Selection and Testing Phage-displayed YHV-binding Peptides for Possible Detection of Yellow Head Virus (YHV) in Shrimp

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ABSTRACT: Yellow head virus (YHV) causes a serious disease that can result in high mortality of penaeid shrimp within 2-3 days after the first appearance of gross signs of disease in rearing ponds. Current detection systems for YHV are based on molecular and immunological techniques. Immunological detection methods require continual production of relatively large, complex proteins using living animals or hybridoma cells. An alternative possibility is to use the phage display technique to find short peptides that can bind strongly with YHV particles and replace antibodies in such tests. Once identified, these peptides could also be tested for efficacy in blocking YHV infection. YHV was purified and then immobilized on microtiter plates for 4 rounds of biopanning against a pool of phage displayed peptide variants. From 89 sequentially generated phage pools, 13 variants were selected for strong binding with YHV by ELISA assay. DNA sequencing led to the deduced amino acid sequences LNAKSRN, KSKKSSS, GPQRKRS, KLKRLSS, RTNKKNA, SNISNAS, SNSKKRN, RTKKMRT, NTKRPAR, GPQRKRS, VSNKKRS, RKKSNAS and GPKKNRS. Preliminary tests using two representative phages (SNSKKRN and GPQRKRS) with high YHV binding activity showed that they were unable to block YHV infection in shrimp. However, when immobilized, these clones could bind YHV from probe solutions, indicating that they have potential for use as YHV detection reagents.

KEYWORDS: phage display, yellow head virus (YHV), *Penaeus monodon*.

INTRODUCTION

Yellow head virus (YHV) is one of the most important causes of mass mortality leading to serious production losses in Thai shrimp aquaculture. It was first reported in Thailand in 1990.[1] Subsequent outbreaks have been reported mostly in Asia[2] but also occasionally in America and India.[3] It can infect shrimp from late post larval stages to adults. Infected shrimp swim near the surface at the pond edge and high mortality occurs within 2-3 days after the first signs of an outbreak. The virus replicates in the cytoplasm of infected cells including those of the lymphoid organ, hemocytes and gills. It infects a range of penaeid species but does not appear to infect many other crustaceans.[4] Rapid diagnostic tools for YHV have been developed. These include reverse transcription polymerase chain reaction (RT-PCR) methods[5,6] and histological methods[4,7] including rapid staining of hemolymph.[8] So far, there are no medications to treat viral-infected shrimp, but culture management techniques are evolving that can lessen the impact of viral diseases.[9] The rapid detection and identification of viruses are crucial in any disease management program.

Phage displayed peptides have been used successfully for numerous applications, including epitope mapping, vaccine development, identification of protein substrates/ligands and identification of peptide mimics of non-peptide ligands. A particularly notable application has been the identification of novel bioactive peptides by panning against immobilized cell-surface receptors.[10] The biopanning procedure selects for those peptides that best bind the target under the panning conditions *in vitro*, regardless of the biological role of the target *in vivo*. In some cases, viral-binding peptides can identify antiviral drug targets. For

instance, a phage-displayed peptide selected from a six-mer library was capable of blocking the interaction of the core antigen protein of hepatitis B viral envelope protein with its target cells and this also blocked infection.[11] This study focused on biopanning to select YHV-binding peptides that might be developed into tools for YHV detection or YHV control by blocking viral infection.

MATERIALS AND METHODS

YHV Polymerase Chain Reaction (PCR) Detection

Gills from YHV-infected shrimp were collected and processed for RNA extraction using a Trizol (Gibco BRL, USA) according to manufacturer's instructions. The PCR detection method employed a commercial, nested-RT-PCR IQ 2000® YHV/GAV PCR detection kit (Farming Intelligene Co., Ltd. Taiwan).

