CHAPTER 2

REVIEW OF LITERATURE

2.1. Cervical cancer

Cancer of the uterine cervix is one of the leading cancer among women worldwide, with an estimated 520,000 new cases and 274,000 deaths reported annually (WHO/ICO Information Centre on HPV and Cervical Cancer- HPV and cervical cancer statistics in India. 2010). About 86% of the cervical cancer cases occur in developing countries, which represents 13% of all female cancers (WHO/ICO Information Centre on HPV and Cervical Cancer- HPV and cervical cancer statistics in India. 2010). Cervical cancer is sub-divided into cervical squamous cell carcinoma and cervical adenocarcinoma (Snijders et al., 2006). Majority of the cases of cervical cancer are squamous cell carcinoma (SCC) and adenocarcinomas are rare. Cervical squamous cell carcinoma (SCC) develops gradually over time from pre-existing non-invasive squamous precursor lesions, also called cervical intra epithelial neoplasias (CIN) or squamous intraepithelial lesions (SIL). The latency period between normal HPV infections to establishment of cancer may take over a decade (Zur Hausen, 2002).
2.2. Etiology

There has been significant progress in the etiology of cervical cancer during the last few decades. Sexually transmitted infectious agents such as gonorrhea, Chlamydia, syphilis, and Herpes simplex virus type 2, were considered to be the causative agents for cervical cancer (Bosch & Munoz, 2002). Between the years 1974 to 1976, Prof. Harold zur Hausen started to postulate and established the link between Human papillomavirus (HPV) and cervical cancer (zur Hausen et al., 1996) and for his contribution in unraveling this link, he was conferred with 2008 Nobel Prize for Physiology and Medicine. During the early 1980s development of technology to detect for the presence of HPV DNA in cellular specimens provided the strong basis for the definite etiological role of HPV in cervical cancer (Bosch et al., 2002; zur Hausen, 1996). The first HPV types isolated from cancer biopsies of the cervix were HPV16 and 18: these were cloned in 1983 and 1984 respectively. Following this, there was an explosion of research on papillomaviruses (Boshart et al., 1984).

Papillomavirus infections in humans are known to cause a variety of benign proliferations; these include warts, intraepithelial neoplasias, anogenital papillomas, oral laryngeal and pharyngeal papillomas (zur Hausen, 1996). Molecular and epidemiological evidence has now established that HPV types associated with anogenital neoplasms,
including condylomata, cervical dysplasia and cervical carcinoma, are almost always sexually transmitted (Lowy et al., 1994). The involvement of HPV in cancers of the vulva, anal canal, vagina and penis is currently being identified in addition to these, the possible infectivity of HPV in cutaneous cancer, oral cancers and other cancers of the upper aero digestive tract is being investigated (Bosch & Munoz, 2002).

In humans, specific papillomavirus types have been associated with over 99% of cervical cancer biopsies (Walboomers et al., 1999). These are considered the “high-risk” types and include, in order of prevalence, HPV types 16, 18, 31 and 45 (Bosch, 1995). HPVs have also been associated with other anogenital lesions and carcinomas, oral and pharyngeal papillomas and skin lesions in a rare genetic disorder called epidermodysplasia verruciformis (EV).

2.3. **Classification and Distribution of Papillomas virus**

Due to the similarity in genome and non-enveloped capsids, papillomaviruses had previously been grouped together with polyomaviruses in one family *Papovaviridae*. Later, it was established that the two virus groups have difference in genome sizes as well as genome organization and have no significant similarities in the nucleotide or amino acid sequences. The International Committee on the
Taxonomy of Viruses (ICTV) has assigned papillomavirus a separate genera (van Regenmortel et al., 2000).

Papillomaviruses (PVs) are epitheliotropic viruses and infect the vertebrates, where they cause neoplasias or exist asymptotically. Papillomavirus isolates are identified as “types” when their L1 gene sequence differs from every other type by at least 10 percent (Bernard et al., 2010). The L1 gene is instrumental for PVs classification, as it is mostly conserved among the PVs, and this is one of the strong reasons for genome based classification PVs (Bernard et al., 2010).

Figure 2.1: L1 gene sequence frequency distribution and percentage of identity of papillomavirus types. (Source: De Villers et al., 2004)
The term “subtype” refers to the rare isolates that differ by 2 to 10 percent from the L1 sequence of any type (Chan et al., 1995). The independent isolates of the same type which show minor differences in L1 gene sequence are referred as “variants”.

De Villiers et al., (2004) detailed the nucleotide sequence comparisons and biologically distinguishing features (host species, target tissues, pathogenicity and genome organization) that determine the PVs classification on the genera level. Greek alphabets are used to name these genera. In 2004, sixteen groups of PVs fulfilled the criterion of genera, and the Greek letters alpha (α) to pi (π) was used to classify them. Human PVs are members of five genera (Alpha (α), Beta (β), Gamma (γ), Mu (μ) and Nu PVs) and two genera (Eta (ε) and Theta (θ) PVs) each comprise of single bird PV. The remaining nine genera contain one or several animal PVs.
Figure 2.2: Neighbour joining phylogenetic tree of 101 PVs based on the nucleotide sequence of the major capsid gene (L1). (Source: de Villers et al., 2004)
2.4. Papillomaviruses - Animal Papillomavirus

2.4.1. Canine oral Papillomavirus

Investigations with canine oral papillomavirus (COPV) demonstrated the narrow host range of papillomaviruses. Oral papillomas infection and transmission in dogs had been reported in the late 19th century and the samples were obtained from an outbreak in foxhound puppies (Lowy, 2007). The papillomas induced by COPV uniformly regressed spontaneously, dogs were the only species susceptible to COPV infection, and inoculation of epithelial sites in the dog other than the oral mucous membranes failed to induce papillomas.

