Cancer of uterine cervix is a significant public health burden across the world. Mortality of women from cervical cancer is reported to be highest in developing countries. Prophylactic vaccine made of the Virus like particles (VLPs) of Human papilloma virus (HPV), though expensive, is effective in preventing onset of homologous infection in vaccinated women. Preparation of cross protective vaccine containing lesser number of recombinant proteins derived from various genotypes of HPV would help reduce the vaccine cost. We report the expression of codon optimized HPV 16 L2 in Pichia pastoris. The expression of HPV L2 protein was verified using RT-PCR and immunoblotting. This manuscript describes initial effort in expressing full length minor capsid protein L2 of HPV16 aimed at developing a broad spectrum prophylactic vaccine for cervical cancer.

Key Words: HPV, Cervical Cancer, Minor capsid protein (L2), Cross Neutralizing Epitopes, Prophylaxis, Vaccine

Introduction

Cancer of the uterine cervix is the second most common cancer in women. Annual incidence of approximately 520,000 new cases and 274,000 deaths have been reported worldwide (1). India alone accounts for approximately 132,000 new cases and 73,000 deaths each year (1) and holds the dubious distinction of accounting for nearly a quarter of all cervical cancer death recorded globally (2). Presently nearly half of all cervical cancer incidences occur in the developing countries; and it is estimated that by the year 2025 the incidence rates in the developing world would increase to nearly 80% of the global rates (1).

Human Papillomaviruses (HPV) are highly epitheliotropic; they establish productive infections only within the stratified squamous epithelia of the skin or the anogenital tract of the humans (3). HPV genome consists of a circular double-stranded DNA of approximately 8kb and encodes for non-structural-proteins (the early genes) and structural proteins (the late genes) (4). Over 200 papillomavirus types have been described, of which around 100 infect humans and are therefore classified as HPV (4, 5 and 6). Nearly 35 different HPV types are found to be associated with cervical cancer in women and these are referred to as ‘high-risk’ HPV types. Persistent infection by high-risk HPV types is the single most common cause for the establishment of the cervical cancer (7).

The HPV major capsid protein L1 and minor capsid protein L2 are synthesized late in infection cycle, whose function is to encapsidate the closed circular double-stranded DNA (8).
Recombinant major capsid protein (L1) self assembles into a structure that mimics the native virus, both structurally and immunologically. Two recombinant vaccines have been commercialized that contain the HPV L1 based VLPs of HPV16 and 18, which cause 70% of all cervical cancer. One of the prophylactic vaccines also contains two additional HPV types that cause benign proliferation (warts) in the genital area of men and women. Clinical studies indicate that these vaccines protect vaccinees from the infection of cognate type HPVs. Given that the available vaccine has a limited spectrum, multivalent VLP vaccines would be required to improve prophylactic efficacy (9). Several other investigators have explored the possibility of using alternate expression platform such as plant (10, 11 and 12), Salmonella (13) vaccinia virus (14) etc. All of these are aimed at reducing the cost of vaccine production or ease of administration, thereby increasing the vaccine coverage in the population.

The HPV L2 is also referred to as minor capsid protein. It plays an important role in the infectivity of HPV (15, 16). The HPV L2 protein contributes to the binding of the virions to the host cell receptor(s), disrupt endosomal membranes and facilitate sub-cellular trafficking of incoming viral genome. HPV L2 also plays a crucial role in the invasion and injection of the viral genome into the host cell. Studies performed in animal PVs demonstrated generation of cross neutralizing antibodies after immunization with the amino terminal peptide of L2 protein (17). These antibodies protected the animals against challenge with cognate papilloma virus types (18, 19). Other investigators have demonstrated that L2 neutralizing epitopes are common among Papillomaviruses, which leads to the possibility of its use in cross protective HPV vaccines (20).

In this manuscript, we describe cloning, integration and expression of codon optimized HPV 16 L2 gene in Pichia pastoris strain GS115. The expression of HPV 16L2 was confirmed using RT-PCR and immunoblotting.

Materials and Methods
2.1 Pichia strain and expression vector : Methylotrophic yeast Pichia pastoris strain GS115 and Pichia expression vector pPICZB were procured from Invitrogen, USA.

