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# ENGINEERED NOROVIRUS-LIKE PARTICLE AS A VACCINE CANDIDATE

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## ABSTRACT

*Noroviruses* account for the bulk of infectious gastroenteritis in adults worldwide. Although Norovirus gastroenteritis is self-limiting, the economic burden of infection is considerable and uncontrolled manifestations can result in death. Noroviruses form an antigenically diverse group of agents and cross-reactive immunity is poor. These features present a considerable challenge for vaccine development. Current approaches have been based on the use of empty virus-like particles produced by self-assembly of the virus capsid protein. In this study we employed a new approach for engineering stable particles of Hawaii virus capsid protein by deleting those immunodominant regions (P2-subdomains) that evoke type-specific responses and bridging the resulting gap with a synthetic poly-glycine chain. This construct was expressed using the baculovirus system and the obtained purified protein was examined by EM which showed that the new engineering approach is permitting some form of structural assembly. Our study points a way to produce a more pan-reactive vaccine in future studies by producing antisera directed predominantly at the most conserved S-domain of Norovirus capsid.

**Keywords:** Engineered Norovirus-like particles, P2-subdomains, Synthetic poly-glycine chain, Vaccine development.

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## INTRODUCTION

Noroviruses (previously Norwalk-like viruses) are now contained in a specific genus called Norovirus (NoV) belonging to the *Caliciviridae* family. Their genome consists of a positive sense RNA strand which is 7.5-8 kb in length, polyadenylated, and organized in three open reading frames (ORFs) **(Green, 2007)**. NoVs account for the bulk of all reported viral gastroenteritis outbreaks (About 96%) all over the world **(Patel *et al.*, 2009)**. NoVs are divided into five genogroups (Gs) according to their capsid and RNA polymerase genes sequencing. Genogroups are further subdivided into genotypes and then strains **(Zheng *et al.*, 2006)**. All human strains are included in GI, GII, and GIV **(Karst *et al.*, 2003)**. Norwalk virus (NV) is considered the prototype strain of GI, while the prototype GII strain is the Hawaii virus which was selected for our study since GII viruses are currently responsible for most of reported NoV outbreaks.

The Hawaii virus capsid has a T=3 icosahedral symmetry (like all NoVs). It is formed of 90 dimers of a major structural protein called VP1. VP1 consists of two main domains, S and P with a flexible link in between. The S domain represents the closed capsid shell, while the P domain represents the protruding arch-like projections on the shell surface. The P domain is divided into P1 and P2 subdomains. The P2 subdomain consists of 126 amino acids and is inserted into the P1 subdomain and hence dividing it into P1-1 and P1-2 **(Nilsson *et al.*, 2003)**.

NoV strains appear to be dynamic and are constantly evolving though frequent point mutation **(Lopman *et al.*, 2008)**. Recombinations between strains leading to the swapping of structural and non-structural genes have also been indentified **(Jiang *et al.*, 1999)**.

The P2 subdomain was found to be the most antigenically variable between different strains of NoVs with sequence similarity reaching up to only 8% **(Kitamoto *et al.*, 2002; Chakravarty *et al.*, 2005)**.

The unavailability of in vitro culture system for human NoVs has shifted the efforts to investigate their recombinant virus-like particles (rVLPs) as possible vaccine candidate. Recombinant VLPs were found to be good immunogenic when administered

parenterally (**Guerrero *et al.*, 2001; Noad and Roy, 2003**).These recombinant particles show several advantages including the repetitive epitope presentation, their size which facilitates their uptake by the dendritic cells resulting in activation of both innate and adaptive immune responses (**Deml *et al.*, 2005**). Also, their particulate nature leads to better induction of the immune response than separate protein antigens (**Jiang *et al.*, 1992**).

The high conservation of the S domain between different strains has pointed out that many antigenic epitopes should be located within that region. Several monoclonal antibody (MAb) mapping studies using NoV rVLPs have proved that most of the antibodies map to the first 70 amino acids of the capsid protein, and were type-specific. Two GI and GII cross-reactive MAbs (1B4, 1F6) were mapped to eleven amino acids within the N-terminus of the capsid protein (**Yoda *et al.*, 2001**). Another broadly reactive MAb (MAb14-1) between GI and GII was identified by **Shiota *et al.* (2007)** to map to the C-terminal P1 subdomain.

For a VLP vaccine to be efficient the final conformation of the capsid protein subunits should resemble that of the wild virus to allow proper exposure of the antigenic determinants (**Gerlich *et al.*, 1996**). We aim in this study to modify the Hawaii virus capsid protein (VP1) by excluding the hypervariable region (P2 subdomain) responsible for type-specific immunity and to bridge the resulting gap with a poly-glycine amino acid α-helix to allow free mobility of the protein subunits and hence assembly of VLPs.

## MATERIALS &METHODS

### Insect cells:

*Spodoptera frugiperda* (Sf21) cells were grown in non-vented tissue culture flasks using TC-100 medium (Gibco, UK) supplemented with 10% heat-inactivated fetal bovine serum (Clontech, UK), 2 mM L-Gutamine (Gibco, UK), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco, UK) at 28°C for five days. Sf21 cells were used to rescue the recombinant baculovirus containing the engineered Hawaii virus capsid protein and also for expressing that protein using high viral multiplicity of infection (MOI).

