chain fragments as expected. Following alignment, six unique clones were identified. They were designated HA2, HB5, HD2, HD6, HF11 and H4A5. Significant differences in the V<sub>H</sub> and V<sub>L</sub> chain CDRs were observed. In comparison with the germline sequence (Reynaud et al., 1983; 1989), there were up to eleven additional amino acids in VHCDR3 indicating that gene conversion had taken place (McCormack et al., 1993). There was evidence of somatic hypermutation in the FW regions; 1 to 3 amino acid variability was found among the clones (Fig. 3.20).

Figure 3.20: Amino acid sequence alignment of anti-HRPII scFvs HA2, HB5, HF11, HD2, HD6 and H4A5 with the chicken germline Ig V<sub>H</sub> and V<sub>L</sub> chain. All clones were different from one another and diversity was mainly concentrated in the CDR regions.
Figure 3.21: ELISA of selected scFvs on HRPII showed specificity to HRPII with no signal against immunising partner ALDO (A) and four scFvs showed stability following incubation at RT, 4°C and -20°C for one month (B).
**ELISA.** The identified clones were further tested in ELISA against HRPII and ALDO to determine their specificity. Each scFv showed specificity for HRPII and did not recognise ALDO or the negative control (2% MP) (Fig. 3.21 A). The stability at different temperatures (RT, 4°C and -20°C) was examined by ELISA after one month incubation. ScFv HB5, HD2 and H4A5 were stable under all conditions. HD6 lost its binding ability after incubation at RT. The other scFvs showed a partial loss of binding ability at 4°C and -20°C while a complete failure to bind was observed after incubation at RT (Fig. 3.21 B).

### 3.4.3 Introduction of a flexible linker

A fifteen amino acid residue linker sequence was introduced into the DNA of selected anti-LDH clones in a two step PCR reaction (Fig. 3.22).

![Diagram](image)

Figure 3.22: Diagram illustrating conversion of “directly” linked V\_H-G-V\_L scFvs to flexible linked V\_H-(G\_4S)\_3-V\_L scFvs using PCR and cloning into the pHEN displaying vector using *SfiI/NotI* restriction sites.
**Sequence analysis.** DNA coding for the newly constructed anti-LDH scFvs was sequenced. Their amino acid sequences were deduced but N-B10 and N-E11 was found to be out-of-frame. N-A2, N-C9 and N-D3 were aligned to their parent clone amino acid sequences (results not shown). N-A2 and N-C9 had similar amino acid sequence to their parent clones with a flexible linker of fifteen residues joining $\text{V}_H$ and $\text{V}_L$ chains. One amino acid was missing in the VH chain of N-D3.

**ELISA.** The newly constructed scFvs designated N-A2, N-B10, N-C9, N-D3 and N-E11, were tested in ELISA (Fig. 3.23). N-A2, N-C9 and N-D3 retained their ability to recognise the target LDH, while N-B10 and N-E11 failed to recognise the target and N-D3 partially lost its binding ability.

![Figure 3.23: ELISA of newly constructed anti-LDH scFvs N-A2, N-B10, N-C9, N-D3 and N-E11. All scFvs retained their binding ability except the two scFvs N-B10 and N-E11 both of which failed to bind to LDH.](image-url)
3.5 Identification of pseudo ψV\textsubscript{L} gene donors

The pseudo ψV\textsubscript{L} genes that donated their sequences during gene conversion were identified by comparing the amino acid sequences of selected scFvs to 25 known pseudo ψV\textsubscript{L} genes (Reynaud et al., 1987). Up to 15 pseudo ψV\textsubscript{L} genes appeared to have donated their sequences during gene conversion events. Between two to five pseudo ψV\textsubscript{L} genes were involved in the maturation of anti-LDH and anti-HRPII light chain clones. The list of pseudo ψV genes that appear to have donated their sequences during the diversification of V\textsubscript{L} chain sequences is shown in Table 2. Where more than one candidate pseudogene appeared to have been involved, all are listed (Fig. 3.24 and 3.25).

Table 2: The list of pseudo ψV\textsubscript{L} genes that were involved during gene conversion events.