2.4.2. Cottontail rabbit Papillomavirus

The cottontail rabbit papillomavirus (CRPV) exhibits number of similar properties of Papillomaviruses (Nasseri & Wettstein, 1984; Danos et al., 1984). Systemic immunization with PV suspension induces neutralizing antibodies without establishing cutaneous infection and protect against cutaneous viral challenge. These investigations established a proof of concept for a prophylactic vaccine against papillomaviruses.

2.4.3. Bovine Papillomavirus

Till date six Bovine Papilloma Virus (BPV) types have been characterized associated with different histopathological lesions. These six genotypes
further divided into three groups namely Xi (ξ), Delta (δ) and Epsilon (ε) (de Villiers et al., 2004). The epitheliotropic BPV types 3, 4 and 6 are classified under the group Xi (ξ), Delta (δ) group have BPV type 1, 2 and group Epsilon (ε) have the BPV type 5.

Infection of Delta-PVs cause papillomatosis, whereas infection by Xi-PVs induces transformation only in the epithelial layer. Virus replication has never been observed in fibroblasts where the BPV genome is present in a nonintegrated episomal form (Jarret, 1985), although the BPV viral gene expression was reported in tumors of mesenchymal origin (Borzacchiello et al., 2007).

BPV types 1, 5 and 6 infect the teats and udders in cows (Campo, 2002). Once the papillomas spread around the primary tumors and the infected cows cannot be milked which may lead to a serious economic problem. BPV type 4 infects the upper gastrointestinal (GI) tract of cattle and causes the papillomas (Campo at al., 1980). The BPV type 1 or 2 involvement in cattle bladder carcinogenesis has been recognized for a long time. BPV-1 is predominantly detected in sarcoid tumors of horses, donkeys and mules (Amtmann et al., 1980).

2.5. Classification – High risk and low risk types of HPV

Genital HPV types are classified into low-risk types and high-risk types. Where low-risk types cause warts and high-risk types, which causes
invasive cervical cancer. Although 13 to 19 high-risk types have been identified, only the 12 HPV types namely 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 are consistently observed clinically (Gravitt et al., 1998). Apart from the high-risk types, six more genotypes ((26, 53, 66, 68, 73 and 82) have been identified as “probably high risk” (Munoz et al., 2006).

2.6. **Cervical cancer prevalence in India**

India has a population of 366.58 million women of ages 15 years or older who may be potentially at risk of developing cancer of uterine cervix. Current estimates indicate that every year approximately 134,000 women are diagnosed with cervical cancer of which more than half (72,825) die from the disease in India (Figure 2.3). Cervical cancer is the most frequent cancer in Indian women and about 7.9% of women in the general population are estimated to harbor persistent HPV infection at any given time.
**Figure 2.3:** Annual number of deaths of cervical cancer in India and southern Asia (Source: WHO Summary report on HPV and cervical cancer statistics in India. 2010).

An estimate suggests number of new cervical cases to increase by the year 2025 to 2,03,757 and estimated number of deaths in 2025 may be 1,15,171, which is almost 70% increase compared to the existing estimates for persistent HPV infection (Figure 2.4; WHO/ICO Information Center 2010).
Bosch et al., after studying cervical cancer patients from 25 countries, reported that HPV types 16 and 18 are detected in more than 70% of cervical cancer cases (Bosch et al., 2003). The HPV type 16 prevalence in India is also reported to be high (~70%) (Das et al., 2008) whereas HPV 18 occurrence differs from 3-20%, followed by other high-risk types such as HPV 45, 33, 35, 52, 58, 59 and 73 (Franceschi et al., 2003; Sowjanya et al., 2005). The HPV type distribution varies depending on geographical regions and also cultural variations (Burchell et al., 2006). Schiffman and Castle (2005) reported that the cervical cancer and HPV infection prevalence in India indicate that the initiation as well as
peak of HPV infection occurs at a slightly older age group (26-35 years) women, when compared to the global incidence (peak in 18-25 years). It is observed that, while in the developed countries there was significant decrease of cervical cancer mortality after incorporation effective screening programs, no reduction in the incidence of cervical cancer was observed during past three decades in the developing countries (Sankaranarayanan et al., 2009).

