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ABSTRACT

The immunogenicity of an antigen can be increased by displaying it in a highly dense, multivalent context, such as on the surface of a virus or virus-like particle (VLP). However, for many viruses, the ability to display diverse antigens on viral surfaces through the construction of recombinant virus particles is limited because peptide insertions are not compatible with virus or VLP assembly. In this thesis, I examined the ability of VLPs of bacteriophage PP7 to have target peptides inserted into a single-chain dimer of its coat protein and form VLP that display the epitope of interest on the particle surface in an immunogenic manner. We found that bacteriophage PP7 is highly tolerant to defined peptide insertions, and a semi-random sequence library. Peptides were displayed on the surface of the VLPs and the recombinant PP7 VLPs were highly immunogenic. Moreover, a PP7 VLP displaying a neutralizing epitope from human papillomavirus (HPV) protein induced a strong antibody response that could protect animals in a mouse genital HPV infection model.

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1. INTRODUCTION

1.1 Viral vaccine advantages and pitfalls

Vaccination is the most effective public health initiative of the twentieth century. Most vaccines are based on attenuated or inactivated viral preparations. Both vaccine types are extremely immunogenic and have the ability to confer strong protection to the individuals to which they are given, but there are numerous disadvantages to them as well. It can be difficult to produce large quantities of certain viruses due to the lack of efficient tissue culture systems that allow for viral propagation (6). There is the risk of viral reversion or incomplete inactivation in given immunizations and this further complicates manufacturing (6). It is due to these issues that creating isolated viral factors through recombinant techniques has been appealing. Unfortunately, viral proteins are typically far less immunogenic than whole virus preparations and usually require large doses and strong adjuvants. There are also difficulties in engineering recombinant proteins that create the same antigenic response as authentic virions (6). Knowing the difficulties of both techniques, it is clear that another method of vaccine development would be greatly beneficial.

1.2 Virus-like particles and their immunogenic properties

Many viral structural proteins are able to self-assemble into virus-like particles (VLPs). These VLPs resemble normal infecting virus but lack viral nucleic acid and are noninfectious (6). On top of this, VLPs can be produced in large quantities through

recombinant methods in expression systems and do not depend on viral replication. Most importantly though, VLPs are strongly immunogenic and can serve as a platform for antigen display. These features make VLPs highly appealing in vaccine development.

What makes VLPs so immunogenic is the manner in which capsid proteins assemble. VLPs are made up of one or more viral proteins that assemble in dense, geometrically repetitive arrays. These formations are typically unique to microbial antigens and are strongly stimulating to the mammalian immune system (6). B cells are able to respond strongly to organized antigen display, in fact, the level of organization helps determine the level of responsiveness of the B cell (2, 6). Multivalent antigens strongly crosslink B cell receptors and lead to B cell activation and proliferation (2). Not only does multivalent display elicit strong antibody responses, it does it at a far lower concentration (as much as 1000-fold) as compared to monomeric antigens (4, 6). All this VLP technology points to the potential of strong vaccine candidates. There are in fact a number of VLP vaccines already on the market, these include the licensed HBV and HPV vaccines (6). The HBV vaccine particles self assemble from the hepatitis B major surface antigen while the HPV VLPs are formed from the major capsid protein L1 (6). These vaccines are extremely effective, safe, and produce extended antibody responses of for up to five years past immunization (8, 11). VLPs are as immunogenic as attenuated and inactivated virus, but without the safety concerns.

1.3 Antigen display on VLPs

VLPs can also be used as scaffolds to display foreign epitopes in a highly immunogenic fashion. Target antigens displayed in this high density manner on the surface of VLPs induces the same strong antibody response as the VLPs themselves. This enables the immunogenicity of antigens to be strongly increased, to the point that even self-antigens become immunogenic when displayed on VLPs (6, 7). Knowing this, VLPs could serve as a platform for display of antigen and create an effective immune response to epitopes that are difficult to target because they are normally poorly immunogenic. There are two main techniques for displaying antigens on VLPs, the first being chemical conjugation which involves attaching peptides to the VLPs using a cross-linker. This method allows for larger, non-linear peptides of any conformation to be attached, but tends to less advantageous from a manufacturing perspective. Genetic insertion is the method used throughout this thesis and involves using PCR and common cloning techniques to insert the antigen epitope into a viral structural protein that can be expressed in an appropriate expression system, forming VLPs that display the target peptide. Chemical conjugation, minor protein fusions, and genetic insertion provide options to attach targets of interest to the VLPs, this makes the particles adaptable to many different and non-conventional targets and allows for great customization (6). It is this customization and flexibility that make VLPs useful in vaccine development. Utilization of VLPs from different phage or various virus species with the same target peptide could vary greatly in effectiveness. Therefore VLPs displaying the same target epitope, but from different phage are worthwhile to create, seeing as one may work better than another.

It is important to note that while current VLP vaccines are highly effective at creating a strong immune response to either hepatitis B major surface antigen or HPV major capsid protein, these antigens were native viral proteins and required no recombinant engineering. Many viruses do not make good display platforms for epitope insertions. Viral structural proteins are commonly intolerant of diverse heterologous peptide insertions without extensive engineering. In addition, many VLPs are extremely limited in the length, size, or other properties of the peptide that can be successfully inserted, and this can strongly reduce their use as a display platform.

1.4 VLPs derived from the bacteriophage MS2

As described above, the genetic insertion of target sequences into viral structural proteins to generate chimeric VLPs is a common method for displaying heterologous epitopes on VLPs and many different VLP types derived from animal, plant, and bacterial viruses have been adapted for this purpose. Our lab has been particularly interested in using RNA bacteriophages for peptide display. We choose to work with phage because they are easily grown in large amounts, structurally simple, and easy to work with. Bacteriophage MS2 is a positive-sense single stranded RNA virus. The viral structural protein, coat protein, forms an icosahedral capsid out of 90 coat protein dimers that are held together by covalent interactions (5). Previously, we attempted to make chimeric VLPs using MS2 by inserting peptides at a surface exposed loop (the AB-loop) on MS2 coat protein. These experiments showed that insertion of foreign peptide sequences into the AB-loop display site caused coat protein to misfold (20). The recombinant proteins were detected only as insoluble aggregates. However, we constructed a version of coat protein in which two copies of coat protein are genetically

fused into a "single-chain" dimer. When foreign peptides were inserted only into the C terminal (downstream) copy of the coat protein, most folding defects were corrected. This single-chain dimer in MS2 bacteriophage had excellent results; the proteins were produced in normal amounts, found in the soluble fraction, and the recombinant protein formed VLPs that were highly compatible with diverse peptide insertions. Moreover, recombinant MS2 VLPs were highly immunogenic, inducing high titer antibody responses against the targeted peptide in mice. In addition, we showed that we could create complex libraries of recombinant MS2 VLPs displaying diverse random peptides. Because individual MS2 VLPs encapsidated the RNA that coded for the peptides displayed on their surfaces, this opened the possibility that these MS2 VLP libraries could be used in affinity selection applications analogous to filamentous phage display.

Knowing that this process worked in MS2 bacteriophage, we wanted to know if the same could be done using another bacteriophage, PP7, seeing as PP7 VLPs are more thermodynamically stable as compared to MS2 outside of the bacterium, where the thiols oxidize to disulfides (5). If the same process could be done in PP7, the library of VLPs to screen against would be larger. Along with those ideas, VLPs from a different bacteriophage may have different immunogenic properties compared to the MS2 particles. Lastly, PP7 particles are not cross reactive with MS2, this may have application when serial administration of VLPs is required.