2.2 Yeast growth and expression media: Components for preparing Pichia pastoris growth and induction media were procured from Hi-Media Labs, India. All other chemicals and fine-chemicals were either sourced from Sigma Chemical Company, USA or Merck, India.

2.3 Codon optimization of HPV 16 L2 gene for expression in Pichia pastoris: DNA sequence coding for the minor capsid protein encoding gene (L2) of HPV 16 was codon optimized for expression in Pichia pastoris. Synthetic gene construct for this purpose was procured from GeneArt (Regensburg, Germany).

2.4 Cloning of the minor capsid protein gene of HPV 16 : The codon optimized L2 gene (1.5 kb) was PCR amplified from the synthetic construct using Pfu DNA polymerase (Qiagen, Germany). The PCR was set-up with the following oligonucleotide sequence and reaction conditions.

16L2 For: 5’ TACCGAATTCTAGAGACACAAGAGATCCGCT3’; and
16L2 Rev: 5’GCTGGAGCTCCAGCCTATTAAACGCCAGAGGA3’.

The PCR reaction mixture contained 100 ng of codon optimized HPV 16 L2 plasmid, 1× PCR reaction buffer (Qiagen, Germany), 200µM of each dNTPs, 50 pico moles each of forward and reverse gene specific primers, three units of Pfu DNA polymerase enzyme. The reaction volume was made to 50 μl using nuclease free water.
PCR was performed using the following temperature cycling conditions: initial denaturation of template was carried out at 94°C for 5 minutes; 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 40 seconds and extension at 72°C for 90 seconds were used for PCR amplification; the final extension was performed at 72°C for 10 minutes. A control reaction using DNA extracted from untransformed *Pichia pastoris* cells were used as negative control. The PCR products were resolved on 1% (w/v) Agarose gel and stained using ethedium bromide.

The amplified HPV 16L2 PCR product and *Pichia* expression vector (pPICZB) were digested using restriction enzymes *EcoRI* and *XhoI* (New England Bio-labs, USA). These restriction enzyme digested 16 L2 gene and the vector were resolved using agarose gel electrophoresis and purified using gel extraction kit (Qiagen, Germany). The insert and vector were ligated using T4 DNA ligase (Genei, India) by incubating at 16°C for 60 minutes. The ligated product was transformed into Top10 *E. coli* competent cells and were selected for Zeocin (100µg/ml; Invitrogen, USA) resistance. The HPV 16L2 gene cloned into pPICZB was verified using restriction analysis and DNA sequencing.

### 2.5 Integration of the HPV 16L2 expression cassette into *Pichia pastoris* Chromosomal DNA:

The pPICZB plasmid clone containing HPV L2 gene insert was used to transform *Pichia pastoris* GS115 (Invitrogen, USA) using *Pichia* EasyComp™ Kit (Invitrogen, USA). Transformants harbouring HPV L2 gene was selected on Yeast-extract Peptone Dextrose (YPD) plates containing 200µg/ml Zeocin (Invitrogen, USA). Integration of HPV L2 gene into *Pichia pastoris* was PCR verified using the promoter specific primers (Alcohol Oxidase primers; AOX) and the 16L2 gene specific primers. AOX primer sequences are mentioned below and 16L2 gene specific sequences were mentioned earlier in Section 2.4.

**AOX For:** 5’GACTGGTCCAA TTGA CAAGAC 3’;

**AOX Rev:** 5’GCAAATGGCATTC TGAC ATCC3’;

PCR reaction was set-up and using 50 ng of codon optimized HPV 16 L2 plasmid, 1× PCR reaction buffer (Bangalore Genie, India), 200μM of each dNTPs, 50 picomoles each of AOX forward and reverse primers, three units of Taq DNA polymerase enzyme. The reaction volume was made to 50 µl using nuclease free water.

