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Display and selection of chicken IgA Fab fragments

Willemien H. Wieland [a,1], Diego Orzáez [a,2], Aart Lammers [b,3], Henk K. Parmentier [b,3], Arjen Schots [a,*]

[a] *Laboratory of Molecular Recognition and Antibody Technology, Wageningen University, P.O. Box 8123, 6700 ES Wageningen, The Netherlands*
[b] *Adaptation Physiology Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, The Netherlands*

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Abstract

Passive immune therapy is regaining interest to prevent and cure infectious diseases both in human and veterinary medicine. Therefore, systems are required that enable efficient targeted selection of antibodies originating from virtually any animal species. Here, a system for the selection of chicken IgA, using phage display, is described. A novel phagemid vector (pChick3) for the display and selection of chicken IgA antibodies in Fab format was developed. The functionality of pChick3 was demonstrated by construction of an immune antibody library using B cells from chickens infected with *Eimeria acervulina*. From this library, 10 different IgA fragments with specific binding to the *E. acervulina* antigen mix, the sporozoite or oocyst fractions were selected. These results demonstrate the efficiency and versatility of the pChick3 vector system that can readily be applied to construct libraries and subsequently select antibodies of the alpha isotype against a wide variety of pathogens and parasites.
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Keywords: Phage display; IgA Fab; Chicken; *Eimeria acervulina*

1. Introduction

The risk of development of drug resistance in pathogens – especially in light of emerging zoonoses – demands development of alternative strategies to maintain a high health status of poultry. Active vaccination with live virulent or attenuated vaccines has been envisaged as a promising strategy. Insufficient immunogenicity in broilers, which is in part caused by their immature immune system, high costs of production, antigenic variability between species

Abbreviations: Calpha1, first domain of the chicken alpha heavy chain; CDR, complementary determining region; PIII, gene III capsid protein of M13 phage; RBS, ribosomal binding site

* Corresponding author. Tel.: +31 317 485261; fax: +31 317 485267.
E-mail addresses: willemien.wieland@wur.nl (W.H. Wieland), Arjen.Schots@wur.nl (A. Schots).
[1] Tel.: +31 317 485551; fax: +31 317 484254.
[2] Present address: Instituto de Biología Molecular y Celular de Plantas, Universidad Politecnica de Valencia, Avda de los naranjos s/n, 46022 Valencia, Spain. Fax: +34 96 3877859.
[3] Fax: +31 317 485006.

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and danger of transfer of genetic material to wild-type pathogen strains represent major drawbacks that have hindered large-scale application. Prevention or reduction of infections of the intestinal tract can also be achieved through passive vaccination by orally administering immunoglobulins. This strategy shows both prophylactic and therapeutic efficacy against viral, bacterial and protozoan infections (Czinn et al., 1993; Enriquez and Riggs, 1998; Offit and Clark, 1985; Winner et al., 1991).

Secretory immunoglobulins form the first line of defence at mucosal surfaces. The ability of immunoglobulins to protect mucosal surfaces is mainly based on immune exclusion, where binding to pathogens prevents attachment and colonization. Several observations suggest a role for chicken IgA in the protection of mucosal surfaces similar to mammalian IgA (Klipper et al., 2000; Muir et al., 2000; Wieland et al., 2004). For therapeutic applications, IgA might be the most desirable isotype because of its stability in the secretory IgA complex. Of all isotypes, IgA is less likely to initiate complement or pro-inflammatory responses (Corthesy and Spertini, 1999; Lamm, 1997; Zeitlin et al., 2000). Furthermore, (secretory) IgA interacts with specialized epithelial M cells in the gut and a role in antigen sampling and processing to induce secretory immune response has been suggested (Heystek et al., 2002; Neutra, 1998; Rey et al., 2004). Passive immunotherapy with orally administrated IgA may be a promising alternative for drug treatments to protect poultry against parasitic, viral and bacterial infections, and in parallel induce specific immune responses. Increased avian mucosal immunity is relevant both for animal welfare as well as for human health. The chances for zoonoses through bacteria such as *Salmonellae* and *Campylobacter* spp., and viruses such as the influenza virus, and the possible presence of drug residues in meat or eggs for human consumption will be reduced.

One of the most abundant infective diseases in chicken is coccidiosis, an intestinal disorder caused by intracellular protozoan parasites belonging to the genus *Eimeria*. These parasites use the oral route of infection and cause diarrhea, morbidity and mortality in poultry (Lillehoj and Trout, 1996; Rothwell et al., 1995). Since coccidiostats will soon be banned in the EU (Regulation (EC) No. 1831/2003), alternatives are needed. The role of secretory IgA in anti-coccidial immunity in chicken has long been described (Davis et al., 1978; Davis and Porter, 1979), especially with respect to secondary infections, but more attention has been paid to active vaccination strategies to enhance cellular-based immunity (Chapman et al., 2002; Jenkins, 2001; Vermeulen et al., 2001). Passive immunoprophylaxis by orally administered IgA could have major benefits especially for young broilers because of immediate effectiveness.