1.5 PP7 VLPs and antigen insertions

Our work focused on the phage PP7 which is a positive-sense single-stranded RNA virus that infects *Pseudomonas*. The PP7 genome is 3588 nucleotides in length and encodes four proteins: replicase, lysis, P25, and coat (22). The coat protein of PP7 phage

serves as the major viral structural protein along with being a translational repressor of the viral replicase (14). Coat protein binds a specific RNA hairpin at the start of the replicase codon to inhibit translation, much like the coat-proteins of other-studied phages MS2 and Qβ, although their RNA loop structure for binding coat protein differ significantly and PP7 coat protein will not repress the operators of these other phage (14). The bacteriophage PP7 capsid is constructed of 180 coat protein monomers that are 127 amino acids long, has a diameter of about 25 nm, and has T=3 symmetry as shown in Figure 1 (22).

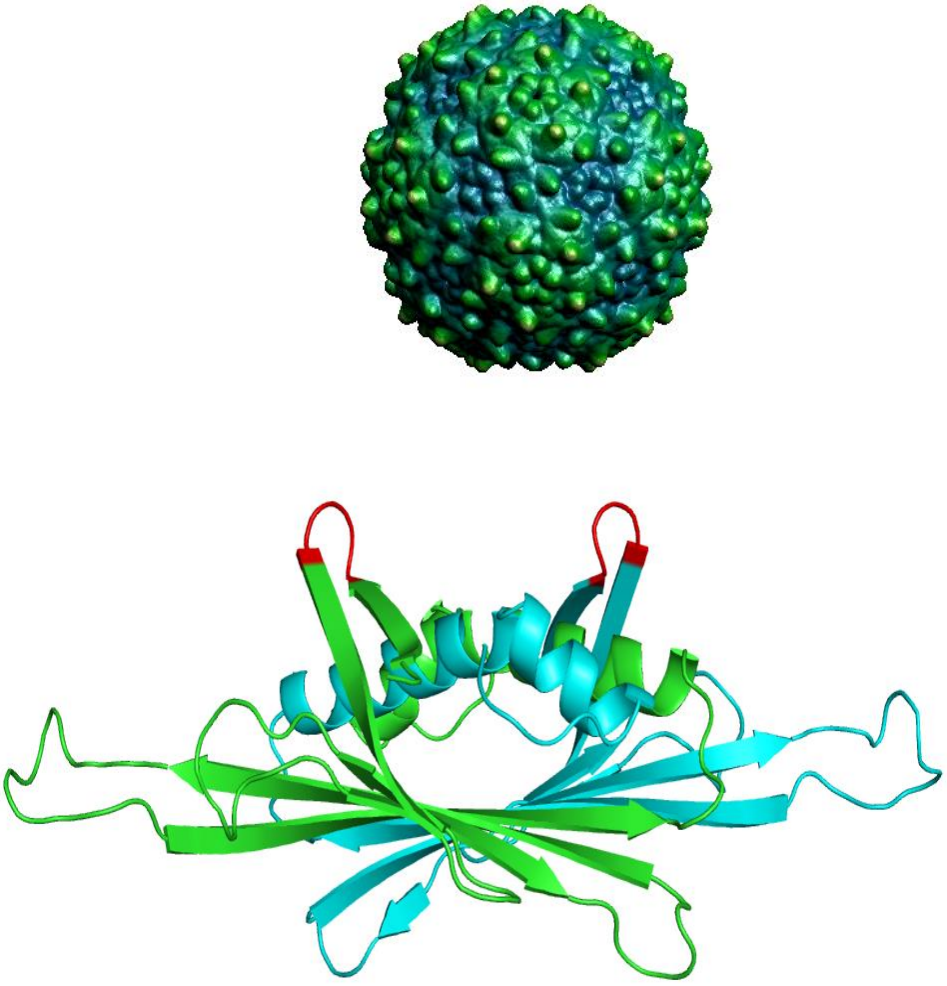


Fig 1. Structural diagrams of PP7 VLPs and coat protein

Rendered surface view of PP7 coat protein dimer. 90 dimers make up the VLP. Also a ribbon diagram of the coat protein dimer with the loop display sites shown in red.

1.6 Peptide Display on PP7 VLPs

Using the same "single-chain dimer" strategy as with MS2, a PP7 plasmid containing the single-chain dimer was created (5, 14). We made four insertions into a surface-exposed loop; these included epitopes from the protective antigen (PA) of *B. anthracis*, the minor capsid protein L2 of HPV, V3 loop antigen from HIV, and the marker antigen FLAG. The PA peptide we chose represents a neutralizing epitope, and, since PA forms the basis of currently anthrax vaccine, serves as a good model (6). L2 is involved in HPV infectivity and is the target of broadly neutralizing antibodies against HPVs (12). V3 is a region of the HIV envelope protein and it core sequence is conserved throughout a limited number of HIV isolates making it an attractive target for a neutralizing antibody response that is easily monitored in HIV neutralization assays. FLAG is an eight amino acid epitope that is often used as a protein tag. Monoclonal antibodies against FLAG are commercially available. We wanted to create chimeric PP7 VLPs that displayed heterologous epitopes that could induce a strong antibody response in mice. We showed that PP7 bacteriophage is compatible with various peptide insertions within the coat protein, has the ability to form functional VLPs that display the target antigen, and are immunogenic and stimulate a vigorous antibody response.

2. METHODS

2.1 Cloning of insert sequence into PP7 coat protein

To create the VLPs that display heterologous peptides we first had clone the sequences into a PP7 expression vector. PCR reactions were conducted using 50ng of p2P7K32 plasmid as template. Forward PCR primers (fig.2) were designed to include a unique Kpn I site downstream of the junction of the two copies of the coat protein designated as the single chain dimer. Five reactions in total were conducted all using the same reverse primer designated 12113 and unique forward primers that were predicted to insert four specific peptides into PP7 coat; the FLAG epitope (DYKDDDDK), an epitope from anthrax protective antigen (VHASFFDIGG), HIV V3 loop derived antigen (IQRGPGRAPV), and an epitope from HPV L2 minor capsid protein (QLYKTCKQAGTCPPD) (12), (5). Successful amplification was verified by agarose gel electrophorsis. Fragments were purified using a PCR clean-up protocol and then digested with the restriction enzymes, Bam HI and KpnI (from New England Biolabs). The PCR products were ligated to BamHI/KpnI cut p2P7K32 vector (fig.3) by ligation and then used to transform *E.coli*.

Forward Primers

FLAG

*ggtacc***gattataaagatgatgatgataaa**gaggctactcgcactctgactgag

D Y K D D D D K

Anthrax Protective Antigen (Atx)

*ggtacc***gtgcatgcgagctttttgatatcggcggc**gaggctactcgcactctgactgag

V H A S F F D I G G

HIV V3

*ggtacc***atccagcgcggcccgggccgcgcgtttgtg**gaggctactcgcactctgactgag

I Q R G P G R A P V

HPV L2

*ggtacc***cagctgtataaaacctgcaaacaggcgggcacctgcccgccggat**gaggctactcg…

Q L Y K T C K Q A G T C P P D

pP7K mutant coat protein sequence (*KpnI site in italics*):

atggccaaaaccatcgttctttcggtc*ggtacc*gctactcgcactctgactgag

Fig.2 List of forward primers for target peptides

List of forward primers 5' to 3'. KpnI restriction site is in italic and the peptide insertions are in bold. The amino acid sequence of the insertions is listed below.