Thermal cycling condition used for PCR amplification were same as mentioned earlier in the methods (section 2.4)

### 2.6 Expression of HPV Minor Capsid protein (L2) in *Pichia pastoris*:

*Pichia pastoris* transformants containing HPV 16L2 gene was grown overnight in a shake-flask with YPD medium supplemented with 100µg/ml Zeocin and the cells were transferred into freshly prepared Buffered Minimal Glycerol (BMGH) medium containing 100 mM potassium phosphate (pH 6.0), 1.34% (w/v) yeast nitrogen base, 0.02% (w/v) biotin, 1% (v/v) glycerol, 100µg/ml Zeocin and 0.004% (w/v) L-Histidine. After an overnight growth recombinant *Pichia pastoris* cells were induced for expression using Buffered Minimal Methanol (BMMH) medium containing 100 mM potassium phosphate (pH 6.0), 1.34% (w/v) yeast nitrogen base, 0.02% (w/v) biotin, 0.5% (v/v) methanol and 100µg/ml Zeocin supplemented with 0.004% (w/v) L-Histidine. To induce expression of the recombinant HPV L2 from *Pichia pastoris*, 0.5% (v/v) of methanol was added every 24 hours to the culture until 72 hours of growth. The cells were harvested after 96 hours of growth and stored at -80°C until further use.

Expression of recombinant minor capsid protein of HPV in *Pichia pastoris*
2.7 Reverse Transcriptase PCR: Methanol induced recombinant *Pichia pastoris* clones expressing HPV16 L2 protein was sampled every 24 hours until 72 hours. Total RNA was extracted from the samples using RNAEasy™ kit (Qiagen, Germany). RT-PCR used to detect the presence of HPV L2 transcripts in the induced *Pichia pastoris* cells using One-Step RT-PCR kit (Qiagen, Germany) and HPV16 L2 gene specific primers. RNA samples from ‘untransformed’ *Pichia pastoris* strain GS115 was used as negative control. The RT-PCR was performed using the following temperature cycle: cDNA synthesis was performed at 50ºC for 50 minutes; Reverse Transcriptase inactivation and *Taq* polymerase activation was performed by heating the reaction at 95ºC for 10 minutes; 40 cycles of denaturation at 94ºC for 30 seconds, annealing at 55ºC for 40 seconds and extension at 72ºC for 40 seconds were used for PCR amplification; the final extension was performed at 72ºC for 10 minutes. A control PCR reaction using identical RNA samples isolated from induced *Pichia pastoris* cells was also set-up to rule out the possibility of genomic DNA contamination of the RNA preparation.

2.8 Immuno Blotting: HPV 16 L2 proteins from *Pichia pastoris* crude cell lysate was resolved on 12% SDS-PAGE gel were transferred to PVDF membrane (GE Healthcare, USA) using Bio-Rad semi-dry protein transfer apparatus. Unused active surface on the PVDF membrane was blocked using PBS containing 2% (w/v) skim milk, 0.1% (v/v) Tween-20. The blot was probed using 1:200 dilution of HPV16 L2 mouse monoclonal antibody produced against amino acids 40-150 of HPV16 L2 (2JGmab#5; Santa Cruz, USA). Goat anti-mouse HRP monoclonal antibody (1:5000 dilution; Sigma, USA) was used as detection antibody and the bands were stained using 3,3′-Diaminobenzidine tetrahydrochloride (DAB) as chromogenic substrate.

**Results and Discussion**

In the present study we used *Pichia pastoris* as a heterologous expression system for expressing recombinant HPV16 L2 protein. Yeasts with the heterologous gene integrated into the chromosome provide an alternate platform for recombinant gene expression. Integration of genes for expression under the Alcohol oxidase (AOX) promoter is known to induce high level of expression in *Pichia pastoris* (21). The necessity of codon optimization for HPV capsid proteins expression in heterologous systems is also well documented (21). HPV 16 L2 gene (Genbank accession number: U34164) was codon optimized and synthesized in-vitro.

Codon optimization was performed to improve the transcription efficiency and transcript stability. This was achieved by improving the overall GC content of the gene, distribution of preferred codon usage along the entire length of the coding sequence (codon adaptation index and codon frequency distribution) and removing negative elements that may for unfavourable secondary structures on mRNA.

The codon adaptation index is graded on the scale of 0.0 to 1.0, with 1.0 indicating the ideal codon usage in a given host. Codon adaptation index on the native HPV 16 L2 was 0.67, while the optimized HPV L2 gene sequence had a codon adaptation index of 0.90 (Fig. 1), indicating that the optimized gene sequence could express well in *Pichia pastoris*. Percentage of codon having a frequency distribution of 91-100 in the native L2 gene was 39% which was significantly improved to 72% in the optimized gene sequence (Fig. 1). The overall GC content which is a measure of transcriptional and translational efficiency was improved from 37.63% to 45.7% upon codon optimization. There were 11 negative CIS elements in the native L2 gene sequence which were reduced to
5 after codon optimization (Fig. 1). Taken together the codon optimized gene sequence of HPV L2 was favourable for expression in *Pichia pastoris*.