<table>
<thead>
<tr>
<th>Clone</th>
<th>FW1</th>
<th>CDR1</th>
<th>FW2</th>
<th>CDR2</th>
<th>FW3</th>
<th>CDR3</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA2</td>
<td>5*</td>
<td>5*</td>
<td>5*/10*</td>
<td>5*/10*</td>
<td>10*</td>
<td>10*</td>
</tr>
<tr>
<td>LD3</td>
<td>10* &amp; 5*</td>
<td>5*</td>
<td>5*/10*</td>
<td>5*/10*</td>
<td>10*</td>
<td>10*</td>
</tr>
<tr>
<td>LB10</td>
<td>24*</td>
<td>4</td>
<td>10*</td>
<td>4</td>
<td>1/19</td>
<td>1/19</td>
</tr>
<tr>
<td>LC9</td>
<td>14*</td>
<td>13</td>
<td>7*</td>
<td>7*/8</td>
<td>8/14*</td>
<td>14*</td>
</tr>
<tr>
<td>LE11</td>
<td>5*</td>
<td>23</td>
<td>24*</td>
<td>17/GL</td>
<td>17/24*/5*</td>
<td>5*</td>
</tr>
<tr>
<td>HA2</td>
<td>13</td>
<td>13</td>
<td>6</td>
<td>12</td>
<td>12/19</td>
<td>12</td>
</tr>
<tr>
<td>HB5</td>
<td>13</td>
<td>10*</td>
<td>13</td>
<td>GL</td>
<td>GL/10*</td>
<td>10*</td>
</tr>
<tr>
<td>HD2</td>
<td>24*</td>
<td>18*</td>
<td>4</td>
<td>4</td>
<td>18*/8</td>
<td>8</td>
</tr>
<tr>
<td>HD6</td>
<td>14*</td>
<td>14*</td>
<td>7*</td>
<td>7*</td>
<td>5*/10*</td>
<td>5* or 10*</td>
</tr>
<tr>
<td>HF11</td>
<td>5*</td>
<td>5*</td>
<td>6</td>
<td>6</td>
<td>6/10*</td>
<td>10*</td>
</tr>
<tr>
<td>H4A5</td>
<td>5*</td>
<td>5*/23</td>
<td>23</td>
<td>GL</td>
<td>5*</td>
<td>5*</td>
</tr>
</tbody>
</table>

(* indicates donors sequences orientated in an inverted fashion; GL = germline)
Figure 3.24: Alignment of anti-LDH Vl chain sequences to pseudo ψVl gene donors. The Vl chain amino acids were assigned to their corresponding donor sequences.
Figure 3.25 continues on the next page
Figure 3.25: Alignment of anti-HRPII V\textsubscript{L} chain sequences to pseudo \(\psi\)\textsubscript{V\textsubscript{L}} gene donors. The V\textsubscript{L} chain amino acids were assigned to corresponding donor sequences.

3.6 Potential use of selected scFvs

Egg yolk antibodies (IgY) specific for LDH and the anti-LDH scFv LC9 were used to determine whether an scFv from the library could be used in modern system as a useful reagent in immunoassay development. Using chicken IgY as a capture antibody and LC9 as the detection antibody, a sandwich ELISA was set up for the detection of LDH antigen (Fig. 3.26). The same IgY was used to inhibit the binding of LC9 to LDH antigen in competitive inhibition ELISA (CI-ELISA). Up to 90% inhibition of LC9 was obtained when the IgY concentration was increased to 1 mg/ml (Fig. 3.27). The IgY directed against all antigens (“cocktail” IgY) was unable to either capture LDH (Fig. 3.26) or block LC9 binding to LDH (Fig. 3.28).
Figure 3.26: Sandwich ELISA using IgY as capture LDH and scFv LC9 specific for LDH as a detection antibody. The blue bar represents ELISA reaction and white bar shows the negative control milk powder.

Figure 3.27: Competitive inhibition ELISA using LDH-specific IgY at different concentrations to block LC9 binding to immobilised LDH. The blue bar represents ELISA reaction and white bar shows the negative control milk powder.
Figure 3.28: Competitive inhibition ELISA using “cocktail” IgY at different concentrations to block the LC9 binding to immobilised LDH. The blue bar represents ELISA reaction and white bar shows the negative control milk powder.