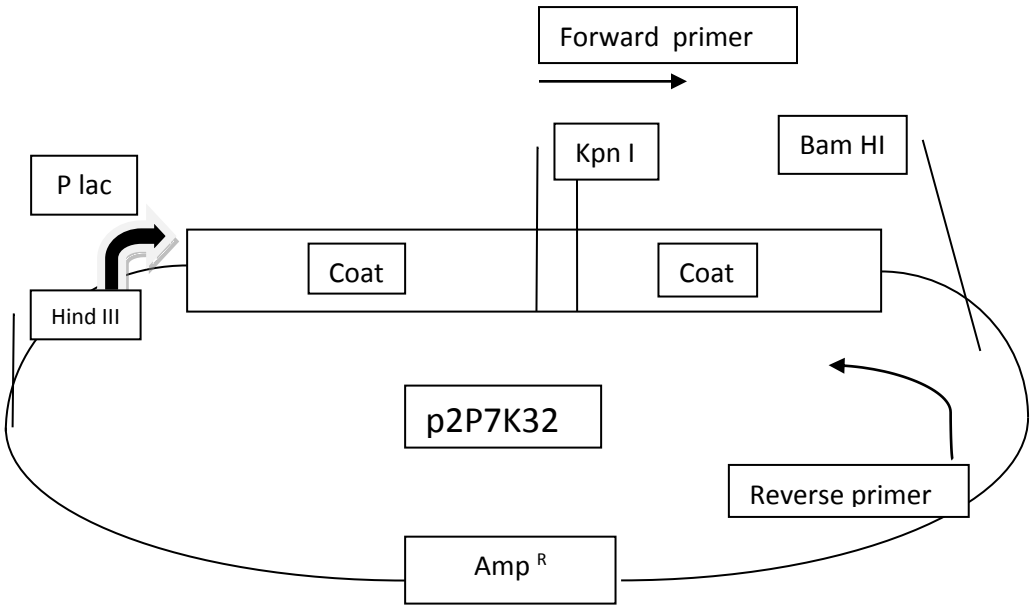


Fig.3 Diagram of the p2P7K32 plasmid

Plasmid p2P7K32. Listed are important restriction sites, the single chain dimer coat protein sequence, primer placement, and resistance cassette.

2.2 Screening and Expression of recombinant PP7 VLPs

The next step was to screen transformants to identify recombinants and to determine whether peptide insertions affected coat protein function. Protein expression and translational repressor activity was determined by transformation (chemically) of the ligation mixtures into *E. coli* strain CSH41F- with the pRZP7 translational repression reporter plasmid (4). Coat protein normally acts as a translational repressor by shutting down synthesis of the viral replicase by binding the ribosome-binding site (14). Through fusion of this sequence on the pRZP7 plasmid upstream of *E. coli lacZ* gene, functional coat protein variants can be easily identified. The cells that contain the plasmid with functional coat protein deliver white colonies while nonfunctional coat protein variants produce blue colonies when plated on 5-bromo-4-chloro-3-indolyl-β-d-galactoside (X-Gal) media. Cells (100µl) were plated on LB medium containing (X-Gal) along with ampicillin (AMP) and chloramphenicol (CAM). These plates grew at 37ºC overnight and were assessed by blue/white screening. To assess whether these clones contained the insert sequence and if they formed VLPs, seven white colonies (those with functional repression) were picked using sterile technique and placed into 1ml if 2xYT or LB media with 50µg/ml of both CAM and AMP and grown at 37ºC overnight while shaking. Plasmids were isolated by mini prep and then sequenced using the aforementioned 12113 primer at the DNA services at the University of New Mexico to verify the predicted sequences. For the capsid purification and western blot, 1ml cultures were spun down at

14000 rpm for 5 minutes, resuspended in 250µl of sepharose column buffer (10mM Tris-HCL, pH7.4, 100mM NaCl, 0.1mM $MgSO_4$, 0.01mM EDTA) (SCB) buffer, then lysed by sonication for 10 seconds. The insoluble material was spun out and then 0.5µl of DNase was added to the supernatant and incubated at 37ºC for one hour. The samples were analyzed on a 1% potassium phosphate gel stained with ethidium bromide to note capsid RNA. It is important to note that the phosphate buffer for the gel must be kept cycling with a pump. The gel was subsequently blotted to nitrocellulose in 1X tris-saline (TS)/BSA for 30 minutes. The blot was incubated with the primary antibody (α-PP7 mouse sera) at a 1:2000 dilution for 90 minutes then washed in TS once and twice in 2X tris-saline-IGEPAc (TSI). The blot was then incubated using AP-labeled goat anti mouse IgG at a 1:5000 dilution for 30 minutes, washed and developed.

2.3 Growth and purification of recombinant PP7 VLPs

To grow large cultures of VLPs that contained the specific insert of interest we selected mini preps that had matching capsid expression in the western blot. We transformed *E. coli* pRZP7 CSH41F- cells (60µl) with the PP7 expression plasmid, containing each of the target antigens and grew in 1ml of LB overnight at 37ºC with AMP and CAM. Then we added this 1ml culture to 100ml of LB and grew for 8 hours at 37ºC. The 100ml cultures were spun down at 8000 rpm for 25 minutes in a Beckman Coulter TJ25 centrifuge. The pellets were suspended in 10ml of lysozyme solution [50mM Tris-HCl, pH8.5, 100mM NaCl, 10mM EDTA, 10mM dithiothreotol (DTT)] along with 0.1g of hen egg lysozyme and incubated on ice for 1 hour. 25µl of deoxycholate (DOC) was added and then incubated on ice for another 30 minutes. The solutions were sonicated 3 times for 45 seconds to aid in lysis. 5µl of both DNase and

RNase (10mg/ml) were added to the samples and incubated at 37ºC for 2 hours. All samples were then spun at 9000 rpm for 30 minutes again in a Beckman Coulter TJ25 centrifuge. The supernatant was collected and ammonium sulfate was added to 80% saturation. Samples incubated on ice for 15 minutes and then were spun down at 9000 rpm for 10 minutes. The pellets were dissolved in 2ml of SCB and spun one more time at 6000 rpm for 5 minutes. The supernatant from the samples was added to a 45cm column of sepharose CL-4B in SCB buffer (19). 5ml fractions were collected and subjected to SDS-PAGE and 1% potassium phosphate gel for capsid verification. Fractions from the column that contained VLPs were pooled and then concentrated by ammonium sulfate precipitation to 50% saturation. This was determined to be the ideal level of saturation based off earlier saturation experiments. Once all the ammonium sulfate was in solution the samples were incubated on ice for 15 minutes and then spun by centrifugation at 8000 rpm for 15 minutes. The pellet was then dissolved in 1ml of SCB, dialyzed versus SCB for 1 hour, and then again with fresh SCB overnight at 4ºC. Samples were then removed from the membrane and the concentration of VLPs was determined using the Bradford assay (3).

2.4 Immunological characterization of recombinant VLPs

We wanted to first ensure that antibodies specific for the inserted epitope bound VLPs. FLAG, L2, or wild-type PP7 VLPs were immobilized overnight at 4ºC onto an ELISA plate (Immulon 2) at 500ng per well (19). The wells were then blocked with PBS and 0.5% non-fat dry milk for two hours at room temperature. Dilutions of an anti-FLAG monoclonal or an anti L2 antibody (RG-1) (7, 10) were added to the wells, and incubated at room temperature for two hours. The reactivity of either FLAG monoclonal to the

VLPs was determined by incubating a horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) at a dilution of 1:5000 in blocking buffer in the wells for one hour at room temperature. The plate was developed with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and the OD measured at 405nm with an OpSys MR plate reader (Thermo Labsystems, Waltham, MA). Values greater than twice the background were considered positive. Antibody titers against target antigens were determined by coating plates with 500ng of target antigen and bound overnight at 4ºC. Plates were blocked with blocking buffer (PBS/0.5%milk) for two hours at room temperature. Collected sera was serially diluted in blocking buffer and then added to the wells for two hours. HRP goat anti-mouse IgG secondary antibody (1:4000) was allowed to incubate in the wells for one hour. Plates were developed with ABTS and OD measured at 405nm.

