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DEVELOPMENT AND CHARACTERIZATION OF HISTIDINE-TAGGED HPV16 L2 AND MS2-ARGININE-TAGGED RECOMBINANT PROTEINS FOR DOWNSTREAM PROCESSES

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DEVELOPMENT AND CHARACTERIZATION OF HISTIDINE-TAGGED HPV16 L2 AND MS2-ARGININE-TAGGED RECOMBINANT PROTEINS FOR DOWNSTREAM PROCESSES

By

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Abstract

Human papillomaviruses (HPVs) are the most common sexually transmitted infections. Persistent infection with HPV can lead to anogenital cancers including head and neck cancers. Three prophylactic vaccines have been approved to prevent against some types of HPV infection. However, the vaccines are HPV-type specific and protect mostly against the HPV types included in the vaccines. To offer broader protection against more HPV types, studies in the field are developing candidate vaccines targeting a conserved minor capsid protein, L2. Nevertheless, reagents for developing and assessing L2 vaccines are limited. For example, antibodies to assess the antigenicity of some L2 epitopes are not available commercially and multivalent platforms to develop and purify clinical grade L2 antigens are limited. In this study, I developed and characterized the immunogenicity of a recombinant Histidine-tagged HPV16 L2 (amino acid 1-130) antigen. In addition to this, I explored the development of a multivalent display platform, a recombinant MS2-bacteriophage coat protein, with a C-terminal Arginine-tag for downstream purification using cation exchange chromatography. All Recombinant proteins (Histidine-tagged L2 and MS2-Arg tagged) were successfully expressed in bacteria. However, only Histidine-tagged L2 proteins were successfully purified in large quantity to homogeneity. Mice immunized with the Histidine-tagged L2 protein elicited anti-L2 IgG antibody titers greater than 10^3. The anti-L2 antibodies generated in this study will be valuable to researchers, in the field, developing L2 vaccines.

Overview

Affinity-tagged proteins are recombinant proteins fused/linked to a polypeptide sequence (an affinity tag) with potentials to bind to a specific chemical or a biological ligand [1, 2]. A number of affinity tags can be genetically fused to a protein and each tag binds to a specific ligand. For example, Histidine tag binds to metal ion ligand (Ni^{2+}-NTA, Co^{2+}-CMA), Arginine tag binds to cation exchanger (SP sepharose and CM saphadex beads), C-myc tag binds to Mab 9E10 antibody, and FLAG tag binds to M1 monoclonal antibody [1, 3] . There are so many advantages of tagging a protein. i) An affinity tag enables the fused protein of interest to be purified from a mixture of contaminating proteins. For example, histidine, glutathione, and arginine affinity tags facilitate protein purifications [1, 2, 4]; ii) Adding an affinity tag to a protein enables the protein of interest to be identified using antibodies that are specific to the tag. For example, c-myc tag on a protein facilitates protein detection using c-myc-specific antibodies; iii) An affinity tag enhances protein solubility. For example, the addition of a glutathione tag to a protein enhances the solubility of the protein.

Affinity tags have been used in the past to purify large quantities of affinity-tagged proteins (from contaminating proteins) for proteomics [1], bio-therapeutics, vaccine studies, etc. [2]. In this study, we explored the ability to express and purify two recombinant proteins [human papillomavirus (HPV)16 L2 protein and bacteriophage MS2 coat protein] from bacterial lysate using histidine affinity tag (Part I) and arginine affinity tag (Part II), respectively. These two tags were chosen because they are very small (6 amino acids) and they are less likely to interfere with the immunogenicity of our

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proteins of interest [1]. Purified HPV16 L2 and MS2 proteins were explored as reagents for vaccine-related studies.

Part I

Development and characterization of the immunogenicity of a recombinant Histidine-tagged HPV16 L2 protein (amino acid 1-130)

1. Introduction

1.1 Human papillomaviruses L2 (aa 1-130)

Human papillomaviruses (HPVs) are the most common sexually transmitted infections [5, 6]. HPVs are non-enveloped viruses with a double-strand circular DNA genome (8 KB). The genome is surrounded by an icosahedral capsid, which is composed of two capsid proteins, the major capsid protein (L1) and the minor capsid protein (L2) [7, 8].

HPVs cause about 5% of cancer cases globally [8-10]. More than 200 types of HPVs have been identified [11]. They can be classified into two genotypes based on the type of lesions that they cause: High-risk HPV (HR-HPV) and Low-risk HPV (LR-HPV). HR-HPVs cause cancer such as anal, vulvar, vaginal, cervical, penile, and head & neck cancers [7, 9]. More than 18 HR-HPVs (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, and 73) [11] have been identified. Globally, HPV16 and HPV 18 cause 50% and 20% of cervical cancer, respectively [5, 7]. The Low-risk HPVs (HPV6, 11, 42) cause warts for example, genital warts.

Worldwide, more than 500,000 people per year are diagnosed with cervical cancer from HPV infection [12]. HPV-associated cancers in addition to HPV-associated genital warts

treatments are very expensive, and may be as high as more than 4 billion dollars annually [13]. This puts a lot of financial pressure on the healthcare system [5, 8, 13]. Fortunately, three vaccines have been developed within the last decade to protect against HPV infections [11]. Cervarix, Gardasil-4, and Gardasil-9 vaccines consist of virus-like particles (VLPs) derived from the L1. Two of these vaccines (Cervarix and Gardasil-4) provide protection mostly against HPV16 and 18. Gardasil can also protect against HPV6 and 11. Gardasil-9 is composed of 9 VLPs and protects against HR-HPV types (HPV16, 18, 31, 33, 45, 52, 58) and LR-HPVs (HPV6 and 11)[11]. Thus, Gardasil-9 can protect against the HPV types that cause 90% of cervical cancer.

Although the available vaccines induce high immune responses, they do not protect against other HPV types not included in the vaccines; the vaccines are type-specific [11]. Given the fact that the vaccines do not protect against all HPV types that cause cancer, medical practitioners still recommend that women who have been vaccinated with the vaccines should continue screening for cervical cancer to make sure that they are not infected with HPV types not included in the vaccines. As an alternative to L1 vaccines, other studies have focused on developing HPV vaccines targeting a conserved protein, the minor capsid, on the capsid. The minor capsid protein, L2, is conserved among HPV types as shown in figure1. Several studies have shown that L2 is able to elicit cross-neutralizing antibodies against most HPV types [9, 14, 15]. For example, immunization with L2 synthetic peptides (derived from HPV16) corresponding to amino acids (aa) 17-36, 34–52, 49–71, 56-75, 65-85, 108-120 elicited cross-neutralized against heterologous HPV pseudoviruses 18, 31, 45, 58 [16-18]. However, the cross-neutralizing antibody titers against these HPV types were less than 200 compared to neutralization of

homologous HPV pseudovirus 16 (with neutralization titers ranging from 400-3200) [16]. In order to enhance the immunogenicity of some of these epitopes, researchers have immunized mice with concatemers of these peptides [19, 20], immunized with L2 peptide conjugated to thioredoxin [21, 22], or immunized mice with virus-like particles (VLPs) displaying the L2 peptides on the surface of the VLPs [6, 23, 24, 25, 26]. Most of these strategies enhanced the immunogenicity of the L2 peptides especially those displayed on VLPs. VLPs displaying L2 peptides elicited broad protection against diverse HPV types after two immunizations with small doses (only 5 µg per immunization) of VLPs [14, 27, 28, 29]. Thus, VLPs are excellent platforms that can be used to enhance the immunogenicity of less immunogenic peptides and they can be used at lower doses with 1-2 immunizations. Despite these advantages, there are some challenges in developing recombinant VLPs displaying HPV L2 peptides. One challenge is purifying the recombinant VLPs from other contaminating bacteria proteins; another challenge is to confirm that L2 peptides are displayed on the surface of the VLPs prior to immunization. The display of L2 peptides on the VLPs is a pre-requisite for a good immune response. Our lab including others in the field have inserted L2 epitopes on the surfaces of VLPs derived from different viruses. . For example, our lab has inserted epitope 108-122 (representing amino acid 108-122) on the coat protein of MS2 bacteriophage. However, we do not know whether this epitope is displayed on the surface of MS2 VLPs following the assembly of recombinant MS2-L2 coat protein into VLPs. This could have easily been demonstrated with enzyme-linked immunosorbent assay (ELISA) using anti-L2 sera (especially against epitope 108-122); however, antibodies targeting this epitope and others are not available commercially. The objectives of this thesis were:

i) develop an L2 protein (amino acid 1-130) from HPV16 and to generate polyclonal antibodies against this protein. Availability of antibodies targeting L2 epitopes will enhance vaccine research that targets L2 protein. To enhance the purification of 16L2 aa 1-130, we decided to generate a recombinant L2 protein fused to a Histidine (His) affinity tag.

ii) Assess the possibility of displaying an Arginine (Arg) affinity tag on the surface of MS2 VLPs that could be used to purify recombinant MS2 VLPs from other contaminating bacteria proteins. An affinity tag on MS2 VLPs will enable the purification of the recombinant VLPs to homogeneity.

1.2 Affinity tags

Affinity tags are a peptides or proteins that bind with high affinity to their respective matrix such as chemicals or even other proteins. The commonly used tags are Histidine (His) tag, Arginine (Arg) tag, Maltose-binding protein, C-myc tag, glutathione S-transferase tag, FLAG tag, etc. [1]. Each tag has a specific matrix for binding. For example: His-tag binds to Ni-nitrilotriacetic acid (NTA) matrix or other metal ion, Arg-tag binds to SP sepharose and CM saphadex beads, Maltose binding protein binds with cross-linked amylose matrix, C-myc binds to Mab 9E10 antibody, glutathione S-transferase tag binds with immobilized glutathione, and FLAG tag bind to monoclonal antibody matrix [1, 3, 30].