3.7 IgY immunoblotting of MCFV proteins

Selection of anti-MCFV scFv antibodies was unsuccessful, therefore IgY polyclonal antibodies were used to detect immunoblotted MCFV proteins to establish if the chicken produced antibodies to any of the known protein epitopes. Of eleven MCFV proteins known to elicit immune responses in animals, only four proteins with molecular weights of 130 kDa, 78 kDa, 24 kDa and ~55 kDa proteins respectively were predominantly recognised by the IgY in the immunoblot (Fig. 3.29).
Figure 3.29: Western blot analysis of virus proteins of partially purified MCFV proteins immunoblotted with chicken IgY. The Pre-Stained Standard protein marker (Seeblue Plus2, Invitrogen) is loaded into lane M, V is the lane loaded with viral proteins and FBT is the lane loaded with the uninfected foetal bovine testes cells.

3.8 Other use of IgY

As with ALDO, initial attempts to enrich phage antibodies against HRPII failed. A new batch of HRPII was purchased (Immunology Consultant Lab, US) and tested in ELISA using “cocktail” polyclonal IgY. The new batch gave a six-fold higher ELISA signal than the old batch (Fig. 3.30) and anti-HRPII antibodies were successfully enriched using the new batch (see Fig. 3.11).
Figure 3.30: ELISA of the new batch of HRPII and comparison to the old batch.
CHAPTER 4: DISCUSSION

4.1 Chicken immune response to antigens

Two chickens, designated “A” and “B” were each immunised with a mixture of five different antigens (LDH, HRPII, ALDO, VSG and MCFV), all of which were emulsified in ISA70 adjuvant. ELISA was used to determine the amount of egg-yolk IgY (Fig. 3.2) produced in response to each of the antigens. Chicken “A” failed to produce antibodies to LDH (Fig. 3.3) while chicken “B” did not produce any antibodies to HRPII (Fig. 3.4) that were detectable in ELISA. This finding suggests that there may be limitations to the extent to which the avian immune system can be exploited and correlates with the prediction by Kaufman (2000) that some chickens infected with pathogens will not present all protective peptides to T cells even though appropriate peptides may in fact exist. In the chicken, only one of each class I and class II major histocompatibility complex (MHC) molecules is expressed. By immunising a chicken with many different target antigens its immune system may therefore become “overloaded” and be unable to simultaneously present peptides representing all the different antigens.

Class I molecules bind antigens that are present in the cell cytoplasm, while class II is associated with extracellular peptides. The human MHC locus has at least 280 genes and up to six different class I molecules and several different class II molecules are expressed. As a result, a large number of different peptides can be presented (Engelhard, 1994). In contrast, the chicken MHC locus, known as the B-locus is simple and compact. It consists of only 19 genes and is therefore approximately 20 times smaller than the human MHC. The chicken MHC determines the resistance and susceptibility to small and large pathogens (Kaufman et al., 1999a; Kaufman, 2000) and as mentioned, there are only two
copies of each class I and class II molecule genes in the B-locus. Only one of each is dominantly expressed (Kaufman et al., 1999a). This means there are fewer chances of an MHC molecule binding a foreign peptide to present to T cells which affects a number of important cell-mediated immune functions and also determines life or death in response to a pathogen (Kaufman et al., 1999b; Kaufman, 2000; Davison, 2003). The preferential dominant expression of a single class I molecule is said to be a ‘suicidal strategy’ which may sometimes be fatal to the chicken (Kaufman, 2000). For example, in the study by Sapats and her colleagues (2006), they reported the isolation of chicken scFvs specific for a very virulent infectious bursal disease virus. They infected chickens with live virus, but could only use spleens from chickens that survived. The bird’s inability to present protective peptide antigen may be a reason for the death of the other chickens. Another example may be evident in the present study where it was found that only four MCF viral proteins including the proposed 130 kDa target protein earmarked for developing a diagnostic test out of eleven (Li et al., 1995a; Adams and Hutt-Fletcher, 1990) were recognised by polyclonal antibodies (IgY) in immunoblotting (Fig. 3.29). The chickens produced antibodies against at least four viral antigens and three unrelated recombinant proteins. This antigenic load may represent a limit to the number of different antigens that the chicken immune system can handle simultaneously.

