CHAPTER 5

DISCUSSION

Persistent infection from Human papilloma virus leads to cancer in the uterine cervix. The socio-economic impact of any disease afflicting women is enormous. Women contribute significantly to the economic progress of this country. Therefore, cervical cancer not only has a direct bearing on the family but also on the economic growth of the country. The disease is set-off by infection of certain papilloma virus types. HPV infection is found in almost all sexually active women. Slow progression of the diseases, absence of typical symptoms and lack of awareness makes cervical cancer go undetected until the infection may irreversibly set in as cancer.

Viruses have been known to cause several types of cancer in humans and animals. Hepato-cellular carcinoma is caused by Hepatitis viruses; T-cell Lymphoblastoma is caused by HTLV-I infection; B-cell lymphoma is caused by Epstein Barr Viruses etc. The rates of these ‘viral cancers’ incidences are lower than their infection rates in the population. Indicating that, cancer may not be the primary result of the infection. Also, since the manifestation of cancer takes several years from the incident infection; it is possible that the abnormalities are gradually built up under certain physiological conditions.
Cancer of the uterine cervix is the second most common cancer affecting women in the world. More than 80% of the global cancer incidents are reported from the developing countries (WHO/ICO Information Centre on HPV and Cervical Cancer; HPV Information Centre, 2010). It is also one of the most common cancer among women in India and nearly a quarter of the world’s cervical cancer incidence is reported from our country. Based on the severity of the disease, HPV is classified as ‘low risk’ and ‘high risk’ types; where infection from ‘low risk’ types mainly causes warts and ‘high risk’ HPV types causes cervical cancer. Amongst the ‘high-risk’ HPV types, HPV 16 and 18 are the genotypes most frequently associated with cervical cancer across the world. Overall HPV type prevalence in cervical cancer in India is found to be in the following order of occurrence HPV 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59 and HPV68 (Sankaranarayanan et al., 2008).

Cervical cancer takes several years to develop from incident HPV infection. The HPV infection leads to morphological changes in cervical exfoliates. Therefore periodic screening of uterine cervix and removal of premalignant lesions can result in a substantial reduction of cervical cancer related mortality. The introduction of large scale cytological screening programs in the developed nations has resulted in the major decline of the cervical cancer mortality (Peto et al., 2004). However, frequent monitoring of cervical exfoliates for abnormalities require a large
number of trained professionals and persistent public funding to support the requisite infrastructure (Sankaranarayanan et al., 2007). In the developing countries, women have limited access to healthcare programme. Also, the screening programs to detect abnormality in cervical exfoliate are absent in rural areas. There are many obstacles in setting up effective screening programs in low resource settings. Though, for developing countries, a promising way of controlling and reducing the cervical cancer related mortality would be to use a prophylactic vaccine. Costs of the available HPV vaccines renders them beyond the reach of most people in the low resource countries, where the disease burden is highest (Sankaranarayanan, 2006).

Most prophylactic viral vaccines existing in the market are made up of live attenuated virus or inactivated viruses. Such preparations are not possible for preparing HPV prophylactic vaccine. Because papillomavirus infections are host restricted. There is no animal model for productive HPV infection or the generation of hyper-proliferative disease after introducing infectious HPVs in any in-vitro culture systems. Though, HPV can be cultured in organotypic raft cultures in-vitro (Sterling et al., 1993). The limitations of the raft cultures for HPV vaccine production are scalability and safety concerns.