An enormous reservoir of idiotypes and isotypes of antibodies with possible therapeutic potential is present in the body. Using combinatorial phage display libraries, such antibodies can be selected on the basis of specific affinity and heavy chain-related functions (Benhar, 2001; Hoogenboom et al., 1998). These phage libraries have originally been described for murine and human antibody repertoires (McCafferty et al., 1990), and subsequently extended to other mammalian organisms like bovine and porcine species (Li and Aitken, 2003; O'Brien et al., 1999). Only recently, the technology was adapted to non-mammalian species like chicken (Andris-Widhopf et al., 2000). Species-specific combinatorial libraries allow selection of recombinant antibodies for therapeutic application in other animal species, including pets and livestock. Following functional selection and characterization, the antibodies have to be produced on a large scale. Recently, novel methods for production of full-size antibodies have been developed (Andersen and Krummen, 2002; Ma et al., 2003; Twyman et al., 2003). These production systems overcome disadvantages like the risk of transfer of pathogens and high costs of production, thus allowing fast and cost-effective production of any desired antibody for therapeutic applications.

For chicken, the application of oral passive immunotherapy using recombinant IgA requires the development of new biotechnological tools specifically designed for birds. Here, we present a new method for the display of chicken antibodies in Fab format. The system contains two novelties: first, it incorporates the possibility to clone chicken V-genes in frame with chicken constant regions; second, instead of the traditional IgY isotype, the constant heavy chain domain corresponds to the alpha isotype, facilitating the subsequent conversion

into full-length IgA antibodies for oral immunotherapy. The applicability of our system has been demonstrated with the generation of an anti-*Eimeria acervulina* phage display library. This combinatorial IgA library was generated from *E. acervulina*-infected chickens. Subsequently, specific antibodies were selected which bound to an *E. acervulina* antigen mixture, or sporozoite and oocyst preparations, respectively. Following this strategy, single *E. acervulina* binding IgA Fab fragments in an anti-*E. acervulina* enriched polyclonal library were obtained.

2. Biological samples, material and methods

2.1. Parasite and antigen preparations

The Houghton strain (Weybridge, UK) of *E. acervulina* was maintained and sporulated using standard techniques (Long et al., 1976). Oocysts were disrupted using glass beads (diameter 0.5 mm). Oocyst wall fragments were prepared as previously described (Karim et al., 1996). Excystation of sporozoites was evoked by treatment with trypsin and bile salts as previously described by Shirley (1995). For collection of all *E. acervulina* antigens, the suspension with oocyst and sporocyst walls and sporozoites was washed in RPMI to remove trypsin and bile salts. The pellet was resuspended in RPMI containing protease inhibitors (Roche) and sonicated. Sporozoites were purified over a DE-52 column as previously described (Shirley, 1995). Antigen preparations were frozen at −70 °C.

2.2. Chicken Infections

For infection experiments, 5-week-old Isa Brown Warren layer pullets were purchased. The birds were orally infected with 2×10^5 sporulated oocysts of *E. acervulina*. Before infection and on the first days after the primary infection the faeces were negative for oocysts, thus severe previous infections with *Eimeria* spp. can be excluded. On day 36, the birds obtained a second oral dose of 2×10^5 *E. acervulina* oocysts. On days 3 and 5 after the second infection two chickens were sacrificed and serum, bursa of Fabricius, cecal tonsils and spleen were collected. Organs were immediately frozen in liquid nitrogen and stored at −70 °C for future RNA extractions.

2.3. Construction of pChick3 phagemid vector

Previously described pHEN2 and fd-tet-DOG1 vectors (Hoogenboom et al., 1991) were used as basic frames for the construction of the pChick3 vector following a similar approach as described by O'Brien et al. (1999). A DNA fragment containing a ribosomal binding site (RBS) and the signal sequence of gene III from M13 bacteriophage was amplified from the fd-tet-DOG1 vector using oligonucleotides AP01CKF1 and AP02CKR1 (Table 1), and subsequently cloned as a *Sal*I/*Sac*I fragment into the pHEN2 frame. In this way, a new open reading frame (ORF2) under the

Table 1
Sequence of the oligonucleotides used for the construction of pChick3 (A) and the amplification of chicken IgL and VL repertoire (B)

Name	Sequence
(A) Construction of pChick3	
AP01CKF1	5′-GATACAGTCGACGGCTCCTTTTGGAGCCTT-3′
AP02CKR1	5′-CCTTTCTATTCTCACAGTGCTAGCGTCCAAGAGCTCGAGCT-3′
AP09F	5′-GTCATCGTGAGCTCCGCCTCCGCCAGCCGCCCGACC-3′
AP012R	5′-GCTCAGG GCGGCCGCTTTGGAGGTGAATATGGGGC-3′
ST01	5′-TGGAGCCGTCGACTAAGGAGGAAATCAACATGAAAAAATTATTATTCGC-3′
MY05CAR1	5′-GTTTTGTCGTCTTTCCAGACG-3′
(B) Amplification of chicken IgL and VL repertoire	
01Ap03IgLF1	5′-CCTCAGGTTCCCTGGCCATGGCAGCGCTGACTCAGCCGKCCTC-3′
01Ap04IgLR1	5′-GAAGAGGTCCGAGTGCTAATAGTCGACCTGGGGATGCAATGT-3′
01Ap02VHF1	5′-CTGGCCGCCCTGCCAGCTAGCATGGCGGCCGTGACGTTGGAC-3′
01Ap01VHR1	5′-CCACGGGACCGAAGTCATCGTGAGCTCCGCCTCCGCCACCCG-3′

Restriction enzyme sites used for cloning are underlined.

