cDNA LIBRARY CONSTRUCTION AND ISOLATION OF GENES FOR CANDIDATE VACCINE ANTIGENS FROM *CHRYSOMYA BEZZIANA* (THE OLD WORLD SCREWWORM FLY)

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ABSTRAK

VUOCOLO, TONY., FLORENTINA SUPRIYANTI, SRI MUHARSINI, dan GENE WIJFFELS. 2000. Konstruksi cDNA *library* dan isolasi gen sebagai kandidat antigen vaksin lalat *Chrysomya bezziana* (*the Old World Screwworm fly*). *Jurnal Ilmu Ternak dan Veteriner (Edisi Khusus)* 5(3): 160-169.

Konstruksi dan pemanfaatan *cDNA libraries* untuk mengisolasi gen pengendali kandidat antigen untuk digunakan dalam produksi vaksin rekombinan terhadap lalat *Chrysomya bezziana* diuraikan. RNA diisolasi dan mRNA dimurnikan dari larva lalat *Chrysomya bezziana* stadium pertama dan ketiga dan dipakai untuk sintesis dua *cDNA libraries* dengan vektor *bacteriophage* λ ZAP *express*®. *Libraries* ini kemudian disekrin dengan menggunakan *Digoxigenin-labelled* DNA *probes* yang diperoleh lewat dua pendekatan yang berbeda. Pertama, pendekatan homolog dengan menggunakan *probes* yang telah didesain berdasarkan gen membran peritrofik yang sebelumnya telah dikarakterisasi pada lalat penyebab myiasis yang masih mempunyai hubungan dekat dengan *Lucilia cuprina*. Kedua, pendekatan *de novo* dengan menggunakan informasi terminal amino dan sekuensi peptida internal yang diturunkan dari protein membran peritrofik lalat *Chrysomya bezziana* yang dimurnikan untuk menghasilkan DNA *probes*. Tiga gen membran peritrofik sudah diidentifikasi dan dikarakterisasi. Cb48 diidentifikasi dengan menggunakan pendekatan homolog, dan Cb15 dan Cb42 diidentifikasi dengan menggunakan pendekatan *de novo*. Identifikasi gen-gen pengendali kandidat antigen terhadap *Chrysomya bezziana* telah memungkinkan untuk memproduksi protein rekombinan yang dapat digunakan dalam uji vaksinasi.

Kata kunci: *cDNA library*, peritrofin, membran peritrofik*, Chrysomya bezziana, Lucilia cuprina,* vaksin

ABSTRACT

VUOCOLO, TONY., FLORENTINA SUPRIYANTI, SRI MUHARSINI, and GENE WIJFFELS. 2000. cDNA library construction and isolation of genes for candidate vaccine antigens from *Chrysomya bezziana* (the Old World Screwworm fly). *Jurnal Ilmu Ternak dan Veteriner (Edisi Khusus)* 5(3): 160-169.

The construction and use of cDNA libraries for the isolation of genes encoding candidate antigens for use in a recombinant vaccine against *Chrysomya bezziana* is described. RNA was isolated and mRNA purified from first and third instar larvae of *Chrysomya bezziana* and used in the synthesis of two cDNA libraries in the bacteriophage vector λ ZAP express®. These libraries were screened using Digoxigenin-labeled DNA probes obtained from two independent approaches. First, a homolog approach used probes designed from previously characterized peritrophic membrane genes identified from the related myiasis fly, *Lucilia cuprina.* Secondly, a *de novo* approach used amino-terminal and internal peptide sequence information derived from purified *Chrysomya bezziana* peritrophic membrane proteins to generate DNA probes. Three peritrophic membrane genes were identified and characterized. *Chrysomya bezziana* peritrophin-48 was identified using the homolog approach and, *Chrysomya bezziana peritrophin*-15 and *Chrysomya bezziana* peritrophin-42 were identified using the *de novo* approach. The identification of these genes as encoding candidate antigens against *Chrysomya bezziana* has allowed the production of recombinant proteins for use in vaccination trials.

Key words: cDNA library, peritrophin, peritrophic membrane*, Chrysomya bezziana*, *Lucilia cuprina*, vaccine

INTRODUCTION

The development of recombinant vaccines against ectoparasites has been documented with the development and commercialization of the world's first ectoparasite vaccine against the cattle tick *Boophilus microplus* (WILLADSEN *et al*., 1995). Similar research has also been undertaken to develop a vaccine against the myiasis flies *Lucilia cuprina* (Sheep Blowfly) (JOHNSTON *et al.,* 1992) and *Chrysomya bezziana* (Old

World Screwworm fly) (SUKARSIH *et al*., 2000). Ectoparasites are generally controlled by insecticides. However, a need for alternative control measures has emerged, due to development of resistance to these insecticides and with more stringent government controls governing their use because of concerns about chemical residues. Recombinant vaccines against ectoparasites could help overcome these problems (ELVIN and KEMP, 1994). The most scientifically demanding step in development of a vaccine is the identification and characterization of antigens able to induce an appropriate protective immunological response in vaccinated animals.

