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Isolation of phage-antibodies against *Eimeria* species that infect chickens

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ABSTRACT

Eimeria is one of the most economically important pathogens in poultry production. Diagnosis of infection has the potential to inform treatment and prevention strategies. Here, phage display technology was used to isolate single chain antibodies (scFvs) that had a broad specificity against oocysts from the seven pathogenic species of *Eimeria* found in poultry. Three such scFvs, representing 2 scFv HCDR3 motifs, were isolated by random picks of clones isolated after five rounds of iterative enrichment (panning) of phage against the seven *Eimeria* species. Phage-antibody binding to *Eimeria* oocysts was also interrogated using next generation sequencing of the HCDR3 region of scFv genes contained with phage particles. This analysis demonstrated that the most abundant scFv found after 5 rounds of panning accounted for over >90 % of scFvs. Furthermore, the three scFvs isolated from random picks of clones were the only antibodies that were enriched through each round of panning. They were also seen to be enriched through the stages of phage panning that included binding to the *Eimeria* oocysts (selection phase) and to be selected against during the stages that consisted solely of phage propagation (growth only phase). The NGS data was further analysed to identify an additional scFv that demonstrated specific enrichment against 3 *Eimeria* species at the third round of panning and had the same pattern of enrichment during the selection and growth phases of panning. Rescue and analysis of this phage-scFv demonstrated a binder with broad specificity for Eimeria species. The four antibodies with broad specificity detected all seven *Eimeria* species in immunoassays. The binding of one such scFv that recognised all species was further validated by fluorescent microscopy.

1. Introduction

Eimeria are protozoan parasites that can cause the disease coccidiosis in chickens. They are a major cause of economic losses in the poultry industry with seven pathogenic species: *E. praecox, E. brunetti, E. mitis*, *E. tenella, E. necatrix, E. acervulina* and *E. maxima* (Williams, 1999; Blake et al., 2020; Bennet and IJpelaar, 2005). Upon infection with *Eimeria*, the parasite invades the epithelial cells of the gut compromising chicken welfare (Shirley et al., 2005) and reducing productivity, often leading to relatively costly interventions. In a recent study by Blake et al. (2020) the economic loss due to coccidiosis was estimated to be £99.2 million in the UK and £10.4 billion worldwide (in 2016). High stocking densities in modern day commercial poultry establishments contribute to the parasite being of growing concern (Blake and Tomley, 2014; Chapman et al., 2013). Traditional methods of diagnosis such as identifying oocyst morphology by microscopic examination of litter or faecal samples, counting oocysts per gram of faeces and lesion scoring after post-mortem are still heavily relied upon, however these methods are subjective and prone to under-diagnosis of subclinical disease (Johnson and Reid, 1970; Haug et al., 2008).

Advances in molecular techniques have led to the development of molecular based diagnostics including polymerase chain reaction (PCR) (Schnitzler et al., 1998), quantitative PCR (qPCR) (Vrba et al., 2010) DNA fingerprinting and random amplification of polymorphic DNA PCR (RAPD-PCR) (Morris and Gasser, 2006; Fernandez et al., 2004). Such assays have been available since the late 1990's but have not gained widespread application likely due difficulties in obtaining template DNA, and the relative high costs including the need for specialist

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equipment. Loop mediated isothermal amplification (LAMP) assays have more recently been developed that can distinguish between the seven *Eimeria* species found in chickens. This offers a molecular assay with the potential of being in-field. However, these assays still require template DNA to be obtained by scraping mucosal cells from intestinal locations where the different *Eimeria* species are likely to be present and the method is therefore labour intensive and requires expertise knowledge (Barkway et al., 2015). There is a need to develop low cost, rapid tests for *Eimeria* diagnosis.

This study aimed to isolate recombinant antibodies against oocysts from all sevens species of *Eimeria* that infect chickens. Antibodies are a crucial component of any diagnostic development platform because of their diverse applications (Kuhn et al., 2016; Ch'ng et al., 2016). Recombinant antibodies have been successfully used in diverse diagnostic platforms including standard laboratory immunoassays and in-field lateral-flow assays (Ch'ng et al., 2016). Such a reagent that binds oocysts from all seven *Eimeria* species of poultry could underpin the development of assays for the direct detection of oocysts in faeces/litter and/or as immunoaffinity reagents to purify and concentrate such oocysts for subsequent analysis by previously reported DNA-based diagnostics.

Phage display is a powerful technology for the isolation of recombinant antibodies that bypasses the use of animals and links the phenotype of a displayed ligand (binding properties) to its genotype. Furthermore, the coupling of phage display and the screening power of next generation sequencing (NGS) has the potential to improve the discovery of novel antibodies against a target (Barreto et al., 2019; Ravn et al., 2013; Ravn et al., 2010; 'tHoen et al., 2012; Naqid et al., 2016b; Naqid et al., 2016a; Zhang et al., 2011). In the reported study, conventional antibody phage display was carried out against *Eimeria* oocysts from the seven pathogenic species of poultry. After five rounds of phage panning, three scFv's representing two HCDR3 motifs were identified that bound to all seven target species. Following the enrichment profile of these binders using NGS analysis, a further candidate binder was identified after three rounds of panning. This scFv demonstrated binding to all seven Eimeria species in both an ELISA and in fluorescent microscopy.