Following binding of tags to their cognate matrix, unbound protein can be washed away and bound tagged-protein eluted from the matrix, yielding a very pure protein/peptide. These tags have been exploited to enhance the purification of proteins by fusing the tags

to proteins of interest [1]. To enhance the purification of L2 protein from other contaminating bacteria proteins, we chose Histidine (His, contains 6 Histidine residues). This tag was chosen because it is very small, may not interfere with protein folding and does not interfere with the immunogenicity of the recombinant protein [1].
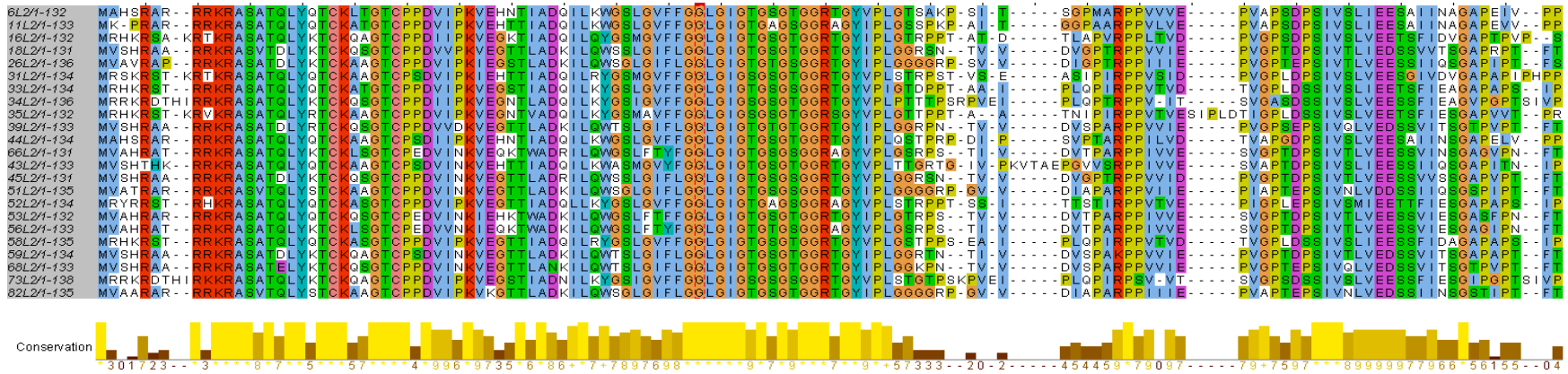
Figure 1. *L2 (aa 1-130) sequence alignment of different types of High and low risk of HPVs. Alignment was done using Jalview software. Conserved residues are shown in yellow bars below*

2. Materials and Methods

2.1 Cloning and screening for the expression of a recombinant His-tagged HPV16 L2 protein (aa 1-130).

An HPV16L2 DNA sequence (representing amino acid, aa 1-130), linked directly at the N-terminus by six Histidine residues (His-tag) was codon-optimized for bacterial expression and was synthesized by Epoch Life Science. To enable cloning of the DNA to a bacterial expression plasmid, two restriction sites (an Nco1 restriction site with a start codon at N-terminus and a BamH1-HF restriction site at C-terminus) were added to the DNA sequence. Two nucleotides were added between the Nco1 site and His-tag to enable in-frame cloning. The synthesized DNA fragment (430 base pair, bp) in figure 2 was used as a template for polymerase chain reaction (PCR).

AGCGCCATGGCTCATCATCACCACCATCACCGTCACAAACGTTCTGCTA
AGCGCACTAAACGTGCATCCGCAACCCAGCTGTACAAGACCTGTAAAC
AGGCTGGCACCTGCCCACCAGACATCATTCCGAAAGTTGAAGGTAAGA
CCATCGCTGATCAGATCCTGCAGTATGGCAGCATGGGTGTGTTCTTCGG
TGGCCTGGGTATTGGTACTGGCTCTGGTACTGGTGGTCGTACCGGCTAC
ATCCCACTGGGCACTCGTCCACCGACTGCGACTGACACCCTGGCACCG
GTACGTCCACCACTGACCGTTGATCCAGTTGGTCCGAGCGATCCGTCTA
TTGTATCTCTGGTGGAAGAAACTTCTTTCATCGATGCAGGTGCTCCGAC
CTCTGTGCCATCTATTCCACCAGATGTGTAAGGATCCTTCT

Figure 2. *Synthesized DNA sequence of His-tagged HPV16 L2 (aa 1-130). The nucleotides with green background are Nco1 (left) and BamH1-HF (right) restriction sites. The nucleotides in red (left) indicates the start codon and the nucleotides in red (right) indicates the stop codon. Six histidine residues (His-tagged; highlighted in yellow background) are linked to N-terminus of HPV16 L2 (sequence in white background between his-tag and stop codon)*

The PCR reactions consisted of a mixture 10µM of forward primer (Table 1), 10µM reverse primer (Table 1), 1X OneTaq Hot Start master mix, 10 ng of DNA template (i.e.

the synthesized His-tagged L2 DNA), and DI water. Negative PCR control was set up as above except that template DNA was not used. The PCR conditions used are shown in Table 2.

Table 1: *Sequences of primers used for PCR amplification of His-tagged L2 DNA*

Primers	DNA sequences	Restriction sites*
Forward primer (CONS. L2 S& (16E5-E7)	5' AGCGCCATGGCTCATCATCACCAC 3'	Nco1
Reverse primer L2(1-130)R	5' AGAAGGATCCTTACACATCTGGTGGAATAGATGGTG 3'	BamH1-HF

Forward primer sequence consisted of Nco1 restriction site highlighted in green background. It also includes the start codon highlighted in red text. Reverse primer sequence contained stop codon (red text) followed by BamH1-HF restriction site (highlighted in green background).

Table 2 : *Thermocycling condition for His-tagged HPV16 L2 DNA*

step	Temperature	Time
Initial denaturation	94 ºC	2 minutes
Denaturation	94 ºC	30 seconds
Annealing	58 ºC	30 seconds
Extension	68 ºC	30 seconds
Final Extension	70 ºC	5 minute
Hold	4 ºC	∞

After PCR, the samples including a 1Kb DNA ladder were run on a 1% agarose gel stained with 0.5 ng/µl ethidium bromide and a photo of the gel was taken following a brief observation on a UV Transilluminator.

2.2 Purification of DNA from the gel and digestion

The DNA band was cut from agarose gel and DNA was extracted from the gel using Qiagen Gel Extraction kit protocol. For cloning to a pDSP62 plasmid, the extracted DNA and the plasmid were separately digested with restriction enzymes as followings:

Purified His-tagged-L2 DNA

-30 µl of amplified DNA

-4.0 µl of 10X cut smart buffer

-0.75 µl of NcoI Restriction enzyme

-0.75 µl of BamHI-HF Restriction enzyme

-4.5 µl DI water

Reaction total volume: 40 µl

Plasmid pDSP62

-1.0 µl of each restriction enzyme (NcoI and BamHI-HF)

-5µ l of 10X cut smart buffer

-24 µl (3000ng) pDSP62 plasmid

-9 µl DI water

Reaction total volume: 40 µl

All reaction mixtures were incubated overnight at 37ºC after which they were separated on a 1% agarose gel. A larger band 3,819 bp from the digested pDSP62 plasmid and a band 409 bp from the amplified His-tagged-L2 DNA were cut from the gel and the DNAs were extracted from gel slices using Qiagen Gel Extraction kit protocol.

2.3 Ligation of DNA to plasmid vector (pDSP62)

The digested His-tagged-L2 DNA was ligated into digested pDSP62 plasmid as follows:

-6 µl of digested His-tagged-L2 DNA

-1.5 µl of digested pDSP62 plasmid

-1 µl of 10X ligase buffer

-0.5 µl of T4 Ligase enzyme

-1 µl of DI water

 Total volume of reaction was 10µl. The ligation reactions were incubated overnight at room temperature or over the weekend at 4ºC.

2.4 Transformation of ligated plasmid to C41 *E. coli* cells

The ligated DNA was transformed into C41 *E. coli* bacteria by heat shock as follows. Ligated plasmid DNA was mixed with C41 *E. coli* competent cells and was incubated in ice for 30 minutes. After that, the mixture was transferred to a 42ºC water bath for 90 seconds and then back into ice for 5 minutes. Eight hundred µl of Luria Bertani (LB) media without antibiotic was added to the mixture and the mixture was shook for 1 hour at 37ºC. The transformation mixture was then centrifuged for 4 minutes at 3000 rpm and 400µl of the supernatant was discarded and the pellet resuspended in the remaining supernatant. One hundred µl of resuspended solution was spread on agar plates

(contained 50 ng/µl kanamycin antibiotic). The kanamycin plates were incubated overnight at 37ºC.

2.5 Screening colonies for protein expression

Colonies were picked up from the agar plates and put in separate tubes with 600µl of LB media containing 50 ng/µl kanamycin antibiotic. The culture tubes were then incubated at 37ºC for 3 hours. When the solution became cloudy, 250µl of cultures were transferred to new tubes and induced with 0.5 µM Isopropyl β-D-1-thiogalactopyranoside (IPTG). The IPTG-induced tubes and uninduced tube were shook for additional 3 hours at 37ºC. After 3 hours, 250µl from one of the uninduced tube was taken as an uninduced negative control and the tube together with culture from induced tubes were centrifuged at 10,000 rpm for 5 minutes. The supernatants were discard and the pellets were resuspended in 70µl of 8M urea to lyse the bacteria. The samples were sonicated to further lyse the cells. From each sonicated sample, 15µl was transfer into small tubes and mixed with 5µl of 5X protein loading dye. This was then heated for 5 minutes at 95ºC to denature the samples. Ten µl of heated samples including the uninduced control were loaded on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel to check for protein expression and the size of the expressed protein. In addition to this, 2µl of protein ladder was loaded to estimate the protein size. The gel was run for 60 minutes at 160 voltage after which the gel was washed and stained with Coomassie Blue dye. The gel was then washed and microwaved with paper towels to destain it.

2.6 Plasmid preparation and sequencing of constructs

After checking for His-tagged-L2 protein expression, the construct with highest protein expression was selected for plasmid extraction and sequencing to confirm the cloned sequence. Plasmid extraction was done using a QlAprep Spin Miniprep Kit and the concentration of the DNA (135 ng/µl) was determined using Nanodrop. A restriction digest was set up using DNA from plasmid prep to confirm that the DNA size released from the plasmid was identical in size to the cloned His-tagged-L2 DNA.

The restriction digest reaction was set up as follows:

-3 µl extracted plasmid DNA

-1.0 µl of 10X cut smart buffer,

-0.25 µl of NcoI Restriction enzyme

-0.25 µl of BamHI-HF Restriction enzyme

-5.5 µl DI water

Reaction total volume: 10 µl

The reaction mixtures were incubate at 37°C for 4 hours after which the digested samples were run on a 1% agarose gel. Also, DNA ladder was run to indicate the DNA size. To confirm the His-tagged-L2 (1-130) DNA sequence, a sample of DNA plasmid was send to Arizona State University for sequencing using T7 promoter primer.

2.7 Purification of His-tagged L2 protein

After ensuring the accuracy of DNA sequence, a large-scale culture from sequenced construct was started for the expression and purification of recombinant protein. First, 50

μl of C41 *E.coli* bacteria expressing the cloned His-tagged L2 protein was used to inoculate 2 ml of LB media in the presence of 50 ng/μl kanamycin and shook at 37°C overnight. Next day, 1ml from the overnight culture was used to inoculate 50 ml of LB media (with 50 ng/μl Kanamycin antibiotic). The cultures were then shook for 4 hours until the cell density reached 600 Optical Density (OD) and became cloudy. Because the His-tagged L2 protein was expressed from a T7 promoter, the culture was induced with 0.5 mM IPTG and shook for additional 3 hours at 37ºC. The induced culture was centrifuged for 20 minutes at 10,000rpm to pellet the cells. The pellet was resuspended in 10 ml of lysis/equilibration buffer (8M Urea, 20mM $Na_2HP_2O_4$, 300mM Na_2Cl, and 10mM Imidazole; pH 7.5) and incubated in ice for 20 minutes. The cells were then sonicated for 5 minutes to lyse them. Fifty μl of lysate from sonicated cells were saved for SDS PAGE to confirm L2 protein expression in total protein fraction. After that, the culture was centrifuged for 10 minutes at 10,000 rpm.

