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# Enrichment of a single clone from a high diversity library of phage-displayed antibodies by panning with *Anopheles gambiae* (Diptera: Culicidae) midgut homogenate

**G.F. Killeen[1,2]*, B.D. Foy[1,3], R.H. Frohn[1], D. Impoinvil[1], A. Williams[4] and J.C. Beier[1]**

[1]Department of Tropical Medicine, Tulane School of Public Health and Tropical Medicine, 1430 Tulane Avenue SL29A, New Orleans, LA 70112, USA: [2]International Centre for Insect Physiology and Ecology, Nairobi, Kenya: [3]Molecular and Cellular Biology Program, Tulane University Health Sciences Center, New Orleans, Louisiana, USA: [4]Cambridge Antibody Technology Ltd, The Science Park, Melbourn, Cambridgeshire, UK

## Abstract

A high diversity library of recombinant human antibodies was selected on complex antigen mixtures from midguts of female *Anopheles gambiae* Giles. The library of phage-displayed single chain variable region fragment constructs, derived from β-lymphocyte mRNA of naïve human donors, was repeatedly selected and reamplified on the insoluble fraction of midgut homogenates. Five rounds of panning yielded only one midgut-specific clone, which predominated the resulting antibody panel. In *A. gambiae*, the epitope was found throughout the tissues of females but was absent from the midgut of males. The cognate antigen proved to be detergent soluble but very sensitive to denaturation and could not be isolated or identified by Western blot of native electrophoresis gels or by immunoprecipitation. Nevertheless, immunohistology revealed that this sex-specific epitope is associated with the lumenal side of the midgut. Severe bottlenecking may limit the utility of phage display selection from naïve libraries for generating diverse panels of antibodies against complex mixtures of antigens from insect tissues. These results suggest that the selection of sufficiently diverse antibody panels, from which mosquitocidal or malaria transmission-blocking antibodies can be isolated, may require improved selection methods or specifically enriched pre-immunized libraries.

## Introduction

The midgut is not only the primary organ for food digestion in many insects but also the site of pathogen entry for the transmission of many arthropod-borne pathogens into their respective vectors (Billingsley & Lehane, 1996). The midgut of mosquitoes has become a particular focus for molecular research in recent years as these insects are vectors for some of the world's most problematic human

*Address for correspondence:
Department of Public Health and Epidemiology, Swiss Tropical Institute, Socinstrasse 57, CH-4002 Basel, Switzerland
Fax: +41 (0)61 2717951
E-mail: Gerry.Killeen@unibas.ch

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diseases, including malaria, dengue, filariasis and yellow fever. Antibodies against midgut antigens can not only allow elucidation of its physiology at the molecular and cellular level but also block pathogen invasion and reduce the fitness of vector mosquitoes (Lal *et al.*, 1994, 2001; Ramasamy *et al.*, 1997; Wang *et al.*, 2001; Almeida & Billingsley, 2002). Recombinant antibodies have the added advantage of being readily manipulated, propagated and delivered to blood-feeding vectors in bacterial hosts (Durvasula *et al.*, 1999; Killeen *et al.*, 2000; Yoshida *et al.*, 2001). Phage-displayed recombinant proteins and peptides can be rapidly selected from large libraries to yield high affinity probes against specific epitopes, antigens or antigen mixtures and subsets (Vaughan *et al.*, 1996, 1998; Osbourn *et al.*, 1998a,b; Edwards *et al.*, 2000), including insect tissues (Marzari *et al.*, 1997; Kasman *et al.*, 1998; Ghosh *et al.*, 2001; Foy *et al.*, 2002). Phage display selection has enabled isolation of a peptide that binds to salivary glands and the midgut lumen, resulting in suppression of malaria parasite invasion of both these tissues (Ghosh *et al.*, 2001; Ito *et al.*, 2002). Furthermore, the availability of diverse panels of recombinant antibodies may enable high throughput screening for molecules with pathogen transmission-blocking or directly insecticidal activities against important arthropods (Killeen *et al.*, 2000). Previously we have used membrane-immobilized mixtures of antigens, from the malaria vector *Anopheles gambiae* Giles (Diptera: Culicidae), to select a phage-displayed antibody specific for carbohydrate moieties on the lumenal surface of the midgut but were unable to generate more diverse panels of antibodies suitable for high throughput screening (Foy *et al.*, 2002).