2.7. Human Papillomavirus (HPV)

2.7.1. Structure and genome organization

Papillomavirus in humans are studied extensively and more than 120 HPV types reported to date (de Villiers et al., 2004). Human Papillomaviruses are epitheliotropic, non-enveloped, double-stranded DNA viruses that infect mucosal and cutaneous epithelia in a wide variety of higher vertebrates. The HPV virion has a diameter of ~55 nm and the genome of virus is approximately 8 kilobases (kb) size (Seedorf et al., 1985). The HPV genome is divided into three regions based on functionality. The long control region (LCR), early region coding for six proteins (E1, E2, E4, E5, E6 and E7) and late region encoding two late proteins (L1 and L2) (Figure 2.5). The early genes are non-structural genes whereas the late genes structural genes responsible for the formation of virus capsid.
2.7.2. Long control region (LCR)

The LCRs of most HPV range in size from 800 to 900 bp and this region contains enhancer and silencer sequences that regulate DNA replication. In the viral genome LCR shows the highest degree of variation (Apt et al., 1996). The 5′ region of the LCR is about 300 bp long and is flanked by the translation termination codon of the L1 gene and the first E2 binding site. It contains a nuclear matrix attachment region (Stunkel et al., 2000), transcription termination and polyadenylation sites for late transcripts, as well as a negative regulatory element that acts at the level of late mRNA stability (Kennedy et al., 1991). The 3′ region of the LCR is about 140 bp in size and contains a single E1 binding site, which identifies the origin of replication. The 3′ region of the LCR is the region between the second E2 binding site and the translation start codon of the E6 gene, which modulate E6/E7 promoter activity i.e.; a binding site for the transcription factor SP1, two binding sites for the viral factor E2 and a TATA box (O’Connor et al., 1995; Chiang et al., 1992).

In the high risk HPV type, viral gene expression is controlled by two promoters. These early viral promoter referred as p97 in HPV 31, as p99 in HPV16 and as p105 in HPV18. These promoters are constitutively active in undifferentiated basal cells as well as most differentiated supra basal cells (Hummel et al., 1992; Rohlfs et al., 1991) and direct transcription of early viral proteins. After cellular differentiation,
activated late viral promoter drives expression of late RNA with a heterogeneous set of start sites located in the early gene E7 (del Mar Pena & Laimins, 2001). The late viral promoter is called p680 in HPV16 and p742 in HPV31 (Grassmann et al., 1996; Hummel et al., 1992). These late promoters direct two set of transcripts; one set terminates at the early poly A site and the other set terminates at the late poly A site located downstream of L1 gene. There were reports indicating that presence of poly A site is required for efficient mRNA translation (Sachs et al., 1997).

Figure 2.5: HPV 16 genome organization showing the long control region (LCR) showing late (P99) and early promoter (P680), early genes and capsid genes.
2.7.3. Human Papillomavirus early proteins

2.7.3.1. E1 Protein

The E1 protein is highly conserved among HPV types. The 73-kDa E1 protein is mediates the replication of virus; it binds to a specific DNA sequence (E1 binding site; E1BS) in the viral origin of replication and forms the hexameric complexes with the help of a second viral protein, E2 (Frattini & Laimins, 1994). The resultant complex has helicase activity and initiates synthesis of progeny DNA (Wilson et al., 2002).

The papillomavirus E1 protein functional regions have been identified. The C terminal has helicase and ATPase activities. Amino acids change in the ATPase domain (P479 → S479) inactivates the ATP binding and disrupts the function of E1 protein (Hughes & Romanos, 1993). The ATPase domain of E1 protein interacts with E2 protein and α subunit p70 of the DNA polymerase (Masterson et al., 1998). A segment of approximately 160 aminoacids immediately upstream of the ATPase/helicase domain is referred as the DNA-binding domain (DBD; White et al., 2001; Titolo et al., 2003). A segment of about 50 amino acids located within the terminus terminal of E1 functions as a localization regulatory region (LRR), which contains a nuclear export sequence (NES) and a nuclear localization signal (NLS) (Deng et al., 2004).
2.7.3.2. E2 protein

The E2 protein has three distinct regions. First the carboxyl terminal, a dimerization domain that initiates the homodimers formation, which recognizes and binds to the 12-bp palindromic DNA sequences (ACCGNNNNCGGT) within the LCR. This region is called E2-Binding Sites (Desaintes & Demeret, 1996). Second, the middle region of E2 - the hinge region, is crucial for regulation of E2 proteins stability and identifying their nuclear localization in others (Zou et al., 2000). The amino terminal domain, which is important for transcription regulation and replication of viral DNA (Desaintes & Demeret, 1996) is the third region.

At higher concentration of E2 protein acts as a transcriptional repressor and at low levels it activates transcription from the viral LCR. Some of the investigators have demonstrated that in HeLa cells E2 protein expression induced the apoptosis by restoring the functions of p53 as a result repression of E6 expression observed (Desaintes et al., 1997). Interaction of E2 with the minor capsid protein L2 leads to the inhibition of the transactivation of E2 for both BPV and HPV proteins (Okoye et al., 2005; Heino et al., 2000).

In the early history of papilloma virus research, there was an ORF designated E3, which was identified later as anomaly. There was no
attempt made later rename all other ORFs. Hence there is no protein designated as E3 in papillomaviruses.

2.7.3.3. E4 Protein

The HPV E4 gene is located in the early genomic region yet it is generally expressed as a late gene with a role in productive infection (Howley, 1995). The E4 protein localizes and aggregates into cytoplasmic and nuclear inclusion granules within the differentiating layer of the infected epithelium and in the basal layer, indicating both an early and late function (Roberts et al., 1993). The E4 facilitates and supports viral genome amplification and also regulates expression of late genes. The E4 protein plays a major role in virus maturation and virus release.