Because the L2 protein had His-tagged inserted in the N-terminal of L2, a Thermo Fisher protocol (Nickel beads; Ni-NTA) was adapted to purify the L2 protein. First, 4 mL of Ni-NTA beads was placed in a bottom-capped purification column. The Ni-beads were allowed to settle down in the column by gravity and the column was then uncapped to drain the supernatant. The Ni-beads were equilibrated in 3ml of lysis/equilibration buffer twice and each time, Ni-beads were, allowed to settle down by gravity. The bottom of the column was then uncapped to drain the equilibration buffer. To bind L2 protein with Ni-beads, 10ml of the L2 protein (from above) was added to the column and rocked for 45 minutes at room temperature using a rotator. After that, the Ni-beads were allowed to settle by gravity and the column was uncapped and flow-through solution was collected

into a tube and labeled as unbound. The column was washed three times each with 3ml of washing buffer (8M Urea, 20mM $Na_2HP_2O_4$, 300mM Na_2Cl, and 20mM Imidazole; pH 6). The column was incubated at room temperature for two minutes in-between washes and all the washes were collected in newly labeled tubes, "washed". Following the last wash, 4ml of Elution buffer (8M Urea, 20mM $Na_2HP_2O_4$, 250mM Na_2Cl, and 250mM Imidazole; pH 8) was added to the column and the bottom of the column uncapped and 1ml fractions of eluted protein were collected in several tubes. To assess which fraction had the protein of interest and its purity, 10µl of all eluted fractions, 10µl of washed supernatant, 10µl of unbound proteins, and 10µl of lysate that was collected before the purification began were each mixed with SDS PAGE loading dye and each mixture heated for 5 minutes at 95°C. The mixtures were then run on an SDS PAGE gel and protein fractions with the highest purity were then combined. To remove urea from the purified protein (a process which can help refold the protein), the protein was put into SnakeSkin Dialysis Tubing [10,000 Molecular Weight Cut Off, MWCO] and the tubing placed in 800mL of 1X Phosphate-Buffered Saline (PBS) buffer. The tubing was incubated and stirred overnight at 4ºC after which the PBS buffer was changed and the protein dialyzed for 8 additional hours at 4ºC. To estimate the concentration of purified His-tagged L2 protein, 5µl and 10µl of the dialyzed protein together with known concentrations (10µg, 5µg, 2.5µg, and 1µg) of hen egg lysozyme (HEL) were loaded in separate walls. The concentration of His-tagged L2 protein was then estimated by comparing the intensity of the His-tagged L2 protein band to those of lysozymes.

2.8 Mice immunization

To induce antibodies against the purified L2 protein, 4 Balb/c mice were immunized intramuscularly (3 times at two weeks interval) with 10μg (per immunization) of His-tagged L2 protein plus alum adjuvant. Another group of mice, control, was not immunized. Sera were collected from the mice two weeks after the last immunization to check for antibodies (described below) against HPV16 L2.

2.9 Enzyme-Linked Immunosobent Assay (ELISA)

In order to determine antibody titers in sera from mice immunized with the His-tagged 16L2 protein, an ELISA was done as follows. An ELISA plate was coated with 500 ng of purified 16L2 (aa 1-130) and the plate was incubated at 4ºC overnight. The plate was then blocked for 2 hours at room temperature with blocking buffer (0.5% nonfat dry milk in PBS buffer). The blocking buffer was then discard and 4-fold serial dilutions of sera from His-tagged 16L2 (aa 1-130) immunized mice or from unimmunized mice were then added to the plate. As a positive control, sera from HPV16 L2 (aa 1-88) was also diluted serially and added to wells in the ELISA plate. The plate was then incubated for 2 additional hours (rocking) at the same temperature. After two hours, the wells in the ELISA plate were washed four times with PBS buffer. Horseradish peroxidase-conjugated goat anti-mouse IgG (secondary antibody) at 1:5000 dilution was added and the plate incubated for 1 hour (rocking) at room temperature. After that, the wells were washed five times with PBS. Fifty μl of 3,3',5,5'-Tetramethylbenzidine (TMB) solution was added to each well to develop ELISA plate. After 13 minutes, the solution changed to blue color, and to stop the reaction, 50μl of 1% of HCl was added to each well. The

OD value of the solution was determined at 450nm using a plate reader. The titers of antibodies were determined as the reciprocal of the highest sera dilution with an OD_{450} greater than 2-fold that of control sera at the same dilution. The values were graphed using GraphPad 5.0 Prism software.

2.10 Western Blotting

Western blotting was done to confirm the ELISA results as well as to confirm the size of the purified His-tagged L2 protein. The assay was done as follows. Two different concentrations (500ng and 1000ng) of His-tagged L2 protein were loaded on an SDS PAGE. Five hundred ng of MS2 also was loaded as a negative control and the gel was run as described above. The gel was then transferred to a Polyvinylidene difluoride (PVDF) membrane. The gel and the PVDF membrane were sandwiched between 3mm Whatman filter papers soaked in 1X transfer buffer (760 ml of Ultra-pure water, 40ml of the mixture: Tris base 18.2 g, Glycine 90.0g, and 500ml of ultra-pure water, and 200ml of methanol). The sandwich (filter paper, gel, membrane, filter papers) was put in an XCell II Blot Module and the module placed in an SDS PAGE electrophoresis tank. The XCell II Blot (middle part of tank) was filled to the top with transfer buffer while outer part of the tank was filled with 650ml of DI water. The protein was then transferred from the gel to the membrane at 30 voltage for one hour. After transferring the L2 proteins to the membrane, the membrane was blocked (rocking overnight at 4ºC) using blocking buffer (5% nonfat dry milk in 1X of Tris buffered Saline with 0.05% Tween-20; TTBS). The membrane was then washed with TTBS buffer and 1:5000 dilution of his-tagged L2 (aa 1-130) sera diluted in blocking buffer was added to the membrane. The

membrane/solution were rocked for two hours at room temperature. The membrane was rinsed four times (each with five minutes incubation) with 1X TTBS. After that, 1:5000 dilution of secondary antibodies (HRP conjugated goat anti-mouse IgG) diluted in blocking buffer was added to the membrane. The membrane was rocked with diluted secondary antibodies for one hour at room temperature. After that, the membrane was washed three times (5 minutes each) and the membrane was developed using a mixture that consisted of equal volumes of SuperSignal West Pico Lumino/Enhance solution and SuperSignal West Pico Stable Peroxide solution. The membrane was incubated for five minutes after adding the mixture. The membrane was then wrapped in saran wrap and scanned using Luminescent Image Analyzer (LAS-4000) mini equipment to detect protein bands that reacted with the L2 antibodies.

3. Results

Recombinant His-tagged HPV16 L2 (aa 1-130) was successfully amplified by PCR. As shown in figure 3, a 430 bp DNA fragment corresponding to the L2 protein (390 bp), 6 his-tag (18 bp) plus restriction site sequences including flanking nucleotides was obtained. There was no amplification of negative control sample thus suggesting that the PCR was not contaminated.
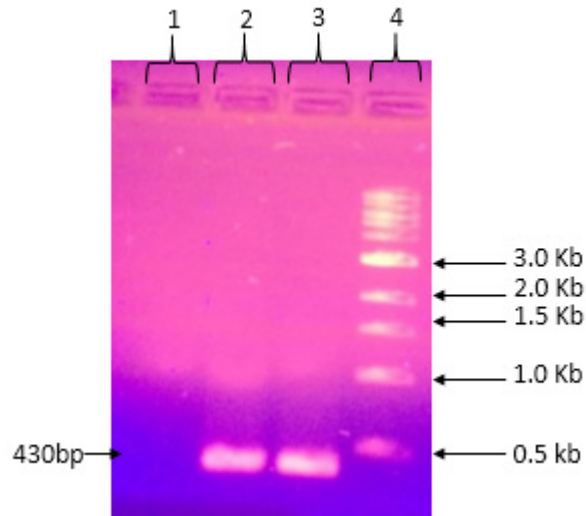
Figure 3. *Electrophoretic mobility of amplified DNA (His-tagged plus HPV16L2 aa 1-130) in 1% agarose gel stained with ethidium bromide. Number 1 is a PCR negative control, number 2 and 3 are recombinant His-tagged plus HPV16L2 (aa 1-130) DNA, and number 4 was 1 Kb DNA ladder*

To clone the amplified His-tagged HPV16 L2 DNA to pDSP62 plasmid, amplified DNA and plasmid were separately digested with Nco1 and BamH1-HF restriction enzymes. As shown in figure 4, the digested plasmid gave rise to two bands (~800bp and 3,800 bp).

Ligation of the circled bands from the gel and transformation of C41 cell gave rise to many bacteria colonies on kanamycin agar plate (data not shown). Plasmid pDSP62 is kanamycin resistant and the growth of bacterial colonies on the agar plate suggested the *C41* cells were transformed with the ligated plasmid.
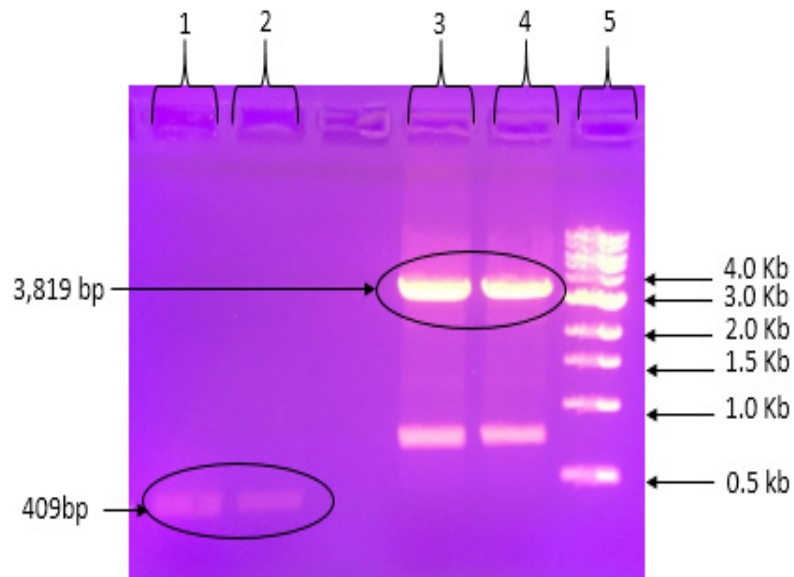
Figure 4. *Agarose gel stained with ethidium bromide showed the size of digested amplified DNA and plasmid using restriction enzymes (NcoI-Bam-H1-HF), Number 1 and 2 are digested His-tagged 16HPV L2 (aa1-130)DNA , Number 3 and 4 , digested plasmid pDSP62; the circled band were purified and ligated. Number 5 was 1Kb DNA ladder*