Papilloma virus major capsid protein (L1) on its own, or in combination with L2, spontaneously assembles into virus-like particles
(VLPs) when expressed in heterologous expression systems. The papillomavirus VLPs induce high titer neutralizing antibodies (Kirnbauer et al., 1992) whose presence is critical in preventing HPV infection. Recently, two VLP based HPV prophylactic vaccines have been introduced into the market; these are quadrivalent ‘Gardasil’ (containing HPV 16/18/6/11) from Merck and bivalent ‘Cervarix’ (containing HPV 16/18) from Glaxo Smith Kline (GSK). Both these vaccines were highly immunogenic, safe and effective in preventing HPV infection of cognate type. To produce affordable HPV vaccine, alternate expression platforms are being explored. In order to meet the global requirements of HPV vaccine it is essential to have HPV production in the developing countries (Sankaranarayanan, 2009). Manufacturing of recombinant Hepatitis B vaccine in India demonstrated the potential of production of high quality vaccine at an affordable cost. The price of Hepatitis B vaccine in early 1980s was $50-80 per dose, which crashed to just $0.3 per dose. It highlighted the importance of regional manufacturing establishments in producing the Hepatitis B vaccine (GAVI Alliance Progress Report, 2007). It is therefore prudent to assume that such an effort may lower the production of HPV prophylactic vaccine price. In addition, a detailed analysis for intellectual property landscape on HPV prophylactic vaccine suggests that intellectual property rights may not be an impediment in manufacturing of the first generation HPV L1-VLP based vaccine if
manufacturers choose different formulations or strains (Padmanabhan et al., 2010). Thus, exploring alternate platform to produce HPV prophylactic vaccine is of significant public health interest.

Several investigators have reported the expression of HPV VLPs in various expression systems. Bimelt et al., (2003) and Varsani et al., (2003) demonstrated the expression of HPV 16 VLPs in plant expression system. Plant expressed proteins have less contaminants of human pathogenic microbes than any other system; also it is an affordable expression platform. The limitation with plant expressed heterologous proteins is that they are less immunogenic compare to other expression system produced proteins (Mason et al., 1992).

Patel et al., (2009) reported the production of HPV 16 VLPs in S. cerevisiae and developed the VLP based ELISA to evaluate the HPV serological association in clinical conditions. Cook et al., (1999) demonstrated the expression and purification of HPV 11 VLPs in S. cerevisiae.

Kirnbauer et al., (1993) and Rose et al., (1993) investigated and reported the expression of HPV VLPs in insect cell expression system. Despite the high level of expression of recombinant proteins, the baculovirus system is relatively expensive. Currently, bivalent HPV vaccine ‘Cervarix’ (HPV16/18) expressed in insect cell system is launched into the market.
Other investigators explored *E. coli* as an expression system for the production of HPV L1 pentamers (Li *et al.*, 1997). Even though in *E. coli* expression systems protein yields are decent, the intracellular milieu is not conducive for the formation of di-sulphide bonds, which in turn will prevent capsomeric L1 assembly to form VLPs.

Earlier Liu *et al.*, (2007) & Bazan *et al.*, (2009) reported the expression of HPV16L1 in *Pichia pastoris*. Detailed characterization of the clone and Immunological, neutralizing studies were not reported in their investigations.

In the present study we chose *Pichia pastoris* as an expression system for expressing HPV major (L1) and minor (L2) capsid proteins. *Pichia pastoris* platform offers many advantages, when compared to bacteria and mammalian systems. They have high growth rate, and can be grown to higher cell densities than bacteria. In addition, being eukaryotic, *Pichia* have the ability to post translationally modify the produced protein and to glycosylate secreted proteins (Hagenson 1991).

There are two types of vectors have been developed for heterologous protein expression in *P. pastoris* and these are autonomous and integrative. Autonomous vectors can replicate outside of the chromosome, but they are generally of low copy number and unstable. Integrative vectors, which are generally preferred, always integrate into the chromosome in order to replicate. *Pichia* with the heterologous gene
integrated into the chromosome provide a stable alternative for recombinant gene expression. Integration of genes for expression under the Alcohol oxidase promoter is known to induce high level of expression in *Pichia pastoris* (Cregg *et al.*, 2000).

Bazan *et al.*, (2009) reported the expression of HPV 16 L1 in *Pichia pastoris* and they employed the strategy of episomal expression of HPV 16 L1 in *Pichia*; which is not stable and requires antibiotic selection for clone propagation throughout the process. These strategies may not be appropriate for manufacturing of proteins at industrial scale.

In our study, we used integrative vectors to create stable clones of *Pichia pastoris* that express HPV 16 L1 or 18L1 major capsid proteins and another expressing HPV 16 L2 minor capsid protein were generated.