YHV Purification

Hemolymph collected into 10% sodium citrate was diluted with one volume of TNE buffer (0.02M Tris-HCl, 0.4M NaCl, 0.02M EDTA, PH 7.4) before centrifugation at 3,000 x g, for 30 min at 4ºC. The supernatant was collected and ultracentrifugation was performed at 100,000 x g for 1 h at 4ºC. The pellet obtained was re-suspended in TNE buffer and layered onto a 15% sucrose solution (sucrose dissolved in TNE buffer containing 20mM Tris-HCl, 1M NaCl, 5mM EDTA, PH 8.0) before ultracentrifugation at 100,000 x g for 90 min at 4ºC. The pellet obtained was washed with TNE buffer and ultracentrifuged at 100,000 x g, 1 h at 4ºC. Finally, the pellet was dissolved in a small volume of TNE buffer[12] and kept at 4ºC. The presence of YHV particles was confirmed by negative staining transmission electron microscopy (TEM). Viral proteins and nucleic acid were examined by Western blot analysis and PCR, respectively. Protein concentration was determined using Bradford reagent (Bio-rad, USA).

YHV Negative Staining for Transmission Electron Microscopy (TEM)

The purified YHV particle preparation (3 µl) was loaded onto a 200 mesh, formvar-coated copper grid and incubated for 15 min at room temperature. The excess liquid was removed using filter paper. The sample was stained with 10 µl of 2% phosphotungstic acid for 3 min and then dried for observation by TEM.

Western Blot Detection

The amount of 5 µl of purified YHV particle preparation was loaded onto a 12 % SDS-PAGE gel. For immunodetection, the blot was blocked with 5% skim milk. Detection was performed using monoclonal antibody (V3-2B) against the N-terminal region of gp116 glycoprotein of YHV. After careful washing, the second antibody (goat anti-mouse conjugated AP) (Roche, Germany) was added to cover the blot. Color was developed by adding NBT/BCIP substrate (Roche, Germany).

T7 Bacteriophage Library

A random heptapeptide (flanked by cysteine residues) phage display library was constructed using the T7 select system from Novagen (Wisconsin, USA). The protocol for library construction was according to the previous report.[13] The protocols for amplification, purification and storage of T7 bacteriophage were according to the T7 select system manual (Novagen, USA). Protein concentrations were measured using Bradford reagent (Bio-rad, USA).

YHV Binding Peptide Screening: Biopanning Protocol

For biopanning the YHV preparation was diluted with coating buffer and 100 µl was applied to microtitre plate wells and incubated at 4°C overnight. The coated wells were washed three times with 300 µl 1X PBS-T and incubated with blocking buffer for 60 min at 37°C. After washing as described above, the initial phage titer was added in 100 µl ($1.79x10^{11}$ pfu/ml). After incubation for 2 h with gentle agitation at room temperature, unbound phages were washed away and bound phages were eluted with 100 µl elution buffer. Then, 10 µl of the eluted phage solution was titered and 90 µl was amplified and purified to constitute sub-library I. The process was called "first biopanning" and sublibrary I that resulted was used for initiation of the second round of biopanning.

Titering of T7 Bacteriophage

E. coli BL21 was inoculated into LB medium and incubated with shaking at 37°C to an $OD_{600} = 1.0$. Top agarose was prepared in 3 ml aliquots using sterile LB medium and melted at 45–55°C in a water bath. A series of dilutions of eluted phage (100 µl) was added to each tube together with *E. coli* BL21 (250 µl) before mixing and pouring onto a pre-warmed (37°C) LB agar plate. After the agar was solidified, the plate was inverted and incubated for 3–4 h at 37°C. The number of plaques was counted and the phage titer was calculated. Percent yield was calculated as the number of phages eluted divided by the input number multiplied by 100. The enrichment factor was calculated as the percent yield of each round divided by percent yield of the first round.