2. Materials and methods

2.1. Quantification and storage of Eimeria oocysts

Oocysts from seven species of *Eimeria* in chickens, *E. necatrix, E. brunetti, E. maxima, E. tenella, E. mitis*, *E. acervulina* and *E. praecox*, were kindly supplied by MSD Animal Health. The oocysts were in suspension within 50 ml of PBS and quantified using a haemocytometer as previously described (Phelan and Lawler, 1997). Amphotericin B was added at 25 μg/ml to prevent fungal growth (Lalonde and Gajadhar, 2009) and oocysts were stored at 4 °C until required.

2.2. Phage display biopanning to identify antibodies to whole Eimeria oocysts

Whole oocysts in solution were used as target antigen and binding antibodies were isolated from the Griffin-1 scFv-phagemid library (MRC, Cambridge, UK). The Griffin-1 human scFv library is a semi-synthetic library (Ch'ng et al., 2016; Strachan et al., 2002; Griffiths et al., 1994). The bacteria used for all phage propagation was *Escherichia coli* TG1 supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5, ($r_K^- m_K^-$) (F′ [traD36 proAB$^+$ lacI^q lacZΔM15]). Helper phage used throughout the study was Exphage (Ex12) which is derived from M13K07 helper but has two amber stop codons within the gIII (Oh et al., 2007).

For panning: approximately 4×10^5 oocysts of each *Eimeria* species were added to microcentrifuge tubes. The oocysts were centrifuged at 2000 ×*g* for 3 min, the supernatant discarded, the pellet resuspended in sterile 3 % (*w/v*) skimmed milk powder in PBS (1 ml) and incubated for 60 min at room temperature. The oocysts were centrifuged as before, the supernatant discarded and the pellet resuspended in 100 μl of sterile PBS. Acid precipitated scFv-library phage was blocked for 1 h with 3 % (w/v) skimmed milk powder in PBS. The phage (10^{13}) were then added to each microcentrifuge containing oocysts and incubated overnight at 4 °C;. Oocysts were centrifuged as before and the supernatant discarded. The oocysts were washed 4 times with 1 ml PBS containing 0.1 % (*v/v*) Tween 20 (PBST), followed by washing once in PBS. The phage were recovered from the oocysts by addition of 1.2 ml mid-log TG1 cells to resuspend the oocyst pellet and then incubation for 30 min at 37 °C. The bacteria were plated out on bioassay dishes containing 2YT agar supplemented with 150 μg/ml ampicillin and 1 % (w/v) glucose. A separate bioassay dish was used for panning against each of the seven *Eimeria* species. Bacteria were grown at 37 °C overnight. Colonies were scraped from the bioassay plates into 5 ml of 2YT media. Bacterial sub-libraries were stored separately as glycerol stocks at −80 °C and mixed prior to phage propagation for subsequent panning rounds. The second to fifth rounds of panning were carried out exactly as the first round except that the input phage was culture supernatant at ~10^{11} phage/ml.

The panning generated 35 bacterial glycerol stocks of phagemid (representing the enriched sub-library for each of the *Eimeria* target species for each of 5 rounds of panning). A further 5 phage samples were also obtained representing the input phage at each round of panning.

2.3. Phagemid clone analysis

The diversity of picked phagemid clones was determined by *Bst*NI fingerprinting as previously described (Gough et al., 1999). Briefly, phagemid DNA was isolated and purified using the Qiagen Spin Mini Prep Kit and the scFv gene PCR amplified with primers LMB3 (5′ CAGGAAACAGCTATGAC ′3) and PHEN (5′ CTATGCGGCCCCATTCA ‘3). Products from *Bst*NI digestion of the amplicons were analysed on a 3 % (*w/v*) agarose gel. A number of phagemid clones were also analysed by Sanger sequencing using primers LMB3 or PHEN to determine the full scFv amino acid sequence.

2.4. DNA extraction and preparation of sample for Ion Torrent sequencing

Phagemid DNA was isolated and purified from each phagemid sub-library (35 samples) using a Qiagen Spin Mini Prep Kit following the manufacturer's instructions. Single stranded phagemid DNA from the five samples representing the input phage for each round of panning was isolated using a Qiagen spin M13 kit following the manufacturer's instructions. A primer set specific to pHEN-Griffin-1 library vector sequences that flanked the HCDR3 gene region was used in a PCR reaction to amplify a ~ 270 bp fragment: VHFR2UniFNEW (5′ CTAGAACATTTCACTTACGGVMARGGNCTKGASTGG 3′) and JHFR4RevNEW 5′ GTAATCCTTGTGGTATCGTGARGAGACRGTGACCRKKGT 3′). PCR was carried out using high fidelity Platinum Taq polymerase with an initial denaturing step at 95 °C for 3 min, followed by a single PCR cycle of 95 °C for 30 s, 55 °C for 30 s and 30 s at 68 °C, then a further 28 cycles at 95 °C for 30 s, 60 °C for 30 s and 30 s at 68 °C. PCR products were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel), eluted within a 20 μl volume. A second round of PCR was performed with 1 μl of purified product from the initial PCR using a second set of primers to provide unique barcode sequences (underlined) for the purpose of sample identification and specific p1 and Akey sequences that are compatible with the Ion Torrent sequencing platform: P1primlinker2 (5′ CCTCTCTATGGGCAGTCGGTGATCTAGAACATTTCACTTAC 3′) and Akey-BC1-lnk1* (5′ CCATCTCATCCCTGCGTGTCTCCGACTCAGCTAAGGTAACGTAATCCTTGTGGTATCG 3′). A distinct barcode was used for each of the 40 samples and the PCR products were quantified by Qubit dsDNA BR Assay Kit, then pooled in equal amounts and resolved by agarose gel electrophoresis. The band of interest (~330 bp) was purified using the NucleoSpin Gel and PCR Clean-up. The product

was then purified with an AgencourtAMPureXP purification kit (Beckman Coulter, UK) following the manufacturer's protocol. DNA amplicon (50 μl containing 100-200 ng DNA) was sequenced via an Ion Torrent PGM service (University of Pennsylvania, US) on a 318 chip.