### PCR:

A clone of cDNA specifying Hawaii virus capsid protein (VP1) in pGEM T-easy was used as the source of DNA from which to engineer the Hawaii capsid protein. Three PCR primer

pairs were designed according to **Apte and Daniel, (2003)** and purchased (SIGMA Proligo, UK) (**Table 1**); two pairs to amplify two segments of Hawaii VP1 gene (Accession number: U07611.2) separately; left hand segment (LH) comprising S and P1-1 domains and right hand segment (RH) comprising P1-2 domain a step by which we could exclude the P2 region. The P2 itself was replaced with a short stuffer of unrelated sequence derived from Lambda phage DNA amplified by the third PCR primer pair. Restriction enzyme sites were chosen to flank the 5' and 3' ends of each segment as follow: BamHI and XhoI to flank the LH segment and HindIII and PstI to flank the RH segment, and HindIII to flank the stuffer segment which is incorporating a native XhoI site in its center. A 100µl PCR reaction typically contained 10µl 10x PCR buffer, 10µl 2mM deoxyribonucleotide triphosphate (dNTP) mixture, 10µl 2.5µM forward primer, 10µl 2.5µM reverse primer, 58µl Milli-Q (Millipore Corporation Quality) water, and 2µl of the target DNA. Five units of Taq polymerase enzyme were added to the mixture, overlaid with 100µl liquid paraffin in reaction tubes and placed in a thermal cycler (TECHNE, Cambridge, UK). The machine was adjusted according to the amplified sequence size and the melting temperatures (Tm) of the primers, as a rough guide extension times were calculated at one minute per 1000 bases and annealing temperatures were 5°C below the predicted Tm of the oligo primers concerned. The thermal cycler was adjusted to run 25 cycles of denaturation [95°C, 1 minute], annealing [51°C, 1 minute], and extension [72°C, 1 minute], after initial denaturation at 95°c for five minutes. A final extension period of 10 minutes at 72°C was allowed to permit completion of incomplete product. DNA was precipitated from the reaction mixture (after removal of paraffin) by the addition of 2 volumes of absolute ethanol and 1/30th volume sodium acetate solution (3M, pH 5.0). Precipitation mixtures were allowed to stand at -20°C overnight (or for 2 hours at -80°C) before the DNA was collected by spinning at 13000 rpm for ten minutes in a microcentrifuge (eppendorf-5415C, California, USA). The supernatant was carefully removed and the pellet was washed twice with 70% ethanol, dried at 37°C for three minutes in a dry heat block and resuspended in 20µl Milli-Q water.

**Table 1. PCR primers**

| Primer/Orientation/Genomic location/Accession number | Sequence |
|---|---|
| LH primer/Forward/5085-5104 [Hawaii ORF2 - U07611.2] | 5'-AGAGGATCCATGAAGATGGCGTCGAATGA-3' |
| LH primer/Reverse/5895-5912 [Hawaii ORF2 - U07611.2] | 5'-GACTCGAGCCCGTGGTGCCCAACAATTC-3' |
| RH primer/Forward/ 6246-6265 [Hawaii ORF2 - U07611.2] | 5'-GCAAGCTTCGGTCTGTTTAACACTGACCA-3' |
| RH primer/Reverse/ 6672-6689 [Hawaii ORF2 - U07611.2] | 5'-GCCTGCAGTTACTGCACTCTTCTGCGCCC-3' |
| Lambda stuffer/Forward/ 33301-33319 [V00638.1] | 5'-GCAAGCTTAGTCTGGATAGCCATAAGT-3' |
| Lambda stuffer/Reverse/33831-33850 [V00638.1] | 5'-GCAAGCTTATTGAAATCGACCATGACGT-3' |

Underline: denotes restriction enzyme site, Box: stop codon, Extra bases are bold: CC after the XhoI site in the LH reverse primer to change the frame translation into glycine and serine (smaller and less antigenic) instead of leucine and glutamic acid (that would result from the added XhoI site), C after the HindIII site in the RH forward primer to keep the ORF as the first A of the HindIII will complete the last glycine (GGA) in the artificial polyglycine nucleotide chain.

**Cloning and plasmid preparation:**

Each PCR product was extracted from 1.2% agarose gel and cloned separately in pGEM T-easy cloning vector. The cloning steps included removal of the stuffer segment by HindIII digestion and its ligation in the RH clone using the newly created HindIII site at the 5' end of the RH segment. Then, the left hand end of the stuffer was removed by digesting this vector with XhoI site contained within the stuffer and PstI flanking the 3' end of the RH segment. This separated the RH segment plus part of the lambda stuffer (terminating in PstI and XhoI restriction sites). Finally, this segment could be ligated in the LH clone after its digestion with XhoI at the 3' end of the LH segment and PstI which exists downstream of multiple cloning site of pGEM T-easy vector to form the final construct. For use, the remaining part of the stuffer was removed by digestion with XhoI and HindIII. The pGEM T-easy plasmid was used to transform *Escherichia coli* (DH5-α) cells during different steps of the cloning strategy and plasmids carrying each insert were prepared using QIAgen Miniprep Kit (QIAGEN, Crawley, UK) according to the manufacturer instructions.