This paper describes an alternative approach, in which the same phage-display library of single chain variable region fragment (scFv) antibody constructs, containing $>10^{10}$ different clones, was selected upon suspensions of midgut tissue homogenate from *A. gambiae*. Although the midgut suspension used to pan the library contained a diverse array of antigens, selection resulted in the enrichment and isolation of one midgut-binding scFv clone with sex specificity for an epitope in the midgut of this mosquito.

## Materials and methods

### *Mosquitoes*

Larvae of *A. gambiae* (strain G3) and *Anopheles freeborni* Aitken (Diptera: Culicidae) were reared on ground TetraTabMin® fish food tablets whereas larvae of *Aedes aegypti* Linnaeus (Diptera: Culicidae) were reared on ground liver powder until pupation. Adults were maintained on cotton balls soaked in a 10% diluted Karo™ syrup. Adult mosquitoes were reared and maintained in an insectary on a 14 h:10 h light-dark cycle at 27°C and ~80% relative humidity and usually kept for 3–10 days before use.

### *Phage display selection of midgut-binding antibody fragments*

Midgut-binding recombinant antibodies were selected by five rounds of affinity panning from a high-diversity library of single-chain variable region fragments (scFv) constructs. The scFv were assembled from heavy and light chain sequences derived from β-lymphocytes of naïve human donors, inserted, expressed and manipulated in the phage display vector pCANTAB-6, as described in detail elsewhere (Vaughan *et al.*, 1996). For each selection round, 25 *Anopheles gambiae* midguts were dissected in phosphate-buffered saline (PBS) containing a cocktail of protease inhibitors (Sigma, USA) and frozen in a 1.5 ml microcentrifuge tube containing 1 ml of PBS plus protease inhibitors. The midguts were thawed and pelleted by centrifuging at $1000 \times g$ for 1 min, following which the supernatant was removed and the pellet ground vigorously on ice with a polypropylene micropestle. The pellet was the recentrifuged briefly after resuspending in 1 ml of cold PBS-inhibitors and ground again. The pellet was then washed twice by resuspending in 1 ml of cold PBS plus 0.1%Tween-20 (PBS-T) plus inhibitors, centrifuging at $8000 \times g$, 4°C for 10 min and removing the supernatant. This preparation of coarse, loosely ground insoluble midgut homogenate was then blocked on ice for 1 h by agitating in 1 ml of 100 $\mu g\ ml^{-1}$ bovine serum albumin, 2 mM EDTA and $10^{15}$ tu $ml^{-1}$ of M13K07 helper phage in cold PBS-T plus inhibitors. EDTA and helper phage were included to prevent non-specific binding by the hexahistidine tag of the scFv construct and by phage coat proteins, respectively. Simultaneously, $10^{12}$ tu of Cesium-banded and purified phage from the full library or subsequently selected subsets were blocked on ice for 1 h in 1 ml of the same mixture. The blocked midgut homogenate suspension was then centrifuged at $8000 \times g$, 4°C for 10 min and the supernatant removed. The resulting pellet was then resuspended in the blocked phage mixture, and agitated on ice for 1 h. The midgut suspension was then pelleted by centrifuging at $8000 \times g$, 4°C for 10 min and unbound phage were then removed by removing the supernatant. Further washes to remove unbound phage were achieved by resuspending in 1 ml of cold PBS-T using a vortex, repelleting and removing the supernatant. This washing procedure was repeated a total of three times with PBS-T and three times with PBS. The suspension was transferred to a fresh microcentrifuge tube for the final pelleting step to remove phage that may have adhered to the inside of the original incubation tube. After removing the supernatant the pellet was immediately resuspended in 1 ml of 100 mM triethylamine and allowed to stand for 15 min. The mixture was then neutralized with 0.5 ml of 1.5 M Tris-HCl, pH 7.4 and stored at 4°C until titred and propagated by transforming log-phase *Escherichia coli* TG1 cells (K12, Δ(lac-pro) supE thi hsdΔ5 [F′ traΔ36 proA$^{+}$B$^{+}$ lacI$^{q}$ lacZΔM15]) as previously described (McCafferty and Johnson, 1996). Note that phage were also titred on 2TY agar plates (McCafferty and Johnson, 1996) containing both 100 $\mu g\ ml^{-1}$ ampicillin and 50 $\mu g\ ml^{-1}$ kanamycin to confirm that no host *E. coli* were co-transformed with both the ampicillin-resistant scFv-bearing phage and the kanamycin-resistant helper phage. This selection process was repeated five times and all handling, maintenance, propagation and purification procedures were carried as previously described (McCafferty & Johnson, 1996). As controls, identical selections were carried out using phage derived from the pCANTAB-6 vector without insert for each round and, as enrichment became apparent after the second round, with scFv-displaying phage but no midgut antigen.