2.5. Analysis of next generation sequencing data

Perl scripts and common bioinformatics tools were used to process NGS data files (scripts used for data processing are provided at https://figshare.com/articles/software/scripts/23775447). Data was initially converted from FASTQ to FASTA format and demultiplexed using Flexbar (Dodt et al., 2012) in order to sort the amplicon sequences according to their barcode identifiers generating an individual FASTA file for each barcoded sample. The Griffin-1 library amplicons were sequenced in the reverse direction hence DNA sequences were reverse complemented using Seqtk (available from https://github.com/lh3/seqtk) before translation. DNA sequences were translated in each of the 3 forward reading frames and concatenated into one file using the Perl script translate.pl which used a custom translation table to differentiate between amber, opal and ochre stop codons in the peptide FASTA output file. N- and C-terminal peptide motifs flanking the HCDR3 region were identified, allowing a single amino acid change to the most common motifs VYYCAR and WGQGT at the N- and C- terminal, respectively. A minimum of 4 amino acids were required to be present in the HCDR3 sequence in order to be carried forward for further analysis, and the N-terminal motif, HCDR3 and C-terminal motif were all required to be in a single reading frame. All amber stop codons were denoted as a lower case q in peptide sequences. The frequency of clones seen at round 3 and round 5 of panning, calculated as a percentage of the total reads, are reported for identified binder sequences. Z scores were generated for each scFv based on the frequency and ratio of binding as described previously by Zhang et al. (2011) and Naqid et al. (2016a, 2016b). The Z scores define relative enrichment for each scFv and in this study the comparison was carried out through progressive rounds of panning, i.e. scFv enrichment against each individual *Eimeria* species when analysing phagemid sequences from bacterial glycerol stocks from round 2 to round 3 of panning were calculated. The scFvs were sifted such that only those that had more than 5 reads, and a Z score of at least 5 were retained for further analysis. Next, data for each of the HCDR3 sequences in phagemid bacterial stocks for each of the seven species were pooled for panning round 2 and also for round 3. Data was compared with input phage for rounds 2 and 3 of panning. The bacterial stocks (output) after round 2 were compared to the input phage for that round to generate Z scores (this phase of the panning is the selection of phage against the target antigen followed by propagation of phagemid in bacteria on a plate; designated the Selection Phase). The input phage for round 3 of panning was then compared to the output glycerol from round 2 of panning to generate Z scores (this phase of panning is the propagation of the phage in bacteria in solution; designated the Growth Phase). The same analyses were also carried out for the next round of panning. This analysis was aimed at identifying scFvs that were consistently enriched during Selection Phases but not during Growth Phases across rounds 2 and 3 of panning.

2.6. Phage-scFv ELISA to detect Eimeria oocysts

Approximately 4×10^5 oocysts in solution of each *Eimeria* species were dispensed into microcentrifuge tubes in duplicates and tubes incubated for 1 h at room temperature with 3 % (*w/v*) skimmed milk powder in PBS. They were then centrifuged at 3000 ×*g* for 3 min, the supernatant was discarded and the pellet resuspended in 100 μl of sterile PBS. Acid precipitated phage (~10^{12} phage/ml), at 400 μl per oocyst sample were blocked for 1 h with 200 μl of 18 % (*w/v*) skimmed milk powder in 6 x PBS. The blocked phage was then incubated with oocyst overnight at 4 °C with gentle rotating. Samples were centrifuged as before and oocysts washed 3 times with 1 ml PBST containing 0.1 % (*v/v*) Tween 20. The oocysts were washed once more with PBS. Anti-M13-HRP (1:5000) in PBS was blocked with 3 % (*w/v*) skimmed milk powder for 1 h before incubating (100 μl) with the oocyst preparation for 1 h. Oocyst samples were washed as before. The oocyst pellets were then resuspended in 150 μl of PBS and 100 μl of the preparation transferred into a well on Nunc Maxisorb® plates. TMB (3,3′,5,5′-Tetramethylbenzidine, 100 μl) was added and after 10 min absorbance was read at 630 nm. Some assays were repeated using filtration (through a 10 μm filter, Mobitec M1002S) to replace the centrifugation during wash steps. Anti-BSA scfv-phage was used as a control for all binding experiments.