It is the larval lifestage of *C. bezziana* that inflicts sub-cutaneous myiasis on its host. Therefore it is appropriate to look at this lifestage in *C. bezziana* for protective antigens. The previously characterized peritrophins from *L. cuprina* have been isolated from larval lifestages as a result of a series of vaccination and parasite challenge trials, leading to the identification of individual proteins showing significant protective activity (TELLAM *et al*., unpublished results; CASU *et al*., 1997). Their expression profile has been determined by RT-PCR and has been documented as being specific to the gut tissues of the larval lifestage, in particular the cardia (ELVIN *et al*., 1996; CASU *et al*., 1997; SCHORDERET *et al*., 1998; TELLAM *et al*., 1999). Vaccination trials *in vivo* and *in vitro* with peritrophic membrane of *C. bezziana* have also shown that striking reductions in larval growth as well as significant mortality are found in larvae feeding on vaccinated sheep (SUKARSIH *et al*., 2000).

The search for protective antigens from larval peritrophic membrane against this parasite has relied on two approaches: the identification of proteins homologous to known antigens from the myiasis fly *L. cuprina* and a *de novo* identification of antigens from *C. bezziana* itself. This paper describes the first major step in the ultimate goal of producing and evaluating a range of recombinant antigens, namely the determination of the candidate genes. These genes, whether identified as homologs of *L. cuprina* antigens or directly from peptide sequence information derived from *C. bezziana* proteins, all coded for proteins of the larval peritrophic membrane.

Gene libraries are an important and integral resource for the identification and characterization of antigens. cDNA libraries with their representation of expressed genes and, unlike genomic libraries, without the complicating presence of introns and intergenic sequences are the gene libraries of choice for this work. Once an antigen is identified, the need arises for its complete cDNA characterization and its subsequent expression as a recombinant protein. Characterization involves obtaining the complete expressed gene sequence from a cDNA library and hence the complete deduced amino acid sequence of the protein. Once the expressed gene sequence is determined, it can be engineered into an expression system for the production of recombinant protein. This recombinant protein can in turn be used in vaccination trials. We report the production of cDNA libraries from *C. bezziana* and their use in identification and characterization of potential antigens through two alternative approaches.

MATERIALS AND METHODS

Isolation of messenger RNA

Freshly hatched *C. bezziana* first instar larvae (3 g) and third instar larvae (5 g) grown under sterile conditions (EAST *et al*., 1993; RIDING *et al.*, 2000) were provided by Dr. Sukarsih (Balitvet, Bogor, Indonesia). This material was processed identically but separately and used to generate two separate cDNA libraries. RNA is readily degraded by RNAses and care must be taken in its isolation and processing. Care was taken to ensure RNAse-free working conditions. Total RNA was extracted from the larvae by snap freezing the live larvae under liquid nitrogen. The larvae were then pulverized to a fine powder in a mortar and pestle sitting in a bath of liquid nitrogen. This larval powder was then transferred to a 50 ml glass homogenizer containing 30 ml of a commercially available guanidine isothiocyanate and acid phenol solution (TRIZOL®, Life Technologies, Gaithersburg MD, U.S.A.). This procedure is based on the CHOMCZYNSKI and SACCHI (1987) method. The larval powder was homogenized to allow complete disassociation of the tissue in the TRIZOL solution. The material was then centrifuged, extracted with chloroform and precipitated with isopropanol in accordance with the manufacturer's specifications. The precipitated total RNA was resuspended in 1 ml of diethyl pyrocarbonate (DEPC) - treated distilled water and then passed twice through a chromatography column containing 100 mg oligo dT cellulose to enrich for messenger RNA. The mRNA was precipitated with 100% ethanol, washed in 70% ethanol and semi-dried. The resulting pellet was resuspended in 50 µl of DEPC-treated distilled water. Absorbance readings at 260nm and 280 nm were taken to determine mRNA concentration and purity (SAMBROOK *et al*., 1989). A 5 µl aliquot of the mRNA was run on a non denaturing agarose gel to confirm its integrity.