4.2 Antibody library construction

Only two sets of primers were necessary to access the diversified avian immunoglobulin genes by PCR amplification of the V_H and V_L gene fragments from the splenic RNA of both chickens as described in previous studies (Davies et al., 1995; Yamanaka et al., 1996; Andris-Widhopf et al., 2000; Sapats et al., 2003; van Wyngaardt et al., 2004;
Finlay et al., 2005). In order to compensate for the failed production of antibodies to one antigen by each of the two chickens, the amplified \( V_H \) and \( V_L \) chain gene fragments were pooled. Two immune phage-displayed scFv libraries were constructed by artificial and random pairing of the \( V_H \) and \( V_L \) gene segments (Fig. 3.6). The essential difference between the two libraries was the length of the polypeptide linker sequence. A “standard” antibody library with a 15 amino acid \([(G_4S)_3]\) linker joining the \( V_H \) and \( V_L \) chains was constructed as a benchmark (size of \( \sim 1.7 \times 10^7 \) transformants), but was not further evaluated. The other library had the \( V_H \) and \( V_L \) chains linked by a single amino acid (G) residue. It had \( \sim 6.7 \times 10^7 \) primary transformants. Both libraries consisted of highly diverse populations of scFv clones with the expected linker length separating the \( V_H \) and \( V_L \) chains (Fig. 3.9 and Fig. 3.10). The scFvs with normal-length flexible linkers are likely to be monomeric, providing the minimal antigen binding site (Plückthun and Pack, 1997). On the other hand, shortening a linker sequence allows the scFv to form diabodies, triabodies or tetrabodies which are multivalent but mono-specific, thereby increasing the avidity (Hudson and Kortt, 1999; Atwell et al., 1999). On the phage surface multivalent antibodies are likely to be formed by association of soluble fragments with intact antibody-pIII fusion protein. These soluble fragments probably result from proteolysis of antibody-pIII fusion protein in the periplasmic space of \( E. coli \) during phage particle morphogenesis (Andries-Widhopf et al., 2000). ScFvs with a shorter amino acid linker are known to perform better than normally linked scFvs when tested in ELISA and are generally advantageous in selection against epitopes associated with a low intrinsic affinity per binding site. The higher avidity is due to the potential for multivalent interaction with separate antigenic determents (Plückthun and Pack, 1997; Hudson and Kortt, 1999; Ravn et al., 2004). While described previously for the construction of a
mouse sub-library by Ravn and colleagues (2004) for panning, this is the first chicken scFv antibody library known to have been constructed by joining \( \text{V}_\text{H} \) and \( \text{V}_\text{L} \) chains with a single amino acid residue. This study mainly focuses on evaluating the short-linker repertoire as a source of antigen-specific scFvs specific for five different antigens.

### 4.3 Antibody selection

There was enrichment of antigen-specific phage antibodies directed against LDH, HRPII and VSG after only two rounds of panning (Fig. 3.11). The rapid selection of specific antibodies suggests that antigen-specific clones are well-represented within the scFv population (Finlay et al., 2005). With “naïve” antibody libraries, it usually takes three to four or more rounds of panning to produce highly specific signals (van Wyngaardt et al., 2004). Although the IgY ELISA indicated the presence of antigen-specific antibodies, no ALDO and MCFV-specific phage antibodies could be obtained even though colony count calculations indicated enrichment after each successive round of panning. The failure to enrich specific phage antibodies against ALDO (Fig. 3.11) may simply have been an artefact due to poor handling of the target enzyme during shipment from the supplier. Initial panning against HRPII also failed, but phage antibodies were obtained after a new batch of antigen was used. Since MCFV is lipid-enveloped, stringent washing with the detergent during panning may be the cause of the failure to enrich viral-specific phage antibodies. In the immunoblot, polyclonal antibodies (IgY) did indeed recognise several viral proteins. Other approaches such as panning on expressed recombinant viral protein may therefore enable anti-MCFV antibodies to be successfully selected and the library may yet be useful in the future as a source of highly specific anti-MCFV scFvs.
Selection of specific scFvs against VSG was suspended due to the limited availability of antigen. Nevertheless, the library yielded several antigen-specific (LDH and HRPII) antibodies that recognised their cognate antigens. DNA sequencing of clones found to be positive in ELISA allowed unique clones to be identified. Five scFvs against LDH and six scFvs against HRPII were identified. Following alignment of their amino acid sequences, considerable hypermutation and extension of CDR regions was noted in comparison with the germline sequence of the \( \text{V}_H \) and \( \text{V}_L \) chains (Fig. 3.14 and 3.21). Compared to CDR3 of both the \( \text{V}_H \) and \( \text{V}_L \) chains, the clones exhibited less variability in their CDR1 and CDR2 regions. Amino acid residues that appear to be antigen-specific were found in CDR2 region of all anti-LDH \( \text{V}_H \) chains (Fig. 3.14), suggesting that antigen-driven gene conversion events had occurred during the \textit{in vivo} immune response. The length of the anti-LDH HCDR3 varied with up to seven additional amino acid residues compared to the germline HCDR3 (Fig. 3.14). The HRPII binders had up to eleven amino acid residues more in their HCDR3s. The amino acid residue extensions found in the HCDR3 regions differed from antigen to antigen suggesting that these regions could be the major determining factor for the antigen binding specificity (Reynaud \textit{et al.}, 1989; McCormack \textit{et al.}, 1993; Nissim \textit{et al.}, 1994; Xu and Davis, 2000; Finlay \textit{et al.}, 2005).