2.7. Fluorescent microscopy to detect Eimeria oocysts

Oocysts of each Eimeria species (10^4) were spun down and resuspended in 500 μl 3 % (*w/v*) skimmed milk powder and incubated for 1 h at room temperature. They were then centrifuged at 3000 ×*g* for 3 min and washed once with PBS. Anti-oocyst or anti-BSA control phage scFv (10^{11} PFU/ ml) were blocked in 3 % (w/v) skimmed milk powder for 1 h and then incubated with the oocysts for 1 h rotating. The oocysts were washed three times with PBST containing 0.05 % (v/v) tween 20 and once with PBS. Rabbit anti-fd (1:2000) was blocked in 3 % (w/v) skimmed milk powder in PBS for 1 h and then incubated with the oocysts for 1 h at room temperature. Samples were washed as before and then incubated with anti-rabbit Alexafluor (1:2000) which had been blocked for 1 h with 3 % (w/v) skimmed milk powder. Samples were washed as before, oocysts resuspended in 100 μl of PBS and viewed using a Leica upright microscope at X40 magnification.

2.8. Statistical analysis

Graph Pad Prism version 7 (Graph Pad Software, Boston MA) was used for statistical analysis. For the comparison of ELISA signals for rescued antibody clones compared to the control scFv, samples were analysed in duplicate and the average value taken. Data were analysed using two-way ANOVA followed by a post hoc Tukey's multiple comparison test; statistical significance was defined as a p value ≤ 0.05. Data are presented as mean ± SD.

3. Results

3.1. Panning for Eimeria binders

Panning was carried out for five rounds. Ten random single colonies were picked from the fifth panning round against each individual *Eimeria* species. The diversity of the clones were assessed by *Bst*NI digestion patterns. Out of the seventy clones, a single pattern was present in 48 of the clones, for 23 that displayed possible diversity in their digestion patterns Sanger sequencing was carried out and three distinct clones were identified. The clones that were identified had HCDR3 sequences of DHTPKWq (present in 68 clones from the Sanger sequencing and *Bst*NI fingerprints), STQLP (1 clone) and STqHP (1 clone). The lower case q denoted a codon for the amber stop codon that was expressed as a Q due to use of an amber suppressor host strain. Of note is that all three clones had similar H chains with variation only in HCDR3 coupled with diverse L chains (Fig. 1).

An ELISA analogous to that described by Abi-Ghanem et al. (2008) for measuring binding to *E. tenella* sporozoites was carried out to measure phage-scFv binding to the seven species of *Eimeria*. For every assay, a control phage-scFv that had known binding activity to bovine serum albumin (BSA) was used. It was noted that oocysts could be lost in the assay during the washing steps and so each assay included duplicate analysis of binding. In addition, the assay was carried out twice and strong binding defined as significant binding above the control scFv in both assays, weak binding was defined as significant binding above the control scFv in one of the two assays and when no assay signal was observed above the control in either assay the scFv was noted as not

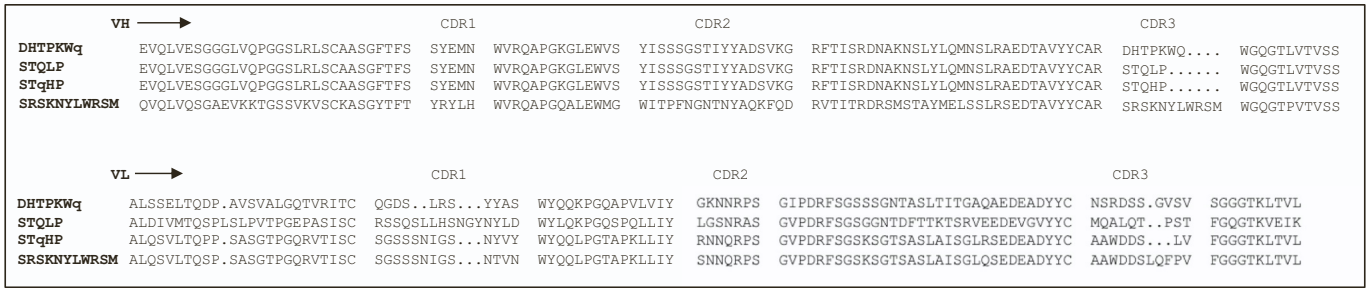

Fig. 1. Deduced amino acid sequences of scFvs against *Eimeria* oocysts. The scFvs were displayed on phage in the order of (N-terminal) VH-linker-VL. The linker was GGGGSGGGGSGGS. Clones designated DHTPKWq, STQLP and STqHP were identified from random picks after 5 rounds of panning. The clone designated SRSKNYLWRSM was identified from NGS analysis of phage isolated at rounds 2 and 3 of panning.

binding to the oocysts of that *Eimeria* species. The DHTPKWq scFv displayed no binding to *E. praecox*, weak binding to *E. acervulina* and strong binding to the other five species (Fig. 2A). The scFvs STQLP and STqHP that share a motif at HCDR3 both bound to all seven *Eimeria* species. STQLP displayed strong binding to six species, and weak binding to *E. mitix* (Fig. 2B). STqHP displayed strong binding to four species, and weak binding to *E. acervulina*, *E. praecox* and *E. mitix* (Fig. 2C). None of the anti-*Eimeria* phage-scFvs bound to the control antigen BSA in conventional ELISAs (data not shown). The ELISA was repeated using filtration to recover oocysts during washing steps (in place of centrifugation), all three scFvs were shown to bind to oocysts from all seven *Eimeria* species (SI, Fig. S1).