2.5 Animal model and inoculation schedule

To assess antibody titers and collect antisera, C57Bl/6 and B10 mice were inoculated with VLPs. Antisera was gathered by intramuscularly inoculating groups of six mice (4 C57Bl/6 mice and 2 B10 mice) with 10µg of VLPs (four VLP types), plus 50µl Incomplete Freund's Adjuvant (IFA) (19). All mice were boosted with the same amount of VLPs two weeks later. Sera were collected after each inoculation and the mice were terminally bled three weeks later (four weeks for the FLAG and L2 mice). All animal care was in accordance with both the National Institutes of Health and with the University of New Mexico guidelines.

2.6 Characterization of antisera

Antibody titers against target antigens were determined by coating plates with 500ng of target antigen and bound overnight at 4ºC. Plates were blocked with blocking buffer (PBS/0.5%milk) for two hours at room temperature. Collected sera was serially diluted in blocking buffer and then added to the wells for two hours. HRP goat anti-mouse IgG secondary antibody (1:4000) was allowed to incubate in the wells for one hour. Plates were developed with ABTS and OD measured at 405nm.

2.7 Genital HPV challenge

Groups of five Balb/c mice were immunized intramuscularly with 10 µg P7-16L2 VLPs (with incomplete Freund's adjuvant) twice at a two-week interval. Two weeks after the second vaccination, mice were challenged with HPV16 or HPV45 pseudovirions encapsidating a luciferase reporter plasmid (pCLucf), as described previously (21). At 48 hours following the introduction of pseudovirus, the mice were each given an intravaginal instillation of 20 µl of luciferin (0.3 mg) and imaged with a Xenogen IVIS 100 (Caliper Life Sciences). Images were taken at 3 min postinstallation of luciferin at medium binning with a 30-s exposure. Images were then analyzed by drawing an equally sized region of interest for each mouse and measuring total flux (photons/second).

3. Results

3.1 Insertion and functional development of target antigens into the AB-loop of PP7 coat protein

It was previously shown that a single-chain dimer version of the MS2 coat protein was largely tolerant of diverse peptide insertion (19) into the surface exposed AB-loop. The three-dimensional structure of PP7 is similar to that of MS2. Both VLPs contain a surface-exposed loop structure (called the AB-loop, shown in Fig. 1) which seemed to be an obvious site for peptide insertion. To create recombinant PP7 VLPs displaying target peptides, PCR primers were designed that allowed for the cloning of five select sequences into the PP7 coat protein; these included, HPV L2 minor capsid protein (QLYKTCKQAGTCPPD), the anthrax protective antigen with three random flanking amino acids on either end (XXXASFFDXXX), the anthrax protective antigen with set flanking sequence (VHASFFDIGG), the HIV V3 loop derived antigen (IQRGPGRAPV), and the marker FLAG epitope (DYKDDDDK). We amplified the insert sequences using a unique forward primer and a common reverse primer under normal PCR conditions and yielded strongly amplified PCR fragments (fig. 4).

The PCR products were cloned into a vector (p2P7K32) that contains a single-chain dimer version of PP7 coat protein using the restriction enzymes Bam HI and Kpn I. After the digestion, the products were run on a 1% agarose gel and isolated for clean up and ligation. Figure 5 shows the large vector segment along with the cut PCR product.

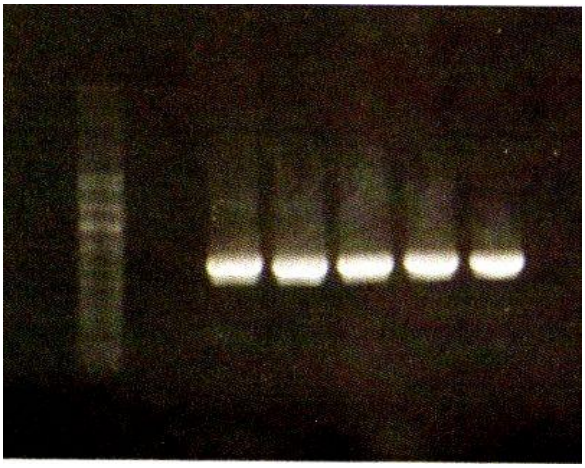


Fig.4 - PCR of target insertions
1% agarose gel of PCR products. These are the epitopes that we wish to display on the VLP surface with L2 being the HPV minor capsid protein, Atx is the anthrax protective antigen, Atx(r) is the protective antigen with random flanking sequence, V3 is HIV derived loop antigen, and FLAG epitope is an engineered marker. All PCR products were designed to be inserted into the p2P7K32 plasmid.

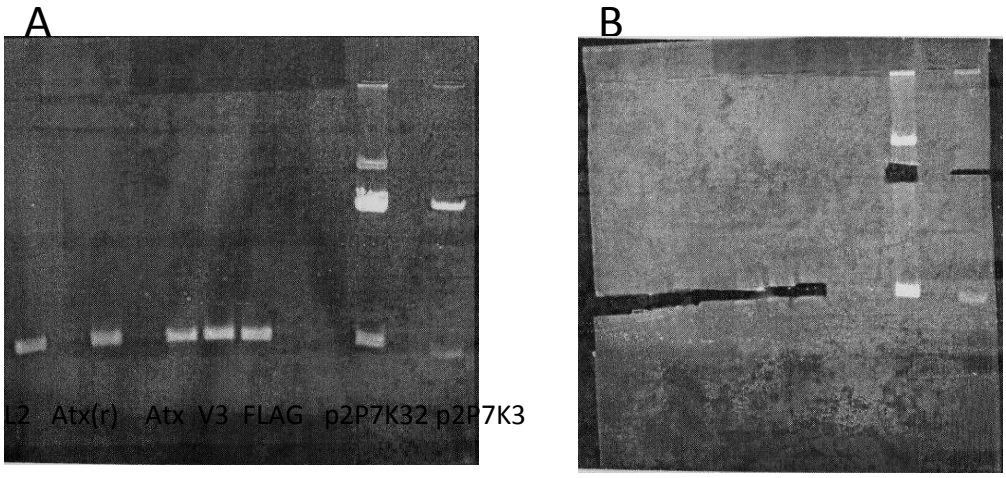


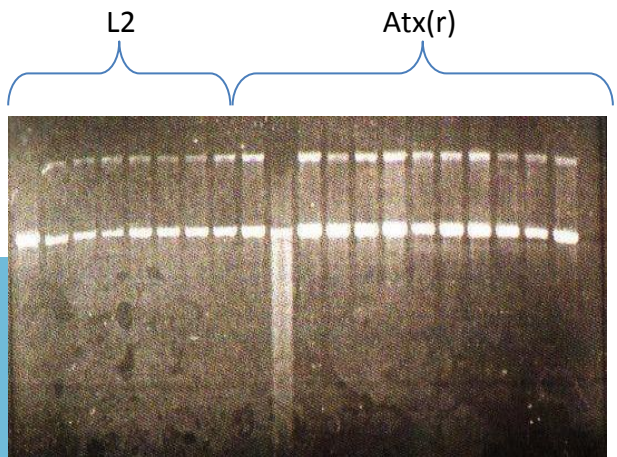
Fig.5A Restriction digest and extraction
1% agarose gel of Bam HI/Kpn I cut amplified PCR products and vector. p2P7K3 serves as a control to verify proper vector digestion.

Fig.5B Restriction digest and extraction
arose gel of Bam HI/Kpn I cut amplified PCR products and vector, post gel isolation. PCR products were later ligated to the p2P7K32 vector.