### *Phage enzyme-linked immunoabsorbant assay (ELISA)*

Midguts from 100 unfed 3–6 day old *A. gambiae* were dissected, frozen and ground in a microcentrifuge tube with a micropestle in PBS plus protease inhibitors. The midgut

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homogenate was supended in 10 ml of ice-cold PBS plus inhibitors and 100 μl allowed to coat overnight at 4°C in each well of a polyvinyl chloride microtitre plate, at an equivalent of 1 midgut per well. As negative controls, replicate plates were coated with PBS-inhibitors alone or with 100 μg $ml^{-1}$ BSA, avidin or bovine α-thyroglobulin. The plates were then washed twice with PBS and blocked in 2% milk solids, 0.1% Tween, 2 mM EDTA in PBS for 2 h at room temperature. In the meantime scFv-displaying and control M13K07 or pCANTAB-6 phage (Vaughan *et al*., 1996) were blocked by diluting to $10^{12}$ tu $ml^{-1}$ in the same blocker for 1 h at room temperature. For screening large numbers of individual clones, crude phage preparations were cultured in microtitre plates and approximately 100 μl of phage-containing culture supernatant was mixed with 100 μl of this blocker at twice the above concentrations (McCafferty & Johnson, 1996; Vaughan *et al*., 1996). The blocked wells were then rinsed once with PBS-T, the blocked phage added and allowed to stand at room temperature for 1 h. The wells were washed six times with PBS-T and then incubated with mouse monoclonal anti-M13-horseradish peroxidase (HRP) conjugate (Pharmacia), diluted 1/5000 in 2% milk solids, 0.1% Tween in PBS (MPBS-T) for 1 h at room temperature. The secondary antibody was then removed and the plate washed six times with PBS-T before developing with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) substrate (Pierce), and the optical density measured at 405 nm.

### *Dot blot detection of phage and scFv reactivity*

Antigen extracts were prepared by thawing frozen midguts or other tissues from 100 mosquitoes in 1 ml of PBS-inhibitors, pelleting them, removing the supernatant and then vigorously grinding with a micropestle in 100 μl of cold 5% Tween-20 in PBS-inhibitors. The suspension was allowed to stand on ice for 15 min, diluted to 1 ml in PBS-inhibitors and centrifuged at 10,000 $\times g$ for 15 min to remove detergent-insoluble material. Aliquots of 10 or 20 μl of this extract, equivalent to one or two midguts were then diluted in 500 μl of cold PBS and immobilized as dots on polyvinylidine fluoride membranes (Amersham, UK) blotted using a vacuum manifold dot-blot apparatus (Bio-Rad, USA). These membranes were blocked overnight in 5% MPBS-T. Membrane strips to be probed with the same phage preparation were cut out and incubated separately under steady agitation for 1 h at room temperature with $10^{10}$ tu $ml^{-1}$ phage diluted and blocked in 3% MPBS-T. The membranes were then washed 6–10 times in 3% MPBS-T, incubating for 15 min with each wash. The membranes were then incubated with pre-blocked anti-M13-HRP (1/2000 in 2% MPBS-T), washed six times in PBS-T and visualized using enhanced chemiluminescent reagents and film (Amersham, UK). Where extracts from different parts of various mosquito species were compared at equivalent protein concentrations, the extracts were diluted to the same protein concentration in PBS-T, using a detergent-compatible Lowry procedure (Bio-Rad, USA) to assay protein concentrations in the original extracts. Antigen dot blots were probed with purified scFv using the same procedure, except that all washes used PBS-T without milk solids and 1/200 mouse anti-c-*myc* 9E10 monoclonal and 1/2500 HRP-conjugated sheep anti-mouse IgG were used as primary and secondary antibodies respectively. ScFv was expressed in 500 ml cultures of the F2 clone using an adapted induction procedure (Killeen *et al*., 2000) and then extracted and purified as previously described (McCafferty & Johnson, 1996; Vaughan *et al*., 1996). All electrophoresis, Western blot, immunoprecipitation and biotin labelling procedures were carried out using standard methods (Ausubel *et al*., 1998).