**Determination of the poly-glycine chain length:**

The distance between the chains entering and leaving the P2 subdomain was measured using Swiss pdb viewer software, and was found to be approximately 28Å apart. This gap could be bridged using a short chain of poly-glycine, an amino acid chosen for simplicity and flexibility and relatively low immunogenicity, and which itself may not promote drastic structural alteration. Number of the required glycine residues was calculated to be 18 if in flexible α-helical conformation (28Å divided by 1.5Å 'length of the impact amino acid'). Accordingly, this artificial sequence was synthesized as sense and antisense strands (SIGMA Proligo, UK) **(Table 2)**. The complementary strands were annealed together to form a dsDNA with sticky termini (XhoI and HindIII) suitable to replace the lambda stuffer segment in the final clone. A 100µl annealing reaction was used containing 10µl 10x annealing buffer, 45µl sense strand and 45µl antisense strand in 1.5ml eppendorf tube which was exposed to heat in boiling bath for two minutes and then transferred to 65°C heating block for five minutes and finally transferred to 44°C water bath for two hours. The samples were left to cool overnight in the water bath after it was turned off. The DNA strands were precipitated and resuspended in 90µl Milli-Q water. The strands were phosphorylated using 100µl reaction containing the 90µl DNA sample, 10µl 10x kinase buffer, 1µl 1mM ATP and 2µl kinase enzyme (which catalyzes the transfer of a phosphate group from ATP to the free terminus of the polynucleotides) and mixed. The mixture was incubated at 37°C for 45 minutes. The annealed DNA strands were extracted from the solution by addition of equal volume (100µl) of phenol-chloroform-isoamyl alcohol to the sample followed by vortex mixing for two minutes. Phases were separated by centrifugation (13000 rpm for two minutes) and the upper (aqueous) layer was harvested to a fresh eppendorf tube. The DNA was re-extracted and precipitated (as before), and resuspended in 50µl Milli-Q water.

**Table 2. Annealed synthetic 18 poly-glycine nucleotide chains (sense, antisense strands)**

| Name | Nucleotide sequence |
|---|---|
| Annealed 18 poly-glycine 'Sense' & 'Antisense' strands | 5'-TCGAGTGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGA-3'<br>3'- CACC TCCTCC TCC TCC TCC TCC TCC TCC TCC TCCTCC TCC TCC TCC TCC TCCTCCTTCGA-5' |

Complementary ends to XhoI & HindIII are underlined.

**Insertion of the poly-glycine chain into the final construct:**

The annealed 18 poly-glycine segment was then used to replace the stuffer segment after it had been removed to produce the final clone in pGEM T-easy vector containing the modified Hawaii capsid gene (18-Hawaii VP1). Ligation reaction included 20ng of the pGEM T-easy vector and sufficient insert DNA to achieve a 3:1 molar excess of insert to vector. A 10µl ligation reaction included 1µl 10x ligase buffer, 1µl 25% polyethylene glycol, 20ng of the vector, and the calculated volume of the insert. Milli-Q water was added to bring the total volume to 10µl. The reaction components were mixed and allowed to stand for two minutes on ice. Then, 1µl of T4 DNA ligase (Promega, Madison, USA) was added and mixed. The mixture was overlaid with 30µl of liquid paraffin and incubated overnight at 14°C in a water bath placed in a 4°C room. After this time the ligation mixture was diluted and the enzyme detached by addition of 1µl 0.2M EDTA (SIGMA, USA) and 9µl Milli-Q water. The Ligation mixture was stored at -20°C until it was used in transformation of competent E.coli cells and then plasmid preparation.

**Confirmation of correct orientation and Sequence verification:**

Correct size of the insert was confirmed following each step in the cloning strategy and also for the final clone by restriction digest reactions using suitable restriction enzymes and results were checked by UV visualisation of the cut segments after agarose gel electrophoresis of digest samples. Fidelity of the final clone in pGEM T-easy vector was confirmed by sequencing of several samples at CoGenics Company using M13 forward and reverse primers and also a designed primer, left Hawaii seq. (SIGMA Proligo, UK) with the following sequence:
**(5'-TTGTCAAATTCTAGATTCCC-3')**
to check the inserted poly-glycine chain and the open reading frame.