After the isolation and subsequent ligation, it was important to verify that there was in fact proper insertion of the PCR product into the vector and that actual capsids are formed. Ligation products were chemically transformed into *E.coli* pRZP7 CSH41F- cells. These cells contain the translational repression reporter plasmid (15) that allows

for a clear and simple blue white screen for functional coat protein activity. Coat proteins normal function is to act as a translational repressor by binding the ribosome-binding site in the viral replicase and of course to act as the subunits of the viral capsule (16-19). With a *lac Z* gene as a reporter, copies of viral coat protein with proper and functional folding will inhibit expression of the *lac* gene and yield white growth colonies. Misfolded coat protein will not stop the expression of *lac* and the subsequent colonies will be blue when plated on X-gal media. All five clones gave predominately white colonies, indicating that the insertions were accepted and still allowed for proper coat protein folding and function.

To confirm our constructions were correct, plasmids were isolated and digested with Bam HI and Kpn I to further verify that the new plasmids showed a proper frame shift based upon their antigen insertion (fig. 6). Those with the correct frame shift were subjected to sequence analysis at DNA services at the University of New Mexico. The resulting sequence analysis that showed epitope insertion into the coat protein was correct for all the samples except the first and sixth lane of V3 and the first lane of FLAG. This indicated that our plasmid construction was correct and we could advance with VLP formation and purification.



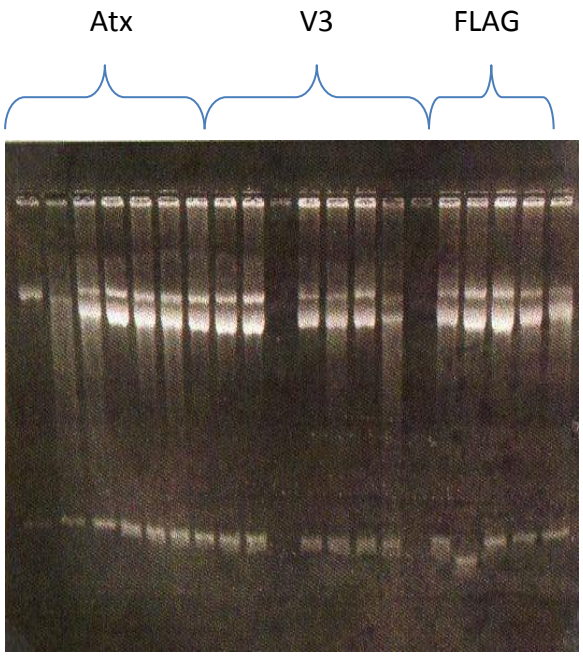


Fig.6 Restriction digest of minipreps
1% agarose 50mM potassium phosphate gel of plasmid minipreps digested with Bam HI/Kpn I compared against p2P7K32 cut with the same restriction enzymes. All but a few of the lanes show a proper frame shift of the samples against the vector (two lanes incorrect in V3 and one in FLAG).

We assessed the presence of VLPs in lysates of cells expressing the recombinant coat proteins. White colonies for each antigen were grown overnight at 37°C in 1ml of LB media. Then the samples were lysed and run on a 1% agarose gel containing 50mM

potassium phosphate. Ethidium bromide staining detected RNA-containing VLPs (fig. 7A). A western blot using anti-PP7 polyclonal sera was also conducted to verify that these bands contained viral coat protein (fig. 7B). Both assays indicated that all five recombinant coat proteins formed VLPs. It is important to note that for the random anthrax insertion (Atx(r)) there was heterogeneity in the migration of VLPs, reflecting the heterogeneity of VLPs with different surface charges.

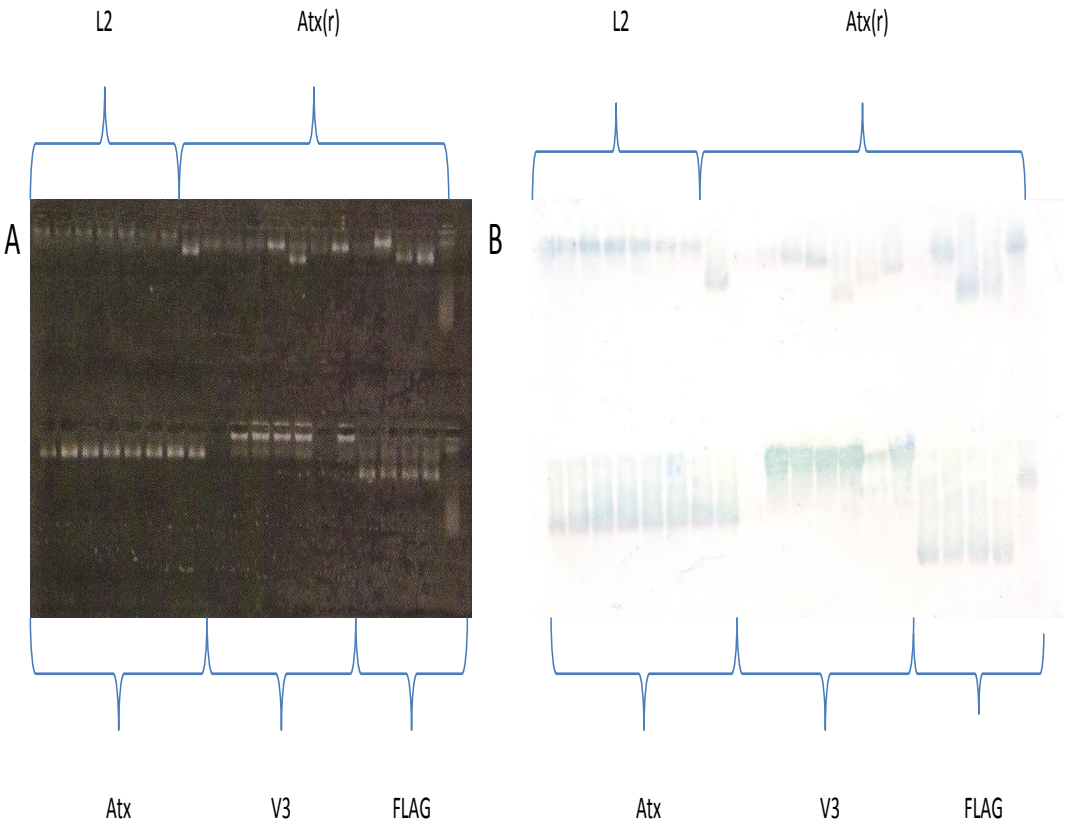


Fig 7A. Phosphate gel electrophoresis of VLPs

1% agarose 50mM potassium gel of capsid RNA for L2, Atx(r), Atx, V3, and FLAG VLPs.

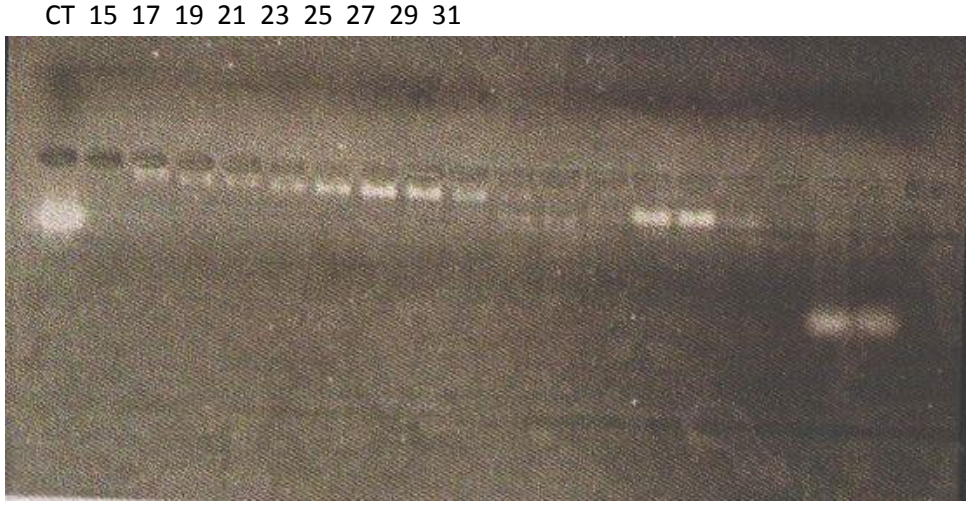
Fig 7B. Western blot of VLPs

Western blot of L2, Atx(r), Atx, V3, and FLAG protein.