### *Clone isolation, insert fingerprinting and sequence analysis*

Individual transformants from the fourth and fifth selection rounds were isolated by picking colonies from agar plates and growing crude phage preparations in culture supernatants as previously described (McCafferty & Johnson, 1996; Vaughan *et al*., 1996). Clones were then individually screened for reactivity with midgut antigens by ELISA as described above, using uncoated wells and pCANTAB-6 phage as negative controls. For genotyping by restriction fragment fingerprinting and sequence analysis, colonies of bacteria harbouring single clones were picked with a sterile pipette tip and put directly into PCR buffer for scFv insert amplification. The insert expression region was amplified by the following vector primers: forward 5′-AGCGGATAACAA TTTCACACAGG-3′, reverse 5′-GTG-GTGTTTCCAGACGTTAGT-3′: with 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by 10 min extension cycle at 72°C. These insert amplicons were then digested and analysed by gel electrophoresis as previously described (McCafferty & Johnson, 1996; Vaughan *et al*., 1996). For clone sequencing, these PCR amplicons were purified with the Concert PCR purification system (Life Technologies, USA) and sequenced using an automated sequencer with the following inner primers: heavy chain, 5′-ACCGCCAGAGCCACCTCCGCC-3′; light chain, 5′-CTCTTCTGAGATGAGTTTTTG-3′.

### *Immunohistochemistry*

Individual midguts dissected in cold PBS-inhibitors, in some cases turned to expose the lumenal surface, were placed in single wells of immunofluorescence assay slides and fixed immediately as previously described (Shahabuddin & Pimenta, 1998). They were then blocked on ice for 1 h with 20 μl of 1% BSA in PBS-T before removing the blocker and replacing immediately with 20 μl of $10^{13}$ tu $ml^{-1}$ pCANTAB-6 or F2 phage in the same blocker. Each midgut was washed six times with 20 μl of blocker and incubated with 20 μl of cold 1/200 anti-M13-HRP in the same blocker for 1 h before washing six times with PBS-T and developing with 3,3′-diaminobenzidine substrate (Sigma, USA). In order to objectively quantify differences in staining intensity, one author (GFK) was blinded to the identity of the phage whereas the other (BDF) was blinded to their arrangement on the slides so that neither knew whether scFv-displaying (F2) or control (pCANTAB-6) phage had been applied to each midgut. Both authors then scored the staining intensity of each entire midgut at 100 × magnification, as an integer value between 0 and 5. Either the haemocoel surface of intact midguts or the lumenal surface of exposed midguts was scored, depending on how each individual midgut was prepared. The staining intensity of midguts stained with F2 and pCANTAB-6 phage were compared statistically using Spearman's ρ correlation test because this non-parametric test makes no assumptions about the structure of the data and only determines whether one group is consistently higher or lower than the other.

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## Results

Enrichment of midgut-binding clones became apparent from the output titres of the second and subsequent rounds of panning (fig. 1a). Indeed, the fifth round of panning produced outputs more than two orders of magnitude higher than equivalent selections without antigen or using pCANTAB-6 phage lacking scFv inserts (fig. 1a). ELISA assays confirmed that only the fourth and fifth selection rounds were sufficiently enriched to react with *A. gambiae* midgut preparations (fig. 1b).

Restriction fragment fingerprinting of individual clones (24 from each from the fourth and fifth rounds) revealed three distinguishable patterns, all coming from amplicons consistent with full-length scFv inserts. Of these, one dominant (31/48 clones) pattern was consistently and specifically reactive with *A. gambiae* midgut preparations as determined by ELISA (fig. 2). The isolate finally stocked for further work was designated F2 and sequence analysis of this clone revealed high sequence similarity with Ig heavy chain variable region germline sequence 3 (IGHV3) and Ig light chain variable region germline sequence 1 (IGLV1).