Bacterial colonies that were screen for protein expression showed that His-tagged L2 (aa 1-130) was cloned into the pDSP62 vector and the protein was expressed. As shown in the SDS PAGE in figure 5, a band of ~15.07KD was observed in samples that were induced with IPTG but not on uninduced sample.
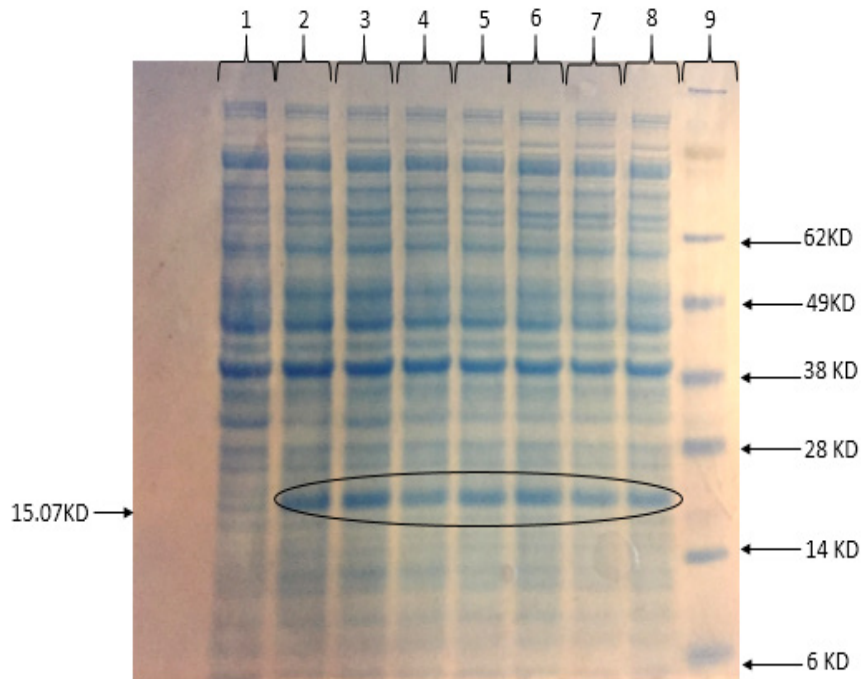
Figure 5. *SDS-PAGE stained with Coomassie blue showing protein expression in different colonies. Number1 is uninduced recombinant His-tagged L2 (aa 1-130) protein. Number 2-8 represent bacteria from colonies induced with IPTG .Number 9 is protein ladder. Circle indicates His-tag L2 (aa 1-130) protein expression*

To confirm the DNA size and the sequence of the cloned His-tagged L2 (aa 1-130) DNA, plasmid preps and restriction digests, using the same cloning enzymes, were set up. As shown in figure 6, a fragment of ~409 bp was released following restriction digest confirming the cloning of His-tagged L2 (aa 1-130). Sequencing confirmed the cloned DNA sequence figure 7.
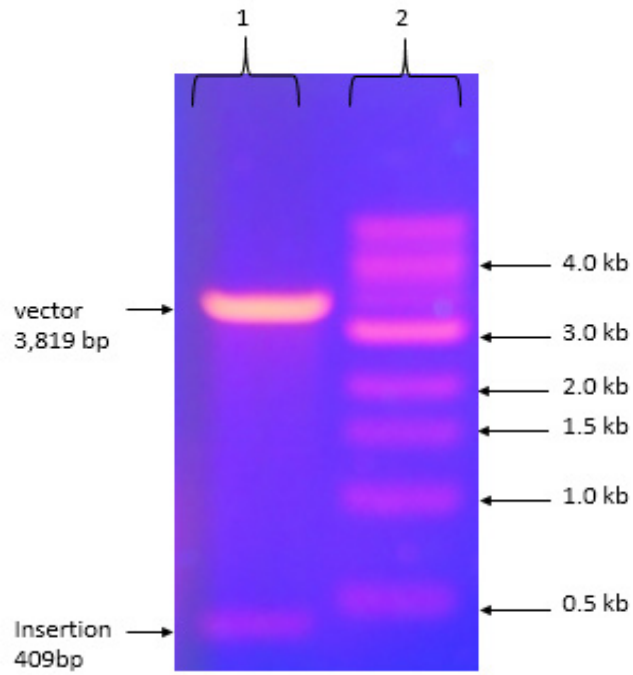
Figure 6. *Plasmid prep. Agarose gel stained with ethidium bromide showed the size of digested amplified DNA and plasmid using restriction enzymes(NcoI and Bam-HI-HF). Lower band in lane 1 showed inserted His-tagged 16HPV L2 (aa1-130) DNA, whereas the upper band in the same lane represented plasmid pDSP62; Lane 2 was 1Kb DNA ladder*

```
Score                    Expect      Identities           Gaps           Strand
780 bits(422)            0.0         422/422(100%)        0/422(0%)      Plus/Plus
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Sbjct  1    CCATGGCTCATCATCACCACCATCACCGTCACAAACGTTCTGCTAAGCGCACTAAACGTG  60

Query  61   CATCCGCAACCCAGCTGTACAAGACCTGTAAACAGGCTGGCACCTGCCCACCAGACATCA  120
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Sbjct  61   CATCCGCAACCCAGCTGTACAAGACCTGTAAACAGGCTGGCACCTGCCCACCAGACATCA  120

Query  121  TTCCGAAAGTTGAAGGTAAGACCATCGCTGATCAGATCCTGCAGTATGGCAGCATGGGTG  180
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Sbjct  121  TTCCGAAAGTTGAAGGTAAGACCATCGCTGATCAGATCCTGCAGTATGGCAGCATGGGTG  180

Query  181  TGTTCTTCGGTGGCCTGGGTATTGGTACTGGCTCTGGTACTGGTGGTCGTACCGGCTACA  240
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Sbjct  181  TGTTCTTCGGTGGCCTGGGTATTGGTACTGGCTCTGGTACTGGTGGTCGTACCGGCTACA  240

Query  241  TCCCACTGGGCACTCGTCCACCGACTGCGACTGACACCCTGGCACCGGTACGTCCACCAC  300
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Sbjct  241  TCCCACTGGGCACTCGTCCACCGACTGCGACTGACACCCTGGCACCGGTACGTCCACCAC  300

Query  301  TGACCGTTGATCCAGTTGGTCCGAGCGATCCGTCTATTGTATCTCTGGTGGAAGAAACTT  360
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Sbjct  301  TGACCGTTGATCCAGTTGGTCCGAGCGATCCGTCTATTGTATCTCTGGTGGAAGAAACTT  360

Query  361  CTTTCATCGATGCAGGTGCTCCGACCTCTGTGCCATCTATTCCACCAGATGTGTAAGGAT  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  CTTTCATCGATGCAGGTGCTCCGACCTCTGTGCCATCTATTCCACCAGATGTGTAAGGAT  420

Query  421  CC  422
            ||
Sbjct  421  CC  422
```

Figure 7. *Sequence alignment of His-tag 16HPV L2 (aa 1-130) DNA (Query sequence; cloned sequence) showed 100% identity with sequence that was synthesized (sbjct, which represents subject sequence). Sequence Alignment was done using nucleotide BLASTN*

To purify the expressed recombinant His-tagged L2 (aa 1-130) protein, Ni^{2+} beads were used. Six His-tagged was attached to L2 to facilitate protein purification using Ni^{2+} beads. As shown in figure 8, when the total protein (Number 2) was added to Ni^{2+} beads in a chromatography column, non-His-tagged bacterial proteins did not bind to the beads (Number 3). Similarly, when the column was washed, non-His-tagged residual bacterial proteins flew out of the column (Number 4) leaving His-tagged L2 (aa 1-130) protein attached to the Ni^{2+} beads. When the protein was eluted with elution buffer (has high

concentration of imidazole), only the his-tagged protein was eluted from the column in

fractions 2 and 3 (Number 6 and 7 on the gel).
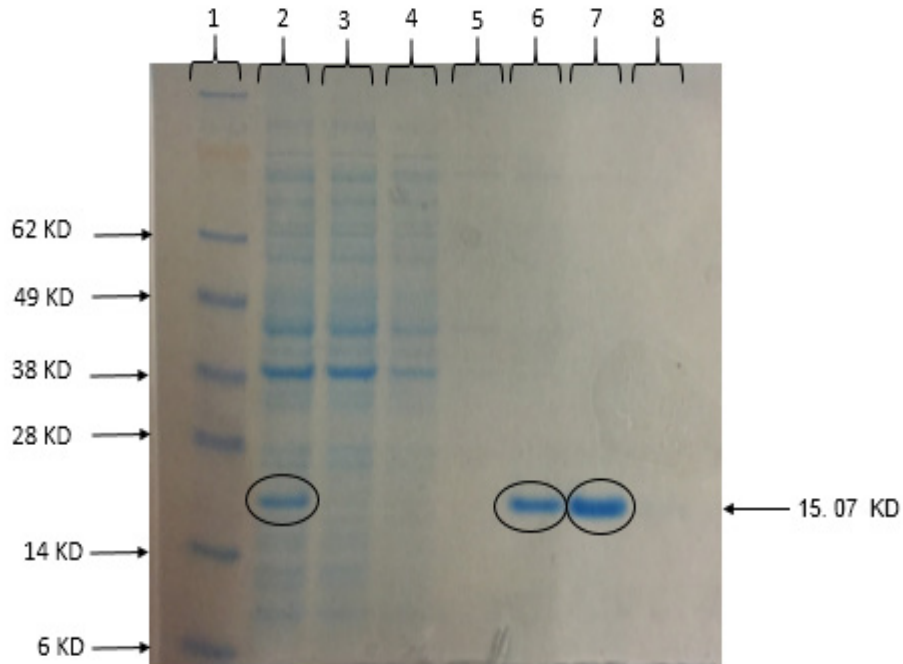
Figure 8. *Purification of recombinant protein* His-tagged L2 (aa 1-130) protein. *The recombinant protein was purified in a chromatograph column using Ni-NTA beads. Number 1 is marker, number 2 is total protein before purification, number 3 unbound protein (flow through) after adding total protein to column, number 4 is flow through of column wash, and numbers 5-8 are fractions 1,2,3,4, respectively of eluted protein*

The concentration of the purified His-tagged L2 (aa 1-130) protein was determined to be

250ng/μl. Figure 9 shows band intensities of protein in comparison to known

concentrations of hen egg lysozyme.
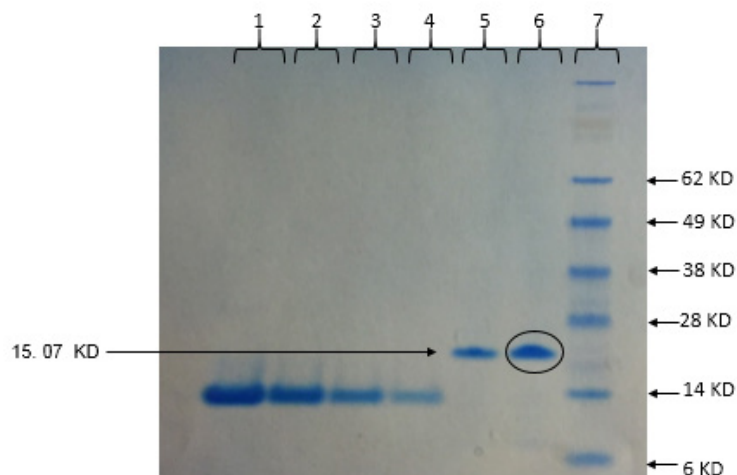
Figure 9. *Estimation of His-tagged L2 (aa 1-130) protein on an SDS PAGE gel based on known concentrations of hen egg lysozyme (HEL). Number 1 is 10 μg, number 2 is 5μg, number 3 is 2.5μg, and number 4 is 1μg of HEL. Numbers 5 and 6 are 5ul and 10ul, respectively, of purified his-tagged L2 (aa 1-130) protein. The circled band had similar intensity with 2.5μg of HEL*