2.7.3.4. E5 Protein

The E5 protein enhances the oncogenic nature of the E6 and E7 (Stoppler et al., 1996). The BPV E5 protein acts as the strong transforming protein. Whereas, in HPV infections; E5 has only weak transforming activity (Schiller & Lowy, 1996). The E5 plays a role in early stage of HPV infection but is dispensable for maintenance of malignant transformation (zur Hausen, 1996). In cultured cells expression of HPV 16 E5 enhanced the activity of epidermal growth factor receptor (EGFR) (Crusius et al., 1998; Pim et al., 1992); co-immuno precipitation studies have demonstrated that HPV 16 E5 can also form a complex with EGFR
when both proteins are over expressed (Hwang et al., 1995). Through activation of EGFR, E5 can interfere with several signal transduction pathways, including the mitogen-activated protein (MAP) kinase pathway (Crusius et al., 1997). HPV 16 E5 also inhibits the process of apoptosis induction by Fas-ligand, tumour necrosis factor related apoptosis-inducing ligand (TRAIL) (Kabsch & Alonso, 2002) and by UV-light (Zhang et al., 2002).

2.7.3.5. E6 Protein

The E6 gene of papillomavirus located at the 5’ end of the viral early region. The papillomavirus E6 gene is the one of the first gene expressed during PV infection. The E6 protein is required for the productive life cycle of the papillomaviruses and has been implicated in supporting the stable maintenance of viral DNA in cultured keratinocytes (Park and Androphy, 2002; Wu et al., 1994). Hudson et al. (1990) and Munger et al. (1989a) demonstrated the major transforming activity of the high-risk HPV E6 protein in cultured cells and its ability to increase immortalization of human keratinocytes in association with the E7 protein.

The HPV E6 binds to the cellular ubiquitin-ligase, called E6 associated protein (E6-AP), which in turn binds to p53. This interaction leads to the p53 degradation and cell cycle disruption (Fehrmann &
Laimins 2003). In addition, E6 binds to various other cellular proteins and these are broadly divided into four classes: proteins involved in cell polarity and motility, transcriptional co-activators, tumor suppressors & apoptosis inducers, and DNA replication and repair factors.

2.7.3.6. E7 Protein

The E7 gene situated at the immediate downstream of the E6 gene. The E7 protein made up of approximately 100 amino acid residues. The HPV16 E7 protein has been studied most extensively as it was the first high-risk HPV oncogene product to be discovered (Kanda et al., 1988; Yutsudo et al., 1988). HPV16 E7 induces elongated S-phase and abrogates the cell-cycle checkpoints (Martin et al., 1998; Peacock et al., 1995). In healthy human cells high-risk HPV E7 protein leads to the chromosomal instability (White et al., 1994). HPV E7 plays a major role in the virus life cycle and is necessary for the stable maintenance of HPV episomes in epithelial cells (Flores et al., 2000; Thomas et al., 1999). The E7 protein is responsible for cellular transformation during the life cycle of high-risk and low-risk HPVs (Oh et al., 2004b; Longworth & Laimins, 2004a, b; Thomas et al., 1999). The differentiation state of the host keratinocyte influences the replicative life cycle of HPVs. The host cellular DNA replication machinery is necessary for HPV genome synthesis, but these differentiated cells do not intrinsically support DNA replication (Stubenrauch and Laimins, 1999). HPV E7 retains
differentiating keratinocytes in a DNA replication competent state (Cheng et al., 1995). The ability of E7 proteins to induce unscheduled DNA replication is important for cellular transformation and it is a necessary factor of the HPV replication strategy.

**Figure 2.6:** Activity of E6&E7 on various cellular targets (Source: Zur Hausen, 2002; Varsani et al., 2003).

The host cell integrated genes E6 and E7 are expressed and their protein products are responsible for the oncogenesis. E7 interferes with transcriptional activity of p53, inactivates the cdk inhibitor p21CIP1 and blocks pRb binding to E2F. The pRb and p53 inactivation interferes with the integrity of important cell checkpoints and the cellular apoptosis mechanism. The E6 protein disrupts apoptosis by binding to p53 and targeting this tumor suppressor for proteolysis via the ubiquitin
pathway. A schematic description of the E6 and E7 function that obstructs normal cell cycle progression is depicted in Fig. 2.6.

2.7.4. **Human Papillomavirus Late proteins (Capsid Proteins)**

2.7.4.1. **Major capsid protein (L1)**

Electron microscopy image analysis of papillomaviruses established that the virion capsids are made up of subunits (capsomeres) arranged in an icosahedral lattice. The T, or triangulation, number nomenclature was derived by Caspar and Klug to explain the possible icosahedral symmetries of capsids of various sizes. Specific icosahedral symmetry means $60 \times T$ identical copies of the capsid subunits PV virions are approximately 55nm in diameter, non enveloped and comprise 360 molecules of the major capsid protein L1, arranged as 72 pentamers or capsomers in a $T=7$ dextro (right handed) icosahedral lattice where 60 pentamers are hexavalent and 12 are pentavalent (Baker *et al.*, 1991; Hagensee *et al.*, 1994). Three dimensional image of cryo-electron micrographs of BPV virions revealed the capsid architecture to $9-A^0$ resolution and a protein density within the pentamer cavity of the pentavalent capsomers was detected (Baker *et al.*, 1991).