**Baculovirus rescue and screening of recombinants:**

DNA to be expressed was recloned from the pGEMT-easy cloning vector using the restriction sites appropriate for transfer into the baculovirus recombination shuttle vector pVL1393 (Invitrogen, USA) as previously described by Nagesha *et al.* (1995). These excisions and reclonings were made with great attention to reading frame. Clones yielding the expected restriction digest pattern and sequencing result were selected and used in co-transfection of Sf21 cells with baculovirus linearized DNA (BD BaculoGold, BD Biosciences Pharmingen). During this reaction baculovirus cannot be rescued unless the linear virus DNA supplied is returned to a circular form. This is only achieved if it could recombine with the cDNA provided in the baculovirus shuttle vector. This recombination inserts the engineered construct into the baculovirus DNA under the control of the polyhedrin promoter. Sf21 cells were grown in 3.5 $cm^2$ dishes to 50% confluence in TC-100 growth medium. The cell monolayer was washed with TC-100 without serum and 0.8ml of TC-100 (serum and antibiotic free) was added to each dish. Meanwhile, the transfection mixture was prepared in a sterile plastic universal, 0.4µg baculovirus linearized DNA and 2µg pVL1393 vector containing the target insert were added and adjusted to 100µl (In general, a molar ratio of 5:1 shuttle vector DNA: baculovirus linear DNA was sought). 10µl lipofectin (GIBCO, Paisley, UK) was diluted in 90µl of serum and antibiotic free TC-100 for each transfection. Then, the diluted lipofectin was added to the DNAs, mixed gently, and allowed to stand at room temperature for 15-20 minutes to allow the association between liposomes and DNA to take place. 200µl of the transfection mixture was then added to each dish dropwise and rocked gently for mixing. Dishes were incubated at 28°C for five hours, then the transfection mixture was removed and replaced with 2ml TC-100 growth medium (containing 10% FBS) and incubation continued in a humidified atmosphere at 28°C for five days, or until cytopathic effect was apparent. The medium was harvested and clarified by centrifugation at 2500 rpm for five minutes, and then stored at -80°C. Recombinant virus was screened by plaque assay in Sf21 cells under agarose overlay. Plaques were visible after 5 days at which time 1.5ml of TC-100 containing X-gal (10%) was added above the agarose to allow the chromogen to diffuse down to the cells overnight. Recombinant plaques did not express lac-Z

enzyme and remained colourless whilst non-recombinant plaques lacking an insert turned blue.
Plaques were also checked for the presence of the 18-Hawaii construct 'recombinant plaques' by PCR using the LH(Hawaii) forward and the RH(Hawaii) reverse primers.

**Expression of recombinant protein using Sf21 cells:**

Expression of the engineered Hawaii capsid protein was attempted using recombinant baculovirus. Infections were carried out at high multiplicity of infection (5-10 pfu/cell) using 6 large ($175cm^2$) flasks seeded with Sf21 cells and grown to 70% confluence. The viral inoculum was added in 4ml TC-100 fresh growth medium per flask and the flasks were rocked at room temperature for one hour. Following this adsorption period 40ml of the growth medium was added to each flask. Flasks were incubated at 28°C for six days when cells were detached using cell scrapers into the medium. Cells were then collected by centrifugation at 2500 rpm for five minutes and both cell pellet and culture supernatant were saved. Both supernatant and cell pellet were sampled for sodium dodycyl sulphate polyacrylamide gel electrophoresis screening (SDS-PAGE) **(Jiang *et al*, 1992)** of the expected protein. The expected molecular weight of the engineered Hawaii virus capsid protein (18-Hawaii) was calculated by OMIGA software (kindly provided by Dr. Mike Carter) to be 48kDa.

**Purification of the engineered Hawaii capsid protein:**

This method was derived from **Jiang *et al.* (1992).** Polyethylene glycol 'PEG' was used to precipitate protein from the pooled Sf21 culture supernatants. Solid PEG (Grade 6000) was added to final concentration of 8% and dissolved by stirring. The flask stood on ice for 1 hour before proteins were pelleted at 1500 rpm for 30 minutes in Mistral-3000i centrifuge (Sanyo, Japan). The supernatant was discarded and the pellet was resuspended thoroughly by syringing in 5ml NT buffer (0.1 M NaCl, 0.01 M Tris pH 7.4) using needles of progressively finer bore (21G, then 25G). Higher speed spinning at 10000 rpm for ten minutes then pelleted the large protein aggregates and debris and the resulting supernatant was diluted using NT buffer and overlaid onto a 5ml cushion of 20% sucrose/NT buffer in a sw28-Ti rotor tube. The samples were spun

at 28000 rpm in Beckman ultracentrifuge (J2-21M/E, Beckman Coulter, USA) for five hours, then the supernatant was discarded and the pellet below the cushion was resuspended in 100µl NT buffer. In this method, protein should not pellet through the sucrose unless particulate, while soluble protein will remain in solution above the sucrose. A pellet sample was boiled in a reducing sample buffer and was run using SDS-PAGE. A sample from the pellet was sent for EM examination.

## RESULTS

In order to promote a more faithful cross-antigenicity, and particle formation of Hawaii virus VLPs, we sought to replace the P2 subdomain responsible for type-specific immunity of the virus and to replace it with a poly-glycine chain to bridge the gap between the emergent and returning polypeptide chains entering and leaving the P1 subdomain and thus allow these chains to move easily to the required distance apart.

**Screening of PCR products**

PCR products were analysed on agarose gels (**Figure 1**) which shows the following band sizes: 549bp, 443bp, and 827bp for lambda, RH, and LH segments respectively.