### 3.1 Cloning and Integration of HPV16L2 gene into Pichia pastoris:

Human papilloma virus 16 L2 gene codon optimized for expression in *Pichia pastoris* was cloned into expression vector pPICZB (Fig. 2). Cloning of the minor capsid protein 16 L2 gene into pPICZB was verified by restriction enzyme analysis using *EcoRI* and *XhoI*. A band of approximately 1.5 kb size was observed on agarose gel indicating the presence of L2 gene (Fig. 3A). The clone was confirmed by restriction analysis using *XbaI* which is located in 16L2 gene sequence as well as pPICZB vector backbone. The restriction digested plasmid revealed a band of approximately 800bp on agarose gel confirming the presence of HPV 16 L2 gene (Fig. 3B). Further, DNA sequencing of the PCR amplicons using Alcohol Oxidase (AOX) primers confirmed the identity of the integrated gene upon BLAST search (data not shown).

HPV 16 L2 gene was integrated into *Pichia pastoris* genome by transforming the chemically competent *Pichia pastoris* cells using the recombinant plasmids. The integration was PCR verified using primers that either bind at AOX promoter site or HPV L2 gene. The PCR products showed the bands size of approximately 1.8 kb and 1.5kb with AOX primers and HPV 16 L2 gene specific primers respectively (Fig. 4A & 4B).

### 3.2 Reverse Transcriptase PCR:

Recombinant *Pichia pastoris* clones expressing HPV16 L2 were induced for protein expression using 0.5% (v/v) methanol. An aliquot of cells were withdrawn after 24, 48 and 72 hours of induction. Total RNA were extracted from these induced *Pichia pastoris*, produced an amplification

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Expression of recombinant minor capsid protein of HPV in *Pichia pastoris*
product of approximately 1.5kb as seen on agarose gel. RT-PCR analysis was performed for recombinant HPV16 L2 *Pichia* clone and the assay confirmed that the gene is transcribed on induction with methanol (Fig. 5). The HPV L2 gene amplification from possible DNA contamination in extracted RNA was ruled out in a PCR reaction that employed identical RNA samples isolated from induced *Pichia pastoris* cells.

**3.3 Immuno Blotting**

The cell lysates of recombinant *Pichia pastoris* were analyzed in Western blot using HPV L2 peptide specific mouse monoclonal antibody, which showed the staining of band size of approximately 55KDa. This experiment proved beyond a reasonable doubt, the expression of HPV16 L2 in *Pichia pastoris*. The mouse monoclonal antibody did not react with naïve *Pichia* cell lysate used as negative control (Fig. 6). In the Western blot apart from the 55KDa band, which is the expected size of L2 a higher molecular weight protein was also observed. There are reports indicating the frequent association of Hsp 70 family proteins with HPV L2. The Hsp70 proteins are thought to act like chaperon that help protein fold or unfold (23). Some investigators observed that apart from HSPs the sumoylation proteins are also tightly associated with HPV L2 (24). The higher size protein band corresponding to HPV L2 observed in this study might be a consequence of the association of any of these chaperones. Further
investigations are required to ascertain these observations in *Pichia pastoris*.

**Conclusion**

The available HPV L1 Virus like particle based vaccines provide type specific protection against the HPV genotypes associated with the cervical cancer. Although there are reports of the VLP based vaccines to provide cross protection to the HPV types that are phylogenetically similar to the vaccine types, these are limited both in spectrum and levels of protection. Development of a vaccine offering cross protection to a wide variety of HPV types that cause cervical cancer remains a challenging task. Some investigators have reported that cross-reactive antigen derived from the minor capsid protein L2 may offer a possible alternative to multivalent L1 VLP vaccines for protection against infection with high-risk HPV types (25). A multimeric L2 peptides derived from different HPV types is under clinical evaluation (26). This work is a preliminary step towards exploring the utility of full length HPV L2 as prophylactic vaccine for cervical cancer.

**References**


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