3.2. NGS analysis of panning enrichment for scFv selection

Colony picks at round five of panning identified 3 scFvs (with 2 HCDR3 motifs, Fig. 1) that bound to all species of *Eimeria*. NGS data was used to describe the enrichment of these 3 scFv binders through the panning process. This analysis confirmed the dominance of DHTPKWq scFv at round 5 where it was seen in 92 to 97 % of sequences across all seven species of *Eimeria* (Table 1). The scFv STqHP was the next most enriched antibody at round 5 contributing 1.29 to 5.30 % of the sequences, scFv STQLP had much lower enrichment at 0.07 to 0.23 % of sequences. Analysis of round 3 of panning revealed that the scFv population was much more diverse, with DHTPKWq accounting for between 6 % and 65 % of sequences recovered after binding to the different species. These data indicate the diversity at round 3 of panning may allow the isolation of further binders.

The study used Z score analysis as an estimation of enrichment of a phage binder (as used by Naqid et al., 2016a, Naqid et al., 2016b) between round 2 and 3 of panning. Using a Z score cut off value of >5.0 to define enrichment, only HCDR3 sequences DHTPKWq (and an scFv with a shared motif, DRTPKWq), as well as STqHP and STQLP were seen to be enriched against all 7 *Eimeria* species (Table 2). In total, 26 HCDR3 sequences were enriched against at least 3 *Eimeria* species. Within these sequences two clear motifs were present where the most dominant sequences were DHTPKWQ, as well as STQHP (SI, Fig. S2). Only 9 HCDR3s unrelated to these motifs were identified. Next, for both rounds 2 and 3, Z scores were determined for the Growth Phases and Selection Phases of the panning cycles. ScFv clones could be enriched due to binding to the target during the selection stage and/or growth advantages during the bacterial growth/phage production stages. We analysed whether these stages were distinct in terms of enrichment for our known scFv binders (Table 3). It was clear that for all three scFvs, they were enriched (Z score > 5.0) in round 2 and round 3 during the selection phases and were not enriched during the growth-only phases. Indeed, their numbers were clearly reduced during the growth-only phases indicating they did not have any growth advantage. Overall, data indicated that for rounds 2 and 3 of panning, the known binding scFvs were enriched against multiple *Eimeria* species and were consistently enriched during Selection Phases and selected against during Growth Phases. Of the 9 further scFvs HCDR3s that were enriched against 3 or 4 *Eimeria* species, only 3 were enriched in the Selection Phases (Table 3). One of these scFvs, HCDR3 sequence SRSKNYLWRSM, was also seen to be selected against during the Growth Phases, therefore matching the enrichment profiles of the known binders. SRSKNYLWRSM was recovered from the polyclonal round 3 phagemids using the method described by Spiliotopoulos et al. (2015). The clone was Sanger sequenced (Fig. 1) and used in an ELISA to assess binding to Eimeria oocysts (Fig. 2D). The scFv produced strong binding against all seven *Eimeria* species and did not bind to the control antigen BSA in a conventional ELISA (data not shown). To further validate this binding, the scFv SRSKNYLWRSM was used to detect Eimeria oocysts using fluorescent microscopy. Analysis clearly demonstrated that this scFv bound to a surface epitope on the oocysts (Fig. 3) and labelled oocysts from all seven species of *Eimeria* (Supplementary Fig. S3).

4. Discussion

Infection of chickens with *Eimeria* parasites can cause serious disease and can lead to significant economic losses (Williams, 1999; Blake et al., 2020). Despite the existence of several sensitive molecular techniques for the diagnosis of chicken coccidiosis, there is a heavy reliance on traditional techniques including faecal microscopy and post-mortem lesion scoring. Traditional techniques are generally subjective, require expertise and are time consuming (Long et al., 1976; Haug et al., 2008). LAMP assays are relatively inexpensive and have the potential for in-field application. However, at present, the LAMP assays for *Eimeria* require template DNA to be obtained from scrapping mucosal cells of the intestines where the different *Eimeria* species are likely to be located, this is labour intensive, requires expertise knowledge and extensive laboratory time (Barkway et al., 2015). With chicken coccidiosis still being a serious disease (Blake et al., 2021), with economic losses estimated globally in excess of £10 billion annually (Blake et al., 2020), it is imperative to develop diagnostics that are relatively inexpensive and that can ideally be applied in the field with minimal specialist knowledge or expertise. The development of specific immunoreagents that can detect all species of *Eimeria* that are pathogens of poultry could form the foundations for the development of relatively low-cost immunoassays.

Phage display technology has been used to successfully isolate antibodies to different targets from diverse libraries and can facilitate the isolation of binders with very defined binding specificity (Rouet et al., 2012). In this study, we have demonstrated that conventional phage display (panning followed by random colony picks) can be used to isolate antibodies that bind to oocysts of the seven economically important species of *Eimeria* in poultry. Furthermore, NGS analysis of phage panning outputs (so called next generation phage display; Barreto et al., 2019, Ravn et al., 2013, Ravn et al., 2010, 't Hoen et al., 2012, Naqid et al., 2016b, Naqid et al., 2016a, Zhang et al., 2011) was applied to describe the enrichment properties of these known binders and