Synthesis of cDNA for cDNA library construction

mRNA was reverse transcribed to produce first strand cDNA with AMV reverse transcriptase and then subjected to second strand synthesis with DNA polymerase 1 using the Riboclone® cDNA synthesis

system (Promega, Madison, WI, U.S.A.). The general quality and representation of the cDNA was tested by PCR analysis prior to cloning into the library vector. PCR was performed on 1 ng of double stranded cDNA using 100 pmol of each of three different sets of oligonucleotide primers based on ubiquitous genes. These genes were ß-actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and a serine protease family (ELVIN *et al*., 1994). The PCRs were performed in the presence of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 3.0 mM $MgCl_2$, 200 µM dNTP and 2.5 U of Amplitaq® DNA polymerase (Perkin Elmer, Emeryville, CA, U.S.A.). The PCR was performed for 35 cycles on a Hybaid™ thermocycler using the following cycling conditions; 60 s denaturation at 94°C, 60 s annealing at 58°C and 60 s extension at 72°C. The sequence of these primers and the expected size of their DNA product are shown in Table 1. The cDNA was size fractionated to remove cDNAs below ~ 300 bp using a spin column containing Sephacryl® S-400 membrane (Promega, Madison CA, U.S.A.). *Eco*R I adaptors were ligated to the cDNA which was then phosphorylated with T4 polynucleotide kinase. Excess adaptors were then removed by passing through a Sephacryl® S-400 spin column.

cDNA library construction

The phage vector, λ ZAP Express® (Stratagene, La Jolla, CA, U.S.A.) was used for cDNA library construction. Briefly, 200 ng of *Eco*R I adaptor ligated cDNA was ligated with 1 µg of *Eco*R I digested λZAP vector. The cDNA ligated vector was then packaged using Gigapack®Gold 111 packaging extracts (Stratagene). The packaged library was titred to determine a primary titre for the library and analysed for number of recombinants. Once the primary library was deemed to be of satisfactory titre and recombinancy, it was amplified. The titre of the amplified library was then determined. The general representation of the amplified library was tested by PCR as previously described for the cDNA analysis.

Homology approach to screening libraries for candidate genes

Digoxigenin (DIG)-labeled DNA probes based on three previously described peritrophin genes from *L. cuprina*; peritrophin-44, peritrophin-48 and peritrophin-95 (GENBANK accessions AAC37261, AAB38414 and AAB70878, respectively), (ELVIN *et al.,* 1996, SCHORDERET *et al.,* 1998; CASU *et al*., 1997) were used to screen the *C. bezziana* cDNA libraries to identify homologous genes in *C. bezziana*. These probes were based in the coding region of these genes. The cDNA libraries were screened under high and low stringency conditions as reported in VUOCOLO *et al.* (2000).

***De novo* approach to screening for candidate genes**

A number of different proteins were isolated from peritrophic membrane of *C. bezziana* larvae and purified peptides generated using *in situ* Endo-Lys C digestion and reverse phase-HPLC (RIDING *et al*., 2000). Peptide sequence information and where possible, amino-terminal sequence, was derived from two native *C. bezziana* proteins, *C. bezziana* peritrophin-42 (Cb42) and *C. bezziana* peritrophin-15 (Cb15) and used to design sense and antisense degenerate oligonucleotide primers. These primers were used to generate DNA fragments from *C. bezziana* cDNA coding partially for the proteins from which the peptides were derived. The PCR fragments were cloned into the plasmid vector, pGEM-T (Promega) and sequenced. DIG-labeled versions of fragments that had been shown to code partially for the native proteins were used to screen the cDNA libraries for full length sequences.

Table 1. Primers used in cDNA and library quality control PCR encoding house-keeping genes

Gene Size	Primer sequence	Orientation	Product
B-actin	cagatcatgtttgagaccttcaac	sense	323 bp
	g(gc)ccatctc(ct)tgctcgaa(ga)tc	antisense	
GAPDH	c(ga)aacatggg(gatc)gcatc(ag)c(gatc)ga(gatc)	sense	290 bp
	a(tc)cc(ca)ttcat(tc)ga(ct)(cgt)t(cg)aa(cg) tacatgg	antisense	
Serine protease	acagaattctgggt(gatc)gt(gatc)ac(gatc)gc(gatc) gc(gatc)ca(tc)tg	sense	~ 450bp
	acagaattca(ag0(gatc)gg(gatc)cc(gatc)cc(gatc) (gc)(at)(ag)tc(gatc)cc	antisense	

DNA encoding Cb15 was obtained by PCR on first instar *C. bezziana* larval cDNA using redundant primers. Redundant sense and antisense primer sequences were based on the Cb15 amino-terminal and internal peptide sequences respectively (RIDING *et al*., 2000). PCR was conducted in 100 µl volumes containing 10 mM Tris-HCl (pH8.2), 200 µM dNTPs, 2 U Amplitaq (Promega), 100 pmol primers and 2 or 3 mM $MgCl_2$. The PCR was conducted through 40 cycles consisting of denaturation at 94°C for 2 min; annealing at 58°C for 1 min and extension at 72°C for 2 min.