Enzyme-Linked Immunosorbent Assay (ELISA)

After the final round of biopanning, individual phage plaques were picked and YHV binding specificity was determined using indirect enzyme-linked

immunosorbent assay (ELISA). Each selected phage (200 μl at a concentration 0.1 μg/μl in coating buffer) was added to one immuno-plate well and left to stand overnight at 4°C. Excess phage was removed and wells were washed three times with PBS-T before blocking with 100 μl blocking solution (1% BSA in PBS) for 1 h in a humidity chamber. Thereafter, 100 μl YHV particles (conc.1:1000 in TNE) was added and incubated at 37°C for 1 h before covering with monoclonal antibody (V3-2B) against YHV. V3-2B is a monoclonal antibody to YHV-gp116 glycoprotein.[14] Internal negative controls (without YHV addition) and experimental negative controls (without bound phages) were run in parallel. The efficiency of monoclonal antibody V3-2B was tested with YHV particles and compared to the negative controls (white spot syndrome virus or WSSV and bovine serum albumin or BSA) and the positive control (expressed gp116 of YHV). Horseradish peroxidase conjugated with goat-anti mouse immunoglobulin antibody (Zymed, USA) was used as a secondary antibody. The color was developed by propidium iodide for 30 min at room temperature. The reaction was stopped by addition of H_2SO_4 to a final concentration of 5N and OD_{490} was measured. Positive clones were selected, amplified and further characterized by PCR analysis and sequencing.

PCR Amplification

Using a sterile loop or pipette tip, a portion of the top agarose of an individual plaque of interest was picked and dispersed in a tube containing 100 μl of 10 mM EDTA, pH 8.0. After vortexing the tube briefly, it was heated at 65°C for 10 min. The suspension was allowed to cool to room temperature before centrifugation at $14,000 \times g$ for 3 min to clarify. The following components were combined in a sterile 0.5 ml PCR tube of 50 μl total reaction volume; 200 ng DNA of phage lysate, 1X Taq buffer (10 mM KCl, 10 mM Tris-HCl, pH 8.3), 2.5 mM $MgCl_2$, 25 pmol each of T7SelectUP Primer and T7SelectDOWN Primer (Novagen, USA), 0.2 mM mixed dNTPs, 2U *AmpliTaq* DNA polymerase (Applied Biosystems, USA). The PCR protocol comprised a pre-PCR step at 94 °C for 2 min followed by 35 cycles of 94 °C for 20 sec, 50 °C for 20 sec, 72 °C for 45 sec and final extension at 72 °C for 4 min. Reaction products were analyzed by agarose gel electrophoresis and positive clones were subjected to nucleotide sequencing by Macrogen (Korea). Construction of a phylogenetic dendogram was carried out using by Clustal W 1.75 Multiple Sequence Alignments (http://www.es.embnet.org/Doc/phylodendron/clustal-form.html).

Dot Blot ELISA

All 13 YHV-binding peptides (10 μg each) were dotted onto nylon membranes (Amersham, Sweden) in two spots, one for the negative control (not exposed to YHV particles) and one for the test (exposed to YHV particles). The test membrane was incubated with 100 μl of YHV particle suspension (conc.1:1000 in TNE) in blocking solution for 1 h. After extensive washing in PBS-T, both membranes were incubated with V3-2B antibody at a dilution of 1:2000. The membranes were washed extensively before incubation with goat anti-mouse IgG antibody conjugated with horseradish peroxidase (Zymed, USA). The protocol used to develop a signal was described above.

Test for Inhibition of Yellow Head Disease

Test shrimp were divided into 4 groups. Group 1 (negative control) was injected with TNE buffer containing no YHV or phage. Group 2 was injected with buffer containing YHV plus phage YBP7-45. Group 3 was injected with YHV plus phage YBP7-48. Group 4 (positive control) was injected with buffer containing only YHV. All preparations were incubated for 1 h at room temperature before injection. The relative concentrations of YHV and phage used were selected based on ELISA test results, and the injection volume was 100 μl/shrimp. The percent mortality was recorded every day post-injection. The experiment was repeated 3 times. Results for experimental and control groups were analysed by ANOVA using software from Jandel Scientific Co., Ltd., and differences were considered significant when $P<0.05$.