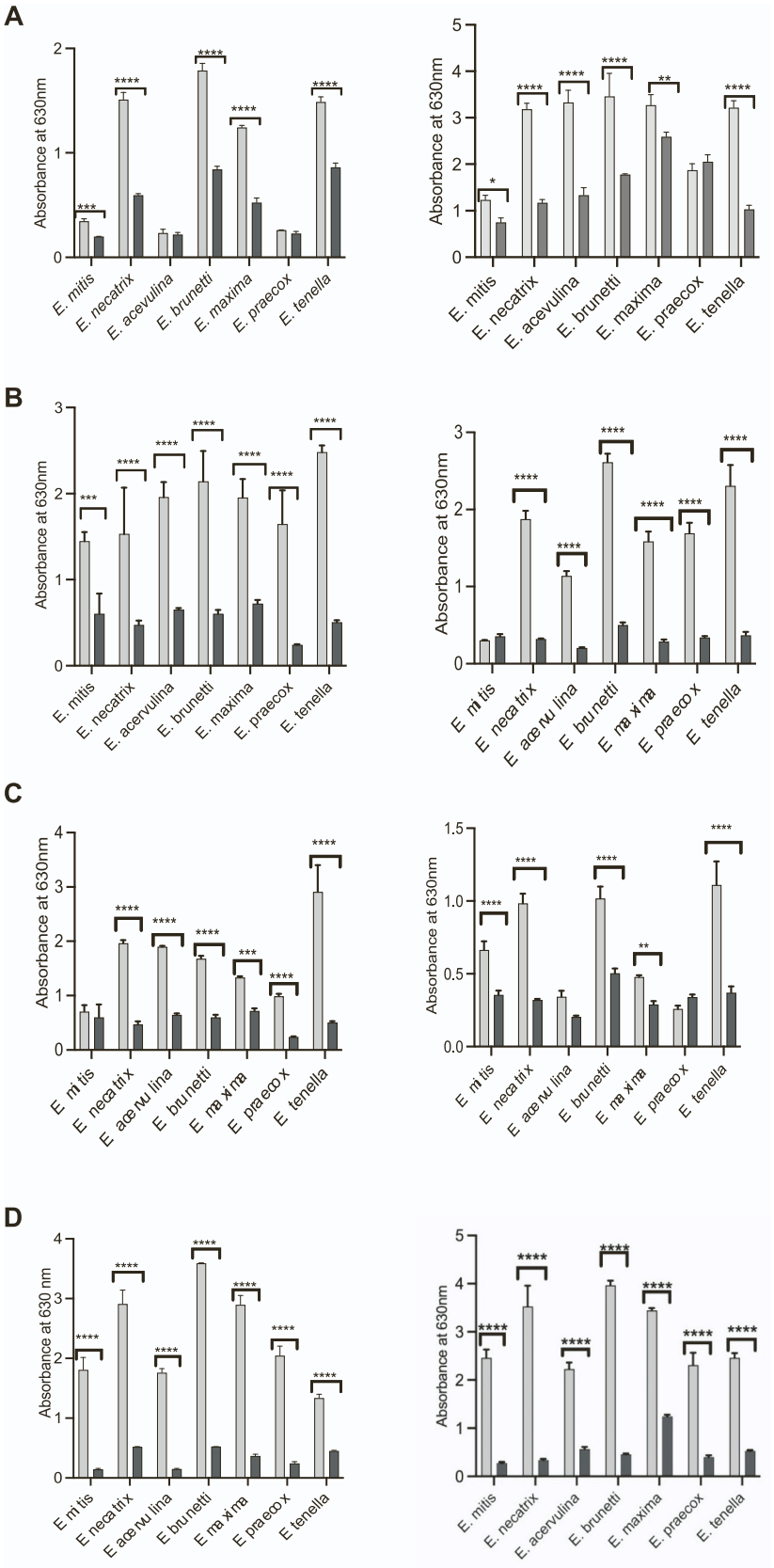

Fig. 2. Binding of scFvs to oocysts from different species of *Eimeria*. scFv with the HCDR3 sequences DHTPKWq (A), STQLP (B), STqHP (C) or SRSKNYLWRSM (D) were produced displayed on phage along with a control anti-BSA scFv-phage. Whole oocysts (10^5) were assayed in duplicate (left and right panels show these repeats) against both the test phage (light grey bars) and anti-BSA control phage (dark grey bars). Bound phage-scFv was detected with anti-M13-HRP. TMB substrate was incubated with samples for 10 mins before absorbance was read at 630 nm. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple

comparisons test to determine significant difference between binding of the scFv from panning and the control scFv. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****). Data represents the mean and standard deviation of two replicates.

Table 1
Frequency of scFvs at round 3 and round 5 of panning.

scFv[1]	Round 3[2]	Round 5[2]
DHTPKWq	6, 48, 25, 65, 17, 8, 64,	95, 92, 92, 97, 95, 96, 93
STQLP	0.01, 0.79, 0.21, 0.35, 0.23, 0.05, 0.58	0.09, 0.23, 0.23, 0.07, 0.17, 0.15, 0.18
STqHP	<0.01, 6.15, 1.45, 2.60, 1.32, 0.56, 7.21	2.10, 5.30, 4.43, 1.29, 2.72, 1.79, 3.91

[1] As defined by the HCDR3 sequence.
[2] Percentage of the total reads for *E. mitis*, *E. necatrix*, *E. acervulina*, *E. brunetti*, *E. maxima*, *E. praecox* and *E. tenella*, respectively.

identified a further scFv with similar enrichment traits that also bound to *Eimeria* oocysts.