The \( \text{V}_H \) chain of the LDH-binding clones, LA2 and LD3 CDR regions are identical with three more residues in CDR3 and there is one amino acid change in the FW1. The major difference in the \( \text{V}_L \) chain was in the FW1 region; a repeat of the first 16 amino acid sequences of the FW1 region was present in the \( \text{V}_L \) chain of LD3 (Fig. 3.14). When aligned, they differed in positions 12 and 14. The two amino acid differences suggest that
the final \( V_L \) chain (LD3) product could have resulted from multiple rounds of gene conversion events with different pseudogene donors (Cohen and Givol, 1983; Reynaud et al., 1987; McCormack et al., 1993, Yamanaka et al., 1996). If true, then the LA2 \( V_L \) chain resulted from the inner primer binding site found in the parent hybrid \( V_L \) chain clone (LD3) as a result of sequence repeats. The CDR regions of the LA2 and LD3 \( V_L \) chains are identical with only one amino acid change in FW2 region. This could possibly have resulted from a PCR error during amplification. Although the role of \( V_H \) chain in antigen binding is well documented, clone LA2 and LD3 \( V_L \) chains may be important in defining the fine antigen binding (Sapats et al., 2003) and the stability of the selected scFv (Fig. 3.15 b). The \( V_H \) chain of LB10 and LE11 were similar in their CDR regions with seven more residues in CDR3 and one amino acid change in the FW3 region. Their \( V_L \) chains differed significantly in both CDR and FW regions. The LC9 scFv had its own unique \( V_H \) (five more amino acids in CDR3) and a \( V_L \) chain that differed from the other scFvs. The anti-HRPII scFvs’ entire \( V_H \) and \( V_L \) chains differed significantly from one another in both CDR and FW regions (Fig. 3.20). HA2 and HF11 have six, HB5 have eleven and HD6 and H4A5 have four more amino acid residues than the germline in HCDR3 (Fig. 3.20).

### 4.4 Stability testing

The supernatant fluids containing the LDH and HRPII binders was stored at different temperatures, namely room temperature, 4°C and -20°C for one month and then tested in ELISA to determine their ability to recognise their antigens. Four (LA2, LB10, LD3 and LE11) anti-LDH scFvs completely lost binding ability after a month at RT. Significant loss (66% reduction of ELISA signal) of binding at 4°C and -20°C occurred in LB10,
LD3 and LE11 while LA2 remained stable. LC9 continued to recognise antigen under all the conditions suggesting the antibody remained stable (Fig. 3.15(b)). Three anti-HRP II scFvs HA2, HD6 and HF11 lost binding ability at RT with a partial loss at 4°C and -20°C for HF11. HB5, HD2 and H4A5 were stable at all tested conditions. The stability of selected scFvs is a key determinant of good candidates for development of a diagnostic immunoassay for field application in areas where storage facilities are not available.

