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ScienceDirect
Veterinary Immunology and Immunopathology 121 (2008) 58–67
Veterinary immunology and immunopathology
www.elsevier.com/locate/vetimm

Phage display selection and characterization of single-chain recombinant antibodies against *Eimeria tenella* sporozoites

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Received 25 April 2007; received in revised form 15 June 2007; accepted 2 August 2007

Abstract

A single-chain antibody library against *Eimeria tenella* sporozoites was constructed by phage display. Antibody-displaying phage was selected in five panning rounds against cryopreserved *E. tenella* sporozoites. A 1000-fold increase in phage output and a 3000-fold enrichment were obtained after three rounds of panning, as the binding clones became the dominant population in the library. Ten clones were randomly selected from the last selection round, and their nucleotide sequences were aligned and compared to chicken germ-line sequences. Analysis of the light chain variable regions revealed possible donor pseudogenes which act as donors in gene conversion events, and contribute to the diversification of the V_L immune repertoire. Possible somatic hypermutation events, a consequence of affinity maturation, were also identified. Soluble antibody was produced in a non-suppressor *E. coli* strain, purified by nickel affinity chromatography, and characterized by immunoblotting. In an immunofluorescence assay, this recombinant antibody showed specific binding to *E. tenella* sporozoites.
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Keywords: Phage display; *Eimeria tenella*; Single-chain antibody; Chicken

1. Introduction

Eimeria tenella is one of the most pathogenic *Eimeria* species, causative agents of coccidiosis and the most important parasites of poultry, in terms of distribution, frequency, and economic losses (McDougald and Reid, 1991). Coccidia belong to the family of Eimeriidiae in the phylum Apicomplexa (Levine, 1970), which also includes organisms that cause serious human and animal diseases such as malaria, toxoplasmosis, and cryptosporidiosis (Augustine, 2001). *Eimeria* parasites invade and multiply in the avian intestinal tract, causing tissue damage that results in blood loss, dehydration, interruption of nutrient absorption, and increased susceptibility to other opportunistic pathogens, such as *Clostridium perfringens* and *Salmonella typhimurium* species (McDougald and Reid, 1991).

Production of recombinant vaccines against *Eimeria* species has been met with little success to date, the major hurdle being the identification of protective antigens (Allen and Fetterer, 2002). Novel vaccination targets can be uncovered using antibodies with known specificity, which are capable of blocking invasion of chicken enterocytes by *Eimeria* organisms. In this study, we used the phage display technology to produce and characterize recombinant antibodies against *E. tenella*.

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doi:10.1016/j.vetimm.2007.08.005

The advent of phage display technology has revolutionized the world of monoclonal antibody production and paved the way for innovations hitherto unheard of. Gene sequences of interest can be inserted into the gene for one of the filamentous phage coat proteins, usually gIII or gVIII. As the phage replicates its DNA inside its host, so does the foreign DNA (Smith and Petrenko, 1997). A peptide displayed on the surface of a filamentous page is thus physically linked to the very DNA that encodes it. This linkage provides for replicability and mutability of the peptide in question. Specific clones can be selected by panning from pools of billions and then amplified, simply by allowing the phage to infect male *Escherichia coli* cells. Since the early 1990s, phage display has been harnessed to mimic the B-cell selection and maturation strategies, i.e., to carry out the immune response *in vitro* (Winter et al., 1994).

The objective of this project was to use the phage display technology to construct an antibody library against *E. tenella* sporozoites, and to select and characterize specific antibodies that could potentially be used for identification of new vaccination targets. The chicken B-cell immune system, with its single heavy and light variable functional domains, makes it possible to amplify the whole B-cell repertoire using a single pair of primers designed around conserved regions flanking these variable segments (Sapats et al., 2003). The library described in this study was constructed using the phagemid vector pComb3X (Andris-Widhopf et al., 2000; Barbas et al., 1991), and was selected against whole *E. tenella* sporozoites. Panning on intact cells offered the advantage of isolating antibodies against native epitopes, but required readily available sporozoites for every round of selection. To that end, we explored the possibility of cryopreservation of *E. tenella* sporozoites, the preparation of which on a daily basis is otherwise practically impossible.