control of the lacZ promoter was generated. This ORF2 was modified with the introduction of a DNA fragment encoding the first constant region of chicken IgA (Cα1). For this purpose, the Cα1 fragment was amplified from chicken cDNA with oligonucleotides AP09F and AP012R (Table 1). The resulting fragment, which incorporated flanking *Sac*I and *Not*I sites, was subsequently cloned as a *Sac*I/*Not*I fragment into the vector frame for the generation of pChick2, an intermediate vector. Further improvements consisted of the introduction of a methionine at the initial position of ORF2 and the modification of the second RBS to give higher expression levels. For this purpose, oligonucleotides ST01 and MY05CAR were used in a directed mutagenesis strategy. Briefly, 15 cycles PCR reaction were conducted in the presence of ST01 and MY05CAR (Table 1) using pChick2 as a template. The resulting fragment was cloned as a *Nhe*I/*Sal*I fragment into pChick2 generating the final version of the chicken IgA Fab phagemid vector pChick3.

2.4. Eckα1 library construction

PolyA+ RNA was isolated from the bursa of Fabricius, cecal tonsils and spleen samples of *E. acervulina*-infected chickens 3 and 5 days after infection using standard techniques and used to synthesize cDNA (cDNA-synthesis kit; Life Technologies, Breda, The Netherlands). First strand cDNA was pooled and the variable heavy chain (VH) and the complete light chain (IgL) of the immunized antibody repertoires were amplified. A two-step strategy as described (McCafferty and Johnson, 1996) was applied for the construction of the Eckα1 library. In a primary step, IgL and VH individual libraries were constructed. IgL was amplified by primers 01Ap03IgLF1 and 01Ap04IgLR1 (Table 1). Bands of correct size were purified from agarose gels, pooled, and digested with *Nco*I and *Sal*I. One microgram of digested and purified IgL was co-incubated with 500 ng of *Nco*I/*Sal*I-digested pGEM-T vector in a 50 μl ligation reaction. Aliquots of 2 μl were used for electroporation into TG1 epicurean competent cells (Stratagene, Amsterdam, The Netherlands). Cultures were incubated for 1 h at 37 °C and grown overnight on 2xYT solid medium containing ampicillin. Serial dilutions were also plated for estimation of library sizes. On the next day, ampicillin-resistant cells were collected in 2xYT medium containing 15% glycerol and kept at −80 °C for further processing (primary IgL library).

In a similar manner, VH bands were amplified using 01Ap02VHF1 and 01Ap01VHR1 (Table 1), purified, pooled, digested with *Nhe*I and *Sst*I, cloned into pChick3 and transformed into TG1 cells for the generation of a pChick3-VH primary library. For combination of both repertoires, representative aliquots of both VH and IgL primary libraries (1 ml each) were grown for plasmid extraction for 6 h in 50 ml 2xYT medium. Ten micrograms of pGEM-IgL plasmid preparation was digested with *Nco*I and *Sal*I and the released band was purified from agarose. In parallel, 10 μg of pChick3-VH plasmid was linearized with *Nco*I and *Sal*I and purified. The two libraries were combined in a 100 μl ligation and transformed into TG1 cells to generate the Eckα1 library. Serial dilutions of the pooled transformation cultures were plated separately for the estimation of the size of the library. Randomly selected, individual clones were used for testing the functionality of pChick3 vector in ELISA and Western blot assays.

2.5. Phage display and panning

An aliquot of viable cells from the library covering approximately 10 times the number of primary transformants was grown and infected with M13-KO7 helper phages (Amersham Biosciences, Roosendaal, The Netherlands) as described (McCafferty and Johnson, 1996). After overnight incubation, the phage-antibody particles were purified by polyethylene glycol/NaCl precipitation and resuspended in a total volume of 3 ml PBS. Selection for binding fragments was performed with overnight coating of *E. acervulina* preparations (50 μg/ml for whole antigen suspension, 10^5 sporozoites/oocysts per well for specific antigen suspensions) in microtiter plates at 4 °C. Wells were blocked with 2% bovine serum albumin (BSA) in PBS buffer containing 0.1% (v/v) Tween 20 (PBS-T) for 1 h at room temperature and subsequently incubated with 50 μl phage suspension and 50 μl 2% BSA/PBS for 1 h at 37 °C. Bound phages were eluted by addition of 100 μl 200 mM glycine. For the amplification of binders, the phage suspension was neutralized with 200 μl 0.2 M $NaPO_4$ buffer (pH 7.0). *E. coli* TG1 cells were infected with

200 μl of the eluted phage suspension. After amplification and purification, phages were selected for three additional rounds using the same protocol. An aliquot of each of the polyclonal phages obtained after each round of selection was stored at 4 °C for further analyses.

2.6. Enzyme-linked immunosorbent assays (ELISA)

To determine expression levels and specificity of phage-produced IgA Fab fragments or *E. acervulina*-specific antibody levels in chicken serum, ELISA was used. Microtiter plates were coated with *E. acervulina* antigens (50 μg/ml for whole antigen ELISA, 10^5 sporozoites per well for sporozoite ELISA) or goat anti-chicken IgA (1:2000; Bethyl, Montgomery, USA) in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). Non-specific binding sites were blocked with 5% low fat milk powder in PBS-T.