DNA encoding Cb42 was also obtained by PCR on first instar *C. bezziana* larval cDNA using redundant sense and antisense primers prepared for each of the two peptide sequences obtained (RIDING *et al*., 2000). PCR using two combinations of sense and antisense primers was conducted in 100 µl volumes containing 10 mM Tris-HCl (pH8.2), 200 µM dNTPs, 2.5 U Amplitaq (Promega), 100 pmol primers, 3 mM $MgCl_2$. The PCR was conducted through 40 cycles of denaturation at 94°C for 2 min; annealing at 54°C for 1 min and extension at 72°C for 2 min.

Synthesis of DIG-labeled DNA probes by PCR

The DIG system is a non-radioactive and effective system for labeling DNA probes for use in screening libraries. Labeled DNA probes were used for screening the cDNA libraries for the genes of interest. All the DNA probes used were labeled by using PCR to incorporate digoxigenin-11-2'-deoxyuridine-5'-triphosphate (DIG-11-dUTP) (BOEHRINGER MANNHEIM GMBH, 1995). DIG-11-dUTP is used as a substrate instead of dTTP by Taq polymerase during PCR. Briefly, 1 ng of purified plasmid DNA containing the probe was used as template in a PCR reaction containing 70 µM DIG-11-dUTP, 130 µM dTTP and 200 µM dATP, dGTP, dCTP each, 100 pmol each of sense and antisense primers specific to the DNA probe, 3 mM $MgCl_2$, 2.5 units of Taq polymerase and its buffer. PCR was performed for 35 cycles. A control reaction containing no DIG-11-dUTP was performed in parallel. The PCR products were run out on agarose gels and the DIG-labeled PCR product excised and purified using a Bresaclean DNA purification kit (Geneworks, Adelaide, S.A., Australia). Successful DIG incorporation into the PCR product was evident by increased size of the PCR product in relation to that of the control reaction containing no DIG.

A DIG-labeled probe for screening the cDNA library for Cb15 was prepared using specific primers whose sequences were based on the 3' and 5' sequences of the Cb15 PCR fragment and the plasmid DNA containing the original PCR fragment as the template. The DIG labeling PCR was conducted in a 100 µl volume under the same conditions as described previously. The thermocycle program was 40 cycles: denaturation at 94°C for 2 min; annnealing at 65°C for 1 min and extension at 72°C for 2 min. A probe for the Cb42 cDNA library screen was produced by DIG labeling PCR using the purified plasmid DNA containing the original PCR fragment, and specific primers whose sequences were based at the 3' and 5' end of the Cb42 fragment. This PCR used the same conditions as described for the Cb15 DIG-labeling PCR.

The purified DIG-labeled probe was denatured at 96^{o}C for 10 min and then added at a concentration of 20 ng/ml to hybridization buffer. Two types of hybridization buffer were used in screening the libraries, a high stringency buffer and a low stringency buffer. The high stringency buffer included 50% deionised formamide, 7% SDS, 5 x SSC and 2% blocking agent (Boehringer Mannheim). The low stringency buffer included 25% formamide, 0.02% SDS, 5 x SSC and 2% blocking agent.

Screening of cDNA library

The *C. bezziana* cDNA libraries were screened with the DIG-labeled DNA probes generated by the homolog approach and the *de novo*/peptide approach. Plaques (500,000) were plated out on a lawn of *Escherichia coli* (XL1-Blue MRF') on LB medium (1% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 0.5% w/v NaCl and 1.5% w/v bacto agar) in petri plates at a density of approximately 50,000 plaques per plate. The plates were incubated for approximately 15 h until they were almost confluent but individual plaques could still be distinguished. Plaque lifts were performed by overlaying plates with Hybond N^{+} nylon membranes (Amersham; Buckinghamshire, England) and the filters processed as described in SAMBROOK *et al.* (1989). Plaques returning a positive hybridization signal were picked from the plates using a sterile wide bore plastic Pasteur pipette or the base of a sterile 200 µl tip and allowed to elute into 500 µl of SM buffer (0.1 M NaCl, 8 mM $MgSO_4.7H_2O$, 50 mM Tris-HCl pH 7.5 and 0.01 % glucose). These plaques were then titred, plated out and screened again until a pure plaque was isolated.