2. Materials and methods

2.1. Parasites

E. tenella oocysts of the WLR-1 strain were propagated by passage through 2-week old broiler chickens (Hyline International, Bryan, TX). Oocysts were isolated from ceca, sporulated, and cleaned from debris, essentially according to established protocols (Tomley, 1997). Sporocyst release was achieved by vortexing sporulated oocysts (3×10^7) with 0.5 cm-diameter glass beads (Sigma, St. Louis, MO) for 2 min. Sporocysts were purified on a 50% Percoll gradient (density 1.13 g/ml, GE Healthcare, Piscataway, NJ) (Dulski and Turner, 1988), resuspended at 10^6 ml^{-1} in excystation medium, and incubated at 41 °C for 90 min with end-over-end mixing. The excystation medium consisted of PBS with 4% (w/v) taurodeoxycholic acid (EMD Biosciences, San Diego, CA), 1 mg/ml tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin, 0.1 mg/ml α-chymotrypsin (Sigma, St. Louis, MO), and 10 mM $MgCl_2$. Freshly excysted sporozoites were washed, resuspended in cell freezing medium, i.e., fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) containing 10% (v/v) DMSO (Sigma, St. Louis, MO), divided in 1-ml aliquots, gradually frozen to −80 °C at the rate of 1° min^{-1}, and stored in liquid nitrogen.

2.2. Immunization

Single comb white Leghorn (SCWL) day-old chicks were obtained from a local commercial hatchery (Hyline International, Bryan, TX), randomized and placed in floor pens. Chicks were maintained at age-appropriate temperatures and given *ad libitum* access to water and a complete chick starter ration formulated to meet or exceed current NRC recommendations for poultry (National Research Council, 1994). On the day of hatch, birds were vaccinated with Coccivac-B (Schering-Plough Animal Health, Omaha, NE), which includes live oocysts of *E. tenella*, *E. mivati*, *E. maxima*, and *E. acervulina*. At 21 days of age, the heaviest 30 birds received a clinical challenge with *E. tenella* oocysts (2×10^5 oocysts per bird). On each of the following 4, 5, 6, and 7 days post-challenge, five birds were euthanized, and their spleens, ceca, and bone marrow were collected and stored in liquid nitrogen. Two weeks after challenge, each of the remaining 10 birds was injected intravenously with 4×10^6 *E. tenella* sporozoites to further activate the splenic B-lymphocytes. Three and five days later, five birds were sacrificed and their spleens were collected and stored in liquid nitrogen.

2.3. Library construction and selection

Generation of scFv fragments, library construction, and panning were performed essentially as previously described (Andris-Widhopf et al., 2000; Rader et al., 2001). Collected organs were ground in liquid nitrogen, and same-organ, same-day samples were pooled. Total RNA was extracted from 1 g of tissue per sample using the Trizol® method (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized from each of the 14 total RNA samples using the RETROscript® kit from Ambion

(Austin, TX), and a cDNA pool was prepared using 10 μl of each cDNA sample. Individual V_H and V_L genes were amplified from the cDNA pool by PCR, using the CSCVHo-F/CSCG-B and the CSCVK/CKJo-B primer pairs (Andris-Widhopf et al., 2000), respectively. V_H and V_L products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA), and were combined via a short linker sequence (GGSSRSS) in a secondary PCR using the overlap primers CSC-F and CSC-B (Andris-Widhopf et al., 2000). The obtained scFv product was purified as described above. Identities of all PCR products were confirmed by agarose gel electrophoresis and sequencing.

The phagemid vector pComb3X was provided by Dr. Carlos F. Barbas III (The Scripps Research Institute, La Jolla, CA). Briefly, the vector (20 μg) and the scFv insert (15 μg) were prepared for cloning by digestion with SfiI (40 U/μl, Roche Molecular Systems, Indianapolis, IN), respectively. Four library ligations were prepared. Each library ligation was electroporated into 300 μl of XL1-Blue electrocompetent cells (Stratagene, La Jolla, CA). Cultures were rescued with VCSM13 helper phage (10^{12} pfu/ml; Stratagene, La Jolla, CA), and phage was precipitated by the addition of 5× polyethylene glycol/sodium chloride (PEG/NaCl). The phage pellet was resuspended in 1% (w/v) bovine serum albumin (BSA) in 0.02 M PBS (pH 7.4), passed through a 0.22 μm filter, and stored at 4 °C with 0.03% sodium azide.

2.4. Panning

The antibody library was taken through five rounds of panning on *E. tenella* cryopreserved sporozoites. Briefly, cryopreserved sporozoites (2×10^6) were washed, resuspended in flow cytometry buffer (PBS containing 20 mM HEPES, 1% BSA, pH 7.4), and incubated with blocked, freshly prepared phage. Sporozoites were washed with flow cytometry buffer four, five, six, seven, and eight times in the first, second, third, fourth, and fifth round of panning, respectively. Bound phage was eluted with 0.1 M glycine–HCl, pH 2.2, neutralized with 2 mM Tris–HCl, pH 9.1, then added to a previously prepared 2 ml XL1-Blue *E. coli* culture, and incubated for 15 min at room temperature. Following helper phage rescue, antibody-displaying phage was precipitated as described above. Phage input and output titers were calculated for each panning round.