The Human papillomavirus major capsid protein (L1) comprises 504 amino acids residues and has a molecular weight of approximately 56 KDa. HPV major capsid protein has two nuclear localization signals
(NLS) consisting of clusters of basic amino acids (Zhou et al., 1991a). The first NLS located at the C-terminal of L1 protein and consists of the sequence KRKKRK (amino acids 499 to 504) and another bipartite NLS (separated by 12 amino acids) overlaps this site, situated at amino acids 484 to 486 (KRK) and 499 to 500 (KR; Zhou et al., 1991a). The NLS is recognized by adapters of the karyopherin α (Kapa) family and by import receptors of the karyopherin β (Kapβ) family that shuttle between the nucleus and cytoplasm (Nelson et al., 2002). Nelson et al. demonstrated that HPV-16 L1 capsomers are translocated into the nucleus by forming complexes with Kapα2β1 heterodimers. The HPV16 L1 major capsid protein enters the nucleus through Kapα2β1 mediated pathway to assemble the virions during the productive stage of infection and also inhibits the Kapβ2-mediated nuclear import of host hnRNP A1 protein thereby favoring virion formation (Nelson et al., 2002).

The 3.5 Å°, structure of T = 1 assemblies of recombinant HPV16 L1 capsomeres defines bonding relationship of within L1 monomers and pentamers (Chen et al., 2000). The monomer of the L1 is composed of 20–382 amino acid residues and appears as an eight-stranded anti parallel β-barrel, in two distinct sheets composed in a classical “jelly roll” β-sandwich (Figure 2.7).
Figure 2.7: HPV16 L1 protein Structural models A: Ribbon diagram of an HPV16 L1 pentamer, in the conformation found in small VLPs (Source: Chen et al., 2001). Three subunits are shown in green, blue and red. The C-terminal arms are in gray, to indicate that these portions of the subunit rearrange when pentamers assemble into virions or into full-sized capsids. B: Atomic structure of HPV-16 L1 monomer showing the $\beta$-sheets (jelly roll sandwich) and the $\alpha$-helices. C: An HPV L1 pentamer viewing from the top. D: HPV L1 pentamer from the side. E: Backbone of a HPV-16 T=7 particle showing the organization of the L1 pentamers (72 pentamers) and their basic interaction with neighboring pentamers (Source: Chen et al., 2000).
In the core domain, three elaborate loop domains (H-I, D-E, and F-G) are located on the outer surface in the assembled pentamer. The amino acid residues 383–475 located at C-terminal to the core domain fold into α-helical and long coiled structures that form the lateral projections from the core comprising the center of a pentamer. The remaining 30 amino acid residues of the C-terminal (476–505) are disordered and likely extend into the interior space of the pentamer or the particle where the basic residues in this region may interact with the viral DNA. Deletions at the C-terminal of up to 30 residues, as well as fusion of additional sequences to the C-terminal (Muller et al., 1997; Paintsil et al., 1996), still permit pentamer formation and thus support the model that this stretch of residues at the C-terminal region is independently oriented and folded.

It has been demonstrated that neutralization epitopes are present in the L1 major capsid protein (Kirnbauer et al., 1992; Christensen et al., 1994a). Both conformational and linear epitopes have been identified on the surface of VLPs. These epitopes are located in three regions of L1; where conformational epitopes localized at amino acids 111 to 130, 174 to 185, and linear epitopes located from amino acid 261 to 280 (Christensen et al., 1996a; Wang et al., 1997). HPV neutralizing antibodies in general recognize conformational epitopes and are type specific and are not cross neutralizing (Giroglou et al., 2001; Christensen
et al., 1996a). Initially it was thought that protection by HPV VLPs was mediated by neutralizing antibodies that recognize conformational epitopes (Christensen & Kreider, 1990; Kirnbauer et al., 1992). However, studies suggest that linear epitopes can also give rise to neutralizing antibodies (Kawana et al., 1999; Roden et al., 2000). In comparison to the type specific conformational epitopes these linear cross neutralizing epitopes are less immunogenic than the type specific epitopes and the antibody levels against linear epitopes are too low for protection against infection (Combita et al., 2002).

2.7.4.2. Minor capsid protein (L2)

Minor capsid protein L2 of HPV has 473 amino acid residues and its molecular weight is approximately 55KDa. The L1 and L2 proteins assemble into capsomers, which in turn forms the capsids (Fehrmann and Laimins, 2003). The L2 nuclear localization signals (NLS) located between residues of 456-461 (RKRRKR; Zhou et al., 1991a).

Day et al. (1998) from their studies on the interaction of L1 and L2 suggested that virion assembly is triggered by interaction of L2 with Nuclear Domain 10 (ND10) and the co-localization of the L1. It is likely that the association of the L1 with ND10 leads to direct interaction of L1 with L2, since stable L1-L2 complexes form in both fully assembled VLPs in vivo and also in partially assembled capsomers in-vitro (Okun et al.,
Although L1 can self-assemble into virus-like particles (VLPs) in the absence of L2 (Kirnbauer et al., 1992; 1993), addition of L2 results in the increase of VLP formation by 4-fold in insect cells and 100-fold in mammalian cells (Hagensee et al., 1993; Kirnbauer et al., 1993). Florin et al. (2002) suggested that synthesis of L2 is initiated prior to L1 in the terminally differentiated keratinocytes. L2 is translocated into the nucleus independently of the L1, inducing the re-organization of ND10 whereas L1 is assembled into capsomers in the cytoplasm and the capsomers are then translocated into the nucleus and recruited to ND10 after L2-induced release of Sp100, a major ND10-associated protein, which is subsequently degraded. HPV L2 protein plays a significant role in the infectivity of HPV (Roberts et al., 2007, Holmgren et al., 2005). The HPV L2 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 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 (Gambhira et al., 2007).