RESULTS AND DISCUSSION

Yellow Head Virus (YHV) Examination

Hemolymph of YHV from 80 shrimp was collected at 48 h to check for YHV by PCR using a commercial two step RT-PCR IQ 2000® YHV/GAV PCR detection kit. The positive bands of 777 bp and 277 bp were detected in all infected shrimp (data not shown). The remainder of infected hemolymph was used to purify YHV particles.
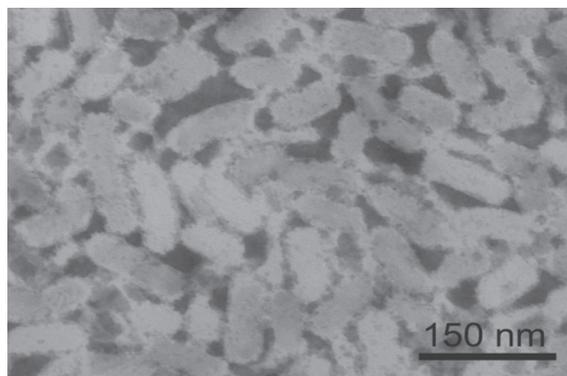
Fig 1. Transmission electron micrograph of negatively-stained, YHV particles purified from shrimp hemolymph.
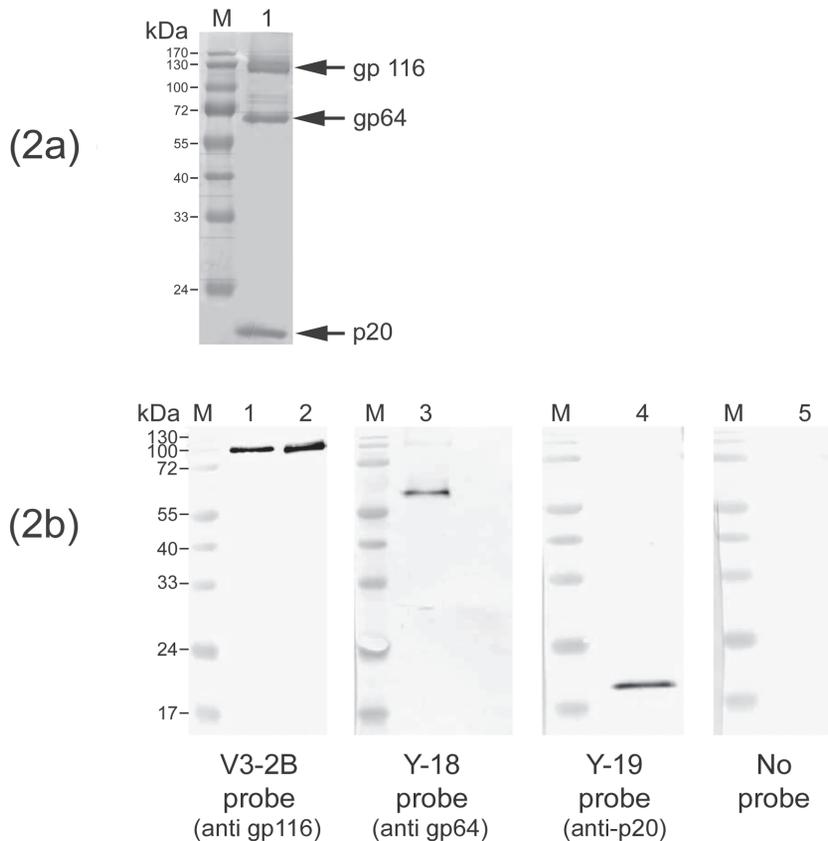
Fig 2. Analysis of purified YHV by SDS-PAGE and western blots. (2a) 12% SDS-PAGE gel of purified YHV stained with Coomassie Brilliant blue. Molecular weight marker proteins are shown in lane M and gp116, gp64 and p20 are indicated by arrows. (2b) Western blots of purified YHV proteins from (2a) probed with MAb V3-2B (1:2000), Y-18 (1:500) and Y-19 (1:500). Lanes marked M = molecular weight marker proteins; Lane 2 = expressed gp116 (positive control); Lanes 2 to 5 = YHV proteins.