To assess the immunogenicity of the purified His-tagged L2 (aa 1-130) protein, mice were immunized with the protein. Sera were collected after the last immunization and IgG antibodies in sera were determined by ELISA. As shown in figure 10, mice immunized with the purified His-tagged L2 (aa 1-130) had antibody titers (greater than 10^3) similar to IgG titers in our positive control sera (from mice previously immunized with 16L2, aa 1-88). However, IgG titer from unimmunized mice was very low (10^2). The ELISA results were confirmed using Western Blotting. As shown in Figure 11, a band of approximately 15.07KD reacted with serum from mice immunized with His-tagged L2 (aa 1-130); serum reacted with His-tagged L2 (aa 1-130) even at a lower concentration (500ng) of the protein. , As expected, the serum did not react with negative control MS2 coat protein confirming that the serum is specific to HPV L2.
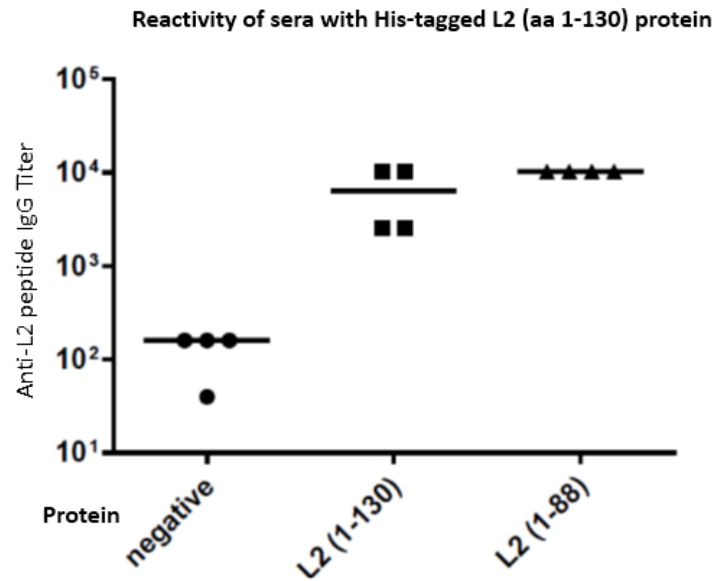
Figure 10. *IgG antibody titers in immunized or unimmunized mice. Four Balb/c mice (per group) were immunized (3 times) intramuscularly with 10ug (per immunization) of recombinant L2 protein plus alum adjuvant. Two weeks after the last immunization, sera were collected and antibody titers were determined by end-point dilution ELISA. Each datum represents serum from an individual mouse*
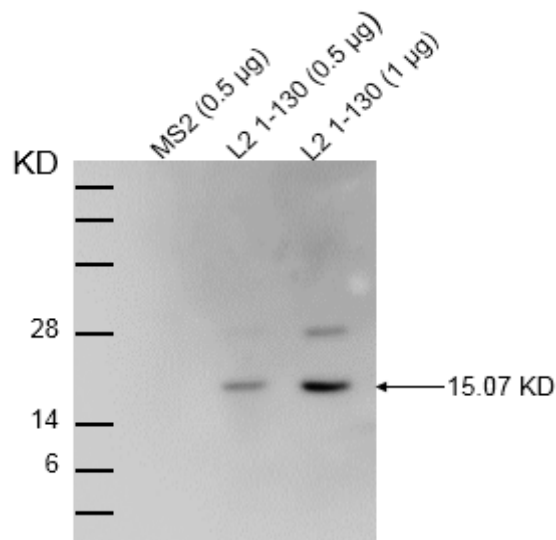
Figure 11. *Reactivity of His-tagged L2 (aa 1-130) sera in a Western blot. MS2 coat protein (negative control), His-tagged L2 (aa 1-130) proteins were probed with 1:5000 dilution of serum from mice immunized with His-tagged L2 (aa 1-130) protein. Arrow indicates His-tagged L2 (aa 1-130) proteins*

4. Discussion

In this study, we successfully cloned and purified His-tagged L2 (aa 1-130) protein from bacterial lysate using Ni-NTA beads. Six Histidine residues attached to the L2 protein enhanced the purification of L2 protein to homogeneity from bacterial cells. Mice immunized with the His-tagged L2 (aa 1-130) protein elicited antibodies (with titers more than 10^3) against the L2 protein. As mentioned earlier, one of the challenges in developing candidate L2 HPV vaccines is to demonstrate that L2 peptides/epitopes are displayed on the surface of a multivalent platform (such as VLPs), which is a prerequisite for a robust immune response. The anti-HPV16 L2 (aa 1-130) antibodies generated in this study will be a valuable reagent for researchers in the field developing HPV vaccines targeting the minor capsid protein, L2. The anti-L2 sera could be used to confirm (by ELISA or Western blot) the display of L2 peptides on the surface of a multivalent platform such as VLPs.

Insertion of an Arg affinity tag on MS2 VLPs to enhance purification of recombinant MS2 VLPs

1. Introduction

As mentioned earlier, virus-like particles (VLPs) are excellent platforms for enhancing the immunogenicity of peptide antigens [31]. VLPs can be derived by overexpressing the coat (capsid) protein of a virus using a plasmid. Following overexpression, the coat protein spontaneously assembles to form VLPs. The VLPs look like the virus from which the coat protein was derived from; i.e. their ability to display epitopes to the immune system in a highly multivalent format. This property makes them very immunogenic [14, 31, 32]. VLPs do not contain the whole viral genome. Thus, they are not infectious and are safe vaccine platforms[33]. Although VLPs can be derived from any virus, our lab is interested in using VLPs from viruses that infect bacteria (bacteriophages: MS2, Qβ, and PP7) as display platforms [14, 31]. They can easily be expressed and purified by gel filtration in large quantities (milligrams) from bacteria. Although gel filtration (using sepharose CL-4B beads to separate protein based on size) removes most contaminating bacterial proteins, the purity of the recombinant VLPs could be improved. In this study, we assessed whether an Arginine (Arg) tag inserted on the C- terminus of MS2 single-chain dimer could be used to purify recombinant MS2 VLPs (by cation exchange chromatography[1]). We chose an Arg tag (tag contains 6 arginine residues) because it is very small, it may not interfere with the assembly of MS2 coat proteins into VLPs or interfere with the immunogenicity of the recombinant protein. Six or five amino acids are commonly used in the construction of Arg tag and the tag can be inserted in the N- or C-

terminus of a protein of interest [1, 3]. The Arg-tagged protein is then purified by cation exchange chromatography using CM-Sephadex or SP Sepharose Fast flow resins as follows: Expressed protein is applied to the resins and only Arg-tagged proteins but not bacterial proteins bind to the resins. The resins are washed and Arg-tagged proteins are eluted from the resins using high salt (sodium chloride; NaCl) concentration [1, 3, 34]. Recombinant proteins fused to Arg tag can yield 44% protein with 95% purity [1]. In this study, were explored whether an Arg tag could be displayed on MS2 VLPs by inserting the tag at the C terminus of MS2 coat protein. An Arg tag displayed on MS2 VLPs will enhance the purification of the VLPs. The C-terminus was chosen to insert an Arg tag because our lab uses the N-terminus for other insertions (e.g. HPV L2 epitopes).

2. Materials and Methods

2.1 Insertion of an Arg tag to C-terminus of MS2 coat protein

Six Arg residues were inserted on the C-terminus of MS2 coat protein by PCR using a reverse primer (MS2-6Arg) that contained 6 Arg residues in-between MS2 coat protein sequence and TAA stop codon. Forward primer, primer Sph1, which binds ~293 nucleotides upstream of the start codon of MS2 coat protein was used in PCR. The reason this primer was used is because we already had the primer in the lab and there was no need to order a new primer close to the start codon of MS2 coat protein. The sequences of reverse primer and forward primer are shown in table 3.

Table 3: *Sequences of the primers that were used to amplified MS2-6Arg*

Primers	Sequences
Forward primer: primer Sph1	5' GATGGTGCATGCAAGGAGATGG 3'
Reverse primer (MS2-6Arg)R	5' AGCAGCCGGATCCTAATTAATTAACCCGGCGTCTATTA <mark>GCG ACG GCG ACG GCG ACG</mark> <u>GTA GAT GCC GGA GTT TGC TGC</u> 3'*

Stop codon is in red text while Arg tag is shown in yellow background. The underlined sequence is the C terminus of MS2 coat protein

The PCR reaction mixture was set up as described above and the PCR was conducted using the following conditions on table 4.

Table 4: *Thermocycling conditions for MS2-Arg-tagged DNA*

Step	Temperature	Time
Initial denaturation	94 ºC	2 minutes
Denaturation	94 ºC	30 seconds
Annealing	58 ºC	30 seconds
Extension	68 ºC	1 minute
Final Extension	70 ºC	5 minutes
Hold	4 ºC	∞

After PCR, the samples were run on a 1% agarose gel, DNA bands were cut from gel and DNA extracted from the gel as described above. A restriction digest was set up as above but using Nco1 and BamH1-HF restriction enzymes. Digested DNA were run and purified from 1% agarose gel, digested DNA were ligated to plasmid pDSP62 (digested with the same restriction enzymes). Ligated reaction (figure 13) was and transformed into C41 cells as described above. To check for protein expression, colonies were picked from agar plates (kanamycin resistant), grown in LB media and induced with IPTG as described above. Control culture was not induced. Colonies that showed protein expression were used to prepare plasmid for restriction digest and sequencing to confirm cloning of recombinant MS2-6Arg tag.