3.2 Growth and purification of VLPs

Now that it had been adequately determined that the cloning had yielded functional plasmids that contained the proper insertions and formed VLPs, we made larger stocks of VLPs. 100ml overnight *E.coli* cultures were lysed and sonicated, and subsequent PP7 VLP stocks were then purified by column chromatography on Sepharose CL4B (19). The collected fractions were subjected to agarose gel electrophoresis to identify the VLP associated RNA (fig. 8A). The same fractions were also run on a 17% SDS-PAGE gel to identify the viral coat protein (fig. 8B). Figure 8 shows these results for the L2 antigen only, the other antigen data is not shown. As shown, VLPs are in fractions 15-31, which is consistent with migration of wild-type PP7 VLPs on the Sepharose column. These fractions containing VLPs were collected, pooled, and concentrated by ammonium sulfate precipitation.

A



B

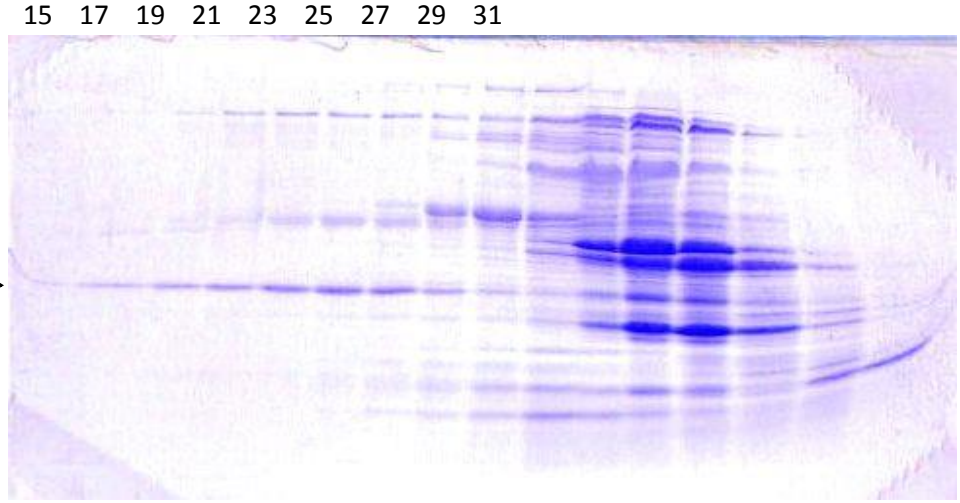


Fig. 8A Phosphate gel electrophoresis of collected fractions

1% agarose 50mM potassium phosphate gel of L2 PP7 VLPs collected in 5ml fractions from Sepharose CL-4B chromatography. CT VLP refers to a conventional MS2 VLP and it extracted RNA.

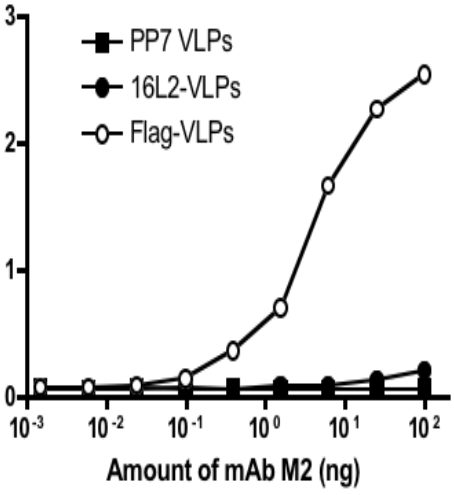
Fig. 8B Phosphate gel and SDS-PAGE gel electrophoresis of collected fractions

17% SDS-PAGE gel display the same column collected L2 fractions. Based on earlier studies it is known that the VLPs come out in the 15-30 range of fractions and have the least amount of cellular protein contamination. Here the arrow indicates the VLP coat protein. It is clear to see that earlier fractions are the cleanest samples.

3.3 Peptides displayed on PP7 VLPs are exposed to the immune system and are immunogenic

The next step was to see if the VLPs we had created displayed the peptides we had inserted on the VLP surface. The first test involved coating plates with 500 ng of FLAG PP7 VLPs, HPV L2 PP7 VLPs, or wild-type PP7 VLPs and then probing the VLPs with monoclonal antibodies against either the FLAG epitope (M2) or the L2 epitope (RG-1). There should be little cross reactivity between either the M2 or RG-1 (10) monoclonal and the non-matching PP7 VLPs. Binding of the monoclonal antibody was detected using a horseradish peroxidase-labeled goat anti-mouse IgG secondary antibody, followed by development with ABTS. Figure 9 shows that the peptides are displayed correctly and on the surface of the VLP and are recognized by the appropriate monoclonal antibodies. It is clear that the antigens are presented well on the AB-loop structure and that there is high specificity between the peptide and monoclonal antibody.

A



B.

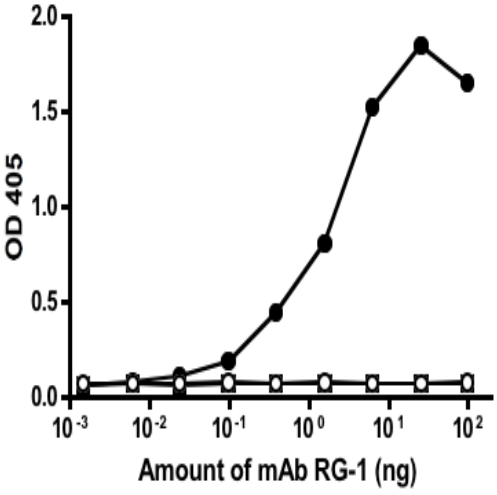
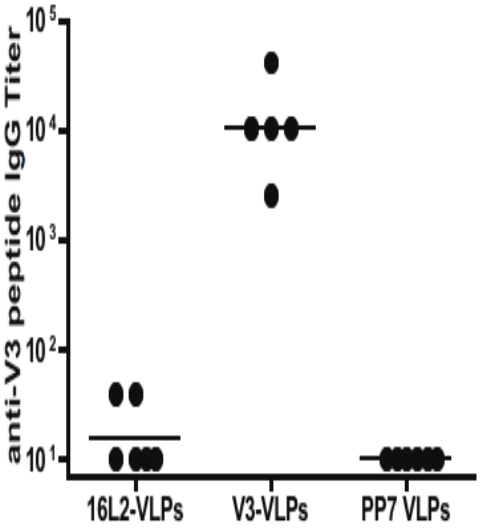


Fig. 9 Binding of monoclonal antibodies to recombinant PP7 VLPs

500 nanograms per well of L2/V3/FLAG PP7 VLPs were mixed with varying dilutions of an **A.** anti-FLAG monoclonal antibody(M2) that binds to FLAG VLPs alone or **B.** anti-L2 monoclonal antibody (RG-1) that binds to PP7 L2-VLPs, but not PP7 V3-VLPs or PP7 FLAG VLPs. Binding was detected using a horseradish peroxidase-labeled goat anti-mouse IgG secondary followed by development with ABTS. Reactivity was determined by measurement of the absorbance at 405 nm (OD 405).