**Confirmed construct in pGEM T-easy vector**

Insertion of the 18-Hawaii construct into pGEM T-easy vector was checked by using two sets of restriction enzymes: (BamHI & HindIII) and (XhoI & PstI). The restriction fragments of the first enzyme set consist of two bands; one corresponds for the vector and the other for LH segment plus 60bp giving final size of 887bp, while, the other enzyme set gives band corresponding for the vector and band for RH segment plus 60bp giving final size of 503bp (**Figure 2**).

**Confirmed constructs in baculovirus shuttle vector (pVL1393)**

Once the construct had been confirmed it was excised from the pGEM T-easy vector using the terminal enzymes (BamHI and PstI) and recloned into the baculovirus shuttle vector (pVL1393). Sites had been chosen to permit easy cloning to produce a final construct in that vector. Once clones had been obtained the presence and orientation of the modified VP1 gene in pVL1393 vector was confirmed by restriction enzyme digestion using the enzyme pairs (BamHI & BglII) and (PstI & XhoI). The fragments released by these treatments were assessed on

an agarose gel. The first enzyme pair shows restriction fragments of the following sizes: 566bp (segment between BamHI and BglII in the LH fragment), 768bp for the segment between the two BglII enzymes including the remaining part of LH, 18 poly-glycine chain and RH segment, and a third band (fragment between BglII and BamHI including the remaining part of the vector) (**Figure 3**), while, the second enzyme pair shows restriction fragments of the following sizes: 503bp including the RH segment and the 18 poly-glycine chain, 3055bp (segment between the two XhoI sites including the LH fragment), and a third band representing the remaining part of the vector (**Figure 3**).

**Sequencing of the final clones**

The 5' and 3' sections of the cloned constructs were checked in pGEM T-easy vector by using the universal M13 forward and reverse primers. To confirm the correct number and sequence of the poly-glycine chain the designed internal primer (left Hawaii seq.) was also used to sequence across the 3' end of the inserted poly-glycine chain and also to check the correct ORF. Two samples were sent to CoGenics Company for 18-Hawaii construct, and the obtained data confirmed presence of the expected sequences (**Figure 4**).

**PCR-confirmed recombinant baculovirus plaques**

After co-transfection of Sf21 cells with linearized baculovirus DNA and the correct clones of pVL1393 containing the 18-Hawaii construct, the transfection medium was harvested and plaqued in Sf21 cells. Several baculovirus plaques were picked and tested for presence of the inserted sequence by PCR using the LH(Hawaii) forward and the RH(Hawaii) reverse primers. The expected size is 1330bp for 18-Hawaii construct. Three out of five tested plaques were proved positive (**Figure 5**).

**Purified 18-Hawaii protein**

Plaques which yielded the correctly sized PCR product were then grown on to produce high titer stocks in order to assess the induction of any recombinant protein. A very pure band of the expected molecular weight (48 kDa) could be revealed on the polyacrylamide gel (**Figure 6**) after several purification steps (above). EM examination of the final particulate pellet showed VLPs, smaller ring structures (T=1), and VLPs aggregates (**Figure 7**).

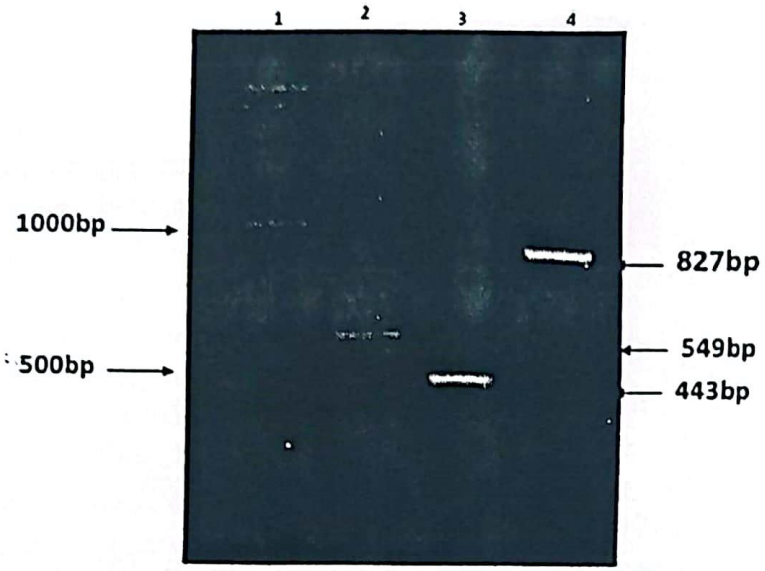


**Figure 1.** Agarose gel (1.2%) showing the PCR products. (1) 1kb DNA ladder. (2) Lambda stuffer [549bp]. (3) RH segment [443bp]. (4) LH segment [827bp].