After purification by ultracentrifugation, TEM showed negatively-stained, whole YHV particles in high quantity, suggesting that the modified protocol developed for purification of YHV particles in this study was effective (Fig. 1). The YHV virions showed a trilaminar envelope with short spikes on the outer surface, similar to previous descriptions.[15]

By 12 % SDS-PAGE and Coomassie brilliant blue-R staining, expected bands for YHV structural proteins gp116, gp64 and p20 [15] were revealed from the purified YHV (Fig. 2a) and were confirmed by Western blot analysis using monoclonal antibodies (MAb) against each epitope (Fig 2b). Whole YHV virions were used as the target for MAb binding in subsequent tests.

Biopanning for YHV Binding Peptides

The target of this experiment was purified YHV (20 µg) incubated with the heptapeptide T7 bacteriophage library to select the population of peptides that could bind to YHV particles. In 5 rounds of biopanning

monitored by titering the eluant from the YHV-coated wells by plaque-forming units (pfu), phage enrichment increased from 2.08×10^5 to 1.94×10^6 pfu (Table 1). Thereafter, the number of phages eluted did not increase, indicating that enrichment was saturated at 9 to 9.3 rounds and that the number of molecular species was stable. A total of 89 individual clones were selected for further analysis of YHV binding specificity by ELISA.

Characterizaton of ELISA-Positive Clones

Of the 89 clones tested by ELISA, 13 gave strong positive reactions (Fig. 3). All 13 were confirmed by PCR to contain recombinant sites of about 129 bp (data not shown). Results of DNA and deduced amino acid sequences of the amplicons from all ELISA-positive plaques are shown in Table 2. They were analyzed for conserved motifs but showed none. The lack of overlap could have resulted from the high variation in available binding sites provided by using whole YHV particles.

Table 1. Selective enrichment of fragments from the library as monitored during biopanning.

Biopanning round	Phage input (pfu/100µl)	Phage eluted (pfu/100µl)	% yield	Enrichment factor
1	$1.79{\times}10^{11}$	$2.08{\times}10^{5}$	$1.16{\times}10^{-4}$	1
2	$1.79{\times}10^{11}$	$5.20{\times}10^{4}$	$2.91{\times}10^{-5}$	0.251
3	$1.79{\times}10^{11}$	$2.09{\times}10^{5}$	$1.17{\times}10^{-4}$	1
4	$1.79{\times}10^{11}$	$2.05{\times}10^{6}$	$1.15{\times}10^{-3}$	9
5	$1.79{\times}10^{11}$	$1.94{\times}10^{6}$	$1.08{\times}10^{-3}$	9.3
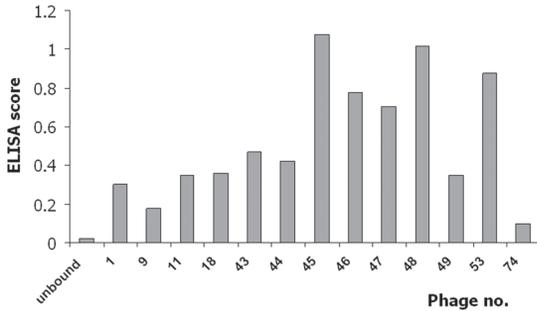
Fig 3. Optical density readings in ELISA tests with various binding phages selected from the fifth round of biopanning.

Table 2. DNA sequences and deduced amino acid sequences of inserts from ELISA-positive plaques.