Plasmid containing the cDNA insert from the purified plaque of interest was obtained as follows. Separate overnight cultures of *E. coli* XL1-Blue MRF' and *E. coli* XLOLR cells were grown in NZY broth (0.1 M NaCl, 8 mM $MgSO_4.7H_2O$, O.5% yeast extract, 1% casein hydrolysate, pH 7.5) supplemented with 0.2% maltose and 10 mM $MgSO_4$. These cells were recovered by centrifugation (1000*g*) and resuspended to an $O.D._{.600}$ of 1.0 in 10 mM $MgSO_4$. The plaque eluate (250 µl) was combined with 200 µl of XL1-Blue MRF' cells at $O.D._{.600}$ of 1.0 and 1 µl of ExAssist helper phage (Stratagene) in a 15 ml Falcon tube. The tube was then

incubated at 37°C for 15 min after which 3 ml of NZY broth was added and the tube incubated at 37°C for 3 h on a rotating culture wheel. After this incubation, the tube was heated at 65°C for 20 min and then centrifuged at 1000 x g for 15 min. The supernatant, which contained the excised phagemid, was collected and 10 and 100 µl of this added to 200 µl of XLOLR cells at $O.D._{600}$ of 1.0 in a 15 ml Falcon tube. These tubes were incubated at 37°C for 15 min and then 300 ul of NZY broth added and further incubated at 37°C for 45 min. The cell mixture from each tube was then plated onto LB-kanamycin agar plates (50 µg/ml). These plates were incubated overnight at 37°C. The following day, individual colonies from the plates were selected and grown in LB containing kanamycin (50 µg/ml). Plasmid DNA was then isolated using a miniprep kit and the cDNA insert subjected to sequence analysis using T3 and T7 primers.

RESULTS AND DISCUSSION

Following vaccination trials with whole and fractionated peritrophic membrane from *C. bezziana*, peptide sequence information for several of the major protein components of this membrane was obtained. In parallel, genes for known antigens from *L. cuprina* were available and it seemed appropriate to ask whether homologs of these antigens could be identified in *C. bezziana*. A core resource for the identification of homologs or the *de novo* identification of *C. bezziana* genes was a cDNA library of high quality from the appropriate life stage.

mRNA isolation and cDNA synthesis

cDNA libraries derived from mRNA should represent only the gene sequences which are expressed and do not contain intronic and intergenic sequence associated with gene libraries prepared from genomic (chromosomal) DNA. This was the governing reason for generating cDNA libraries. Further, the protein components of larval and adult *L. cuprina* peritrophic membrane appear to be largely stage specific (TELLAM *et al*., unpublished observation). For this reason, the larval stage was selected for isolation of mRNA and subsequent cDNA synthesis. Two separate cDNA libraries were synthesised from two larval life-stages of *C. bezziana*, these being newly hatched first instar and mid third instar. Two libraries from different larval lifestages were generated to address the issue of differential expression and provide a better chance of identifying candidate genes. The extraction of mRNA and synthesis of cDNA of high quality suitable for library production proved a difficult task and was repeated several times due to degradation of the RNA. The most important factor affecting the quality of cDNA is the quality of the mRNA. Particularly for large transcripts, it is essential to start with the highest quality RNA available. The inclusion of internal controls to check and monitor quality of the mRNA and cDNA proved invaluable. Figure 1 displays the RNA isolated from the larval tissue and its enrichment for mRNA. mRNA, representing approximately 1-3% of the RNA in total RNA, was isolated and purified on oligo dT cellulose. The processing of the total RNA through the oligo dT cellulose column shows an enrichment for mRNA with a corresponding reduction in ribosomal RNA. The smearing of RNA up the lane of the gel indicates the presence of a range of transcript sizes including large transcripts up to ~ 10 kb. The cDNA produced was analysed for general quality by PCR (Figure 2). The house keeping genes, ß-actin, GAPDH and the family of serine proteases are expected to be present in the cDNA and the libraries. The results shown in Figure 2 demonstrate the presence of these 'house keeping' genes in the cDNA at the expected size, confirming the quality of the cDNA and libraries.

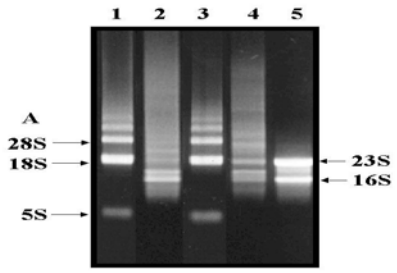

Figure 1. Non denaturing agarose gel depicting the isolated total RNA and purified mRNA from *C. bezziana* larvae. Lane 1, 5 µg of total RNA isolated from 1st instar larvae; lane 2, 5 µg of mRNA isolated from 1 st instar total RNA; lane 3, 5 µg of total RNA isolated from 3rd instar larvae; lane 4, 5 µg of mRNA isolated from 3rd instar total RNA; lane 5, bacterial ribosomal RNA standard containing 16S- and 23S-ribosomal RNA. [(A) highlights the 18S- and 28S-ribosomal RNA present in total eukaryotic RNA which is removed during the mRNA isolation]