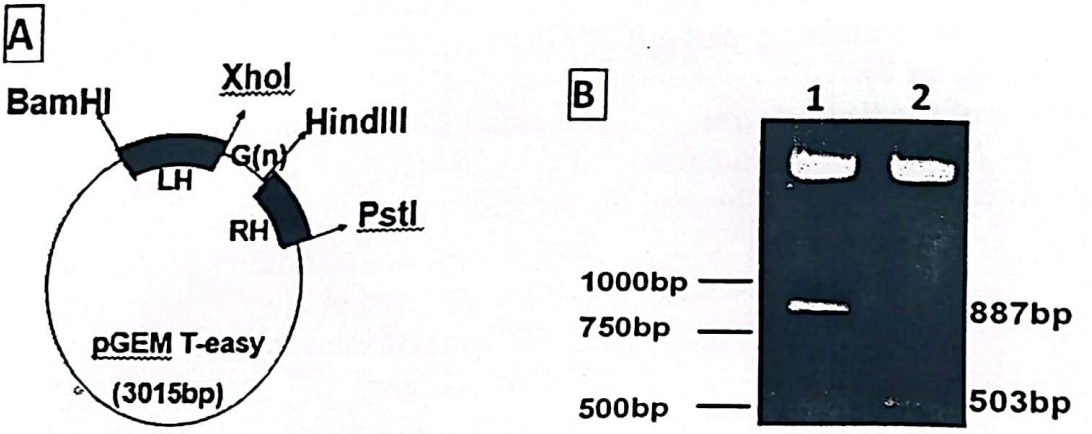


**Figure 2.** (A) Schematic diagram showing the final clone of the Hawaii capsid protein after substitution of the P2 subdomain with 18 poly-glycine chain (Gn) in pGEM T-easy plasmid vector and showing different restriction enzyme sites used for screening of the correct insertion of the constructed nucleotide sequence in that vector. (B) Agarose gel (1.2%) showing the restriction fragments confirming the correct cloning of the modified Hawaii capsid gene in pGEM T-easy. (1) Restriction fragments of (BamHI & HindIII). (2) Restriction fragments of (PstI & XhoI).

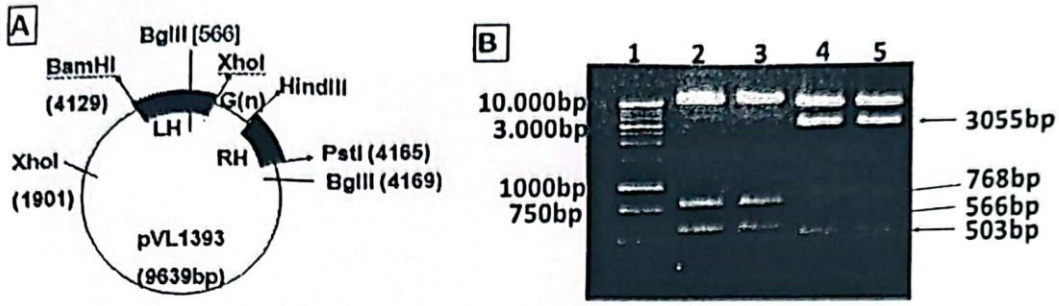


**Figure 3.** (A) Schematic diagram showing the final clone of the Hawaii capsid protein after substitution of the P2 subdomain with 18 poly-glycine chain (Gn) in baculovirus shuttle vector (pVL1393) and showing different restriction enzyme sites that were used for screening of the correct insertion of the constructed nucleotide sequence in that vector. (B) Agarose gel (1.2%) showing the restriction fragments confirming the correct cloning of the modified Hawaii capsid gene into the baculovirus shuttle vector (pVL1393). (1) 1kb DNA ladder. (2) & (3) Restriction fragments of (BamHI & BglII) of clones 1 and 2 of 18-Hawaii construct respectively. (4) & (5) Restriction fragments of (PstI & XhoI) of clones 1 and 2 respectively.