2.2 Assessing the solubility of recombinant MS2-Arg-tagged coat protein

Assembly of MS2 coat protein to virus-like particles (VLPs) is a pre-requisite to elicit a robust immune response (antibody titers ~10^4) at low doses (≤ 5 μg) of VLPs. Ninety copies of the MS2 single-chain dimer assemble to form a VLP and the ability of coat protein to form VLPs can be predicted following lysis of protein with lysis buffer. If protein is likely to form VLPs, it will be in the supernatant (soluble) fraction after lysis and centrifugation; otherwise, it will be in the pellet. To assess whether the insertion of 6-Arg residues on the C-terminus will affect the solubility of the recombinant coat protein (potential to fold to VLPs), 3 colonies with high levels of MS2-Arg coat protein expression were used to start 2ml overnight cultures (per colony) with LB growth media and was subsequently used to separately inoculate 50 ml LB growth media (with 50 ng/ul

kanamycin) in duplicate flasks. The cultures were grown shaking for 3 hours at 37 ºC after which the cultures were induced with IPTG (at OD_{600} - 0.6) as described above. One group of culture (from duplicate) was grown shaking for an additional 4 hours at the same temperature and the other group was induced shaking overnight at room temperature. After that, the cultures were divided into two tubes to assess solubility with two different lysis and purification buffers. All the tubes were centrifuged at 10,000rmp for 20 minutes to pellet the cells. The supernatants were discarded and the pellets saved at -20ºC. On the next day, the pellets were dissolved into two different buffers to assess which buffer the protein was soluble in. One group of the pellets were resuspended in 5ml of lysozyme solution with of 0.01g of hen egg lysozyme while the other group was resuspended in 5ml of 10mM borax buffer pH 9. The two groups of tubes were incubated for 45mintues in ice. Then, 1μl of 10% of deoxycholate was added to each tube to aid lysis and kept in ice for 30 minutes. The cells, were the sonicated for 10 minutes (with 30-second pulses in-between sonication). After sonication, 2 ng/μl of DNase and 2mM $MgCl_2$ were added to the lysate and then incubated at 37ºC for one hour. Fifty μl of total protein (lysate) was taken from each tube and saved for protein analysis in SDS PAGE gel. The rest of the lysate was centrifuged at 3,700rpm for 30 minutes at 4ºC to separate soluble fractions from pellet. The supernatants were transfer to new tubes and labeled "supernatant". SDS PAGE gel was run to confirm the expression and solubility of MS2-Arg-tagged protein. After confirming the solubility of the protein in borax buffer, the soluble protein was tested for purification with 10mM borax at different pH conditions (pH 7, 8, and 9) as described below.

2.3 Purification of MS2-Arg-tagged protein by an ion cation exchange chromatography using CM-Sephadex C-25 and SP Sepharose beads.

The ability of MS2-Arg-tagged protein to be purified by cation exchange chromatography was assessed using two types of beads (SP Sepharose and CM Sephadex C-25). The pH of a protein (an ultimately its charge) is very crucial for its purification by cation exchange chromatography. In cation exchange chromatography, a positively charge protein binds to negatively charged resins in a column. The net charge of the protein depends on the pH of the buffer used in lysing the protein. If the pH is less than the isoelectric point (pI; pH at which net charge is zero), the net charge on the protein will be positive [35]. In our case, the pI of MS2-Arg was 8.73 (calculated using http://isoelectric.ovh.org). Thus, to assess the pH at which MS2-Arg could be purified using cation exchange chromatography, lysis/solubility of the protein was determined using borax buffer at three pH conditions (pH 7, 8 and 9). Lysis and binding of protein to SP Sepharose and CM-Sephadex C-25 resins was assessed as follows: 0.07g of SP Sepharose resin beads were equilibrated with 400μl of borax buffer (10mM borax at pH 7, 8 and 9) whereas CM-Sephadex C-25 beads were resuspended in 400μl of borax buffer (at pH 7, 8 and 9) and the tubes were inverted for a minute. The tubes were centrifuged at 1000rpm and supernatant discarded. The whole process was repeated twice. One ml of MS2-Arg-tagged protein (from section 2.2 above) was mixed with beads and the mixture was incubated rocking for 3 hours at room temperature. The beads were then spun at 1000 rpm and the supernatant was collected into a new tube as "unbound". Three hundred μl of washing buffer (10 mM of borax, at corresponding pH) were used to wash

the beads twice (5 minutes per wash). After each wash, the beads were spun down at 1000 rpm and the supernatant was collected as "washed". To get pure protein, the beads were eluted with 500μlof 400 mM NaCl elution buffer (at pH 7). SDS page gels were run using supernatant that were collected before the purification steps, unbound proteins, washed proteins from the matrix, and elusion fractions of MS2-arg-tagged protein that were collected.

3. Results

Following PCR using pDSP62 as a DNA template, a DNA fragment of expected size (1,100bp) was amplified (figure 12).
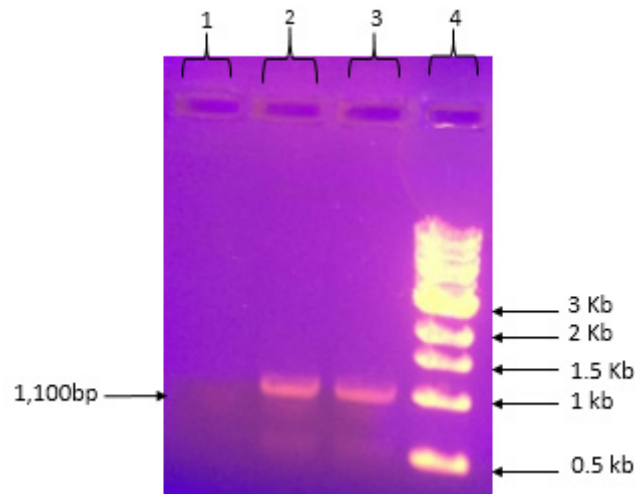
Figure 12. *Electrophoretic mobility of amplified MS2-Arg-tagged DNA in an ethidium bromide-stained 1% agarose gel. Number 1 is a negative control, numbers 2 and 3 are MS2-Arg DNA (1,100 base pair), and number 4 is 1 KB DNA ladder.*

The DNA fragment consisted of MS2-Arg coat protein (807bp) plus a (293bp) sequence upstream of MS2 coat protein. Restriction digest (BamHI-HF/NcoI) of PCR product

release the MS2-Arg fragment (807bp; figure 13), which was then cloned into pDSP62 plasmid.
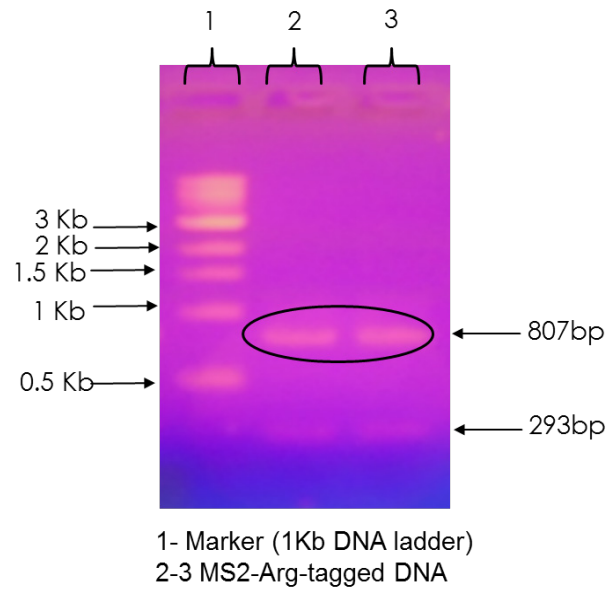
Figure 13. *Agarose gel of digested amplified DNA using restriction enzymes (NcoI and BamHI-HF). Number 1 is 1KB DNA ladder, numbers 2 and 3 are digested MS2-Arg DNA. The circled band (MS2-Arg; 807bp) was cut, digested and purified for ligation*

The colonies picked from the agar plate showed protein expression of the expected size (~28KD). As shown in figure 14, the cultures from four colonies were highly expressed following induction with IPTG while uninduced culture (panel A, lane 1) was not expressed.
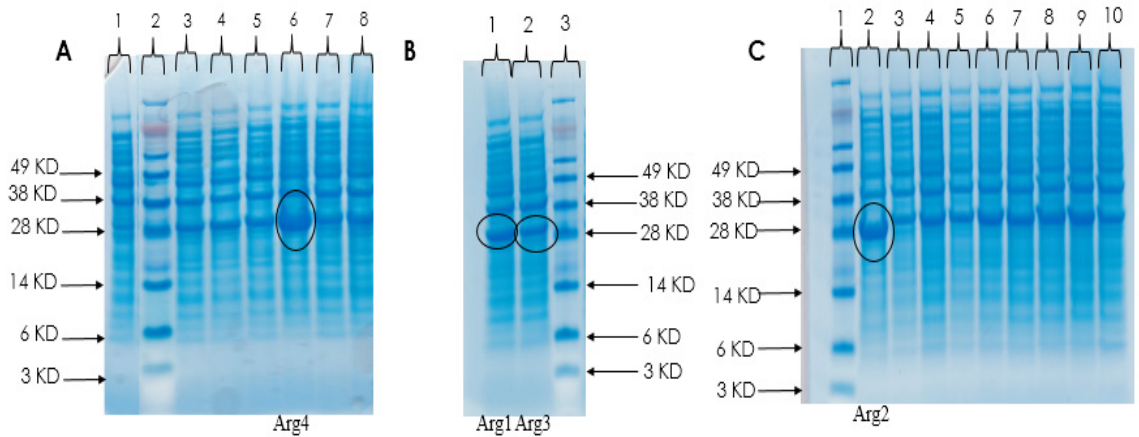
Figure 14. *Expression of MS2-Arg-tagged coat protein on SDS-PAGE gel. Protein expression was induced using IPTG. Circles indicate high expressed protein (~28KD). Number 1 in panel A is uninduced colony. Number 2 in panel A, number 3 in panel B and number 1 in panel C are protein markers. The remaining numbers in panels A, B, and C represent bacteria from individual colonies. Plasmid prep was done from colonies that showed expression (circled on the gel; Arg1-Arg4)*

Restriction digest confirmed the size of the cloned DNA fragment. As shown in figure 15, a 807bp fragment was released from cloned plasmids (pDSP62-Arg). Three of the constructs (pDSP62-Arg1, pDSP62-Arg3, pDSP62-Arg4) were sequenced to confirm MS2-Arg nucleotide sequence.
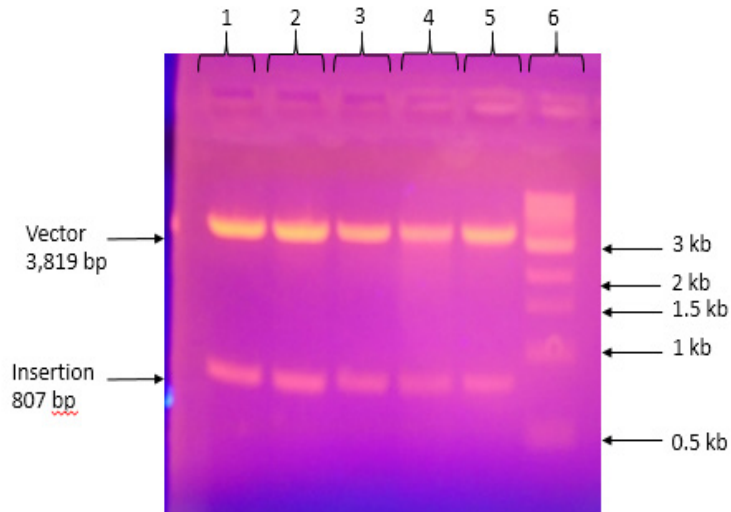
Figure 15. *Restriction digest of plasmid prep sample ran on a 1% agarose gel (stained with Ethidium bromide). Number 1 is a control pDSP62 plasmid, numbers 2,3,4,5 represent digestion of plasmid preps from 4 colonies with high expression. Number 6 is 1KB marker*

Figure 16 shows that cloned MS2-Arg sequence (query) of pDSP62-Arg1 and pDSP62-Arg3 have 100% identity with the known sequence (subject sequence). This thus confirms the insertion of 6 Arg residues on the C-terminal of MS2. pDSP62-Arg4 also show insertion of 6 Arg residues; however, there is an adenosine to a cytosine point mutation in the MS2 sequence of this construct (figure 17); which changes Alanine amino acid to Aspartate amino acid. Thus, the sequence identity of this construct is 99%. pDSP62-Arg2 had lots of mutation in the MS2 sequence and was not pursued further.