There are reports indicating the presence of neutralizing epitopes on HPV minor capsid protein (L2). These epitopes are located within the N-terminal region of amino acids 108 to 120 (Kawana et al., 2001b;
Roden et al., 2000). The amino acid sequence of this region is highly conserved among other HPV types (Kawana et al., 1999), which leads to the hypothesis that they may be used in HPV cross protective vaccines (Kawana et al., 2003).

2.8. Virus-like particles

Conventionally most prophylactic viral vaccines are made of live attenuated virus or inactivated virus. Understanding of the biology of papillomavirus infection was hindered by the lack of tissue culture systems to propagate the viruses. Papillomavirus infections are remarkably species restricted; hence, there are no animal models for productive HPV infection, or the generation of hyper proliferative disease after application of infectious HPVs. However, in the late 1990s organotypic raft cultures of primary human keratinocytes (PHKs) achieve stratification and squamous differentiation in-vitro (Sterling et al., 1993), suggesting that they may support the productive phase of papillomavirus infection (Broker and Botchan, 1986). The limitations with these Keratinocyte raft cultures for HPV production are scalability and the cost of manufacturing.

One of the very important discoveries in papilloma virus research was that L1 protein on its own, or in combination with L2, spontaneously assembles into virus-like particles (VLPs) when expressed in-vitro. This
phenomenon was first demonstrated by over expression of HPV L1 and L2 proteins in mammalian cells, eventually VLPs formation was observed in other eukaryotic expression systems (Zhou et al., 1991, Kirnbauer et al., 1992; Hagensee et al., 1993; Rose et al., 1993).

The HPV type 11 virus particle assembly shows that it is nucleated by a dimer of pentamers and proceeds by addition of one pentamer at a time (Casini et al., 2004). It is known that redox environment (correlating with disulfide formation), pH, and ionic strength all influence HPV VLP assembly (Sapp et al., 1998).

**Figure 2.8:** Schematic representation of papilloma virus VLPS (Source: Salunke et al., 1986).
The papillomavirus VLPs induce high titer neutralizing antibodies (Kirnbauer et al., 1992) and the response has been shown to be type-specific but long lasting (Carter et al., 2000; Wang et al., 1997). Small quantities of VLPs administered to rabbits and mice have been effective immunogens, even without adjuvants. The neutralizing epitopes displayed on the VLP surface, together with the high stability of VLPs, possibly contributes to the highly immunogenic nature of HPV VLPs (Schiller, 1999). Multiple copies of immunogenic peptides displayed on VLP surface triggers host immune response via PAMP: PRR (pathogen associated molecular pattern: pattern recognition receptors). The interaction of VLPs with B-cells will induce their activation through the oligomerization of VLP-specific antibodies as proposed for hepatitis B core particles (Milich, 1987).

2.9. Natural course of HPV infection

HPV infection, unlike many genitourinary infections, is not usually associated with immediate symptoms such as itching, burning, and vaginal discharge (Mao et al., 2003). Rather, the majority of those infected with HPV will not develop clinical disease or symptoms because the host immune system resolves most infections. In one study, only 24.8% of women infected with HPV 6 or 11 actually developed genital warts (Mao et al., 2003). A large, prospective 10-year cohort study of more than 20,000 women enrolled in a health maintenance organization
found that the incidence of CIN 3 or cancer was approximately 7% in HPV-positive women for the duration of the study (Sherman et al., 2003). Thus, only the minority of patients with HPV infections develop serious clinical complications. The exact mechanism by which HPV infection is cleared by the host immune system is currently unknown. A number of factors are associated with an increased risk of initial infection and/or clinical advanced stage such as genital warts, CIN or invasive cancer.

Figure 2.9: Progression of cervical cancer of HPV infection (Source: Burd EM. 2003).
2.10. HPV Transmission

Carcinogenic genital HPVs are mainly sexually transmitted through contact with infected cervical, vaginal, vulvar, penile or anal epithelium. There are reports indicating the non-sexual transmission of HPV and this may be due to direct contact with the skin or mucosas or indirectly transmitted through contaminated objects, or during the perinatal period (Sinclair et al., 2005).

Perinatal transmission may occur: directly, in delivery by cesarean section by transferring infection from the vaginal canal, after a premature rupture of the amniotic membranes (Tenti et al., 1999). Also, during the time of fetus passage through the birth canal and on coming into contact with infected maternal secretions (Rintala et al., 2005). Indirectly the virus may be transmitted during vaginal delivery from contaminated objects; and intrauterine transmission at the time of fertilization from sperm carrying latent HPV (Lai et al., 1996); ascending infection from secretions of the maternal genital tract; and transplacental transmission (Favre et al., 1998).

2.11. HPV Clinical Manifestation

Depending on the presence of HPV type, HPV infection may lead to one of three possible results. The first is anogenital warts on or around the genitals and anus in both men and women. The second result is latent or
infection, in which remarkable symptoms are observed and the infected area remains cytologically normal. The third is active infection associated to high risk HPV types in which the virus causes changes in infected cells eventually it will lead to urethral, penile, vaginal or cervical intraepithelial neoplasia (Burd, 2003).