Detection of the binding phage-antibody particles was determined with peroxidase-labelled anti-M13 (1:2000; Amersham Biosciences, Roosendaal, The Netherlands). Functional association of the immunoglobulin heavy chain fragment with the light chain was determined using a sandwich ELISA. For this purpose, microtiter plates were coated with goat anti-chicken IgA (1:2000; Bethyl) to capture recombinant phages and blocked as described. As a second antibody, an anti-chicken IgY (peroxidase-conjugated; 1:5000; Bethyl) recognizing the chicken lambda chain was used. ELISA reactions were developed with ABTS (Roche, Mannheim, Germany) and colour development was determined after 1 h at room temperature.

2.7. Western blot analysis

For further characterization of IgA Fab expression of the phages, samples were mixed 1:1 with buffer containing 50 mM Tris–HCl (pH 6.8), 2% (w/v) SDS, 10% (w/v) glycerol, 0.01% bromophenolblue and DTT (40 mM). Samples were boiled for 5 min and subsequently cooled on ice. All samples were centrifuged for 3 min at 13,000 rpm. Supernatant was collected and resolved on 7.5% polyacrylamide gel. Total protein content of the samples was determined by staining of the gels by Coomassie blue. Protein concentrations of the samples were equalized, run on SDS-PAGE gels and finally the proteins were blotted onto nitrocellulose membranes. After overnight incubation at 4 °C in PBS-T buffer containing 5% ELK, blots were washed shortly in PBS-T. To detect chicken IgA, the blots were incubated at room temperature for 1 h in PBS-T with 5% ELK containing goat anti-chicken IgA-AP (Bethyl) or goat anti-chicken IgY-AP (Sigma). The reaction was visualized by incubation of the blot in developing solution (1 M diethanolamine–Cl (pH 9.8) with 4 mM $MgCl_2$, 4 mg of 5-bromo-4-chloro-3-indolyl phosphate and 5 mg of Nitro Blue Tetrazolium) for 30 min.

2.8. Screening of individual clones

Individual clones from positive phage pools were grown in 5 ml TYE and infected with helper phages as described in Section 2.6. Antibody-phage particles were purified by polyethylene glycol/NaCl precipitation and resuspended in a total volume of 200 μl PBS. Serial dilutions of the phage solutions were tested for binding to *E. acervulina* antigens (total antigen, sporozoite or oocyst fraction) and to BSA by ELISA. The variable regions of these clones were sequenced and subsequently analysed with Vector NTI Suite, Version 8 (InforMax).

3. Results

3.1. Display of chicken IgA antibodies in Fab format

For the display and selection of chicken IgA antibodies in Fab format a new system based on the pChick3 phagemid, derived from pHEN2 (Hoogenboom et al., 1991), was designed (Fig. 1). By introducing a new ribosomal binding site in the pHEN2 vector, the monocistronic operon directed by the LacZ promoter was converted into a dicistronic operon. This strategy allows the coordinate expression of two genes encoding heavy and light chains under the control of a single promoter. The first gene in pChick3 contains the pelB secretion signal and is intended for introduction of chicken IgL. The second open reading frame uses the secretion signal of the