YHV-binding phage	DNA sequence	Amino acid sequence
YBP7-1	CTCAATGCGAAGAGCCGTAAT	LNAKSRN
YBP7-9	AAGTCTAAGAAGTCTAGTTCT	KSKKSSS
YBP7-11	GGTCCTCAGCGTAAGCGTTCT	GPQRKRS
YBP7-18	AAGCTCAAGAGGCTCAGTTCT	KLKRLSS
YBP7-43	CGTACTAATAAGAAGAATGCT	RTNKKNA
YBP7-44	AGTAATATAAGCAATGCGTCT	SNISNAS
YBP7-45	TCTAATTCTAAGAAGCGTAAT	SNSKKRN
YBP7-46	CGTACTAAGAAGATGCGTACT	RTKKMRT
YBP7-47	AATACTAAGCGTCCTGCTCGT	NTKRPAR
YBP7-48	GGTCCTCAGCGTAAGCGTTCT	GPQRKRS
YBP7-49	GTTTCGAATAAGAAGCGTAGT	VSNKKRS
YBP7-53	CGTAAGAAGTCTAATGCTTCT	RKKSNAS
YBP7-74	GGTCCTAAGAAGAATCGTTCT	GPKKNRS

Other investigators have reported no more than random similarity between peptide isolates, indicating that multiple peptides can be equally effective at recognizing and binding to a single molecule.[16]

The clones YBP7-11 and YBP7-48 had identical sequences indicating that the number of molecular species was stable, and this corresponded to the biopanning saturation result. A dendogram was constructed from the deduced amino acid sequences
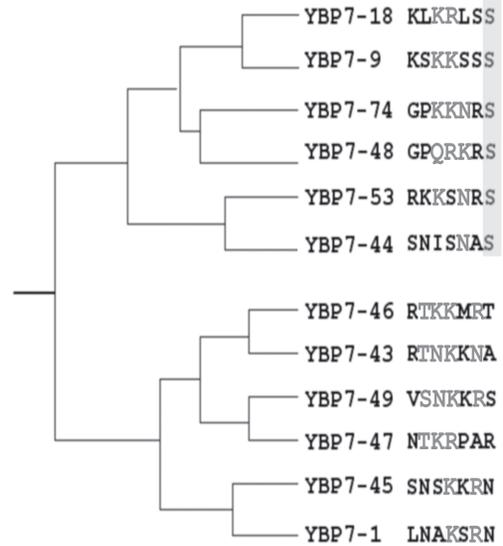
Fig 4. Dendrogram showing the relationships of the representative YHV binding phage insert sequences using http://www.ebi.ac.uk/cgi-bin/clustalw. The gray letters show aligned amino acids in the same group. The shaded, gray letters show identical, aligned amino acids.

using the distance between the two sequences. Criteria for comparing sequences were similarity scores (for amino acid sequence), isoelectric point and molecular weight. The tree contained two main clades (Fig. 4) and six sub-clades of 2 sequences each that shared 3-4 identical amino acids, except for YBP7-47 and YBP7-49 that shared no identical amino acids but only similar amino acids at three positions. For further tests, one representative of each sub-clade was selected on the basis of the highest ELISA score (i.e., YBP7-45, YBP7-48, YBP7-53, YBP7-46, YBP7-47 and YBP7-18).

Random phage display peptide libraries (PDPLs) typically contain a small population of phage clones expressing peptides that bind selectively to the target protein. Phages from this group are "target-specific binders" that can be further sub-divided into scarce "high-affinity" binders and much more abundant "low affinity" binders. The PDPLs also contain a population of "target unrelated peptides" (TUP) that may react with constant regions of the target protein or other components of the screening system such as the beads, plates or capturing molecules (e.g., streptavidin, protein A, etc.). In this study, all binding phages were compared with previously reported TUP[17] and none of the 13 selected clones were found to have similar DNA sequences.

Neutralization of YHV Infection by YBP

Neutralization of viral infectivity is a complex and poorly understood phenomenon. Studies on functional domains of protein suggest that neutralization sites

Table 3. Cumultive shrimp mortality after injection of virus mixed with phage preparations or with buffer solution alone. Values given are means of 3 replicates. 1 = negative control shrimp injected with buffer only; 2 = shrimp injected with YBP7-45 plus YHV; 3 = shrimp injected with YBP7-48 plus YHV; 4 = shrimp injected with YHV in buffer.