BLASTN for MS2-Arg1 and MS2-Arg3

Score	Expect	Identities	Gaps	Strand
1434 bits(776)	0.0	776/776(100%)	0/776(0%)	Plus/Plus

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Query  1    CCATGGCAAGCAATTTCACGCAATTTGTATTGGTAGATAACGGGGGTACGGGGGATGTTA  60
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Sbjct  1    CCATGGCAAGCAATTTCACGCAATTTGTATTGGTAGATAACGGGGGTACGGGGGATGTTA  60

Query  61   CGGTAGCACCTTCAAATTTTGCAAATGGTGTAGCAGAGTGGATATCAAGCAATAGCAGAA  120
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Query  121  GCCAAGCATATAAGGTTACGTGCTCAGTAAGACAATCAAGCGCTCAAAACAGAAAGTATA  180
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Sbjct  121  GCCAAGCATATAAGGTTACGTGCTCAGTAAGACAATCAAGCGCTCAAAACAGAAAGTATA  180

Query  181  CGATAAAGGTAGAAGTTCCGAAGGTTGCTACGCAAACGGTAGGTGGTGTTGAATTGCCGG  240
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Sbjct  181  CGATAAAGGTAGAAGTTCCGAAGGTTGCTACGCAAACGGTAGGTGGTGTTGAATTGCCGG  240

Query  241  TTGCAGCTTGGAGAAGCTATCTCAACATGGAGTTGACGATACCTATATTTGCAACCAACA  300
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Sbjct  241  TTGCAGCTTGGAGAAGCTATCTCAACATGGAGTTGACGATACCTATATTTGCAACCAACA  300

Query  301  GTGATTGTGAATTGATAGTAAAAGCTATGCAGGGGTTGTTGAAGGACGGTAATCCTATAC  360
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Sbjct  301  GTGATTGTGAATTGATAGTAAAAGCTATGCAGGGGTTGTTGAAGGACGGTAATCCTATAC  360

Query  361  CGAGCGCTATAGCTGCTAATAGTGGCCTCTACGGCAACTTTACTCAGTTCGTTCTCGTCG  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  CGAGCGCTATAGCTGCTAATAGTGGCCTCTACGGCAACTTTACTCAGTTCGTTCTCGTCG  420

Query  421  ACAATGGCGGTACCGGCGACGTGACTGTCGCCCCAAGCAACTTCGCTAACGGGGTCGCTG  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  ACAATGGCGGTACCGGCGACGTGACTGTCGCCCCAAGCAACTTCGCTAACGGGGTCGCTG  480

Query  481  AATGGATCAGCTCTAACTCGCGTTCACAGGCTTACAAAGTAACCTGTAGCGTTCGTCAGA  540
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Sbjct  481  AATGGATCAGCTCTAACTCGCGTTCACAGGCTTACAAAGTAACCTGTAGCGTTCGTCAGA  540

Query  541  GCTCTGCGCAGAATCGCAAATACACCATCAAAGTCGAGGTGCCTAAAGTGGCAACCCAGA  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  GCTCTGCGCAGAATCGCAAATACACCATCAAAGTCGAGGTGCCTAAAGTGGCAACCCAGA  600

Query  601  CTGTTGGTGGTGTAGAGCTTCCTGTAGCCGCATGGCGTTCGTACTTAAATATGGAACTAA  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601  CTGTTGGTGGTGTAGAGCTTCCTGTAGCCGCATGGCGTTCGTACTTAAATATGGAACTAA  660

Query  661  CCATTCCAATTTTCGCCACGAATTCCGACTGCGAGCTTATTGTTAAGGCAATGCAAGGTC  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661  CCATTCCAATTTTCGCCACGAATTCCGACTGCGAGCTTATTGTTAAGGCAATGCAAGGTC  720

Query  721  TCCTAAAAGATGGAAACCCGATTCCCTCAGCAATCGCAGCAAACTCCGGCATCTAC  776
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721  TCCTAAAAGATGGAAACCCGATTCCCTCAGCAATCGCAGCAAACTCCGGCATCTAC  776
```

Figure 16. *Sequence alignment of MS2-Arg-tagged DNA (Query sequence) showed 100% identity with known (sbjct, which represents subject sequence). Sequence Alignment was done using nucleotide BLASTN*

BLASTN MS2-Arg 4

Score	Expect	Identities	Gaps
1428 bits(773)	0.0	775/776(99%)	0/776(0%)

```
Query   1    CCATGGCAAGCAATTTCACGCAATTTGTATTGGTAGATAACGGGGGTACGGGGGATGTTA   60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   1    CCATGGCAAGCAATTTCACGCAATTTGTATTGGTAGATAACGGGGGTACGGGGGATGTTA   60

Query   61   CGGTAGCACCTTCAAATTTTGCAAATGGTGTAGCAGAGTGGATATCAAGCAATAGCAGAA   120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   61   CGGTAGCACCTTCAAATTTTGCAAATGGTGTAGCAGAGTGGATATCAAGCAATAGCAGAA   120

Query   121  GCCAAGCATATAAGGTTACGTGCTCAGTAAGACAATCAAGCGCTCAAAACAGAAAGTATA   180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   121  GCCAAGCATATAAGGTTACGTGCTCAGTAAGACAATCAAGCGCTCAAAACAGAAAGTATA   180

Query   181  CGATAAAGGTAGAAGTTCCGAAGGTTGCTACGCAAACGGTAGGTGGTGTTGAATTGCCGG   240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   181  CGATAAAGGTAGAAGTTCCGAAGGTTGCTACGCAAACGGTAGGTGGTGTTGAATTGCCGG   240

Query   241  TTGCAGCTTGGAGAAGCTATCTCAACATGGAGTTGACGATACCTATATTGCAACCAACA   300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   241  TTGCAGCTTGGAGAAGCTATCTCAACATGGAGTTGACGATACCTATATTGCAACCAACA   300

Query   301  GTGATTGTGAATTGATAGTAAAAGCTATGCAGGGGTTGTTGAAGGACGGTAATCCTATAC   360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   301  GTGATTGTGAATTGATAGTAAAAGCTATGCAGGGGTTGTTGAAGGACGGTAATCCTATAC   360

Query   361  CGAGCGCTATAGATGCTAATAGTGGCCTCTACGGCAACTTTACTCAGTTCGTTCTCGTCG   420
             ||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   361  CGAGCGCTATAGATGCTAATAGTGGCCTCTACGGCAACTTTACTCAGTTCGTTCTCGTCG   420

Query   421  ACAATGGCGGTACCGGCGACGTGACTGTCGCCCCAAGCAACTTCGCTAACGGGGTCGCTG   480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   421  ACAATGGCGGTACCGGCGACGTGACTGTCGCCCCAAGCAACTTCGCTAACGGGGTCGCTG   480

Query   481  AATGGATCAGCTCTAACTCGCGTTCACAGGCTTACAAAGTAACCTGTAGCGTTCGTCAGA   540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   481  AATGGATCAGCTCTAACTCGCGTTCACAGGCTTACAAAGTAACCTGTAGCGTTCGTCAGA   540

Query   541  GCTCTGCGCAGAATCGCAAATACACCATCAAAGTCGAGGTGCCTAAAGTGGCAACCCAGA   600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   541  GCTCTGCGCAGAATCGCAAATACACCATCAAAGTCGAGGTGCCTAAAGTGGCAACCCAGA   600

Query   601  CTGTTGGTGGTGTAGAGCTTCCTGTAGCCGCATGGCGTTCGTACTTAAATATGGAACTAA   660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   601  CTGTTGGTGGTGTAGAGCTTCCTGTAGCCGCATGGCGTTCGTACTTAAATATGGAACTAA   660

Query   661  CCATTCCAATTTTCGCCACGAATTCCGACTGCGAGCTTATTGTTAAGGCAATGCAAGGTC   720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   661  CCATTCCAATTTTCGCCACGAATTCCGACTGCGAGCTTATTGTTAAGGCAATGCAAGGTC   720