Two lambda-ZAP Express cDNA libraries were produced from *C. bezziana* first instar and third instar larval lifestages. The λ ZAP Express vector has been designed to allow *in vivo* excision of the cDNA insert to

form a pBK-CMV phagemid containing the cloned insert. This is a relatively simple and efficient procedure and by-passes the need for isolation of lambda-DNA and sub-cloning. The procedure for this excision involves the use of the helper phage, ExAssist (Stratagene) and the *E. coli* strain XLOLR (Stratagene). The helper phage contains an amber mutation that prevents replication of the phage genome in the non-suppressing XLOLR cells allowing only the excised phagemid containing the cDNA insert to replicate. This allows for rapid characterization of the insert in a plasmid system. This vector was also chosen for construction of the *C. bezziana* libraries because it can be screened with either DNA probes or antibody probes. A first instar library representing 800,000 primary clones with 96% recombinants and a third instar library representing 400,000 primary clones with 85% recombinants were produced. The cDNA libraries contain the house keeping genes, ß-actin, GAPDH and the serine proteases as determined by PCR (result not shown). Insert sizes ranging from 0.8 kb to in excess of 4 kb were also shown to be represented in the libraries. These libraries were subsequently amplified to produce working stocks for screening.

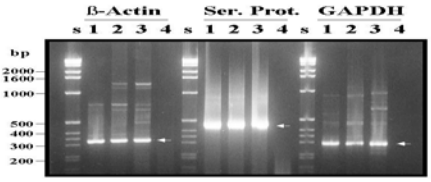

Figure 2. Agarose gel depicting amplified products obtained from PCR of cDNA with three different sets of primers specific for ß-actin, serine proteases, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Lane S, 1kb DNA ladder standard (Life Technologies); lane 1, *C. bezziana* 1st instar cDNA template; lane 2, *C. bezziana* 3rd instar cDNA template; lane 3, *L. cuprina* 1st instar cDNA template (positive control); lane 4, no DNA template (negative control)

Identification of potential vaccine candidates by homolog screening

The first approach to the identification of candidate genes for vaccine development was the homolog approach. This is based on the assumption that the homolog of a protective antigen in one species has a high probability of being protective in another closely related species. A number of antigens have been shown to be partially protective in vaccine trials against *L. cuprina*. Three of these; peritrophin-44, peritrophin-48 and peritrophin-95 have been characterized at the gene level. DIG-labeled DNA probes based on the *L. cuprina* peritrophin sequences were synthesized and used to screen the *C. bezziana* libraries. Figure 3 shows a diagramatic representation of the location of the probes with reference to the cDNA sequences of *L. cuprina* peritrophin-44, peritrophin-48 and peritrophin-95. The probes were designed to represent segments of the coding sequence of these genes and did not include the non-coding 5' or 3' untranslated regions (UTRs), since the UTR sequences are less likely to be conserved between species than is the coding sequence. Attempts using high stringency hybridization buffers and washes proved unsuccessful in isolating any peritrophin homologs from the *C. bezziana* libraries. In light of these negative results, the screening protocol was changed to low stringency hybridization buffers and washes. A *C. bezziana* peritrophin-48 homolog (Cb48) was identified using this procedure and is discussed in detail in VUOCOLO *et al*. (2000). Attempts to identify peritrophin-44 and peritrophin–95 homologs in *C. bezziana* have been unsuccessful to date.

L. cuprina peritrophin-95 protein contains two distinct domains. The first domain contains a sub-set of five similar cysteine-rich domains described as peritrophin-A domains (TELLAM *et al.*, 1999), whilst the second domain is completely different and contains a proline and threonine-rich repeat domain (Figure 3). In view of this domain structure of peritrophin-95, a variety of different peritrophin-95 probes were used independently in the screening process. Initially, a full-length peritrophin-95 probe was used (probe A, Figure 3) and a number of strong positive signals were returned from the library screen. The cDNA inserts from these positive plaques were sequenced but found to be false positives, unrelated to the peritrophin genes. The false positive results from the screening process were found to be due to non-specific binding of the probe encoding the proline- and threonine- rich repeat region of peritrophin-95. This was determined by producing independent peritrophin-95 probes based on the proline- and threonine- rich repeat domain only and the peritrophin-A domain (probes B and C respectively, Figure 3). Figure 4 shows the DIG-labeled probe generated for the peritrophin-95 repeat region (probe B, Figure 3). This result is indicative of DIG-labeling by PCR and highlights the size increase expected for the labeled product (lane 1) compared to the unlabeled control product (lane 2). The repeat region probe returned false positives in the screening process whilst

the peritrophin-A domain probe did not return any positives. This result highlights the pitfalls of this homolog approach and the need for caution in using probes containing repetitive sequences due to the potential for detecting non-specific targets. The peritrophin-44 probe did not return any positive results.