### 5.1. Cloning, expression and Purification of HPV 16 L1, 18 L1 and 16L2 proteins in *Pichia pastoris*

The work was initiated by amplifying HPV 16 L1 coding sequence from DNA samples obtained from cervical exfoliates of female donors. The HPV 16 L1 was sequence verified and expressed in *Pichia pastoris* as recombinant protein. Western blot analysis of the recombinant *Pichia pastoris* lysate expressing native HPV16 L1 yielded a discernable, albeit faint, protein band of approximately 56KDa size. The expression levels of native HPV 16 L1 was poor to undertake with any further studies. The
idea of amplifying the HPV 16 L1 gene from cervical exfoliates was to obtain HPV type specific to the geographical area. However, the success of quadrivalent vaccine introduced into the market by Merck provided us with an insight that the variant types may not really be required to produce an effective prophylactic vaccine. We began to explore the possibility of expressing HPV 16 L1, 18 L1 or 16 L2 genes that were specifically codon optimized for expression in *Pichia pastoris* whose protein sequence were available in the public sequence repository (GenBank). Thus the HPV capsid protein genes were synthesized *in-vitro* by altering the nucleotide sequence to favor expression in *Pichia pastoris*. A similar approach was also tested by several other investigators, emphasizing the impact of codon optimization for HPV genes (Sandhu *et al.*, 2008).

HPV 16 L1, 18 L1 and 16 L2 genes were codon optimized and synthesized *in-vitro*. Codon optimization of these genes 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 form unfavorable secondary structures on mRNA. Taken together the codon optimized gene sequences of HPV 16 or 18 L1 and 16L2 were found favorable for expression in *Pichia pastoris*. 
Recombinant clones of *Pichia pastoris* that express HPV 16 or 18 L1 major capsid proteins and another expressing HPV 16 L2 minor capsid protein were generated in this study. Stability of HPV 16 or 18 L1 clones was assessed by measuring L1 expression up to four consecutive passages using Western blot analysis and densitometry. All passages showed similar band density of L1 protein indicating that clones were stable.

The presence of HPV16L1 mRNA transcripts in the methanol induced cells of recombinant pPICZB16L1 *Pichia pastoris* clone was confirmed by RT PCR and Northern blot analysis. A clear band at approximately 1.5kb was observed in both RT PCR and autoradiograph. Since the Northern-blot and RT PCR results of HPV 16 L1 correlated well we choose to verify pPICZB18L1 and pPICZB16L2 constructs using RT PCR alone. The expressions of proteins were analyzed in Western blot using monoclonal antibodies and the results further confirmed the expression of recombinant HPV capsid proteins.

In the Western blot for HPV16 L2 probed with a monoclonal antibody (2JGmab#5), a higher molecular weight band was also observed apart from the 55Kda band. There are reports indicating the frequent association of Hsp 70 family proteins with HPV L2. The Hsp70 proteins are considered to act like chaperon that help protein fold or unfold (Reading *et al*., 1989). Some investigators observed that apart from HSPs
the sumoylation proteins are also tightly associated with HPV L2 (Martina et al., 2010). The higher sized 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*.

We noticed that conventional method of protein sample preparation for SDS PAGE, where samples are denatured by boiling at 95°C resulted in the L1 specific band that subscribed to a much higher size (~90 Kda) than the expected ~56 kda. The tendency of L1 proteins to multimerize upon boiling has been suggested in previous reports (Cook et al., 1999) therefore protein samples were processed for electrophoresis at 75°C in this study.

Though the purification of HPV VLPs reported in this study is part of proof-of-principle; the yields obtained were 9.5 mg/Liter and 6.4 mg/Liter of HPV 16 and HPV 18 VLPs respectively. Process scale optimization is being taken up to improve the quality and quantity of HPV 16 and 18 VLPs reported in this thesis.

Bazan et al., (2009) demonstrated the purification of HPV 16 VLPs using heparin sepharose chromatography. Where as in the present study, HPV 16 and 18 VLPs was also purified using heparin sepharose chromatography.
Studies performed in animal PVs demonstrated generation of cross neutralizing antibodies after immunization with the amino terminal peptide of L2 protein (Gambhir et al., 2007). Kawana et al., (2003) demonstrated that L2 protein neutralizing epitopes are common among Papillomaviruses, which leads to the possibility of its use in cross protective HPV vaccines. Based on these investigations we proposed to incorporate L2 protein in our studies.