Low quantities of scFv could be induced and purified from bacterial cultures of the F2 clone and although this scFv reacted strongly with dot-blotted *A. gambiae* midgut extracts, scFv-displaying phage proved to be a reagent which was much more readily prepared, purified and stored in large quantities. Furthermore, phage-displayed scFv exhibited far greater sensitivity in dot-blots and ELISA, a format in which scFv alone failed to generate any useful signal. F2 phage reacted specifically with preparations from midguts of *A. gambiae*, *A. freeborni* and *Aedes aegypti* but not bovine serum albumin (BSA), avidin or bovine α-thyroglobulin, in a manner very similar to that of the phage pool from which it was isolated (fig. 2).

Dot blot analysis using phage showed that F2 reacted with an epitope which was predominantly located in the head of female *Anopheles gambiae* with smaller amounts in the midgut and abdomen (fig. 3a). Interestingly, although the epitope is found in the heads and abdomens of both sexes, its expression in the midgut is sex-specific and occurs only in females (fig. 3a). Although specific to the midgut of females, it does not seem to be expressed in higher quantities in blood-fed females (fig. 3b). Nevertheless, the abundance of this epitope increased steadily in the first three days after eclosion (fig. 3c), coinciding with maturation of the adult mosquito and the beginning of the teneral period, suggesting that it may have a role to play in bloodmeal digestion.

As previously described for another scFv that binds to carbohydrate moieties (Foy *et al.*, 2002), immunohistology revealed that the lumenal side of the *A. gambiae* midgut was stained more intensely by F2 than by pCANTAB-6 phage ($P$ = 0.017 and 0.002, Correlation coefficient = 0.671 and 0.807, by Spearman's ρ correlation test (n = 12) for intensity scoring by authors GFK and BDF, respectively). Although the haemocoel side of midguts stained consistently darker than the lumenal side, this appeared to be largely non-specific and no statistical difference between the test and control phage was observed (P = 0.268 and 0.113, Correlation coefficient = 0.348 and 0.481, by Spearman's ρ correlation test (n = 12) for intensity scoring by GFK and BDF, respectively).

Although the antigen could be stably extracted with Tween-20 and Triton-X100, it was otherwise very sensitive to denaturation by a variety of other detergents, including sodium dodecyl sulphate. The epitope also lost reactivity if exposed to methanol concentrations above 1% and all attempts to probe Western blots of native gels transferred with these low methanol concentrations, using a range of detergent concentrations and buffers, as well as electrophoresis and transfer protocols, failed to detect the antigen. Similarly, a variety of procedures to immunoprecipitate the cognate antigen of F2, using either phage or scFv, failed. Following failure of standard protocols using immobilized protein G and the anti-c-*myc* antibody to precipitate the scFv and its bound antigen from Tween extracts, several additional approaches were explored in