2.5. Sequence analysis

Ten clones from the third round of panning were randomly picked from the output plates of the last selection round. Each colony was grown in LB/carbenicillin broth, and cultures were incubated overnight at 37 °C and 250 rpm. Phagemids were extracted from the bacterial pellets using the Miniprep kit (Qiagen, Valencia, CA), and were sequenced using the ompseq and gback vector-specific primers (Andris-Widhopf et al., 2000). Sequencing was performed using the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) at the Gene Technologies Lab, Institute of Developmental and Molecular Biology (Department of Biology, Texas A&M University, College Station, TX). Sequence homology and alignment were performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST), and the CLUSTAL W program (European Bioinformatics Institute, Cambridge, UK), respectively.

2.6. Production of soluble antibody fragments

Antibody fragments without pIII were produced by electroporating phagemid DNA (20 ng) into TOP10F' cells (Invitrogen, Carlsbad, CA), and plating the transformed cells on LB/carbenicillin plates. The bacterial culture was incubated at 37 °C and 250 rpm for 5 h ($OD_{600} \sim 0.5$), induced by adding isopropyl-ß-D-thiogalactopyranoside (IPTG, Sigma, St. Louis, MO) to 2 mM, and further incubated overnight at 37 °C and 250 rpm. Culture supernatants were harvested and checked for the presence of antibody fragments by immunoblotting. Samples were subjected to SDS-PAGE and were then transferred to a polyvinylidene fluoride (PVDF) membrane as previously described (Bhaskaran et al., 2005), except that the membrane was blocked with 5% (w/v) skimmed milk, then sequentially probed with mouse anti-$(His)_6$ (1:1000) (Covance, Berkeley, CA), biotinylated goat anti-mouse IgG (1:80,000), and alkaline phosphatase-conjugated streptavidin (1 μg/ml) (Jackson Immunoresearch, Laboratories, West Grove, PA). All washes were done in TBS containing 0.05% Tween-20. Following appropriate color development using the NBT/BCIP substrate system (Sigma, St. Louis, MO), the membrane was washed extensively in distilled water, dried, and imaged on an HP flatbed scanner.

2.7. Antibody purification

Antibody-containing supernatant from an induced culture was harvested at 5000 × *g* and 4 °C for 15 min. The supernatant was further clarified through a 0.45 μm filter (Pall Corporation, East Hills, NY). The cell pellet

was frozen at −20 °C, resuspended in 12.5 ml of B-PER® Bacterial Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL), rocked gently for 10 min, and centrifuged at 15,000 × *g* and 4 °C for 20 min. The supernatant (soluble fraction) was filtered as above. The histidine-tagged antibody was purified from the culture supernatant and from the cell pellet's soluble fraction under native conditions on a HIS-Select Nickel Affinity Gel (Sigma, St. Louis, MO), essentially according to the manufacturer's instructions. Purified proteins were eluted with 250 mM imidazole, and were detected by immunoblotting as described above.

2.8. Immunochemical analysis of antibody fragments

2.8.1. Enzyme-linked immunosorbent assay

Antibody fragments produced in XL1-Blue *E. coli* cells were analyzed for binding to *E. tenella* sporozoites. All washes and dilutions were made in flow cytometry buffer. Sporozoites (1×10^5 well^{-1}) were incubated with culture supernatant, soluble fraction obtained from the cell pellet, or with flow cytometry buffer (negative control) for 1 h with gentle rotation. Sporozoites were then pelleted, washed three times, and resuspended in mouse anti-HA (1 μg/ml; Roche Molecular Systems, Indianapolis, IL). After washing, sporozoites were incubated with a 1:10,000 dilution of peroxidase-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA). Detection of bound antibody was completed by addition of BD OptEIA™ TMB substrate (BD Biosciences, San Diego, CA). The reaction was stopped by the addition of 10% sulfuric acid. Sporozoites were pelleted, the supernatants were transferred to a flat-bottom ELISA plate, and absorbances at 450 nm were read with a Wallac plate reader (Perkin-Elmer, Boston, MA).