4.5 Donor sequences

The \( \psi V_L \) chain pseudogene \((\psi \psi V_L)\) donor sequences have been well characterised for the chicken (Reynaud et al., 1987). This made it feasible to trace those pseudogenes which had donated their sequences during \( V_L \) chain gene conversion. By comparing the CDR and FW region amino acid sequences to the germline, non-germline amino acids were assigned to \( \psi V_L \) donors where possible. More than one \( \psi V_L \) donor was involved in diversification of each of the selected \( V_L \) chain clones indicating that the selected clones had undergone several rounds of gene conversion during \textit{in vivo} affinity maturation. Based on the number of potential donors, two for LA2, LD3, HB5 and H4A5; three for HF11; four for LE11, HA2, HD2 and HD6; and five for LB10 and LC9 gene conversion events had occurred (Fig. 3.24 and 3.25). The boundary between donor sequences and germline sequences is not well-defined. In some cases germline sequences were found in CDR2 regions (LE11, HB5 and H4A5). An inability to trace the origins of other amino acid residues is likely to be due to extensive somatic hypermutation that resulted in mutations occurring evenly around the rearranged V genes (Gearhart and Bogenhagen, 1983; Parvari et al., 1990). An overlap of donor sequences from different \( \psi V_L \) donors in a single region (either CDR or FW) was found. McCormack and Thompson (1990) also
reported an overlap between two gene conversion events which prevented an accurate description of either ends of the \( V_L \) chain sequences. A sixteen amino acid residue repeat was found at the beginning of FW1 region of clone LD3. The origin of these sequences was traced using amino acids at positions 12 and 14 to enable allocation of possible donors. Corresponding sequences belonged to \( \psi V_L \) 10 and \( \psi V_L \) 5. The fact that the LD3 clone consists of amino acids from only two \( \psi V_L \) donors supports the suggestion that FW1 is a hybrid of two donor sequences formed during two separate gene conversion events.

An examination of the \( V_L \) chains amino acid sequences showed that the pseudo \( \psi V_L \) gene donors that were closer, and those in an inverted orientation regardless of their location (closer or distant) with respect to the functional \( V_L \) locus, were predominantly used during gene conversion. Ten of the eleven selected \( V_L \) clones had at least one donor sequence in an inverted orientation. The \( V_L \) chain gene conversion events that took place in this study are therefore similar to those reported previously (Reynaud et al., 1987; McCormack and Thompson, 1990; McCormack et al., 1993). Although the origin of \( V_H \) chain sequences could not be traced because the \( \psi V_H \) sequence had not been characterised, evidence of gene conversion events is found in the HCDR3 (Fig. 3.14 and 3.20) and HCRD2 of LDH clones (Fig. 3.14).

### 4.6 Introduction of a flexible linker

In order to evaluate the reported superiority of scFvs joined with a single amino acid residue, a flexible fifteen amino acid linker was introduced into the scFvs. N-A2 and N-C9 recognised LDH in ELISA (Fig. 3.23) since these scFvs were in-frame and have
correct parent sequences. N-D3 had partially lost its binding ability with N-B10 and N-E11 unable to bind LDH. N-D3 had a missing codon in V_H and N-B10 and N-E11 were completely out-of-frame. Therefore no conclusion could be drawn due to the fact that sequence analysis revealed changes in nucleotide sequences which may have resulted from PCR errors.

4.7 Potential use of selected scFvs

The usefulness of selected scFvs in diagnostic reagents was tested by setting-up sandwich and CI-ELISAs using an LDH-specific IgY prepared by Wouter Wyngaardt (Fig. 3.26 and 3.27) and anti-LDH LC9 scFv. The “cocktail” IgY failed to either trap LDH or block scFv binding (Fig. 3.26 and 3.28). This may be due to the broader spectrum of this serum as it consists of antibodies against three different proteins and at least four other viral proteins. The stable scFv LC9 was chosen from the selected anti-LDH antibodies stored at 4°C or -20°C. The fact that binding to LDH by LC9 was inhibited, also demonstrates the specificity of the selected scFvs from the constructed library. Therefore, these scFvs have the potential to be of use in developing diagnostic immunoassays aimed at capturing antigens or detecting serum antibodies.