HPV infection may be latent, subclinical, or clinical. It may take the pathway of low viral-load infection without clinical disease, or high viral-load infection with clinical disease (Moscicki et al., 2004). Such disease may manifest as genital warts and within about two years 90% women clear the HPV infection caused by either high or low risk types (Ho et al., 1998; Koshiol et al., 2008). The median time to clearance of genital warts after treatment is about six months (Winer et al., 2005). In women, up to 30% of cases of genital warts spontaneously regress within four months (Lacey, 2005). It is not clear whether this immune mediated regression eliminates infection or suppresses it permanently, but in both the cases the virus stops to manifest lesions. Minor percentages of women who fail to clear the infection are at the risk of progression of malignancy (Moscicki et al., 2004).

2.12. HPV Viral life cycle

Human papillomavirus infect keratinocyte in the basal epithelium. The productive life cycle of HPVs depends on the host epithelial tissue
differentiation (Howley and Lowy, 2001). After virus binds and enters, virions shift to the nucleus and establish their genomes as multi-copy extra chromosomal plasmids. These are maintained at approximately 20 to 100 copies per infected basal cell (Stubenrauch & Laimins, 1999). The E1 and E2 proteins with the mediation of host cell proteins initiate the replication of viral genomes (Ustav & Stenlund, 1991; Lambert, 1991). Further, one of the daughter cells moves away from the basal layer and starts differentiation. In general, uninfected keratinocytes exit the cell cycle after leaving the basal layer. Whereas, HPV-infected cells undergo differentiation but remain active in the cell cycle (Stubenrauch and Laimins, 1999). This allows highly differentiated supra-basal cells to re-enter S-phase and facilitates high-level productive viral DNA replication. The E7 protein mediates the cells differentiation to undergo cell cycle progression (Flores et al., 1999; Halbert et al., 1992). Late viral promoter of HPV activates the differentiation-dependent amplification of viral genomes in suprabasal cells (Hummel et al., 1992). Progeny virions are assembled in highly differentiated cells and then released to the extracellular environment (Frattini et al., 1996; Howley and Lowy, 2001; Meyers et al., 1992).
2.13. HPV Immunology

2.13.1. Cell mediated immune response

Both early and late gene products of PVs have been proven to induce T-cell responses. The L1 protein of papillomaviruses elicits cell-mediated immunity; detailed information has come from the study of animals immunized with VLP and direct *in-vitro* analysis of the interaction between VLP and immune cells. De Bruijn *et al.*, demonstrated the ability of papillomavirus VLPs to protect from tumor challenge in a mouse model (De Bruijn *et al.*, 1998). Primates were immunized with monovalent or quadrivalent (HPV 6, 11, 16, 18) VLPs to evaluate for antibody, cytokine, and CTL responses to immunogens (Palker *et al.*, 2001) and results indicated that there was weak CTL response along with some type-specific induction of cytokines but antibody production predominantly dominated the response to these immunogens. Dasilva *et al.* (2001) demonstrated the physical interaction of HPV-16 VLP with antigen presenting cells including dendritic cells, macrophages and B cells. VLP-mediated activation of dendritic cells (Lenz *et al.*, 2001), along with the uptake and presentation of VLP by dendritic cells have been documented (Rudolf *et al.*, 2001).

The early gene products, the E6 and E7 proteins are consistently expressed in PV-induced lesions, thus becoming likely targets of cell-
mediated responses. The induction of localized CD4\(^+\) T cell-mediated inflammation, known as delayed-type hypersensitivity reactions to the E6 (Chambers \textit{et al.}, 1994) and E7 (McLean \textit{et al.}, 1993) proteins have been demonstrated.

The observation that HPV-related disease could be correlated with specific human leukocyte antigen (HLA) alleles (Wank & Thomssen, 1991; Apple \textit{et al.}, 1994) also implicates T cell-mediated immunity in monitoring infections. Additionally, regressing genital warts in humans exhibit infiltrates of T cells and macrophages (Coleman \textit{et al.}, 1994); this was also demonstrated in ROPV (Christensen, 2000) and COPV infection, where the infiltration of CD4\(^+\) and CD8\(^+\) T cells was pronounced (Nicholls \textit{et al.}, 2001).

The IL-2 and interferon gamma (IFN-\(\gamma\)) are responsible for regression of HPV lesions and activates CTLs (Stellato \textit{et al.}, 1997). Woodworth \textit{et al.} (1992) demonstrated that IFN-\(\gamma\) repressed the HPV-16 expression in immortalized cell lines. Some other investigators showed that in eukaryotes interferons interfere with viral replication and form an early cytokine barrier against viral infection also inhibit the HPV-infected cells differentiation (Rockley and Tyring, 1995). HPV infected keratinocytes secrete the cytokine TNF-\(\alpha\) (Arany \textit{et al.}, 1993) and this TNF-\(\alpha\) inhibits the HPV replication. TNF-\(\alpha\) facilitates the antigen
presentation to CD4+ T cells and result in the clearance of infection (Al Saleh et al., 1998; Coleman et al., 1994). Further, TNFα enhances the migration of Langerhans cells (LCs) into the lymph nodes and present the antigen to CD8+ T cells (Cumberbatch and Kimber, 1992). TNFα mediated natural killer cells eliminates the tumor cells (Glas et al., 2000; Kashii et al., 1999). HPV infected cells are eliminated by cell-mediated immune response and the failure of T cell secretion cause persistent infection and eventually leads to the development of cancer.