2.8.2. Immunofluorescence

Cryopreserved sporozoites were thawed, washed, dried on glass slides, and then fixed with 4% paraformaldehyde/0.05% glutaraldehyde for 15 min. Slides were thoroughly washed in PBS, and incubated with either the purified anti-*E. tenella* scFv or with TBST, overnight at 4 °C. After washing with TBST, slides were incubated for 1 h with mouse anti-$(His)_6$ at 1:1000, washed, and then incubated with FITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA) at 1:300. Slides were then washed and mounted with HardSet Vectashield mounting medium (Vector Labs, Burlingame, CA), and imaged with a Zeiss Axioplan fluorescence microscope.

3. Results

3.1. Library construction and transformation

Variable regions of the heavy and light antibody genes were successfully amplified and were used in a secondary PCR reaction to generate scFv fragments. Identities of V_H, V_L, and scFv products (400, 350, and 750 bp, respectively) were confirmed by agarose gel electrophoresis (Fig. 1) and sequence homology with other immunoglobulin genes in the GenBank database (data not shown). Four library ligations were prepared, precipitated, and introduced into XL1-Blue *E. coli* cells by electroporation. The combined library size, i.e., the total number of independent transformants obtained from the four ligations, was 7.4×10^7. The background ligation level, as indicated by self-ligation of the vector, was less than 5%. Successful cloning was ascertained by SfiI digestion (data not shown).

3.2. Panning

The antibody library was taken through five rounds of panning on *E. tenella* sporozoites. Parasites had been previously cryopreserved and were thus available for each day of panning. After incubation of the library with the sporozoites and washing away unbound and weakly bound phage, phage particles were eluted and used to infect an *E. coli* culture. Phage was then rescued by the addition of helper phage which provided the proteins and enzymes needed for replication. At this point, the addition of carbenicillin and kanamycin ensured the survival of only those cells containing both the helper and phage genomes. At the end of each panning round, phage was precipitated by the addition of PEG/NaCl solution. The phage was then re-amplified by repeating the panning cycle. In this manner, specifically binding clones were selected and amplified. Input and output titers from each round are shown in Table 1. A 1000-fold increase in output was obtained after three rounds of panning. There was clear enrichment of the positive clones over the panning rounds, with the increased washing stringencies selecting for highly specific clones. The third panning round resulted in a 3000-fold enrichment over the first round, as the binding clones became the dominant population in the library. Phage was precipitated from individual clones picked from the output plates of this round. Phagemids purified from these clones and digested with SfiI yielded scFv and vector fragments of the expected size (750 bp and 3.5 Kbp, respectively; data not shown).

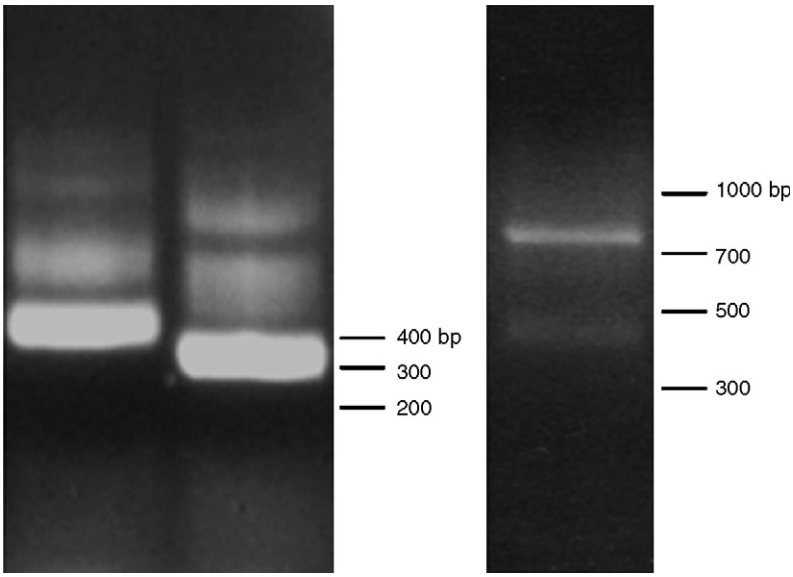

Fig. 1. Amplification of V_H, V_L, and scFv fragments. (Left panel) V_H (400 bp) and V_L (350 bp) fragments; (Right panel) single-chain antibody fragment (750 bp).

3.3. Sequence analysis

Nucleotide sequences of V_H and V_L genes from the 10 selected clones were compared to published germline sequences from the inbred CB strain (Reynaud et al., 1987, 1989), and appeared to represent three independent clones, designated S2, S8, and S9 (Fig. 2). Deduced amino acid sequences of heavy and light chains are shown in Fig. 3. As expected, differences were chiefly present in the complementarity-determining regions (CDR). In the light chain of clone S8 (S8L), an insertion of three nucleotides (AAC), and a deletion of six nucleotides (AGTACT) were observed in the CDR1 and CDR3, respectively. Another triplet deletion (AGT) was observed in CDR3 of S9L. To reveal evidence of gene conversion events, the V_L nucleotide sequences were further compared to the 25 pseudogenes (ψV1–ψV25) of the CB strain (Reynaud et al., 1987). Multiple substitution events were observed, and two, three, and six pseudogenes were identified as possible gene donors for clones S2, S8, and S9, respectively (Fig. 4). In some cases, more than one pseudogene could be identified as a donor candidate. For example, either of ψV8 or ψV24 could act as a donor for CDR2 of S9L. Similarly, the CDR3 of S8L could have received sequence blocks from either ψV6 or ψV11. Following identification of donor pseudogenes, four, three, and two single nucleotide substitutions were observed in clones S2, S8, and S9, respectively.

3.4. Production and purification of soluble antibody fragments

Antibody fragments without pIII were produced by introducing phagemid DNA from selected clones into TOP10F' cells. When this non-suppressor *E. coli* strain is infected by phagemid DNA, the amber stop codon located just upstream of gIII is respected, and soluble proteins (in this case scFv) are secreted into the culture

Table 1
Results of panning rounds of the antibody library against *E. tenella* sporozoites

Round	Input	Output	% phage bound ($\times 10^{-4}$)	Enrichment
1	1.65×10^{12}	2.4×10^{4}	0.015	
2	1.8×10^{11}	1×10^{5}	0.556	38.2
3	5×10^{11}	2.2×10^{7}	44	79.2
4	2×10^{11}	7.6×10^{7}	380	8.6
5	7.5×10^{11}	8.5×10^{7}	113	0.3

%Phage bound = (output/input) × 100. Enrichment = fold increase of % phage bound compared to the previous round.

```
Heavy chain

GL GCCGTGACGTTGGACGAGTCCGGGGGCGGCCTCCAGACGCCCGGAAGAGCGCTCAGCCTCGTCTGCAAGGCCTCCGGGTTCACCTTCAGCAGTTACAACATGGG
     Forward Primer                                                                                   CDR1
S2L A*******************************************G*************************************************TCC*****T
S8L ******************************************G**A*********************************T*TA*****G*************
S9L A******************************************G**A*****************************************T**************

GL TTGGGTGCGACAGGCGCCCGGCAAGGGGCTGGAGTTCGTCGCTGGTATTGACAACACTGGTAGATACACAGGCTACGGGTCGGCGGTGAAGGGCCGTGCCACCA
                                                                                CDR2
S2L G*********************************A*GG************AG***TGA**************A**T****C************************
S8L ****************************T****A***A****CA*****AG************T*******AT**T***G************************
S9L ******************************************A*C****AG**G*************************************************

GL TCTCGAGGGACAACGGGCAGAGCACAGTGAGGCTGCAGCTGAACAACCTCAGGGCTGAGGACACCGGCACCTACTACTGCGCCAAAGCTGCTGGTNNNNNNNNN
                                                                                                   CDR3
S2L ************************************************************T***C*******************CA*******TGTACTACT
S8L ********************************************************************************T******AG*******ACGGTACAT
S9L ***********G*****************************************************************T******AG*******ACGGTACAT

              D segment                                          JH gene
GL NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNACTGCTGGTAGCATCGACGCATGGGGCCACGGGACCGAAGTCATCGTCTCCTCC
                                                                                Reverse Primer
S2L TGGTGTTGGGGTTATACTGGT            *A**T**A*********************************************
S8L CTGATGCCTACACTTTTTCTATCTTTCCTGCCCT*****A*********************************************
S9L CTGATGCCTACACTTTTTCTATCCTTCCTGCCCT**T**A*********************************************

Light chain

GL  GCGCTGACTCAGCCGTCCTCGGTGTCAGCGAACCCGGGAGGAACCGTCAAGATCACCTGCTCCGGGGATAGCAGCTAC---TATGGCTGGTACCAGCAGAAGG
       Forward Primer                                                          CDR1

S2L **C**************************A**********A*************************G*G*TG**AG*---***********T*********T
S8L **C*************************************A*******G*****************AG***TG**A**AAC*********************T
S9L **C***************************A*****A****A*************************G****TATAG*---***********T***********

GL  CACCTGGCAGTGCCCCTGTCACTGTGATCTATGACAACACCAACAGACCCTCGAACATCCCTTCACGATTCTCCGGTTCCAAATCCGGCTCCACAGCCACATT
                                    CDR2

S2L *T*******************************T*****GA***G*********G*****************************************G********
S8L *T*****************************A*G******G*****************************************************AG******
S9L ******************************************************************************C***T*********AA******

                                                                                          JA gene
GL  AACCATCACTGGGGTCCGAGCCGACGACAATGCTGTCTATTACTGTGCGAGTACAGACAGCAGCAGTACTGCAGGTATATTTGGGGCCGGGACAACCCTGACC
                                                                   CDR3                          Reverse Primer

S2L ************************G***G*G**********T*****G****GA***********C*TA***T******************************
S8L *****************A******T***G*G**********T*****G****G**********------**GT*C******************************
S9L *****************A**********G*G**********T*****G****G***********---TA***T******************************

GL  GTCCTAGGT
S2L *********
S8L *****T***
S9L *********
```

Fig. 2. Nucleotide sequences of heavy and light chains of anti-*E. tenella* scFv. Germ-line sequences (GL) from the CB strain are shown on top. CDR (complementarity-determining regions) and PCR primers are underlined in the germ-line sequences. Nucleotide identity is denoted with an asterisk (*) and deletions with a dash (−). The sequence to be filled with the D segment in the germ-line heavy chain is shown with Ns.

supernatant. The scFv was detected by western blotting using an antibody against the histidine tag, and was found to have the expected molecular weight of approximately 35 kDa (Fig. 5, left panel). Nickel affinity chromatography was used to purify anti-*E. tenella* antibody from the supernatant of an induced culture (Fig. 5, right panel). The yield of purified scFv was 30 mg/l of bacterial culture.

3.5. Immunochemical analysis of antibody fragments

3.5.1. Enzyme-linked immunosorbent assay

The antigen specificity of the produced scFvs was tested by ELISA. Except for clone 2, all antibody fragments harvested from *E. coli* culture supernatants tested positive (Fig. 6). Antibody fragments obtained

```
Heavy chain

GL   AVTLDESGGGLQTPGRALSLVCKASGFTFSSYNMGWVRQAPGKGLEFVAGIDNTGRYTGYGSAVKGRATISRDNGQSTVRLQLNNLRAEDTGTYY
                                     CDR1                      CDR2

S2L  T**************G****************P*V***********W****S*D****DF*P****************************SA***
S8L  **************GT**********SI*G****************I*S*S***S**D**A*********************************
S9L  T**************GT*********************************A*SS********************D********************


GL   CAKAAGTNNNNNNNNNNNNNNNAGSIDAWGHGTEVIVSS
               CDR3

S2L  ***H**CT----TWCWGYTGNVD***************
S8L  ***S***VHLMPTLFSIFPAL*D***************
S9L  ***S***VHLMPTLFSILPALVD***************


Light chain

GL   ALTQPSSVSANPGGTVKITCSGDSSY-YGWYQQKAPGSAPVTVIYDNTNRPSNIPSRFSG
                          CDR1                      CDR2

S2L  *************E********GGGS-*******S**********YNDK***D*******
S8L  *************E**E*****S*GNN*******S**********ES*K***********
S9L  *************E********GGYS-***F****************************


GL  SKSGSTATLTITGVRADDNAVYYCASTDSSSTAGIFGAGTTLTVLG
                             CDR3

S2  *K**************E*E***F*G*E***TY**************
S8  ******S*******Q***E***F*G*A***--**************
S9  *T****N*******Q***E***F*G*A***-Y**************
```

Fig. 3. Deduced amino acid sequences of heavy and light chains of anti-*E. tenella* scFv. Germ-line sequences (GL) from the CB strain are shown on top. CDR (complementarity-determining regions) are underlined in the germ-line sequences. Amino acid identity is denoted with an asterisk (*) and the absence of corresponding residues with a dash (−). Amino acid residues to be filled with the D elements in the germ-line heavy chain are denoted by Ns.

from bacterial cell pellets produced absorbance readings comparable to the negative control (results not shown).

3.5.2. Immunofluorescence

Purified scFv stained *E. tenella* sporozoites intensely and specifically (Fig. 7). In some instances, oocyst shells were also stained. No fluorescence was obtained with the negative control sporozoite preparations.

4. Discussion

The objective of this study was to produce recombinant antibodies against *E. tenella* sporozoites. An immune library against *E. tenella* sporozoites (7.4×10^7 total transformants) was constructed by phage display and was panned against intact cryopreserved sporozoites. Specific, likely high-affinity antibodies were

Germ-line

FW1	CDR1	FW2	CDR2	FW3	CDR3

S2L

ψ10	ψ10	ψ7	ψ7	ψ10	ψ10

S8L

ψV23	ψV23	ψV23	ψV5	ψV5	ψV6 or 11

S9L

ψV8	ψV14	ψV8 or 24	ψ8 or 24	ψV3	ψV11 and 7

Fig. 4. Diversification of V_L chains by gene conversion from donor pseudogenes. Variable lambda chains of clones S2, S8, and S9 are compared to the germ-line sequence. Pseudogenes are identified with the 'ψ' symbol. CDR, complementarity-determining region; FR, framework region.