Despite several attempts to purify HPV16 L2 protein, we remained unsuccessful. Purification of recombinant HPV 16 L2, to the best of our knowledge, without the use of any affinity tag has not been described thus far. Further studies need to be performed to determine optimum condition for purifying HPV L2 protein.

Christensen et al., (1996) have developed a panel of monoclonal antibodies that reacts only with correctly folded HPV VLPs. Pichia expressed HPV 16 and 18 VLPs folding and conformation was assessed by ELISA using the same mouse monoclonal antibodies i.e., H16V5 and H18G10 respectively. These VLPs also showed reactivity to the conformation specific monoclonal antibodies of HPV 16 and 18 VLPs. The reactivity was concentration dependent; neither HPV16 nor HPV18 VLPs cross reacted with the monoclonal antibodies specific to the conformation of the other HPV types. The assay was intended to detect the correctly folded VLPs. A prophylactic vaccine containing only the correctly folded
VLPs would elicit neutralizing antibody response in vaccinees but not the capsomeres or higher L1 aggregates (Kirnbauer et al., 1992).

Pichia expressed HPV 16 and 18 VLPs were visualized under Transmission Electron Microscope (TEM) and these VLPs showed variable particle size having a mean of approximately ~53nm. The variability of VLP size has been reported earlier (Yufang et al., 2008). There were more number of capsomeres than VLPs in the HPV 16 VLP preparation and similar observation was reported earlier in the case of S. cerevisiae derived VLPs (John Schiller, personal communication). Mach et al., (2006) demonstrated the disassembly and reassembly methods to fold the capsomeres to VLPs and similar strategy needs to be adopted to increase VLPs yields in our preparations.

5.2. Immunological characterization of Pichia pastoris expressed HPV 16, 18 VLPs

Hagensee et al., (1994) and Kirnbauer et al., (1993) reported the antigenic properties of the HPV VLPs. The VLPs were immunologically and structurally similar to the native virion.

To verify immunogenicity of the Pichia purified VLPs, the Pichia purified HPV 16 and 18 VLPs were adsorbed onto aluminum hydroxide gel and administered to mice either singly or in combination. Taking a
clue from our observation of the high immunogenicity with HPV18 VLP in mice, where the VLPs were found immunogenic, we decided to use lower the dose of HPV18 VLP compared to the dose of HPV16 VLP in bivalent composition. Mice immunized with these formulations elicited high serum IgG response against each VLPs. Mice immunized with HPV 16 VLPs showed some dose concentration dependent response and the mice immunized with 20μg of HPV16 VLP showed greater than a 4 log serum IgG titer. Mice immunized with HPV 18 VLPs did not show any dose dependency, and even the mice immunized with 1μg of 18 VLP were able to generate similar serum antibody titers as higher concentration VLP groups, indicating that the HPV18 VLP preparations were highly immunogenic.

A group of mice received 3μg doses of HPV18 VLP. This group was used to evaluate whether one booster immunization was sufficient to elicit geometric mean serum antibody titers ($\log_{10}$) of $10^{4.36} \pm 0.11$. The results indicate that mice receiving just one booster generated equally strong antibody response. We had performed a comparative analysis of immune response between the commercial vaccine and our preparations in mice. The result, clearly demonstrates that the HPV 16, 18 VLPs expressed in *Pichia pastoris* elicits comparable immune response in mice.
Mice immunized with HPV 16, 18 VLPs in combination (bivalent vaccine) elicited geometric mean titer ($\log_{10}$) of serum antibody on 35$^{th}$ day post primary immunization in mice against HPV 16 VLP was $10^{3.75} \pm 0.44$, while the titer against HPV 18 VLP was $10^{3.86} \pm 0.38$. This remained even on 190 days post primary immunization. The results indicated that the VLPs expressed in *Pichia pastoris* could elicit long lasting serum antibody titers in mice. There was difference in antibody titers from mice immunized by the combination of VLPs (bivalent formulation) versus individually formulated HPV VLPs. Even mice immunized with the positive control (commercial vaccine) showed difference in titers despite of having adjuvant in combination studies. This observation need to be addressed in our future studies. Aluminum hydroxide gel formulated *Pichia* based hepatitis B vaccine (Elovac®) from Indian Immunologicals was established in the market. As the safety and efficacy of this vaccine was proven, we decided to incorporate the same aluminum hydroxide gel in our HPV VLP formulations. Further studies need to explore for validating the appropriate adjuvants in HPV 16, 18 VLPs formulation.