The next step was to show that these VLPs were immunogenic. To test the immunogenicity, we intramuscularly inoculated C57Bl/6 and B10 mice with two 10µg doses of L2 PP7, V3 PP7, or wild type PP7 VLPs, and then collected sera from the mice two weeks after the second immunization. Sera was tested for IgG antibodies against the V3 or the L2 peptide by ELISA (fig. 10). Mice immunized with the recombinant VLPs elicited high titer IgG responses (> 10^4) against the appropriate peptides, whereas no reactivity was seen in the negative controls. Thus, the recombinant PP7 VLPs were capable of inducing high titer antibody responses against the inserted peptides.



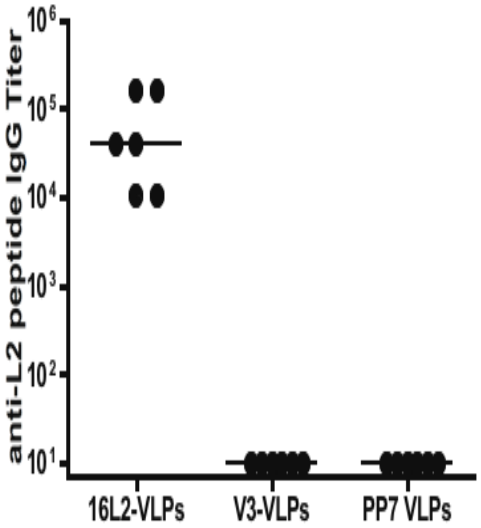


Fig.10. Antibody responses to immunization with PP7 VLPs

IgG antibody responses in groups of mice immunized with wild-type PP7 VLPs, V3-VLPs, or 16L2-VLPs. Data shows end-point dilution ELISA titers against a peptide representing the HIV V3 peptide linked to KLH (left panel) or a peptide representing amino acids 14-40 from HPV16 L2 conjugated to streptavidin (right panel). Results are from sera obtained two weeks after the second vaccination. Each datum point represents the antibody titer from an individual mouse. Lines represent the geometric mean titer for each group.

3.4 Mice immunized with 16L2-VLPs are strongly protected from genital HPV pseudovirus infection

The 16L2-VLP vaccine we designed contains amino acids 17-31 from HPV16 L2, a region shown to contain one or more highly cross-reactive neutralizing epitopes (1, 10), suggesting that the 16L2 VLPs could potentially protect against HPV challenge. We assessed whether 16L2-VLPs could protect mice from HPV challenge using a HPV pseudovirus/mouse genital challenge model (21). Groups of five Balb/c mice were immunized intramuscularly with 10 µg PP7-16L2 VLPs (with incomplete Freund's adjuvant) twice at a two-week interval. Two weeks after the second vaccination, mice were challenged with HPV16 or HPV45 pseudovirions encapsidating a luciferase reporter plasmid (pCLucf) (13, 21). Two days following the introduction of pseudovirus, the mice were each given an intravaginal instillation of 20 µl of luciferin and imaged. Images were then analyzed and total flux (photons/second) was measured. The more illumination that is seen indicates more infection. Mice immunized with the adjuvant Alum serve as the positive control and 100% infection, while mice that were mock-challenged with pseudovirions serve as an uninfected control. It is clear that the L2 VLPs provide fairly strong protection (fig. 11). Mice immunized with 16L2-VLPs were strongly (>98%) protected from infection with the homologous pseudovirus, HPV16, whereas mice immunized with Alum were not protected. We also tested whether vaccination with 16L2-VLPs could protect mice from genital infection with a heterologous HPV type. We chose HPV45 pseudovirus because its L2(17-31) sequence varies from the HPV16 sequence at three of the fifteen amino acid positions. As shown in Figure 11, 16L2-VLP-immunized mice were also protected (~83%) from genital infection with HPV45

pseudovirus. Thus, 16L2-VLPs have potential as a pan-HPV vaccine. Because the L2 particles were designed from the HPV 16 minor capsid protein, it makes sense that the best protection is seen in the HPV 16 strain. We also saw a fairly strong response to the HPV 45 strain. This cross reactivity between strains could allow for a vaccine that protects broadly. One could even imagine a VLP that displays several strains of the minor capsid protein to provide very broad protection.

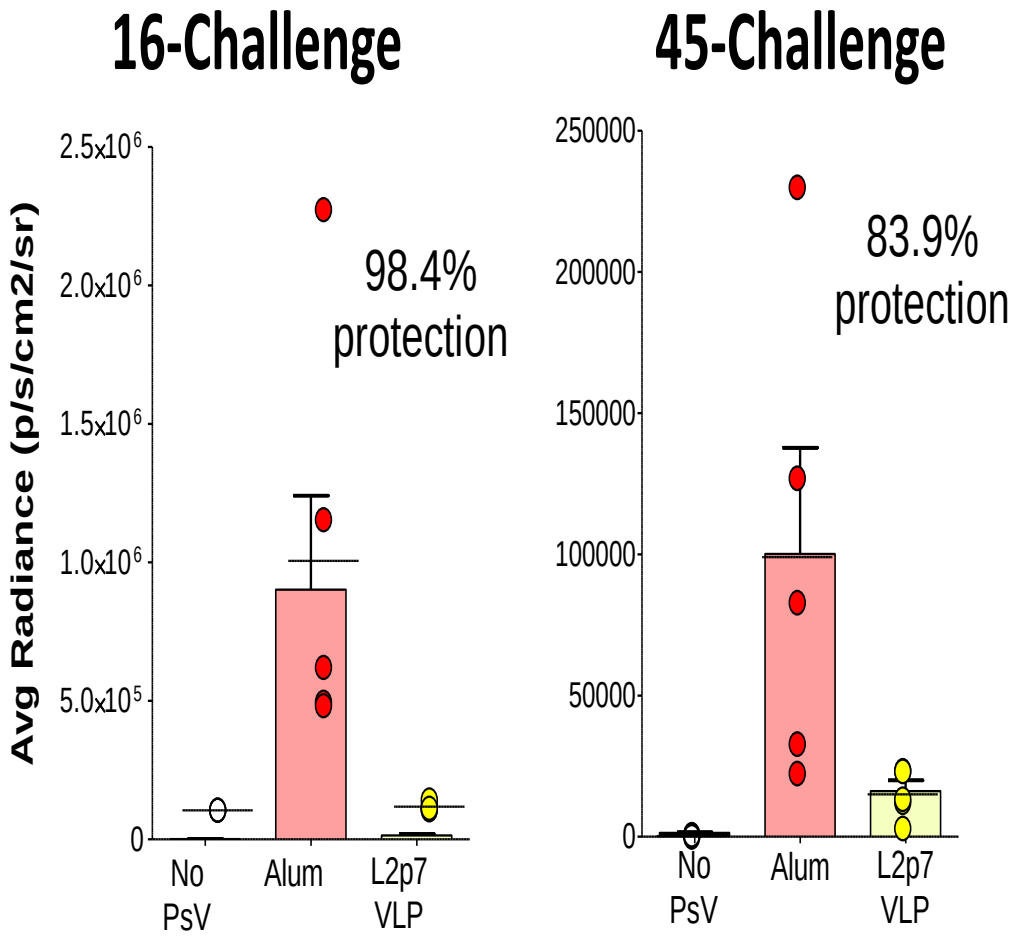


Fig. 11 VLP immunization protect mice from challenge with HPV pseudovirus

in vivo luciferase assay of mice vaginally challenged with HPV16 or HPV45 pseudovirions. Groups of five mice were immunized two times with 16L2-VLPs or, as a control, Alum. Two weeks after the second immunization mice were challenged with either HPV16 pseudovirus (left panel) or HPV45 pseudovirus (right panel) containing a luciferase reporter. As a control, a group of five mice were uninfected. Luciferase activity was quanititated 48 hours after infection.