Hours after injection	Cumulative mortality ± SD (%)			
	1	**2**	**3**	**4**
24	3±10 (13)	2±1 (9)	3±3 (13)	2±1 (8)
44	4±3 (20)	10±9 (50)	13±8 (67)	13±8 (65)
48	4±3 (20)	13±6 (63)	19±1 (93)	16±6 (82)
50	4±3 (20)	19±1 (95)	20±0 (100)	20±0 (100)

and virus attachment sites are often distinct.[18] There are only a few examples of neutralization epitopes that are also involved in attachment of the virus to its cellular receptor.[19] In our phage neutralization tests, we chose YBP7-45 and YBP7-48 as they were in different main clades of the dendrogram and both gave high ELISA scores. The results showed that shrimp in all groups died at 48 h (100% mortality) after injection (Table 3) so there was no significant difference of shrimp mortality in all groups (P>0.05). Our negative results on protection contrasted with the positive inhibition results recently reported for shrimp challenged with white spot syndrome virus (WSSV) that had been incubated with a decapeptide motif <u>YAY</u>NN<u>S</u> from a phage display library specific for WSSV.[20] A possible reason for our negative protection results might be that our two tested phages attached to sites non-essential for infection. Alternatively, binding may have occurred at essential sites but been reversible and too weak to prevent infection. More extensive testing using variable amounts of phage and viral inoculum would be necessary to ensure that our negative test results were not due to an imbalance in the amount of YHV and phages or other variable factors. The other 11 clones could also be tested for protective ability.

Dot Blot ELISA of YHV-Binding Peptides

None of the 13 selected phage clones showed detectable binding in Western blots of YHV viral preparations subjected to 12 % SDS-PAGE (data not shown). On the other hand, all clones did bind with whole viral particles using the dot blot technique (Fig. 5). The signals for the clones differed in intensity suggesting that affinity to YHV was not equal. Similar results to those shown in Fig. 5 were obtained when the membrane was first dotted with individual phages and then probed with whole YHV particles (data not shown). The lack of binding in the western blot tests may have been the result of protein denaturation during the SDS-PAGE step in the analysis.

Although the two phage clones tested in this study were ineffective for blocking YHV infection, the dot-blot and ELISA tests suggest that one or more of the clones may be useful in developing improved diagnostic tools for YHV detection in shrimp. In addition to dot-blot and ELISA formats, they could be tested for replacement of expensive polyclonal antibodies currently used at the trap line in the lateral flow, gold-labeled, monoclonal antibody (MAb) detection strip developed for YHV in Thailand (Shrimp Biotechnology Business Unit, BIOTEC, Thailand). This substitution could reduce the cost of the kit and make it a more affordable for shrimp farmers who wish to confirm YHV infections themselves. It is also possible that the combined clones could increase the sensitivity of the test strip. For example, it has been shown with WSSV that ELISA detection is substantially improved by using two complementary MAb that target different epitopes of a single viral coat protein.[21] A similar phage display approach might be applied for improvement of the similar detection strip for white spot syndrome virus (WSSV) of shrimp that uses a gold-labeled MAb specific to the 28 kDa envelope protein VP28[22] and a polyclonal antibody at the trap line.
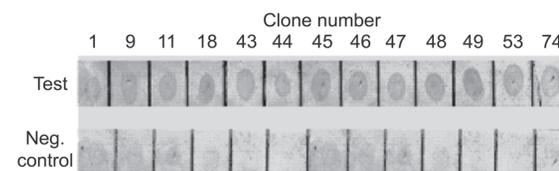
Fig 5. Results from dot-blot ELISA tests with all 13 YBPs (in two replicates) dotted on membranes. The upper membrane was incubated with a solution of YHV particles and the lower membrane with buffer. After extensive washing, both strips were reacted with monoclonal antibody V3-2B followed by the secondary antibody conjugated with horseradish peroxidase.

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