4.8 Conclusion

The successful isolation of recombinant antibodies from a single multiple targeted immune library illustrates the power of phage-display technology for producing potentially useful diagnostic and therapeutic reagents. The rapid enrichment of antigen-specific phage antibodies suggests that the spleen may have been enriched in affinity matured antibody clones or could have been due to the multivalency of tri- or tetrabodies
that resulted from joining of V\textsubscript{H} and V\textsubscript{L} chain with a single amino acid linker. The avian immune system has been shown to have some potential use in raising recombinant antibodies against multiple target antigens. This can be achieved by immunising a single chicken with a cocktail of different target antigens. Depending on the combination and complexity of antigens used, useful and stable antigen-specific scFvs to each of the immunising partners can be selected in a relatively short time frame. Several unique and stable antibodies were identified that might be useful for diagnostic applications. The ability of selected scFvs for use in development of diagnostic immunoassays was demonstrated in sandwich ELISA and CI-ELISA. The resulting libraries are likely to remain a rich source of highly-specific single chain antibody fragments to a number of MCFV proteins as shown in the immunoblot using IgY (Fig. 3.28). In addition, the polyclonal antibodies (IgY) raised in the course of the study can be used in research. This is the first study in which it was demonstrated that a single chicken can be used to raise recombinant antibodies against more than two different target antigens. This study therefore extends work by Finlay and colleagues (2005) where a single chicken was used to raise antibodies against two target antigens. The approach described here can be seen as an alternative to the traditional construction of a single immune antibody library for a single target antigen. It makes minimal use of animals and may therefore be cost-effective. Lastly, high levels of antigen-driven gene conversion events were shown in this study. In support of the previous reports, it was found that there is indeed a preferential usage of pseudogene donor sequences during gene conversion which appears to be determined by its proximity and its orientation in relation to the functional variable gene locus.
Future studies should establish more precisely the number of recombinant antigens that the chicken immune system can handle simultaneously. The immunised birds are a potentially important source of antibodies as a result of *in vivo* affinity maturation. For therapeutic applications chicken antibodies can be converted to complete human antibodies by the process called humanisation (Nishibori *et al.*, 2006).
CHAPTER 5: APPENDICES AND REFERENCES

5.1 Appendices

5.1.1 Appendix A: Buffers and stock solutions

Ampicillin stock (100 mg/ml)

Dissolve 1 g of ampicillin in 10 ml dH₂O. Filter sterilise using 0.2 μm filter. Aliquot and store at -20°C.

Coomassie brilliant blue

1.25 g Coomassie blue
225 ml methanol
50 ml acetic acid
225 ml dH₂O

Destaining stock solution

400 ml acetic acid
600 ml dH₂O

20% Glucose

200 g glucose
600 ml dH₂O

Adjust volume to 1l.
**1M H₂SO₄**

Add 29 ml of concentrated H₂SO₄ in 493 ml dH₂O to make 2M H₂SO₄ and use to stop ELISA reaction.

**0.1M IPTG**

Dissolve 1.19 g isopropyl β-D-thiogalactopyranoside in 40 ml dH₂O and adjust volume to 50 ml. Filter sterilise and store at -20°C.

**Kanamycin stock (25 mg/ml)**

Dissolve 250 mg kanamycin in 10 ml dH₂O. Filter sterilise, aliquot and store at -20°C.

**2% MP**

Dissolve 2 mg of Elite milk powder in 10 ml of 1x PBS.

**5M NaCl**

Dissolve 29.22 g of NaCl in 80 ml dH₂O and adjust volume to 100 ml with dH₂O, autoclave and store at RT.

**10x PBS stock solution**

80 g NaCl
2 g KCl
26.8 g Na₂HPO₄- 7 H₂O
2.4 g KH₂PO₄
800 ml dH₂O
Adjust pH to 7.4 with HCl and adjust volume to 1l. Autoclave and store at RT.

**PBS-0.5% Tween 20**

Add 500 μl Tween to 100 ml of 1x PBS and store at RT.

**20% PEG/2.5M NaCl**

200 g polyethylene glycol

500 ml 5 M NaCl

Adjust volume to 1l with dH₂O and autoclave. Store at RT.

**Sucrose gradients**

Dissolve sucrose in PBS and store at -20°C.