The Griffin-1 scFv phage library was panned against whole intact individual *Eimeria* oocysts of the seven pathogenic species of chickens. Whole oocysts were the preferred target for this study because they are the most abundant form of the parasite and are present in poultry litter, making them a readily available sample type for immunoassays (Waldenstedt et al., 2001; Long et al., 1975; Gadelhaq et al., 2018). Panning on intact cells has also been reported to have the advantage of isolating antibodies against native epitopes of antigens in as close as possible state to that found in the field (Abi-Ghanem et al., 2008). Random colony picks were performed from the round 5 panning output glycerol stocks. A total of seventy single clones were picked and analysed for diversity by *Bst*NI fingerprinting and Sanger sequencing. This analysis revealed that a single clone accounted for ~97 % of the clones. Three binders were identified in total, all of which had similar VH domains with variation only in HCDR3 but were coupled with diverse VL domains. All three scFv bound to all seven species of oocysts in

Table 2
Enrichment of scFvs between round 2 and 3 of panning.

scFv[1]	*E. mitis*[2]	*E. necatrix*	*E. acervulina*	*E. brunetti*	*E. maxima*	*E. praecox*	*E. tenella*
DHTPKWq	172	723	486	291	392	245	1206
STqHP	54	105	105	20	99	66	248
STQLP	12	29	40	11	41	19	66
INCPL	31	19	37	5	30	–	–
LFQPq	–	–	35	–	–	17	14
pRSIP	–	11	8	–	9	7	–
PSHDQqFIP	–	–	10	5	38	–	–
QMPTG	21	7	–	–	14	–	–
RPVIHYAYKEG	27	–	–	–	34	–	15
RSYAEVV	–	10	8	–	6	–	–
SGRECFPYD	81	78	79	8	100	57	–
SRSKNYLWRSM	–	20	–	18	43	–	–

[1] Defined by the HCDR3 sequences. Sequences with Z scores > 5.0 between Rounds 2 and 3 for at least 3 Eimeria species are shown. This included the DRTPKWq, STqHP and STQLP sequences (in bold) identified from Round 5 picks as well as 9 unrelated sequences.
[2] Z scores are shown generated by comparing the sequence frequency for each HCDR peptide detected in round 3 of panning compared to round 2. Only Z scores greater than 5.0 are shown and are interpreted as defining enrichment, – indicates no enrichment.

Table 3
Enrichment of scFvs between phases of panning.

scFv[1]	Selection1[2]	Growth1[2]	Selection2[2]	Growth2[2]
DHTPKWq	372	-255	1365	-337
STQLP	67	-61	123	-62
STqHP	185	-109	341	-88
SRSKNYLWRSM	68	-66	62	-27
pRSIP	14	-5	27	1
RSYAEVV	7	3	20	4
PSHDQqFIP	-3	28	-9	0
INCPL	-9	42	-24	7
LFQPq	-6	75	-60	42
QMPTG	-24	49	-43	25
RPVIHYAYKEG	-2	36	-14	23
SGRECFPYD	-35	141	-104	118

[1]Defined by the HCDR3 sequence, sequences of known binders identified from round 5 picks are in bold.
[2]For rounds 2 and 3, Z scores were determined by comparing the phage input to output bacterial stock panning step (panning selection following by bacterial growth on plates; designated the selection phase, Selection1 for panning round 2 and Selection 2 for panning round 3) and the bacterial stock to phage generation step (bacterial growth in culture and phage propagation; designated growth phase; Growth 1 for panning round 2 and Growth 2 for panning round 3). Z scores > 5.0 was used to indicate enrichment (grey boxes).

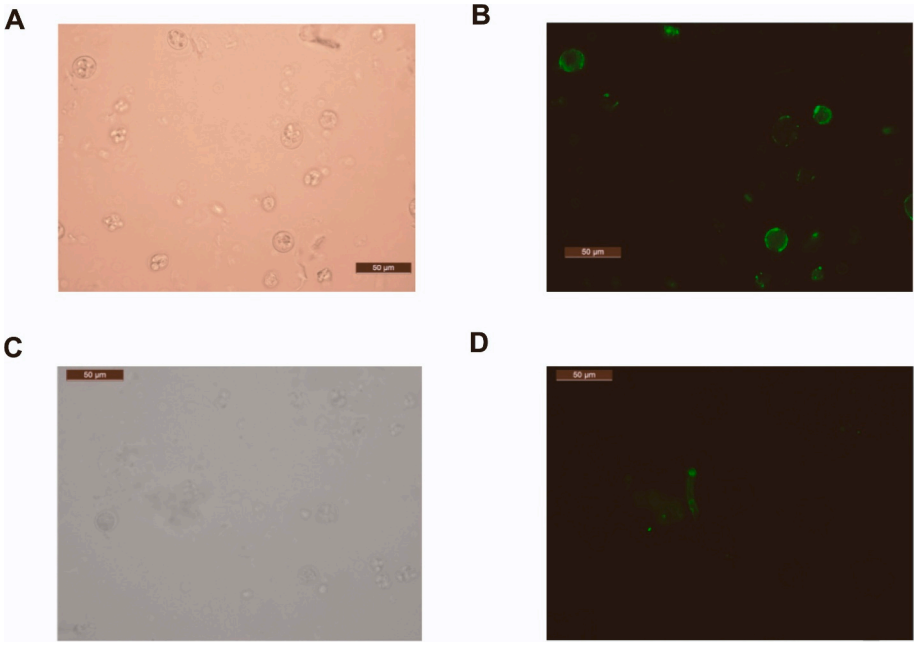

Fig. 3. Binding of scFv SRSKNYLWRSM to a surface epitope of *Eimeria* oocysts. The phage scFv was incubated with a pool of Eimeria oocysts from all seven species. Bound scFv-phage were labelled with rabbit anti-phage polyclonal antibody and then Alexafluor 488 goat anti-rabbit IgG. Oocysts were viewed and imaged using a Leica upright microscope. Binding of scFv SRSKNYLWRSM is shown in B and binding of the control anti-BSA scFv is shown in D. Scale bar (50 μm) is shown. Light field images are also shown (A and C).

immunoassays.