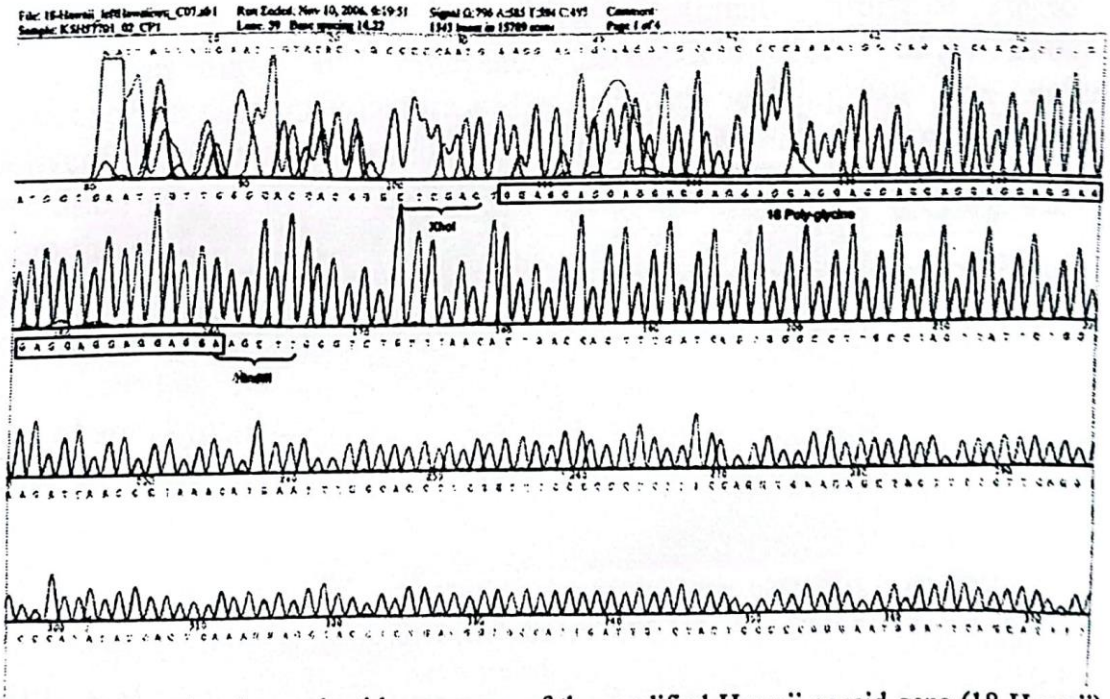

**Figure 4.** Showing the nucleotide sequence of the modified Hawaii capsid gene (18-Hawaii) using the internal primer (left Hawaii seq.) showing the 18 poly-glycine amino acids (boxed) flanked by the added restriction sites (XhoI & HindIII).

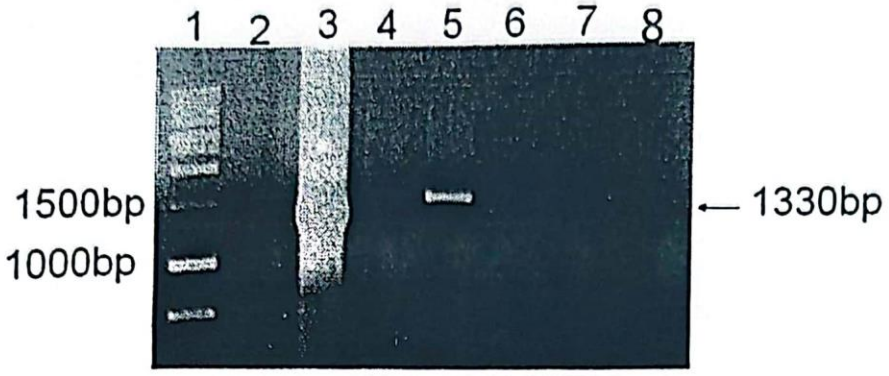


**Figure 5.** Agarose gel (1.2 %) showing PCR screening of the picked plaques of the recombinant baculovirus with the modified Hawaii capsid gene. (1) 1kb DNA ladder. (2) Negative control. (3) Positive control (pVL1393 plasmid with insert). (4), (5), (6), (7) and (8) PCR products of five different plaques with the expected size band (1330bp) for 18-Hawaii construct shown in lanes 4, 6, and 7.

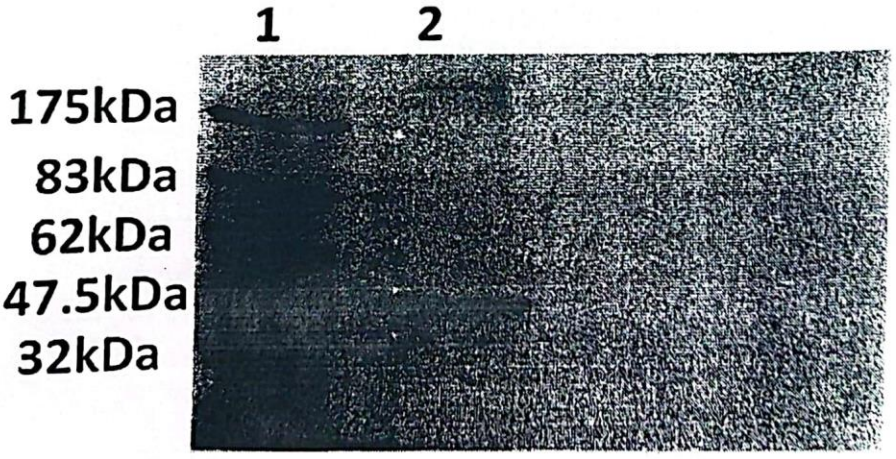


**Figure 6.** SDS polyacrylamide gel. (1) Prestained broad range protein marker. (2) Purified 8-Hawaii capsid protein with the expected size '48 kDa'.

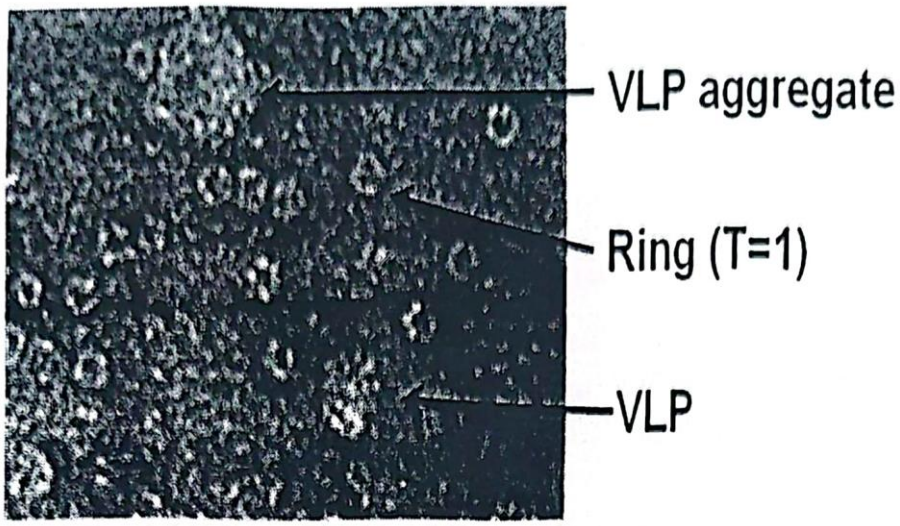


**Figure 7.** Electron micrograph of the purified Hawaii capsid protein (after modification) showing potential VLPs, small protein rings and also larger structures representing attached multiple VLPs.

## DISCUSSION

Constraints on different aspects of vaccine manufacture and delivery have been responsible for exclusion of about 20% of infants from the compulsory immunisation programme with the result of two million deaths all over the world every year **(Langridge, 2000)**. The fear of spread of infectious diseases from un-immunised persons makes it necessary to find an alternative that is easy to deliver, cost effective, safe and socially acceptable to provide protection **(Fifis *et al.*, 2004)**.

Although live attenuated vaccines are efficient immunogens, they carry a pathogenicity risk due to different virulence factors **(Streatfield *et al.*, 2001)**. Adjuvant-boosting of the safer inactivated vaccines can overcome their less potent immunogenicity, however their antigenicity is compromised on oral administration **(Tacket and Mason, 1999; Langridge, 2000)**. When these vaccines are given through the oral route they are mostly affected by the harsh gastric conditions and enzymatic activity which dramatically distorts the structure and hence the immunogenicity of the delivered antigenic determinants allowing only few intact antigens to pass

through the epithelium. Therefore, higher doses of orally administered inactive vaccines are usually required to compensate for this antigen loss **(Lamm, 1997)**.

Using VLPs as vaccines can add more advantages for protection of the antigen **(Jennings and Bachmann, 2008)**. Survival at acidic pH has been reported for some viruses including noroviruses, adenovirus type-5 **(Rexroad *et al.*, 2006)** and poliovirus type-I which infect the gut. This particle stability can be explained as an adaptation of viruses to their natural route of infection **(Krell *et al.*, 2005)**. Also, due to their particulate nature and repetitive antigen display to the immune cells VLPs enhances the immune response with better induction of cytotoxic T-lymphocytes than other separate proteins which may induce immunotolerance with suppression of the immune response **(Garside and Mowat, 1997)**.

Thus, in an attempt to both remove the source of type-specific immunity of Hawaii virus capsid protein and also to retain the ability of the capsid protein subunits to self-assemble we deleted the P2 subdomain by exclusion while using PCR-amplification of the remaining capsid domains as two separate segments which were put together during the cloning strategy with a temporary stuffer segment in between. As the P2 is a large subdomain consisting of 126 amino acids we sought to replace it with a poly-glycine chain to bridge the gap between the emergent and returning polypeptide chains leaving and entering the P1 subdomain and thus allow these chains to move freely while keeping the natural distance between individual protein subunits. This engineering work was followed by cloning and expression of the final construct using the baculovirus system.

In the study of **Bertolotti-Ciarlet *et al.* (2002)** they investigated the role of different domains of NV in the formation of intact VLPs. They showed that only the 20 N-terminal residues of the S domain can be excluded without affecting the assembly of intact T=3 particles with native virus size. However, particle assembly was compromised by further deletion in the N-terminus. Also, deletions in the C-terminal P1 region (interacting with the S domain) affected both stability and size of the particles. Deletion of the whole P domain (P1 & P2) resulted in change in the

native dimensions of the protein subunits and the produced particles appeared structurally heterogenous with smooth surface and smaller size than expected by electron microscope. They also tried internal deletions of 10-15 residues in the P2 domain (responsible for dimeric contacts of protein subunits) with resulting significantly larger size heterogenous particles in low yield. In our study, we deleted the whole P2 subdomain (126 amino acids), and as this deletion would be expected to have a deleterious effect on VLP formation we replaced the resulting gap with 18 poly-glycine amino acids chain. Glycine was chosen as it is the smallest of the amino acids and is known to be ambivalent so as it would not affect the protein folding or antigenicity. After confirmation of the identity of the purified protein by reaction with the rabbit anti-Hawaii capsid protein (unmodified) antibodies in a Western blot, a sample was examined with EM. The obtained electron micrograph (**Figure 6**) shows particulate structures like those appearing in case of natural infection including VLPs. This result shows the possibility of excluding the hypervariable region in frequently evolving viruses while preserving their rVLPs formation by bridging the resulting gap with suitable length poly-glycine amino acid chain to keep the native dimensions of protein subunits. Bridging the gap can also be tried in future studies with independently folded domain of unrelated virus provided that the distance between the insertion residues does not exceed the gap length.

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