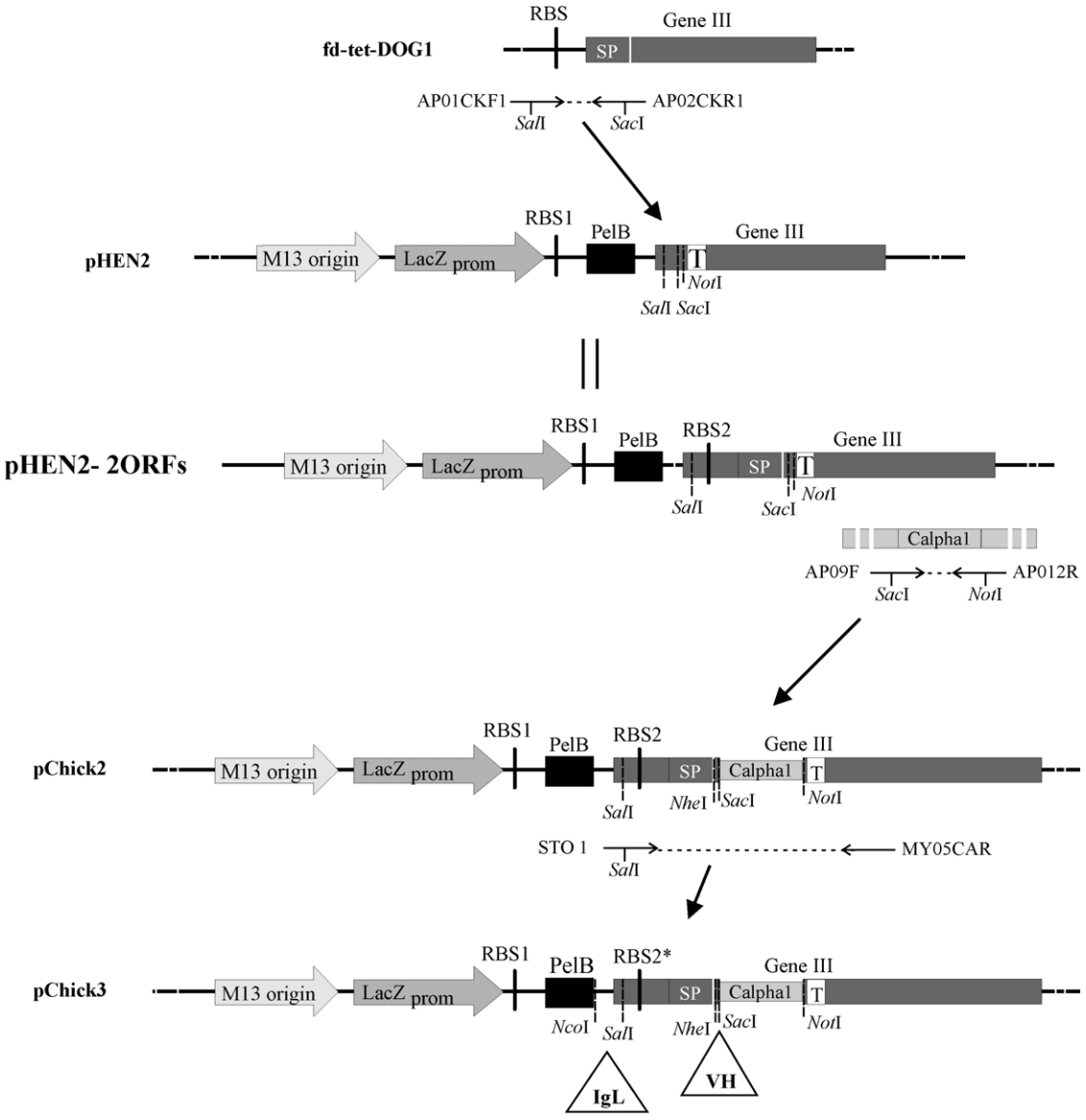

Fig. 1. Schematic representation of the pChick3 phagemid vector construction for the display of chicken IgA Fab fragments. The LacZ promoter ($LacZ_{prom}$) directs the expression of a bicistronic operon. The two ribosomal binding sites are indicated as RBS1 and RBS2. The first cistron is originally present in the vector pHEN2 (Hoogenboom et al., 1991) and comprises the PelB signal sequence for secretion of the chicken light chain. The second cistron was constructed by insertion of the RBS (RBS2) and secretion signal sequence of the pIII protein (SP) derived from fd-tet-DOG2, and modified by addition of the first constant domain of the chicken alpha heavy chain (Calpha1). In a final step, RBS2 and the initial start codon were modified by directed mutagenesis (depicted by an asterisk). The final construct comprises the VH-Calpha1 peptide fused to two tag sequences, i.e. c-myc and His6 (T), followed by an Amber stop codon and the pIII coat protein of M13 phages.

gene III capsid protein (pIII) of M13, and incorporates the Cα1 domain of chicken IgA (Mansikka, 1992) fused to a penta-His tag, a c-myc tag and to the M13 gene III capsid protein. An amber stop codon separates Cα1/penta-His/c-myc from pIII, allowing the expression of the recombinant Fab fragments either fused to pIII or not. Additionally, the second ORF contains a cloning site for the introduction of chicken VH. Since

no data were available on the structure and stability of chicken IgA Fab fragments and their behaviour in phage display, we first tested the performance of pChick3. Chicken variable heavy and light chain repertoires were amplified with specific oligonucleotides from cDNA samples from chicken lymphoid tissues and cloned into pChick3. The resulting phagemids were transformed into *E. coli* and the production of recombinant phages was induced with IPTG. The expression of IgL and VH-Cα1 either or not fused to pIII was assessed in a western blotting experiment (Fig. 2A). Both IgL and VH-Cα1-PIII were found when expressed in TG1 cells (Fig. 2A, "S" lanes). A band of approximately 25 kDa was found next to the VH-Cα1-PIII band. This band is likely free VH-Cα1 as it corresponds to the band in the N lanes wherein the ability to express free Fab fragments was shown in a SupA^{-} *E. coli* strain. In vivo association between chicken light chain and VH-Cα1-pIII fusions was tested in a sandwich ELISA assay. As shown in Fig. 2B, phage preparations gave positive signals in ELISA when sandwiched between anti-IgA