Conventional random colony picks have a limitation of providing a small perspective of the complete diversity of binders and can easily be dominated by nonspecific sequences or limited numbers of true binders (Liu et al., 2015). Clonal dominance may reflect selection for limited numbers of high affinity binders or binders to a common epitope, but may also reflect certain clonal properties such as superior growth advantage or expression (Rouet et al., 2018). It is not uncommon for high affinity binders to be present at low frequency, and these may not be detectable with conventional random picks (Rouet et al., 2012). It is now well established that the application of NGS analysis of antibody genes isolated after panning can increase the number of positive clones obtained compared to conventional screening by random picks (Yang et al., 2017; Ravn et al., 2010; Ravn et al., 2013; Barreto et al., 2019; Ljungars et al., 2019). Positive clones have been described in phage populations at abundances of 0.01 % (Yang et al., 2017) or 0.005 % (Ljungars et al., 2019) demonstrating that random picks would be unlikely to identify such clones. In terms of which round to analyse, it has been suggested that the second and third panning rounds may be the most informative in retaining relatively high diversity compared to later rounds whilst having undergone sufficient enrichment compared to earlier rounds to enable identification of binders (Zambrano et al., 2022). Here, clone diversity was very limited after 5 rounds of panning and so analysis was carried out on rounds 2 to 3 to identify scFvs that were reproducibly enriched between these panning rounds against multiple *Eimeria* species. Furthermore, bioinformatics analysis removed all clones that had a frameshift within the HCDR3 or that contained an opal or ochre stop codon. As previously reported, the present study used the HCDR3 sequence to represent the scFv as this region usually has the highest contribution to antibody binding (Mitchell and Colwell, 2018). This approach does present a limitation for NGS approaches that analyse short reads. Other regions will contribute to binding and are not being represented in the data. As such the approach may miss potential binders that are enriched through non-HCDR3 regions. Recent advances have looked to overcome this, Barreto et al. (2019) report a method to strip out the framework regions and directly link CDR regions before sequencing; whilst Moura-Sampaio et al. (2022) applied Illumina's cluster mapping capability to pair non-overlapping reads of V_L and V_H regions allowing selection of binders based on whole recombinant antibody sequences. Also, Z scores were used as a measure of relative enrichment (Zhang et al., 2011; Naqid et al., 2016a; Naqid et al., 2016b), in this case when comparing sequential rounds of panning or sequential phases of the panning cycle. Data demonstrated that 26 HCDR3 sequences were enriched against at least 3 *Eimeria* species between rounds 2 and 3, but within these sequences only 9 were unrelated to the three sequences identified by random picks at round 5.

The removal of target-unrelated clones is more challenging, these clones can be enriched during panning due to binding to blocker or any solid supports or due to propagation advantages. Here, analysis was carried out to determine whether the scFvs that were repeatedly enriched between rounds 2 and 3 were enriched during the selection phases of panning or during the growth phases. When analysing the known binders isolated by conventional picks, it was clear they had consistent enrichment traits, they were highly enriched against all seven species between rounds 2 and 3, they all had high level enrichment during the selection phases of panning over these rounds and their relative abundance was reduced during the growth phases. Here, only 1 of the 9 distinct enriched HCDR3s had this same enrichment profile, SRSKNYLWRSM. This clone was recovered and shown to produce binding against all seven *Eimeria* species in both ELISA and fluorescent microscopy. Interestingly, this scFv was not enriched from round 3 to 4, or from round 4 to 5, (data not shown) presumably as it was outcompeted by the dominant DHTPKWq clone due to high affinity, binding to a common epitope, superior growth advantage or expression, or a combination of these factors.

To our knowledge, this is the first report considering phage-antibody panning stages to inform antibody clone selection, however this has recently been reported for peptide-phage display where motifs could be seen to be enriched through either growth-related or target-specific selection pressures (Braun et al., 2020). Together, these studies indicate that such analysis may provide another tool to improve the identification of true binders before rescue and testing. Using conventional and NGS based scFv selection, the study also successfully identified multiple recombinant phage-antibodies that have broad specificity against all

seven *Eimeria* species that are pathogens in poultry. Such antibodies will require further development as soluble antibody formats, for instance to overcome expression limitations due to amber stop codons (Wu et al., 2007; Barderas et al., 2006). Once developed further, they could underpin the development of rapid, cost-effective assays to this pathogen.

CRediT authorship contribution statement

Mary T. Angani: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Jonathan P. Owen:** Writing – review & editing, Methodology, Formal analysis. **Ben C. Maddison:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Kevin C. Gough:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2024.113759.

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