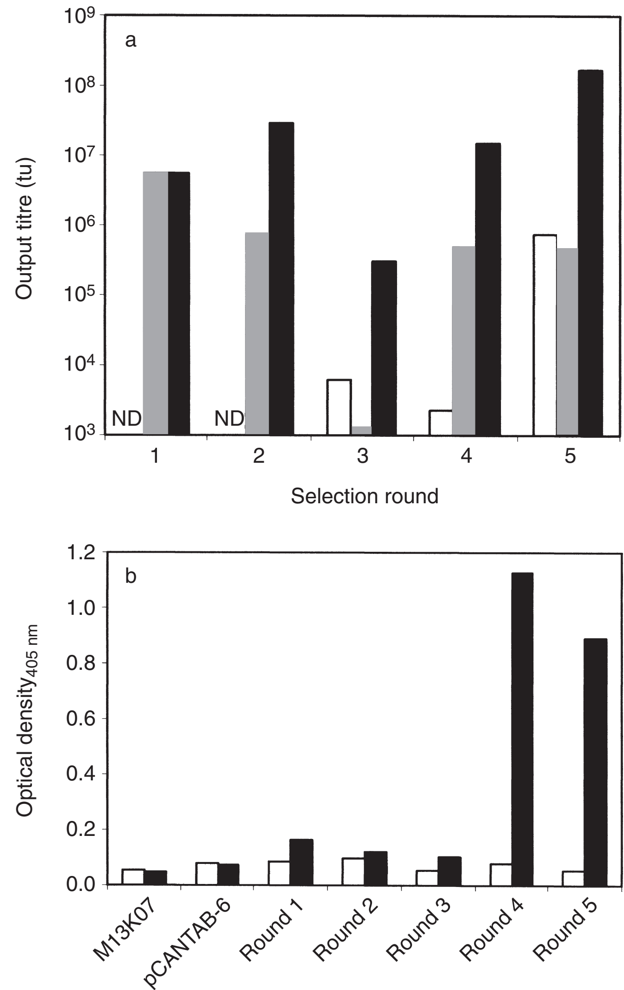


Fig. 1. Enrichment of *Anopheles gambiae* midgut-binding scFv-displaying phage clones by successive rounds of panning. (a) Output titres of each selection round: empty bars, no antigen control plus scFv-displaying phage; grey bars, antigen plus pCANTAB-6 control phage; solid bars, antigen plus scFv-displaying phage; ND, not determined. (b) ELISA reactivity of control phage (pCANTAB-6 and M13K07) and phage from each selection round: empty bars, no antigen controls; solid bars, midgut antigen coated.

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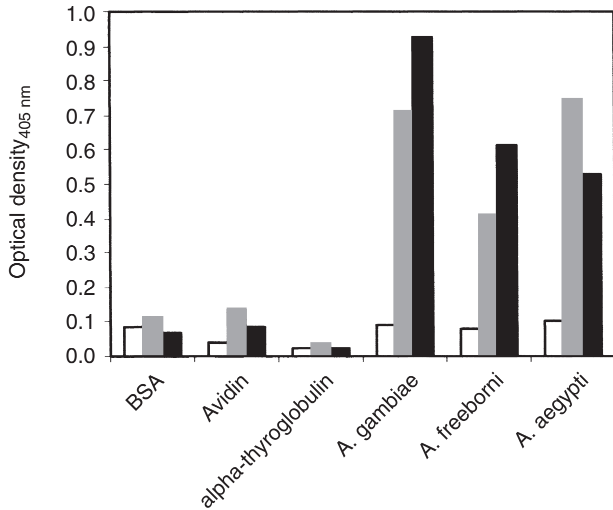


Fig. 2. Antigen specificity of F2 scFv-displaying phage compared with control phage lacking an scFv insert (pCANTAB-6). ELISA reactivity with control antigens and homogenates of midguts from female *Anopheles gambiae*, *A. freeborni* and *Aedes aegypti* mosquitoes. Empty bars, antigen plus pCANTAB-6 control phage; grey bars, antigen plus mixed scFv-displaying phage from the fifth selection round output; solid bars; antigen plus F2 scFv-displaying phage.