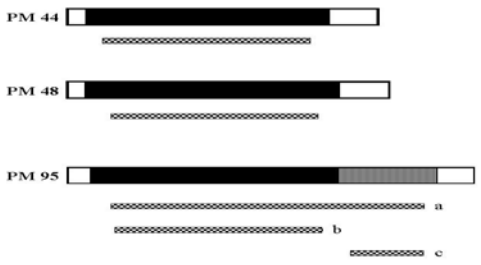

Figure 3. Conceptual diagram showing the general location of DNA probes generated from *L. cuprina* peritrophin-44, peritrophin-48 and peritrophin-95. The DNA probes used for cDNA library screening are represented by (▚). The shaded region ( ) represents the coding sequence. The unshaded regions ( ) represent the non-coding 5' and 3' untranslated region. The partially shaded region of peritrophin-95 (▥) represents the coding region containing proline/threonine repeat sequence.

Analysis of the Cb-peritrophin-48 homolog showed substantial sequence divergence from its *L. cuprina* counterpart with only its putative deduced protein structure conserved (VUOCOLO *et al.*, 2000). It is possible that homologs of peritrophin-44 and peritrophin-95 do exist in *C. bezziana*, but isolating them through a direct homolog probe approach may be more difficult than anticipated.

De novo approach to screening for candidate genes

Two candidate antigens, Cb15 and Cb42, were successfully isolated from the larval peritrophic membrane. Cb15 generated both an amino-terminal sequence and two internal peptide sequences. Degenerate sense and antisense primers derived from the amino-terminal sequence and the sequence of the longest internal peptide produced a single 160 bp product in a PCR containing 2 or 3 mM $MgCl_2$ (Figure 5, lane 4). The 3 mM $MgCl_2$ PCR produced the same 160 bp band, but several other minor products were evident as well as primer-dimers (Figure 5, lane 8). The water only control (which contained both primers but no template) produced the primer-dimers (Figure 5, lanes 1 and 5). The single primer control reactions did not produce the 160 bp product although several other products were present, but only evident in these reactions. The 160 bp product was ligated into pGEMT-Easy for cloning into XL1-Blue cells for subsequent DNA sequencing. The DNA sequence contained an open reading frame which encoded the Cb15 amino-terminal sequence and both peptide sequences.

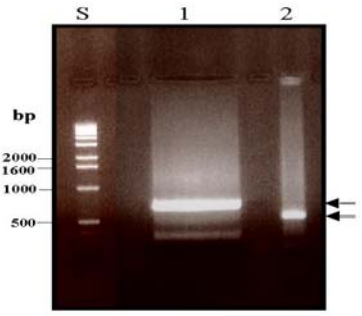

Figure 4. DIG-Labeling by PCR. Agarose gel showing peritrophin-95 repeat region DNA probe generated by PCR. Lane S, 1 kb DNA ladder standard (life technologies); lane 1, DIG-labeled peritrophin-95 repeat region fragment; lane 2, same peritrophin-95 repeat region fragment without DIG. The arrows highlight the size difference created by incorporation of digoxigenin into the DNA probe.

Having ascertained that the 160 bp product encoded part of Cb15, the DNA was DIG-labeled by PCR and used to screen the cDNA library. In the first round of screening, many plaques were positive. Fifty plaques were randomly picked, and the eluants of 19 plaques were processed for secondary screening. All 19 returned positive plaques. The eluants were tested by PCR (using primers located in the cloning sites of the vector) to determine the insert size of the clones. Four clones were chosen and the phagemid for each clone was isolated by *in vivo* excision and then transformed into XLOLR cells. The DNA was purified and digested with *Eco*R I or *Xho* I. Three of these clones; S3.4, S4.1 and S4.2 were sequenced.

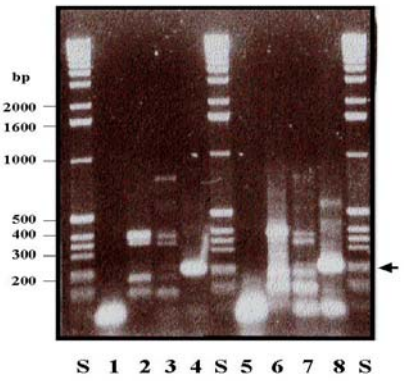

Figure 5. PCR of cDNA partially encoding Cb15. PCR was conducted on third instar larval *C. bezziana* cDNA using 2 and 3 mM $MgCl_2$ in lanes 1–4 and 5–8, respectively. In lanes 1 and 5, PCR was performed in the presence of the sense and anti-sense primers, but in the absence of template. All other reactions contained *C. bezziana* cDNA, and the following primers: the sense primer only: lanes 2 and 6; the anti-sense primer only: lanes 3 and 7; and both primers in lanes 4 and 8. The molecular size standards (1kb ladder, Life Technologies) are indicated. The DNA products were resolved in a 1% agarose gel and stained with ethidium bromide. The arrow indicates the specifically amplified Cb15 fragment in lanes 4 and 8.