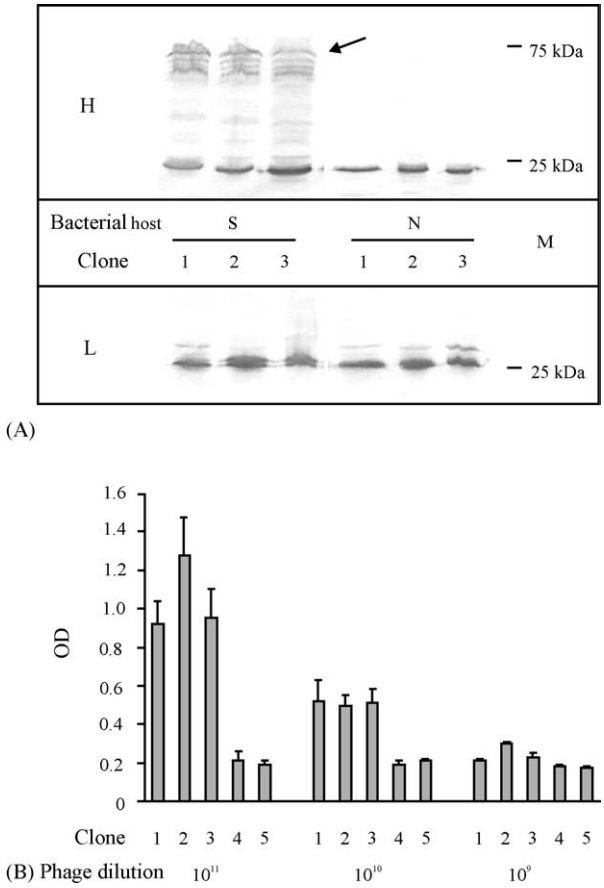

Fig. 2. Demonstration of the ability of pChick3 vector to produce recombinant chicken Fab fragments. (A) Western blot analysis showing expression of the VH-αCH$_1$ fragment (Panel H) and the light chain (panel L). Numbers (1–3) represent randomly chosen bacterial colonies. "S" lanes correspond to SupA+, TG1 host cells, expressing the VH-αCH$_1$-pIII fusions (see arrow in panel A); "N" lanes correspond to SupA-TOP10 cells expressing only the free VH-αCH$_1$ form. Molecular weight of the proteins is shown by the size marker (lane M). (B) Sandwich ELISA showing functional association of heavy and light chains. Recombinant phage preparations were captured with anti-chicken IgA and detected with anti-chicken IgY antibodies fused to peroxidase (recognizing the chicken light chain). Numbers represent individual colonies expressing pChick-transformed, randomly selected colonies (1–3) and unrelated scFv recombinant phages (4 and 5). Serial dilutions of phage-containing supernatant are indicated.

and anti-IgL antibodies, demonstrating formation of stable Fab fragments resulting from the association of light chains with VH-Cα1-pIII.

3.2. Construction of anti-E. acervulina Fab library

To obtain IgA fragments with specificity for *E. acervulina* parasites, an antibody library from lymphocytes isolated from *E. acervulina*-infected chickens was generated. To determine the optimal time-point for collection of antibody-coding mRNA, the serum antibody response following *E. acervulina* infection was monitored. After primary infection, *E. acervulina*-specific antibody levels were low (data not shown). Three days after secondary infection an increase of both oocyst and sporozoite-specific antibodies was found (Fig. 3). Maximum levels of specific antibodies were found 1 week after secondary infection. In mammals, the germinal centres of lymphoid organs contain the largest repertoire 2 days before serum immunoglobulin levels reach their maximum, therefore bursa, spleen and cecal tonsils were isolated 3 and 5 days after secondary infection. To synthesize cDNA, mRNA was isolated from these lymphoid organs. Following this approach, the library was thought to comprise antibodies induced both systemically and mucosally. Amplified IgL and VH cDNA fragments were incorporated into pGEMT and pChick3, respectively. In this fashion, two primary

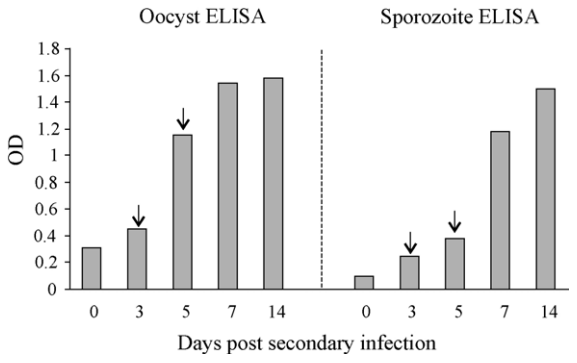

Fig. 3. *E. acervulina*-specific antibody levels (serum dilution 1/200) from chicken infection experiments detected with anti-chicken Ig, conjugated to peroxidase. Chickens used for library construction were sacrificed on days 3 and 5 after the secondary infection (depicted with arrow), just before maximum serum levels of specific antibodies were reached.

libraries were constructed: a pGEMT-IgL library containing 2.0×10^6 clones, 95% of them harbouring an insert; a pChick3-VH library consisting of 4.4×10^6 clones, with 97% insert-containing colonies. Both libraries were combined into the pChick3 frame, generating a secondary library named Eckα1. The Eckα1 library comprises 1.5×10^7 independent clones, 82% of them showing both IgL and VH inserts. Diversity of the library was analysed by PCR and fingerprinting of the immunoglobulin variable regions and showed high diversity among the clones (>80% different clones with *Bst*NI digestion, data not shown).

3.3. Selection of E. acervulina binding IgA Fab fragments from Eckα1

To demonstrate the applicability of the library, Fab fragments specific for antigens of different *E. acervulina* stages were selected. Three approaches were undertaken: (i) selection against a suspension containing all *E. acervulina* antigens (oocyst- and sporocyst walls and sporozoites), (ii) a sporozoite and (iii) an oocyst antigen suspension. The antigens were immobilized in microtiter plates and incubated with phage preparations derived from Eckα1. Prior to selection, little binding to *E. acervulina* antigens could be found (results not shown). After four selection rounds, a gradual rise in *E. acervulina*-specific binding was observed in the selection against sporozoites and oocysts (Fig. 4B and C), whereas only a weak increase in absorbance was found when binding of phages to BSA was tested. Panning against the suspension containing all *E. acervulina* antigens showed the highest increase in binding already after the second panning round (Fig. 4A). The rise in polyclonal binding affinity by specific selection procedures shows the feasibility to enrich the Eckα1 library within two or four selection rounds for specific *E. acervulina* binding Fab fragments. Further characterization of five clones selected on a mix of *E. acervulina* antigens (2, 8, 10, 11 and 14; second selection round), three on sporozoites (A2, C3 and C4; fourth selection round) and two on oocyst walls (E4 and G5; fourth selection round) was performed. These 10 clones were isolated and the presence of both VH and IgL inserts was confirmed by PCR. ELISA results show significant anti-*E. acervulina*-specific binding activity (Fig. 5). The nucleotide sequences of the variable regions of the