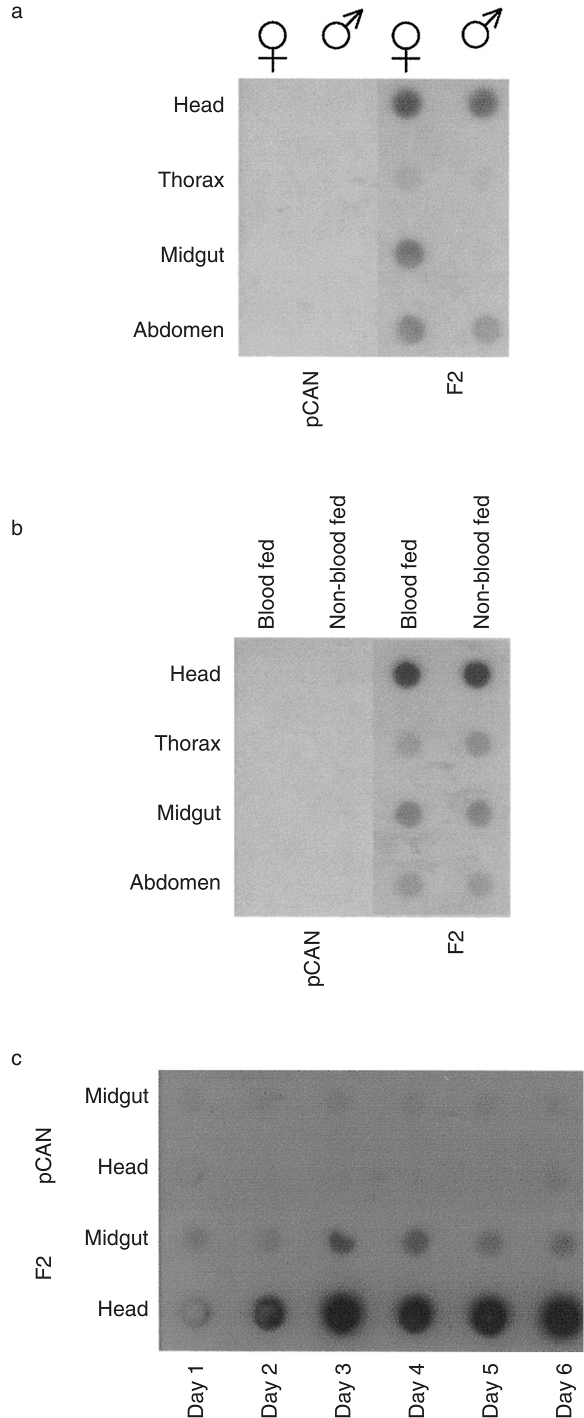


Fig. 3. Tissue localization, sex specificity and ontogeny of the cognate epitope of F2 scFv-displaying phage within *Anopheles gambiae* mosquitoes. Reactivity of F2 and pCANTAB-6 control phage with dot blotted antigen mixtures from different parts of: (a) males and females (both 8 days old); (b) blood fed (24 h after feeding) and non-blood fed females of the same age (10 days), (c) females of different ages. For (a) and (b), protein concentration in each extract was measured and 0.5 µg applied to each dot whereas in (c) all protein extracted from respective tissues were blotted at an equivalent of two mosquitoes per dot.

order to precipitate sufficient protein for sequence analysis and possibly cloning. The antigen could be successfully biotinylated without compromising reactivity, as shown by trapping biotinylated antigen extracts on avidin-coated ELISA plates and probing with F2 phage. Nevertheless, a variety of protocols failed to yield any specific bands in Western blots of immunoprecipitates probed with avidin-HRP. Similarly, gold stained electrophoresis of extracts precipitated with biotinylated F2 phage and streptavidin-coated beads did not yield any identifiable bands associated with the specificity of the scFv.

## Discussion

The F2 clone is the first recombinant antibody the authors are aware of with specificity for a sex-specific epitope in an insect midgut. However, additional probes for this antigen, with specificities for more robust epitopes, will clearly be required to study this antigen in more detail. One of the major shortcomings encountered in the selection of phage-displayed antibodies on insect tissues is the lack of clonal diversity found in enriched pools after several rounds of panning. Other quite different procedures for selecting of antibodies (Foy *et al.*, 2002) and peptides (Ghosh *et al.*, 2001), have also yielded a single cognate clone, despite the huge diversity of antigens and epitopes in the panning materials. This is not a problem unique to panning upon antigen mixtures from insect tissues and results from severe bottlenecking of phage population diversity as they are repeatedly selected and reamplified (Norohna *et al.*, 1998; Mutuberria *et al.*, 1999). The limitations of such homogenous antibody selections are clearly illustrated by the example

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reported here. Selection on mixed midgut antigens failed to yield a diverse panel suitable for large-scale bioactivity screening (Killeen *et al*., 2000). Furthermore, although the only reactive clone isolated did indeed react with an antigen of potential interest, this antigen could not be identified because of the sensitivity of the target epitope to denaturation.

The availability of more diverse antibody panels against insect tissue antigens could circumvent many such problems by offering alternative probes for particular molecules and allowing directed selection against targeted structures and antigens (Cai & Garen, 1995; de Kruif *et al*., 1995; Edwards *et al*., 2000; Osbourn *et al*., 1998a,b; Tordsson *et al*., 1997). The rationale behind conventional efforts to induce mosquitocidal or transmission blocking immunity in experimental animals is that, by immunizing with antigens that are normally concealed from the immune system, vulnerable epitopes within the midgut can be targeted. Thus the low level of pre-existing antibody clones within naïve libraries, and the necessity to carry out large numbers of panning steps to enrich them, is likely to result in extreme bottlenecking and low diversity in the selected panels, regardless of the diversity of the original library. The results presented here suggest that the selection of sufficiently diverse antibody panels, from which malaria transmission-blocking or mosquitocidal antibodies can be isolated, may require improved selection methods or specifically enriched pre-immunized libraries.

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