4. DISCUSSION

Throughout this thesis, we made it our goal to show that PP7 can be used to display target peptides. We started by creating primers to four target epitope sequences, and then cloned them into the PP7. Insuring that heterologous peptides could be inserted into the coat protein and still allow for proper folding and display was the next step. We found through blue/white screening, restriction digest, and sequencing that we got proper insertion of peptides. To verify that VLPs were forming we preformed gel electrophoresis and western blotting to show the presence of RNA and coat protein. Finally the VLPs were grown in large scale and collected by column chromatography.

We needed to insure that these VLPs displayed the target peptides, were not cross-reactive, and induced high antibody titers. ELISA of VLPs against serial dilutions of either monoclonal antibody or antisera showed that the PP7 VLPs were in fact able to display the target epitope, induce high titers, and were not cross-reactive. A luciferase assay provided proof of concept by showing that the 16L2 particles provided strong protection against pseudo infection by HPV 16, and HPV 45. Again, the near complete protection seen in HPV 16 infection is to be expected as the VLP was designed from HPV 16 minor capsid protein, but the 83% protection seen against HPV 45 even given the strong variation in sequence would suggest that these VLPs could be broadly neutralizing.

Traditional study of viruses has looked at their role as infecting agents and the cell biology associated with infection; but current VLP study and vaccine development have allowed the virus to become a nanoplatform (9). VLPs could be used in the same manner as filamentous phage. The components that make VLPs so appealing for vaccine development involve being immunogenic and being able to display varying target epitopes, along with encapsidating their RNA/DNA for recovery and epitope identification. A library of random inserts could be created that expands across bacteriophage species, and tested against sera from a virally infected individual. Positive VLPs could be selected and their RNA sequences recovered. Mutable rounds of selection could be conducted till a highly immunogenic VLP is produced and used directly as a vaccine candidate.

It is essential that the VLP display the target peptide in a dense array to create strong antibody cross-linking and immune response, but it is also important for the particle to encapsidate its RNA sequence. If the VLP cannot encapsidate its DNA/RNA then it is impossible to recover sequences by affinity selection for epitope discovery. It is clear that both components are necessary for VLPs to serve as an effective display platform that also can be used for epitope discovery.

Typically filamentous phages are used for epitope discovery; as a library of peptide variants can be screened and target sequences recovered through affinity selection. This process is typically done in M13 filamentous phage. Random insertions are cloned into the pVIII gene, which makes up the capsid or into the pIII region, which makes blunt end of phage (23). The issue with this system is that filamentous phage do not display antigen in a dense array, making direct vaccination typically ineffective. The

pIII region is not wide enough and does not have enough display sites to create strong cross-linking of peptide, while insertions into the pVIII region must be made sparingly, or proper capsid formation will fail. Because of this, once the peptide is recovered by affinity selection, it must be synthesized and then inserted into a display platform; unfortunately when out of its originally identified context, these antigens tend to be poorly immunogenic; due to thermodynamic issues, 3D structure, or the actual epitope domain being displayed.

So for VLP based vaccines to really be effective, they must serve as both the discovery and display platform. In order for that to happen, there are three things a VLP must be able to do; first, it must be able to display foreign antigen in dense arrays that can elicit a strong immune response, second, it must have a tolerant display site that can retain normal protein function even with insertion of diverse peptides, and lastly in must encapsidate the nucleic acid that encodes the viral protein-peptide fusion for recovery sequence by affinity selection (19). This process has been done and shown to be effective in MS2 bacteriophage, our goal was to test various peptide insertions into PP7 phage and see if they were compatible with VLP assembly, exposed on the virus surface, and immunogenic. After seeing that PP7 VLPs can display epitope insertions on the particle surface and are immunogenic, the next step would to create the afore mentioned library of PP7 VLPs to go in parallel with the MS2 libraries already created (19). We have already created a semi-random library of PP7 VLPs based upon the anthrax protective antigen with random flanking sequence. These insertions form particles and could be used as part of a library scan (fig.12). It would be important to try selecting a VLP for a particular target using the libraries and see how many rounds of selection are needed to

recover a particle that provides sufficient protection in an animal model with a vaccine already in circulation to serve as a control. Showing the genotype phenotype link would be necessary. Also it will be essential to generate VLPs to random, unknown targets and see if we can again produce high titer levels of antibody using our VLPs in an animal model. Truly getting a sense of the time required to go from unknown target to large scale VLP production will be important as well. The future possibility of mechanized production should also be considered. Along with vaccine development, VLP platforms could be used in many potential different scenarios, including, drug delivery, and cell imaging. While current technologies provide many effective vaccines, they are slow in development and involve some degree of risk. It is clear that a new method of vaccine development that is fast, safe, inexpensive, and effective is needed to combat the ever present threat of viral pandemic not just in the United States, but in the developing world as well.

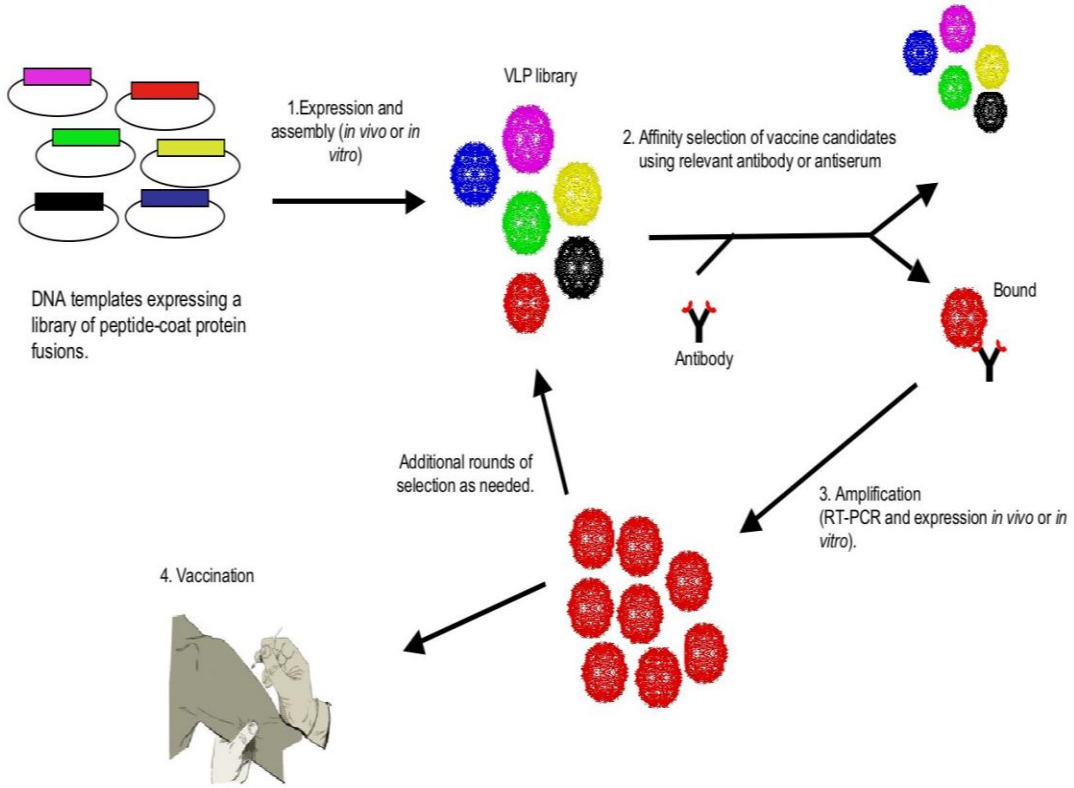


Fig. 12 Diagram of library screening

Diagram of the VLP library screening process, selection of target particles, amplification through RT-PCR, and finally, direct vaccination. This process applies to all VLP types.

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