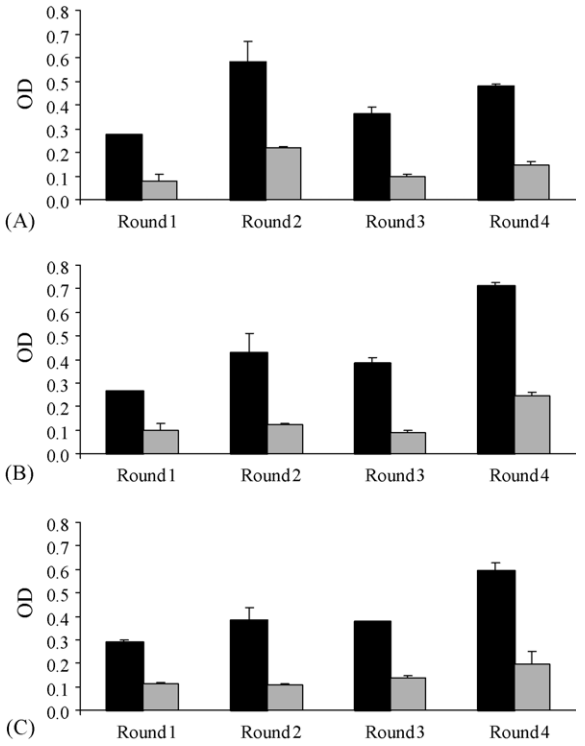

Fig. 4. Binding of selected and unselected polyclonal phage suspensions panned against: (A) *E. acervulina* whole antigen suspensions, (B) sporozoites and (C) oocysts. Dark bars represent specific ELISA, light bars show reactivity against BSA.

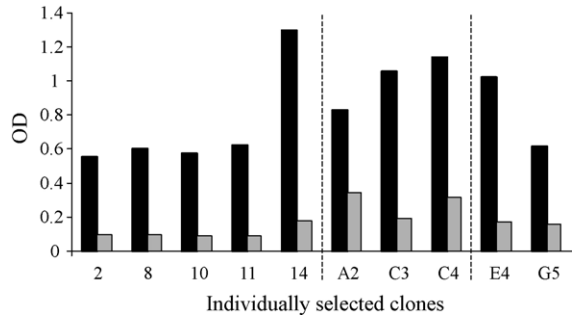

Fig. 5. Phage preparations derived from individual colonies after selection show specific binding in an ELISA against crude *E. acervulina* antigen preparations, sporozoites or oocysts as depicted (dark bars) using BSA coated wells as a control for unspecific binding (light bars). Five phage clones (2, 8, 10, 11 and 14) were chosen from panning against the whole antigen preparation, three and two clones were picked from selection against sporozoites (A2, C3 and C4) or oocysts (E4 and G5), respectively.

light and heavy chains originating from the 10 binders were determined. The 10 clones were found to be very different with variation mainly located in the CDR regions, as expected. Alignment of the CDRs of the deduced protein sequences of the variable domains is shown in Fig. 6A and B. It is worth noting that amino acid sequences of individual CDRs of several different clones are identical. For example, the CDR1 of the light chains of clones 11 and G5 are identical, whereas the CDR2 of clone 11 matches the CDR2 of clone 8, and the CDR2 of clone G5 matches that of clone 14. Most variation in amino acid content, caused by changes of nucleotide sequences such as nucleotide substitutions, insertions and deletions, was found in the CDR3 of both light and heavy chain sequences. Variation in the heavy chains is higher than in the light chains.

4. Discussion and conclusions

A new system for the expression and selection of chicken IgA Fab fragments was developed. Using this system, antibody fragments of the alpha isotype with specificity against parasite antigens of different parasitic stages can be isolated and transferred to suitable expression systems for future use in immune therapeutic applications. The use of antibodies in prevention and treatment of infective diseases has regained interest (Dunman and Nesin, 2003). This renewed interest is a consequence of the development of resistance of pathogens against antibiotics and other drugs, demanding the development of alternative immunotherapeutic strategies. Antibody-based therapies can be useful, for example, to prevent *Eimeria* infections, where antibodies are known to play a role in protection against the parasite. Shortly after infection B cells found in the intestinal tract of mature chickens produce parasite-specific antibodies. Biliary sIgA antibodies are detected within 1 week after oral infection, and correlate with low parasite numbers (Lillehoj and Lillehoj, 2000). In this study, an increase of oocyst-specific serum antibodies at day 5 after secondary infection with *E. acervulina* was found, with a maximum after the first week. For sporozoite-specific antibodies, a significant elevation was found after 1 week, with a maximum after 2 weeks. Preceding the increase of parasite-specific