<table>
<thead>
<tr>
<th>[ ] %</th>
<th>sucrose (g)</th>
<th>1xPBS (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td>25</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>30</td>
<td>12</td>
<td>28</td>
</tr>
<tr>
<td>35</td>
<td>70</td>
<td>130</td>
</tr>
<tr>
<td>40</td>
<td>16</td>
<td>24</td>
</tr>
</tbody>
</table>

**10x SDS running buffer**

10 g sodium dodecyl sulphate

30.3 g Tris

144.1 g glycine

Dissolve in 800 ml dH₂O and adjust volume to 1l. Store at RT.
1 M Tris pH 7.4

Dissolve 12.11 g of Tris (hydroxymethyl) aminomethane in 80 ml of dH₂O. Adjust pH to 7.4 with HCl and then adjust to 100 ml with water. Autoclave and store at RT.

5.1.2 Appendix B: Media

2xTY broth

Prepare 1 litre:

16 g Tryptone

10 g Yeast extract

5 g NaCl

Adjust to 1 litre with distilled water. Autoclave and store at RT.

TYE plates:

10 g Tryptone

5 g Yeast extract

8 g NaCl

15 g Agar

Dissolve in 600 ml dH₂O and adjust to 900 ml with dH₂O. Autoclave, cool to 50°C, then add 100 ml of 20% glucose to make 1 litre and 1 ml of ampicillin (100 μg/μl), then pour onto plates and store at 4°C.
5.2 REFERENCES


Atwell J.L., K.A. Breheney, L.J. Lawrence, A.J. McCoy, A.A. Kortt and P.J. Hudson. 1999. scFv multimers of the anti-neuraminidase antibody NC10: length of the
linker between $V_H$ and $V_L$ domains dictates precisely the transition between diabodies and triabodies. *Protein Engineering, 12*(7):597-604


**Berek C. and C. Milstein. 1998.** The dynamic nature of the antibody repertoire. *Immunological Review, 105*:5-25


**Burrows P.D., R.P. Stephan, Y. Wang, K. Lassoued, Z. Zhang and M.D. Cooper. 2002.** The transient expression of pre-B cell receptors governs B cell development. *Seminars in Immunology, 14*:343-349

**Burrows P.D. and M.D. Cooper. 1997.** B cell development and differentiation. *Current Opinion in Immunology, 9*:239-244

**Carrington M. and J. Boothroyd. 1996.** Implications of conserved structural motifs in despurate trypanosome surface proteins. *Molecular and Biochemical Parasitology 81*:119-126


Jukes T.H., D.T. Fraser and M.D. Orr. 1934. The transmission of diphtheria antitoxin from hen to egg. *Journal of Immunology, 26*(5):353-360


Polson A. 1990. Isolation of IgY from the yolks of eggs by a chloroform polyethylene glycol procedure. *Immunological Investigations, 19*(3):253-258
Ratcliffe M.J.H. 2006. Antibodies, immunoglobulin genes and the bursa of Fabricius in chicken B cell development. Developmental and Comparative Immunology, 30(1-2):101-118


Reynaud C., A. Dahan and J. Weill. 1983. Complete sequence of a chicken λ light chain immunoglobulin derived from the nucleotide sequence of its mRNA. Proceedings of the National Academy of Science of the United States of America, 80:4099-4103


polymerase chain reaction and detection of a high prevalence of mixed infections.

_Molecular and Biochemical Parasitology, 58_:283-292


_Thompson C.B. and P.E. Neiman. 1987._ Somatic diversification of the chicken immunoglobulin light chain gene is limited to the rearranged variable gene segment. _Cell, 48_:369-378


_Todd W.J. and J. Storz. 1983._ Morphogenesis of a cytomegalovirus from an American bison affected with malignant catarrhal fever. _Journal of General Virology, 64_:1025-1030


Warr G.W., K.E. Magor and D.A. Higgins. 1995. IgY: clues to the origin of modern antibodies. *Immunology Today*, 16(8):392-398


World Health Organisation. [http://www.who.int/malaria/rdt.html]

Xu J.L. and M.M. Davis. 2000. Diversity in the CDR3 region of V\textsubscript{H} is sufficient for most antibody specificities. *Immunity*, 13:37-45

5.3 Publication

Paper: