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Production of immunogenic human papillomavirus-16 major capsid protein derived virus like particles

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***Background & objective*: Recombinant DNA technology allows expression of the human papillomavirus (HPV) major capsid protein (L1) in heterologous expression systems and the recombinant protein self assembles to virus-like particles (VLP). We took up this study to produce recombinant HPV-16 L1 in yeast, establish the process of recombinant L1 derived VLP preparation and develop an ELISA using VLP as the antigen for serological evaluation of anti HPV-16 L1 antibody status.**

***Methods*: Complete HPV-16 L1 was amplified from genomic DNA of an esophageal cancer biopsy, cloned and the protein was expressed in a galactose-inducible *Saccharomyces cerevisiae* expression system. Self assembled VLP was purified by a two-step density gradient centrifugation process and the VLP preparation used to test its suitability in developing an ELISA.**

***Results*: The recombinant protein was predominantly a ~55 KD species with distinct immunoreactivity and formed VLP as confirmed by electron microscopy. An ELISA using the VLP showed its efficacy in appropriate immunoreactivity to serum/plasma IgG.**

***Interpretation & conclusions*: Recombinant HPV-16 capsid protein derived VLP was produced and the VLP antigen based ELISA can be used to probe serological association of HPV with different clinical conditions. The VLP technology can be improved further and harnessed for future vaccine development efforts in the country.**

Key words Human papillomavirus type-16 - major capsid protein - recombinant protein expression - VLP - yeast expression system

The high-risk HPV types (HPV-16, HPV-18, HPV-31, HPV-33 and HPV-45) have been linked to several epithelial cancers in humans including cancer of the uterine cervix, head and neck and esophageal cancer[1]. Many viral envelop or capsid proteins have the ability to spontaneously self-assemble into non-infectious empty VLP with appreciable immunogenicity in different culture systems when expressed by recombinant DNA

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technology[2,3]. L1, the major capsid protein of HPV, alone or co-expressed with the minor capsid protein L2, also forms recombinant VLP that exhibit ordered repetitive display of neutralizing epitopes. Both prokaryotic and eukaryotic expressions systems have been employed to generate empty HPV VLP that are morphologically similar to native virions[4-8]. It is known that recombinant HPV VLP based ELISA can help to indicate the association between HPV infection and neoplasia in question[9-11]. In this study we report the cloning and expression of immunoreactive L1 protein in the yeast *Saccharomyces cerevisiae* and production of VLP. Purified VLP exhibited typical morphology by electron microscopy and also showed consistent sero-reactivity proving its utility in ELISA.

Material & Methods

Cloning of HPV-16 L1 gene in yeast expression vector pGal426: Full length L1 sequence was PCR amplified from genomic DNA of one HPV-16 positive biopsy using the primers described earlier[5]. Amplification was performed with 10 pmoles of each primers and 1 U of Taq DNA polymerase (Fermentas, Lithuania) for 30 cycles consisting of initial denaturation at 94°C for 1 min, annealing at 48°C for 1 min, and an elongation step at 72°C for 1.45 min. The amplified product was purified by PCR purification kit following manufacturer's instructions (Qiagen, Germany) and cloned first in the TA cloning vector pTZ57R (Fermantas) followed by subcloning in the yeast shuttle expression vector pGal426 that contains the galactose inducible promoter Gal1 and uracil synthesizing gene (*ura3*) as selection marker[12,13]. Briefly, pTZ57R-L1 was sequentially digested with SacI, polished with klenow and digested with HindIII to release the insert that was cloned at SmaI and HindIII sites of pGal426. Recombinant ampicillin resistant pGal426-L1 clones were confirmed by restriction digestion analysis. The insert was sequenced using an ABI PRISM 3100 automated sequencer (Applied Biosystems, USA) and the full length L1 sequence (GenBank accession no. DQ155283) was compared to the prototype HPV-16 complete genome sequence (GenBank accession no. K02718).

Transformation of host and expression of protein: *S. cerevisiae* strain INVSc-1 (Invitrogen, USA) was used as host system to express the L1 protein by transformation of yeast following manufacturer's instructions, which comprised of inoculation of 10 ml Yeast-peptone-dextrose (YPD) medium with a colony of INVSc-1 followed by incubation while shaking at 30°C overnight. Optical density at 600 nm (OD_{600}) of the overnight culture was determined and culture was diluted to an OD_{600} of 0.4 in 50 ml of YPD medium and grown for additional 3 h. Cells were pelleted at 1500 *g*, washed in 40 ml Tris-EDTA buffer, *p*H 7.5 (TE), resuspended in 2 ml of lithium acetate (LiAc; Sigma)/0.5×TE and incubated at room temperature for 10 min. For each transformation, 1 µg plasmid DNA and 100 µg denatured sheared salmon sperm DNA (Life Technologies, USA) were mixed together with 100 µl of yeast suspension. 700 µl of LiAc/40 per cent polyethylene glycol-3350/TE was added to above mixture and incubated at 30°C for 30 min. 88 µl of dimethyl sulphoxide was added to the mixture and heat shock was given at 42°C for 7 min. Cells were microcentrifuged for 10 sec, supernatant removed and the pellet further resuspended in 1 ml TE and re-pelleted. Finally the cell pellet was resuspended in 50 µl TE, spread on selective complete medium lacking uracil (SC−Ura) plates and incubated at 30°C for 3-4 days.

Transformants were inoculated in 5 ml of SC−Ura, grown at 30°C overnight, OD_{600} was determined and volume of overnight culture necessary to obtain an OD_{600} of 0.4 in 5 ml of induction medium (SC−Ura+2% galactose) was calculated. The required volume of culture was removed and kept for induction in a 5 ml reaction for 12 h at 30°C. Yeast cells were harvested by centrifugation at 3000 *g* for 5 min at 4°C, washed in sterile distilled water and stored at −80°C. All subsequent steps were carried out at 4^0C. The cell pellet was resuspended in ice-cold break buffer (20 mM Sodium phosphate, *p*H 7.2, 100 mM NaCl, 1.7 mM EDTA) containing 2 mM PMSF and equal volume acid washed glass beads (425-600 micron; Sigma). The yeast-glass bead mixture was lysed in a bead beater (Biospec Products, USA) and the lysate clarified by centrifugation at 23,500 *g* for 10 min at 4°C. Protein was estimated using Bradford reagent and bovine serum albumin as a standard (BSA; Sigma).

Western blotting: Recombinant HPV-16 L1 (rL1) expression profile from the crude cell lysate of the yeast clones was obtained by immunoblot. For immunoblotting, 20 µg protein in each lane was resolved on a 10 per cent SDS-PAGE gel and transferred onto nitrocellulose membrane (Sigma) using a trans-blot apparatus (Bio-Rad, USA). The blot was blocked with 3 per cent BSA in phosphate buffered saline (PBS), *p*H 7.4 at 37°C for 2 h, washed once in PBS, once in blotting buffer (20 mM Tris, *p*H

7.5, 100 mM NaCl, 0.3% Tween-20, 0.5% non fat dry milk powder) and subsequently immunoblotted with anti-HPV-16 L1 monoclonal antibody Camvir-1 (BD Pharmingen, USA) at the suggested concentration of 2 μg/ml in blotting buffer for 4 h. The blot was washed thrice in blotting buffer and reacted with horseradish peroxidase (HRPO) conjugated rabbit anti mouse IgG (Sigma; 1:1000 in blocking buffer) for 1 h. After three washes with blotting buffer and further three washes with PBS, the blot was reacted with substrate solution consisting of 0.05 per cent 3, 3'-Diaminobenzidine tetrahydrochloride (DAB; Sigma), 50 μl of 8 per cent $NiCl_2$ (Sigma) and 5 μl of 30 per cent H_2O_2 till (dark blue) color development.

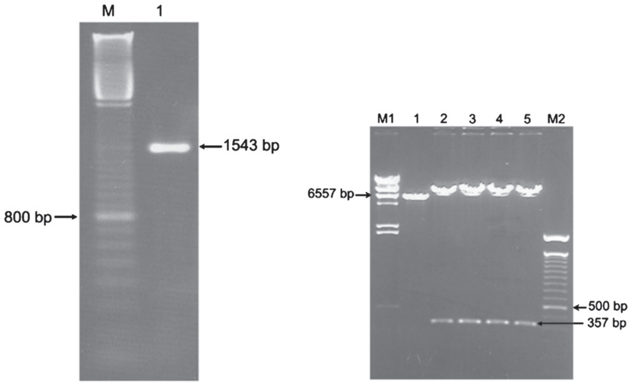
Purification and characterization of VLP from scaled up culture: The transformed yeast cells were streaked on the SC−ura plates, incubated at 30°C for 48 h, cells from a colony inoculated in 50 ml SC−Ura medium and further incubated for 24 h while shaking at 30°C. 40 ml of this saturated culture was inoculated in 1.6 L of fresh medium and incubated at 30°C for 24 h followed by induction with 2 per cent galactose (Sigma) for 72 h. Harvested yeast cells were lysed as described above and the clarified cell lysate was layered onto a cushion of 45 per cent (wt/vol) sucrose in break buffer containing 0.01 per cent Tween-80. The sucrose suspension was centrifuged at 100,000 *g* in a Kontron TST 28.38 swinging rotor at 4°C for 4 h using the Centricon T-1080 ultracentrifuge (Kontron AG, Switzerland). The resulting whitish pellet was resuspended in 1 ml of 27 per cent (wt/wt) cesium chloride (CsCl)-PBS and centrifuged to equilibrium at 141,000 *g* in a Kontron TST 60.4 swinging rotor at 10°C for 20 h. The visible band was collected carefully, and density of the fraction was determined by a refractometer (PZO Warszawa RL1, Poland). To remove residual CsCl and to concentrate the VLP preparation, the fraction was diluted 1:5 with PBS and VLP were pelleted at 188,000 *g* in a Kontron TST 41.14 swinging rotor at 4°C for 2 h. The pellet was resuspended in 100 μl of PBS and used for detection of rL1 protein by immunoblotting where the reaction signals were detected using the chemiluminescent ECL system following manufacturer's instructions (Amersham, UK). Briefly, increasing amount of protein in the range of 5-20 μg was resolved, probed and washed as for immunoblotting and reacted with the same secondary antibody (1:4000 in blocking buffer) and signals detected over 30 sec-1 min exposure.

Transmission electron microscopy: Transmission electron microscopic examination was carried out on negatively stained VLP specimens under 100 KV operating voltage (Tecnai 12 Biotwin, FEI Netherlands).

Sero-reactivity of VLP: The self-assembled HPV-16 L1 VLP fraction made in yeast was used to ascertain its utility in developing an in-house ELISA with reference to specific plasma/serum IgG reactivity. ELISA immuno-stripes (Nunc, Denmark) were coated with 50 ng of VLP in 100 μl PBS/ well by incubation at 4°C overnight. To remove unbound reagents, wells were washed thrice with PBS buffer and nonspecific binding sites were blocked with PBS containing 0.5 per cent non-fat milk powder for two and half hours at 37°C. To remove the reactivity with yeast proteins that co-purified with VLP fraction, 1:50 diluted sera samples were pre-absorbed with 50 ng of control yeast lysate (yeast transformed with empty vector) for 1 h at room temperature. The plates were incubated with 100 μl of pre-absorbed plasma for two and half hours at room temperature followed by five washes with PBS. 100 μl HRPO-anti human IgG (Sigma) diluted 1:10000 in dilution buffer was added per well, incubated for 1 h at room temperature and washed identically. Finally, 100 μl ortho-phenylene diamine (OPD; Sigma) in substrate buffer (10 mg in 20 ml PBS, *p*H 6.0) supplemented freshly with 5 μl 30 per cent H_2O_2 was added in the wells and incubated in the dark for 30 min. The reaction was stopped by the addition of 100 μl 1 N HCl per well and the OD at 492 nm against the reference of 650 nm was measured using ELISA plate reader (Molecular Devices, USA).

Results

A 1543 base pairs (bp) PCR amplified product containing the complete HPV-16 L1 was obtained using the genomic DNA of a HPV-16 positive esophageal cancer biopsy (Fig. 1a). First the PCR amplified product was cloned in the TA cloning vector pTZ57R (data not shown) and subsequently subcloned in the yeast shuttle vector pGAL426. To confirm the clones, the recombinant plasmids were digested with EcoRI/ HindIII. The release of a 357 bp fragment from the plasmid indicated the presence of HPV-16 L1 insert in proper orientation in pGAL426 (Fig. 1b). The cloned insert was further confirmed by nucleotide sequencing and was found to be almost identical to the published prototype HPV-16 L1 sequence except 3 mutations at positions 6240 (C to G), 6432 (A to G) and at 6860 (T to C) (the nucleotide number is on the basis of prototype sequence). The observed nucleotide changes



(a) (b)

Fig. 1. (a) PCR amplification of HPV-16 L1. M: 100 bp marker; 1: amplified HPV-16 L1; **(b)** Confirmation of pGal426-L1 clones by EcoRI/HindIII digestion. M1: λHindIII marker; 1: Linearized pGal426; 2-5: EcoRI/HindIII digests of four clones; M2: 100 bp marker.

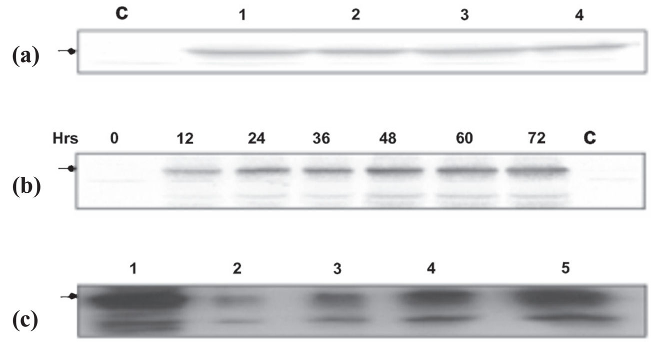


Fig. 2. Western blots using the monoclonal antibody Camvir-1. **(a)** Crude lysates of yeast clones harbouring pGal426-L1. C: 25 µg crude lysate of host strain transformed with only pGal426 vector; 1-4: 25 µg crude lysate from four yeast clones; **(b)** Time point profiling for induction of recombinant HPV-16 L1 protein. C: 25 µg crude lysate of host strain transformed with only pGal426 vector; 0-72: 25 µg crude lysate of a yeast clone from indicated time points; **(c)** Density gradient purified VLP fraction. 1: 20 µg crude lysate; 2-5: 5, 10, 15, 20 µg of purified fraction respectively.

results in a change in histidine to aspartate at amino acid 202, threonine to alanine at amino acid 266 and no change at amino acid 408 respectively (Table). L1 protein expressed in *S. cerevisiae* in small scale culture was first analyzed for yield and specificity by immunoblot analysis of crude cell extracts prepared from four recombinant yeast clones using the HPV-16 L1 monoclonal antibody (Fig. 2a). In all cases, the antibody reacted strongly to an ~55 KD species, as well as to some lower molecular weight proteins, which may result from the degradation of the full-length L1 or premature termination of translation. The antibody did not react with cell lysate from yeast transformed with empty vector. All four yeast clones appeared to express comparable amounts of recombinant L1 protein and hence one of the clones was selected for further expression. The expression of HPV-16 L1 in the selected yeast clone was analyzed by immunoblotting after incubation for 0, 12, 24, 36, 48, 60, 72 h in a shake-flask culture to optimize induction time for optimum expression of recombinant protein. Expression of HPV-16 L1 protein showed a gradual increase from 12 h to 48 h of induction and expression of protein level maintained till 72 h (Fig. 2b). The enriched, gradient purified fraction of HPV-16 VLP was shown to have a density of 1.29 g/ml and immunoblotting of purified VLP fraction also showed the presence of HPV-16 L1 at ~55 KD (Fig. 2c). The protein content in the purified fractions in different batches was found to be in the range of around 100-150 µg/L of culture, measured by Bradford's method (data not shown). Electron microscopy analysis of the fraction revealed the presence of HPV-16 L1 VLP with the average diameter of the spherical particle around 35-50 nm with distinct uniform VLP architecture (Fig. 3a and b).

Standardization of HPV-16 VLP based ELISA: The absorbance for all the samples was assayed twice, in duplicates, on different days. In initial experiments, eight different plasma were used (three from healthy individuals, three from cervical cancer cases and two from ESCC patients) to titrate the concentration of VLP as antigen for ELISA. The lowest concentration of VLP preparation that gave maximum absorbance using these plasma (diluted 1:50 in dilution buffer containing PBS with 0.5% non-fat milk powder) was 50 ng/well (data not shown). Once the conditions of ELISA were

Table. Amino acid sequence comparison between cloned HPV-16 L1 with prototype sequence. (Boldface letters indicate differences from the prototype sequence).

HPV-16 Strain	Amino acid at position														
	76	78	83	97	176	181	194	202	266	282	319	346	353	422	474
Prototype K02718	His	Pro	Phe	Arg	Thr	Asn	Val	His	Thr	Ser	His	Ala	Thr	Thr	Leu
Cloned HPV-16 L1 DQ155283	His	Pro	Phe	Arg	Thr	Asn	Val	**Asp**	**Ala**	Ser	His	Ala	Thr	Thr	Leu

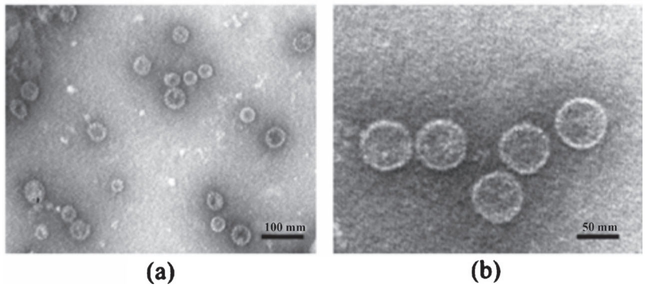


Fig. 3. Transmission electron microscopy of HPV-16 VLP. **(a)** Field showing VLP ranging in 35-50 nm sizes; **(b)** A cluster of uniform sized VLP.

standardized, ELISA was validated by running three different reactive plasmas (from healthy individuals with no information on their HPV status whatsoever) on different days to check the change in their absolute absorbance. As shown in Fig. 4, comparable pattern of absorbance profiles were found for all three plasma samples on 10 different days.

Discussion

Even though HPV-16 capsid is composed of major (L1) and minor (L2) protein, recombinant major capsid protein L1 alone is sufficient for assembly of VLP, which resemble morphologically to native virions and elicits anti-HPV antibody response. The antibodies primarily recognize the conformational epitopes on L1 of intact virions[4-6]. The complete ORF of HPV-16 L1 was amplified from esophageal cancer biopsy derived genomic DNA and verified by sequencing. A mutation of histidine to aspartate at position 202 is in agreement with earlier observations suggestive of a prerequisite mutation for L1 protein self assembly. In addition, a threonine to alanine mutation at amino acid 266 was also reported, which did not show any adverse effect on assembly of VLP[6]. These observations indicated that the cloned HPV-16 L1 was appropriate for expression of the candidate protein. Immunobloting analysis showed expression of ~55 KD recombinant L1 proteins in yeast, which is in proper agreement with the theoretical calculation on the basis of nucleotide number and even with other published reports where molecular weight of HPV L1 was documented around 55-58 KD[4-7]. As gradual increase in protein expression was observed till 48 h of galactose induction and high level of recombinant protein was maintained over 72 h in our system, we continued induction till 72 h point. The gradient purified fraction showed ~35-50 nm size VLP and the morphology was very distinct implying empty shell conformation. Diameter of the papillomavirus virion has been reported as 55 nm, but most reports of heterologous expression of HPV VLP documented around 40-50 nm size of the VLP[4-7]. One study also reported assembly of HPV-11 VLP, from S. cerevisiae system, ranging in a very wide size range with diameter from 32 to 97 nm[14].

The overall yield of VLP fraction containing protein using the given method was apparently low in our hand. However, we are currently trying recently reported simplified and cheaper methods with several times higher yield of pure VLP fractions[15-16]. The potential to use the HPV-16 L1 derived VLP for developing a useful ELISA procedure was validated by checking the change in absolute absorbance of three different reactive control sera on different days, which showed comparable profile of absorbance for all three sera samples on different days. This observation implied that the VLP preparation can be used as an antigen for appropriate serology study. We are currently using the ELISA for evaluating HPV-16 L1 antibody profile in the sera/plasma from ESCC patients reporting to this hospital. Excellent efficacy of HPV VLP as a prophylactic vaccine in recent times has opened a major scope for further improvements in yield and more appropriate delivery[17-20].

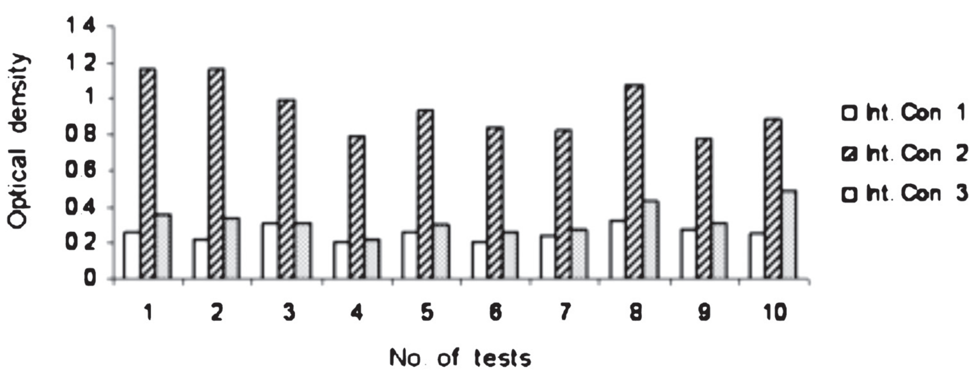


Fig. 4. Validation of HPV-16 VLP based ELISA by checking the reactivity of three internal control plasmas on 10 different days.

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