(A)

```
          →---CDR 1--←   →----CDR 2----←   →-------CDR 3-----------←

10        SGYNMQWVRQAP   QISSTGRYTEYGAAVK   CAKASGSG.CS....GYGCYG.YTASID
C4        -S-ALG------   S--N--SG-W----M-   ---TTY--.-...SV---G..VAG---
A2        -SFY-F------   G-NAANT--G--P---   ---SAAGFS-A....NGWC.D.-AGQ--
14        -S-D-L------   G-HC--NSKY-AP--R   ----ARGY.-GW..T.....PYT-DI--
11        -S-G-N------   G-GN-----G--S---   ---SAV-D.--...S-.....WRAG---
8         -S-A-N------   G-NPA-S--A------   ---GATGYS.............-DGN--
G5        -S-A-N------   A---D-SS-A------   ---NA.................-A-N--
E4        -S-G-H------   G-Y-GSS-MY-AP---   ---DD-IT.............PDAGE--
C3        G--I-H------   G-GN-----G--S--R   ---SAYG-S.W...SY.....TTA-F--
2         -S---G------   A--ND-SW-G-AP---   ---TT-N-Y-AWWASPL--G-YTIGT--
```

(B)

```
          →----CDR 1----←   →CDR 2←   →CDR 3←
10        SG..GSYS.....YGW   SNNNRPSD   SSYV..GI
C4        --..----.....---   --DK---N   G-ST...A
8         --..D-DY.....---   N-------   --ST..AA
11        --..D-SY.....---   N-------   --ST..D-
G5        --..D-SY.....---   A-T----N   --NT...A
14        --..VAAMMEVIT---   A-T----N   -TDA...A
2         --..D-SW.....---   D-DK---N   --SA.A--
C3        LR..---T.....---   N-DK---N   --SA.A--
E4        --GGS-SY.....---   DSTST---   --GTYG-M
A2        R-..--S.....---   N--K----   --G....-
```

Fig. 6. Alignment of the deduced amino acid sequences (shown using the single letter code) from the 3 CDRs of VL (A) and VH (B) of 10 *E. acervulina* binding clones. Sequence gaps are indicated by dots, identical residues are depicted as dashes. Clone numbers correspond to those shown in Fig. 5.

serum immunoglobulins, chickens are known to generate germinal centres in their lymphoid tissues where secondary diversification of the antibody repertoire takes place. Our observations on the rise of specific immunoglobulin levels in the sera justify the time-point for mRNA collection at days 3 and 5 after secondary infection in order to generate an optimal *E. acervulina*-specific antibody library. Inclusion of cDNA from the main mucosal lymphoid sites, the bursa of Fabricius, spleen and cecal tonsils ensured coverage of both the systemic and mucosal anti-*E. acervulina* immunoglobulin repertoire.

The applicability of the Eckα1 library was demonstrated with the selection of polyclonal and monoclonal antibody preparations directed against either a mix of *E. acervulina* antigens, or against more specific antigens like oocyst- or sporozoite fragments. The selected clones revealed high variability with more homogeneity in the light chains than in the heavy chains. This is in contrast to the findings by Andris-Widhopf et al. (2000), who showed sequence variability being predominant in the light chain repertoire. For mice, however, combinatorial light chain variability alone is able to build up a sufficiently complex repertoire to mount protective responses against a variety of pathogens (Senn et al., 2003). The predominance of heavy chain diversity in our anti-*E. acervulina* enriched libraries can possibly be explained by the complexity of the antigen mix used in the selection. This might also explain the lack of very strong binders among the individual clones studied.

High costs and safety restrictions limit commercial application of live parasite vaccination. Oral passive immunization using polyclonal antibodies directed against conserved epitopes of the genus *Eimeria* could represent a promising alternative. By oral delivery of a protective antibody mix, the natural mucosal immune response is mimicked preceding the host's own immune response and (partial) protection against several *Eimeria* strains can be provided by direct inactivation of invading parasites. The Eckα1 library presented here can be used to isolate binding antibodies against conserved epitopes of several *Eimeria* species. A comparable strategy has been

described recently, indicating that polyvalent neutralizing antibody formulations targeting epitopes on defined antigens may provide optimal prevention of cryptosporidiosis, a diarrhoeal disease caused by the *Eimeria*-related parasite *Cryptosporidium parvum* (Riggs et al., 2002; Schaefer et al., 2000). It has to be kept in mind, however, that little is known of the possible immunomodulating characteristics of orally administered IgA in poultry. But unlike the use of live vaccines, the controlled administration of IgA constructs does not impose any safety risks to the environment.

The pChick3 vector incorporates chicken constant domains in a combinatorial antibody library approach demonstrating that phage display technology can be extended to chicken IgA antibodies. The incorporation of the Cα1 sequence into the phagemid serves a double purpose. On the one hand, when IgA is the final goal, it could be of benefit to perform the selection steps in a format that closely matches the final product. On the other hand, it facilitates mass transfer of binders into IgA full-size antibodies intended for oral protection.

The present design responds to our global objective to extend passive oral immunotherapy to poultry, which requires in this case the expression of specific polymeric IgA complexes. In order to be therapeutically active in oral passive immunization approaches, chicken IgAs need to be produced as full-length antibodies, together with their partners in the sIgA complex, the J-chain and the secretory component (Corthesy and Spertini, 1999; Ma et al., 1998). In a next step, a system was constructed to rapidly transfer the Fab genes to vectors allowing expression of full-size, polymeric antibodies in tobacco plants (Wieland, 2004). Ongoing research focuses on further evaluation of the plant-expressed, secretory IgA for their parasite neutralizing activities and in vivo stability and functionality when administered orally.

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