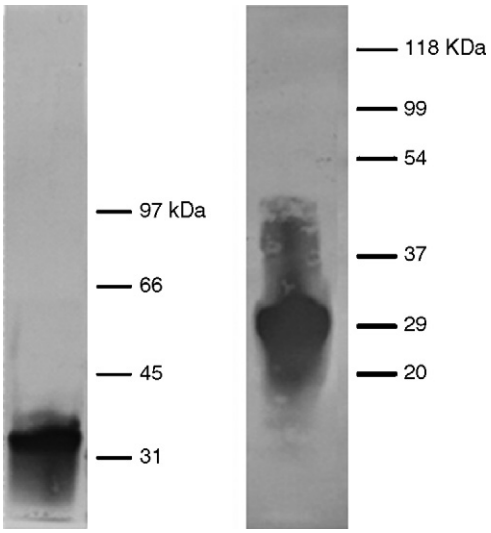

Fig. 5. Immunoblotting of soluble anti-*E. tenella* scFv produced in TOP10F' cells and purified by nickel affinity chromatography. Soluble antibody was detected using mouse anti-$(His)_6$, biotinylated goat anti-mouse IgG, and alkaline phosphatase-conjugated streptavidin. (Left) Soluble scFv detected in culture supernatant (~35 kDa); (Right) affinity-purified scFv.

obtained, an advantage bestowed by immunization (Burton, 1995). To accommodate for the tedious panning process, we resorted to cryopreservation of *E. tenella* sporozoites. A readily available daily supply of whole sporozoites was thus provided. Frozen sporozoites could be thawed and used immediately. More than 50% of the frozen sporozoites remained viable, and even dead ones remained intact (data not shown), and thus were still useful for panning. Thawed sporozoites have been reported to develop into mature schizonts when

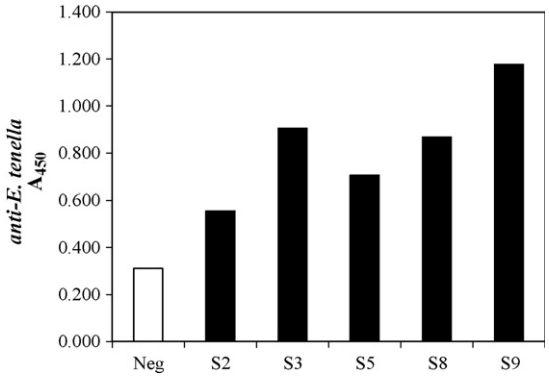

Fig. 6. ELISA testing of the specificity of antibody fragments to *E. tenella* sporozoites. The fragments were obtained from five different clones picked from the last panning round. Binding was detected using an anti-HA antibody and peroxidase-conjugated anti-IgG. Each vertical bar represents the average of duplicate absorbance readings at 450 nm. Open bar (Neg), negative control; black bars (S), antibody fragments from cell culture supernatants.