Query   721  TCCTAAAAGATGGAAACCCGATTCCCTCAGCAATCGCAGCAAACTCCGGCATCTAC   776
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   721  TCCTAAAAGATGGAAACCCGATTCCCTCAGCAATCGCAGCAAACTCCGGCATCTAC   776
```

Figure 17. *Sequence alignment of MS2-Arg-tagged DNA (Query sequence) showed 99% identity with known (sbjct, which represents subject sequence). The highlighted nucleotides in red shows the point mutation. Sequence Alignment was done using nucleotide BLASTN*

The solubility (assembly potential) of recombinant MS2-Arg coat protein was assessed

by lysing bacteria with two different lysis buffers and the supernatant was run on an SDS

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PAGE gel to check for presence of recombinant protein in the supernatant. Bacterial cultures that were induced at 37 ºC were insoluble when lysed with lysozyme or borax buffer (data not shown). Similarly, bacteria cultures that were induced overnight at room temperature were not soluble in lysozyme lysis buffer (figure 18A). However, when the bacteria were lysed using 10% borax buffer (pH 9), two of the constructs representing MS2-Arg3 and MS-Arg4 appeared to be soluble (figure 18B). MS2-Arg3 was soluble in borax at pH 7 and 9 but not at pH 8 (figure 19).
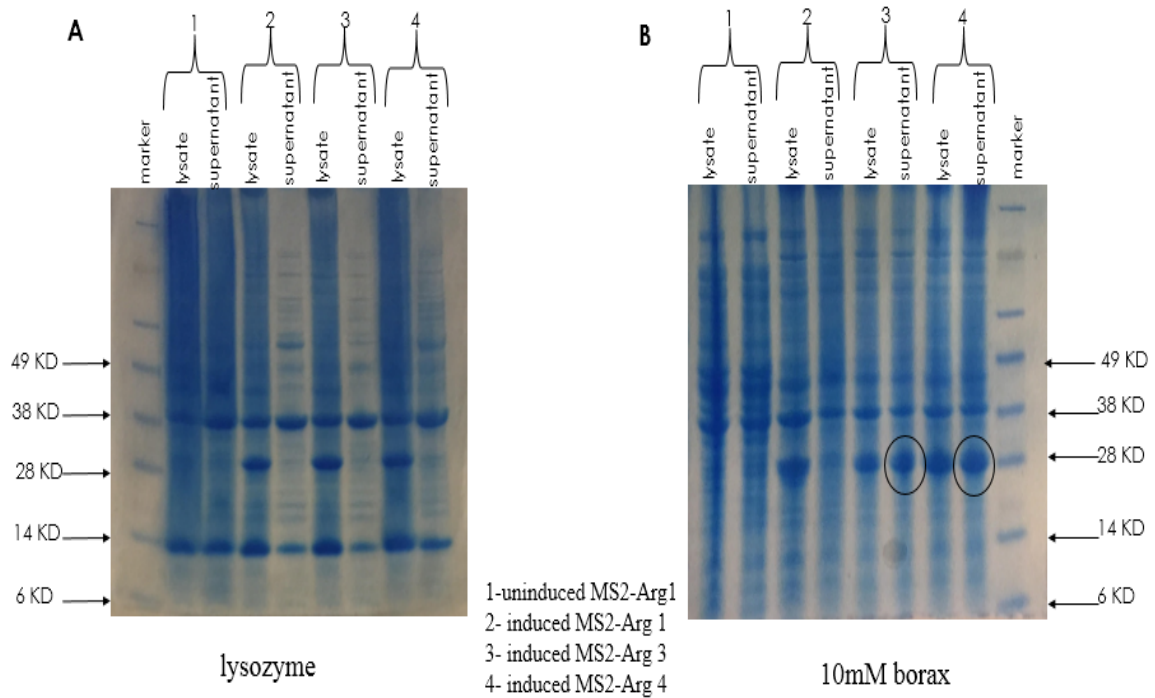

Figure 18. *Assessing the solubility of MS2-Arg-tagged coat proteins. A) lysis of bacteria pellet with lysozyme solution and B) lysis of bacterial pellet with 10mM borax buffer. Number 1 is uninduced MS2-Arg 1, number 2 is induced MS2-Arg 1, number 3 is induced MS2-Arg 3 and number 4 is induced MS2-Arg 4*
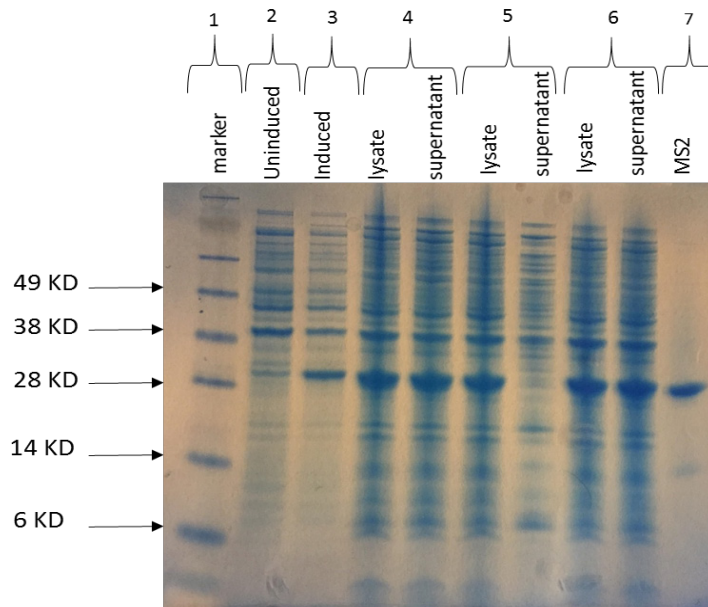
Figure 19. *Assessing the solubility of MS2-Arg3-tagged coat proteins at different pH conditions. Number 1 is Marker. Number 2 is uninduced Arg3, number 3 is induced Arg3 lysate (lysed with urea), number 4 is Arg3 lysate and supernatant lysed with 10mM borax at pH7, number 5 is Arg3 lysate and supernatant lysed with 10mM borax at pH 8, number 6 is Arg3 lysate and supernatant lysed with 10mM borax at pH 9, and positive control MS2*

whereas MS2-Arg4 seemed to be soluble at pH 9 and to a less extend pH 7 (figure 20).

Thus, borax at pH 7 was explored with SP Sepharose and CM-Sephadex C-25 beads to

purify MS2-Arg3. MS2-Arg4 was not explored for purification because it was soluble at
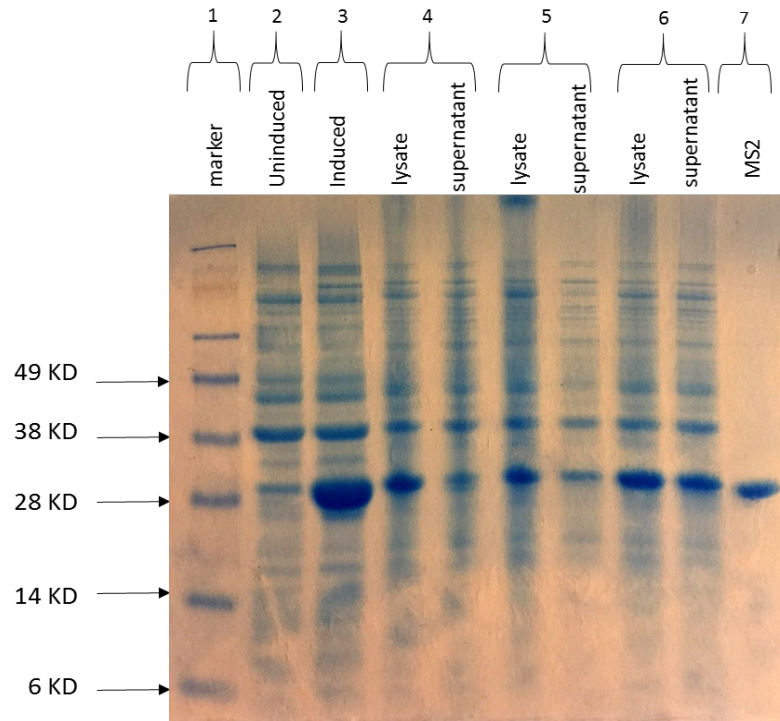
pH 9, which is above the pI.

Figure 20. *Assessing the solubility of MS2-Arg4-tagged coat proteins at different pH conditions. Number 1 is Marker, number 2 is uninduced Arg3, number 3 is induced Arg3 lysed with urea (positive control), number 4 is Arg 4 lysate and supernatant lysed with 10mM borax at pH 7, number 5 is Arg4 lysate and supernatant lysed with 10mM borax at pH 8, number 6 is Arg4 lysate and supernatant lysed with 10mM borax at pH 9, and positive control MS2*

While small amount of MS2-Arg3 could be purified using CM-Saphadex C-25 resins, the protein could not be purified using SP Sepharose. As shown in figure 21, a small fraction of MS2-Arg3 was eluted from CM-Sephadex C-25 resins following elution with 0.4M NaCl.
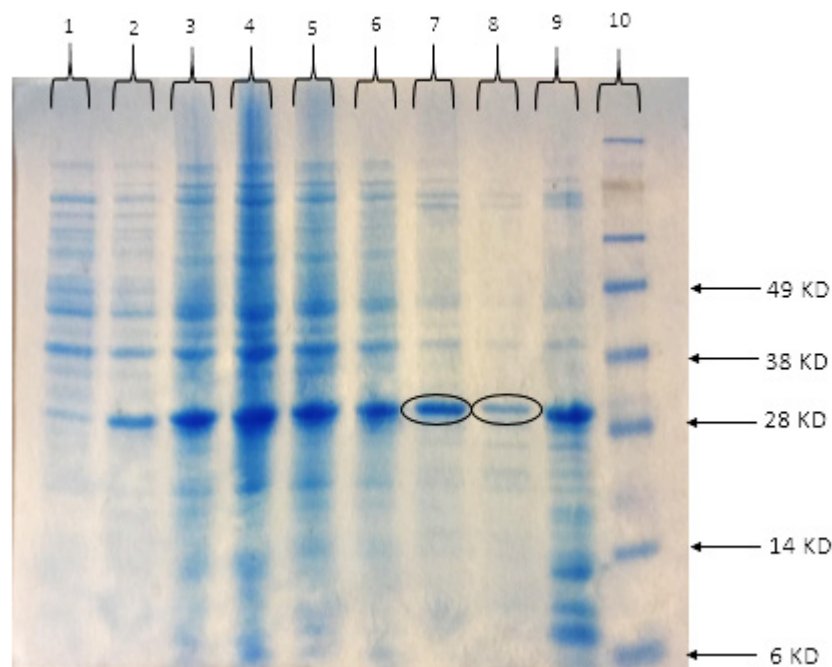
Figure 21. *Purification of recombinant protein MS2-Arg3 protein. The recombinant protein was purified by cation exchange chromatograph using CM-Saphadex C-25 beads. Number 1 is uninduced Arg3, number 2 is induced Arg3 lysed with urea (positive control), number 3 total protein before purification, number 4 unbound protein (flow through) after adding total protein to column, numbers 5-6 is flow through of column washes, numbers 7 and 8 are fractions after elution, number 9 CM-Saphadex C-25 beads after elution, and number 10 is a marker.*

Purification using SP Sepharose resins was unsuccessful. Most of the protein did not bind to the SP Sepharose beads (figure 22); MS2-Arg3 protein was present in the unbound fraction. Additionally, the small fraction of MS2-Arg3 that was bound to the beads was washed during the wash step (figure 22).
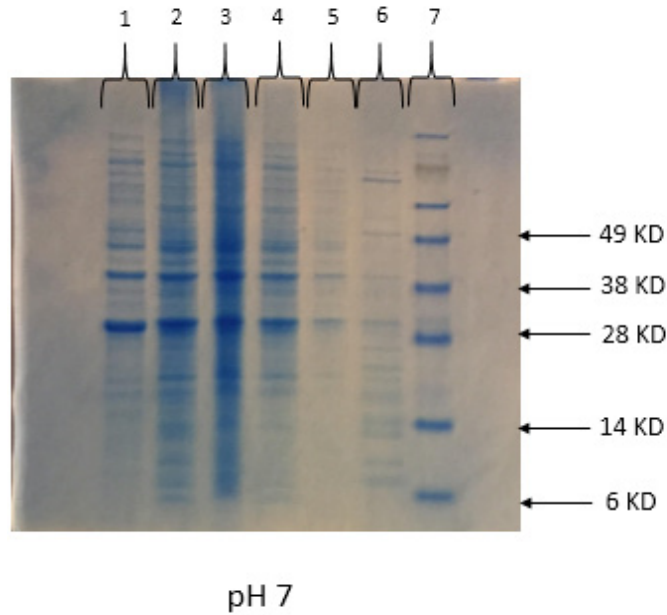
pH 7

Figure 22. *Purification of recombinant protein MS2-Arg3 protein by cation exchange chromatograph using SP-Sepharose FF beads. Number 1 is Arg3 lysate (lysed with urea; positive control), number 2 is total protein before purification, number 3 is unbound protein (flow through) after adding total protein to column, numbers 4-5 is flow through of column washes,, number 6 is fraction after elution, and number 7 is a marker*

4. Conclusions

To develop a platform that could enhance the purification of recombinant MS2 VLPs, we inserted, successfully, 6-Arg residues on the C-terminus of MS2 bacteriophage coat protein. Solubility studies (ability to form VLPs) showed the MS2-Arg is soluble in 10mM borax and can be purified in small quantity, using CM-Sephadex C25 resins (at pH 7), from contaminating bacterial proteins. Future studies are needed to assess (using transmission electron microscopy) whether the purified MS2-Arg can assemble into VLPs. Recombinant bacteriophage VLPs displaying an Arg tag will enhance VLPs purification following gel filtration purification and will be a valuable tool for vaccine development.

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