Kirnbauer *et al.*, (1992) reported the papillomavirus VLPs ability in inducing neutralizing antibodies. The neutralizing epitopes display of on the VLP surface, their stability contributes to the highly immunogenic nature of HPV VLPs (Schiller, 1999). The presence of neutralizing antibodies is critical in preventing HPV infection by VLP vaccines.
Bazan et al., (2009) demonstrated the efficacy of HPV 16 VLPs using Hemagglutination Inhibition Assay (HAI), which lacks sensitivity, at best measuring only a subset of HPV neutralizing antibodies, and the test is extremely subjective. Cell based assays for measuring efficacy are far more sensitive and demonstrate specific virus neutralization.

In order to efficiently prevent the onset of virus infection from homologous HPV type, it is desired that the vaccine elicit type specific neutralizing antibody response. These were analyzed using in-vitro HPV pseudovirus neutralization assay. We noted that the pseudovirus neutralization titers elicited by HPV18 VLPs ($10^{4.65}, 10^{5.06}$ and $10^{5.48}$) were higher than those elicited by HPV16 VLPs ($10^{3.51}, 10^{4.77}$ and $10^{4.65}$; Figure 4.24). This trend was similar to those obtained for VLP binding antibody titers.

Neutralizing antibody response in mice immunized with Pichia purified HPV 16 or 18 VLPs were compared with mice immunized with bivalent commercial vaccine (Cervarix®). Geometric mean titers ($\text{log}_{10}$) of serum neutralizing 35th day post primary immunization of group of mice immunized with Pichia expressed 2 μg of HPV 16 VLP was $10^{3.66}$ and 1 μg of HPV 18 VLP was $10^{5.16}$ while neutralizing antibody titer of mice immunized with Cervarix® vaccine of 2 μg HPV 16 VLP was $10^{5.34}$ and 1 μg HPV 18 VLP was $10^{4.85}$ (Figure 4.25). Interestingly, neutralizing antibody titers in mice immunized with commercial vaccine were
comparatively lower for HPV18 VLPs than *Pichia* expressed 18VLPs. These results indicate that the *Pichia* purified 18 VLPs purity is better than the 16 VLPs. The lower reactivity may also be due to more number of capsomeres found in HPV16 VLP preparation. This further strengthens the observation that VLPs and not capsomeres are important for conferring protection in the vaccinees.

Given that HPV is epitheliotropic virus, infecting mucosal lining of the genital tract, one may argue that effective neutralization of the virus is possible only if secretory IgA are present in the cervical mucosa. It is established that the IgA secretion is not mandatory to prevent onset of HPV infection (Schiller and Davies, 2004). Based on recent studies in mouse and monkey cervicovaginal challenge models is reasonable conclude that exudation of systemic antibodies at sites of trauma is the major mechanism of protection by HPV VLP vaccines (Roberts *et al.*, 2011). Consistent with the conjecture is the finding that the quadrivalent commercial vaccine provides excellent protection against external genital lesions in both women and men that occur on cornified skin that is not bathed in cervicovaginal mucus. Thus measurement of systemic neutralizing antibodies, as reported herein, is likely to be most relevant in conferring protection from genital infection in people.

In the present study we have demonstrated conclusively, that *Pichia pastoris* can express VLPs that elicit neutralizing antibody, using
the *in-vitro* assay that a WHO advisory group as designated as the gold standard. The neutralizing antibody response was quantified in this well validated and sensitive assay, which is significant step forward in the validation of the *Pichia pastoris* expression system.

The work is further being extended by optimizing production and purification parameters on a pilot scale. Well optimized production and purification process will be used for clinical evaluation of the VLPs for use in human subjects. We remain optimistic that an affordable prophylactic vaccine for cervical cancer can be made available to a large number of people.