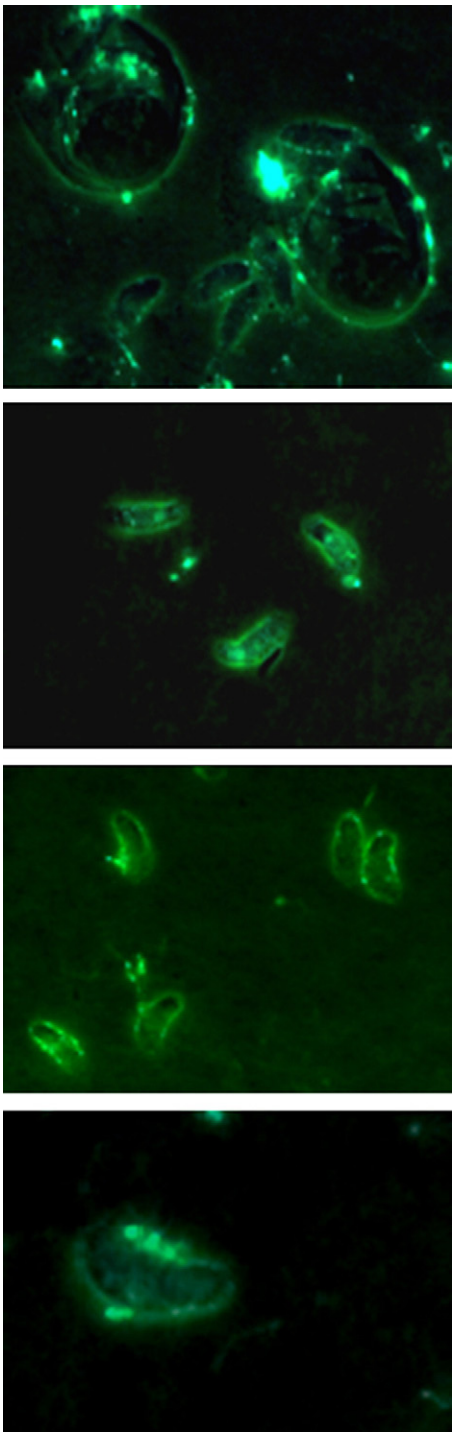

Fig. 7. Immunofluorescence staining of *E. tenella* sporozoites with purified anti-*E. tenella* scFv. Oocyst shell staining is also observed (top panel). Detection was achieved using mouse anti-$(His)_6$ and FITC-conjugated goat anti-mouse IgG.

inoculated into bovine embryonic kidney cells (Doran and Vetterling, 1968). Enrichment of the library with specific clones was evident after three rounds of panning, and specific antigen-binding antibodies were isolated. Antibody-displaying phage pools from all five rounds of panning were analyzed for binding to sporozoites (data not shown). Specific binding increased along the panning rounds, attained a maximum at the third round, and then decreased slightly, reaching a plateau after five selection rounds. This finding was consistent with the observation that the highest enrichment of positive clones was obtained after the third panning round.

Nucleotide sequence analysis of the lambda chain revealed vestiges of gene conversion, the main contributor to diversification of the chicken immune repertoire. As previously reported, the boundaries of the donor pseudogene and the germ-line genes were not clear (Reynaud et al., 1987), and in some cases, more than one candidate pseudogene could be identified as a sequence donor (McCormack et al., 1991). Blocks of nucleotide sequences were transferred from a total of 10 pseudogenes into the three different V_L genes. Single nucleotide substitutions were also observed. Although PCR-generated errors cannot be excluded, these substitutions may represent somatic hypermutation events, which occur following activation with antigen, and are coupled with affinity maturation in B-cells (Maizels, 2005). Gene conversion also occurs in V_H chains, sometimes extending into the D segment (McCormack et al., 1991). It is, however, difficult to trace sequence donors, since the complete set of V_H pseudogenes has not been fully determined yet (Reynaud et al., 1989).

Antibody fragments showed specific binding to *E. tenella* sporozoites, as confirmed by ELISA and immunofluorescence. The latter also revealed binding of the anti-*E. tenella* scFv to oocyst shells, an observation in line with previous reports of antigenic profile similarity between sporulated oocysts and sporozoites (Tomley, 1994a).

Cell surface panning offered the advantage of isolating antibodies against corresponding antigens in a state as close as possible to that found *in vivo*. This option was the only one that could theoretically yield antibodies capable of blocking invasion of enterocytes by sporozoites. It should be noted, however, that excystation *in vitro* does lead to the loss of some antigens (Wisher and Rose, 1987), possibly due to proteolysis by trypsin present in the excystation medium (Tomley, 1994b).

Future investigations will focus on purification and further characterization of antibodies selected from the constructed library. These antibodies can be tested, alone or in combination, for their ability to block *in vitro* the invasion of chicken enterocytes by *E. tenella* sporozoites. Such antibodies can potentially be mass-produced in plant bio-factories, using a variety of crops that are normal components of animal diets, such as maize, soybean and wheat (Artsaenko et al., 1998; Berghman et al., 2005; Fischer et al., 1999; Prins et al., 2005). Plantibodies that can block the invasion mechanism of *E. tenella* sporozoites can possibly constitute a novel, cost-effective approach to control coccidiosis in poultry. Moreover, production of antibodies with known specificity and guaranteed reactivity with live *E. tenella* sporozoites can be obtained by panning against biosynthetic *Eimeria* proteins, thus identifying possible vaccine candidates against coccidiosis.

Acknowledgements

This work was supported by a Pilgrim's Pride Corporation Graduate Endowed Fellowship and a Tom Slick Graduate Research Fellowship. The authors wish to thank Dr. Carlos F. Barbas III for providing the pComb3X vector, Ms. Dana Dean for assistance with the photomicrographs, and Mr. Sid Anderson for assistance with the oocyst production.

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