The DNA sequence of S3.4 clearly encoded Cb15. This clone contained DNA sequence encoding a classic signal sequence, the sequence of the mature protein, the stop codon and polyA adenylation sequence (Figure 6). The other two clones, S4.1 and S4.2 did not contain sequences encoding Cb15 or even related proteins, but rather heat shock proteins.

Cb42 did not return an amino-terminal sequence but yielded two internal peptides after *in situ* digestion with endo-Lys-C (RIDING *et al.,* 2000). With no amino-terminal sequence available, any attempt to derive a probe for Cb42 by PCR had to rely solely on internal peptide sequence information. However, the positions of these peptides relative to each other within the native protein were unknown. Hence, both sense and antisense degenerate primers were synthesized for both internal peptides and different combinations of the primers were trialed in PCR on cDNA derived from first instar larvae. One combination of primers produced a 194 bp fragment, which was not present in any of the controls (data not shown). The DNA sequence of this fragment contained an open reading frame incorporating both peptides. A DIG-labeled DNA probe was generated by PCR using specific primers and the cloned 194 bp fragment used in screening the cDNA library. On primary screening of the library, 26 plaques were positive and 12 were randomly selected for processing through secondary and tertiary screening. In the third screen, all plaques were positive demonstrating that the clones were pure. The phagemid from eight clones was isolated by *in vivo* excision. The phagemid DNA of three clones, K1.1, K3.2 and K7.1 were purified and partially sequenced.

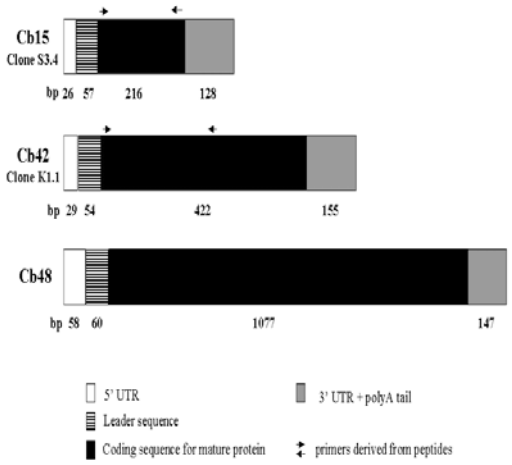

Figure 6. Schematic diagram of the cDNA structure of Cb15, Cb42 and Cb48. The insert of clone S3.4 was sequenced and found to encode for Cb15 and clone K1.1 was found to encode for Cb42. Several clones were sequenced and found to encode Cb48 and a consensus sequence deduced. The cDNA sequences encoded a signal (or leader) sequence, the mature protein sequence, the 5' and 3' UTRs, a polyadenylation site and polyA tail. The primer sites are also depicted where applicable

Partial sequencing of the clones verified that each contained sequences encoding Cb42 (results not shown). Clone K1.1 was fully sequenced. As shown in Figure 6, this clone contained sequence encoding a short 5' UTR, a classic signal sequence, the sequence of the mature protein, the stop codon and poly-A adenylation signal sequence and poly-A tail.

Cb15 and Cb42 are both cysteine rich proteins probably containing multiple internal disulphide bonds. These proteins are probably members of the same

family. The presence of these disulphide bonds is a common feature of all larval peritrophins isolated and characterized to date. However, the domain structures of Cb15 and Cb42 are not reminiscent of the peritrophin A and B domains identified in other peritrophins (TELLAM *et al.*, 1999). In fact, their unique $CX_{8-9}CX_{17-21}CX_{10-11}CX_{12-13}C$ motif has been termed the peritrophin C domain (TELLAM *et al.,* 1999). Cb15 and Cb42 encode proteins of 7.9 and 15.2 kDa, respectively. These predicted sizes are inconsistent with their migration in SDS-PAGE. This suggests that these proteins fold into very stable and robust structures that do not denature easily despite reduction of their multiple disulphide bonds.

CONCLUSION

In this study, two approaches were taken in finding DNA encoding candidate antigens for a *C. bezziana* recombinant vaccine. These approaches were, firstly, the search for homologs of useful proteins previously identified in the development of an *L. cuprina* vaccine and, secondly, the identification of cDNA clones based on amino acid sequences from proteins tightly associated with the larval PM. Full length cDNA sequences of Cb48, Cb15 and Cb42 were successfully obtained by isolation of clones from the *C. bezziana* larval cDNA libraries.

These cDNA clones have provided DNA sequence information required for the engineering of constructs for the expression of recombinant proteins. Both bacterial and yeast expression systems have been used for the production of recombinant proteins for use in vaccination trials. The generation, production and use in vaccination trials of these antigens are described in the following papers (WIJFFELS *et al*., 2000; MUHARSINI and VUOCOLO, 2000).

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