2.13.2. Humoral Immune response

Epitopes present on the VLPs elicit the humoral immune response against HPV infection. HPV L1 assembled VLPs shows the similar antigenic properties of the native virion (Hagensee et al., 1993, 1994; Kirnbauer et al., 1993; Rose et al., 1993). These VLPs have been a very important tool in the development of detection assays of an immune response to HPV infection and also in the preparation of vaccines against HPV (Breitburd et al., 1995; Suzich et al., 1995; Koutsky et al., 2002; Harper et al., 2004).

Investigations illustrated the correlation between sero-positivity and HPV disease (Van Doornum et al., 1994; Wikstrom et al., 1995b) also other studies demonstrated the HPV antibodies response was not matched with other parameters of HPV infection (Jenison et al., 1990;
Kochel et al., 1991b). Nonnenmacher et al. (1995) evaluated the humoral response against the HPV-16 E6 and E7 proteins and also observed the difference in antibody levels between early proteins and capsid protein antibody levels, the results however were not well correlated. HPV early protein peptides were used to demonstrate the correlation between E6 or E7 antibodies levels and role in the tumor progression (Fisher et al., 1996; Dillner et al., 1997). The results of these studies confirmed the presence of E7 antibodies at late-stage of cervical cancer.

Humoral immune response has not been detected in every women infected with HPV and women with repeated HPV infection have greater chances of seroconversion (Wideroff et al., 1995; Carter et al., 2000). Kirnbauer et al., (1994) report that approximately 50–60 percent of infected women seroconvert although other studies report as high as 88 percent (Nakagawa et al., 2002). In general it may take 6-8 months for seroconversion (Carter et al., 2000; Ho et al., 2004), while sero-prevalence in men are found to lower than women (Svare et al., 1997; Slavinsky et al., 2001).

In general humoral immune response against HPV elicits IgA response first followed by a secondary response of IgG antibodies. Some studies indicated that in every case immune response against HPV may not show this pattern. Ho et al. (2004) observed that the presence of the IgG and IgA antibodies at the same time. Wang et al. (2000)
demonstrated the presence of IgG antibody levels reflect earlier infections where as detection of IgA antibodies indicates the recent infections.

The growth characteristics of HPV limited the study of humoral immune response until the production of virus-like particles. These VLPs allowed to study in detail of the humoral immune response against HPV infection.

2.14. Prevention of HPV and cervical cancer


A number of different strategies for cervical cancer prevention have been identified, including cervical cytology methods, HPV DNA testing, and a variety of iterations of direct visual inspection of cervix. In developing countries, no clinically significant reduction in the incidence of cervical cancer has occurred during the past three decades (Ferlay et al., 2004; Sankarnarayanan et al., 2001). Poor nutrition and general health status resulting in impaired immune function and other social, behavioral, and possibly genetic factors may also contribute to the high prevalence of virally associated tumors in the developing countries. In the developed world, by contrast, there has been a major decline in cervical-cancer mortality after the introduction of large-scale cytologic testing. HPV Screening by Papanicolaou (Pap) smears in the United States have resulted in a 70% reduction in the mortality from cervical cancer during
the past 50 years (Bosch et al., 2002; Plummer & Franceschi, 2002). Cuzick et al., demonstrated that the cost, coverage and acceptability, infrastructural requirements, and complexity of technology and implementation (i.e., number of visits required to effect treatment) are crucial in HPV screening (Cuzick et al., 2008).

HPV Screening techniques that are more appropriate to low resource settings are currently being investigated as opposed to the conventional cytology based methods (Sankaranarayanan et al., 1998). As an alternative to the cytology based detection tests several other tests have been developed. Among these the widely used method is visual inspection of cervix. This is performed in two approaches i.e; visual inspection with acetic acid (VIA) and visual inspection with Lugol’s iodine (VILI). The first method, VIA is also called as direct visual inspection (DVI) or colposcopy, in this method cervix is examined visually using bright light after one min application of 3-5% acetic acid using cotton swab. If aceto white areas observed near to squamocolumnar junction (SCJ) then indicates test is positive. Aceto-whitening is thought to be due to reversible coagulation of intercellular proteins followed by acetic acid application. The second visual inspection approach, in which cervix is examined visually after application of Lugol’s iodine also known as VILI. If the test is positive mustard-yellow areas on the cervix is observed. The
advantage of visual inspection approach is that it gives immediate result for treatment.

In addition, ineffective screening programs to identify lesions at a precancerous, treatable stage leads to the high incidence of cancer in the developing world. In the developing countries like India, few screening strategies have shown population level effectiveness over the long term. Indeed, effective HPV prophylactic vaccination in these populations would result in the greatest impact on the global cervical cancer burden.

2.14.2. Human papilloma virus Vaccines

Two different strategies may be employed for the development of vaccines against HPV based cancers and these are prophylactic and therapeutic vaccines.

2.14.2.1. Prophylactic Vaccines

Currently, two prophylactic HPV vaccines have been developed commercially (Harper et al., 2004; Villa et al., 2005; Harper et al., 2006; Garland et al., 2007). One of the vaccine is quadrivalent (HPV 6,11, 16 and 18) Gardasil® (produced by Merck and Co.) and another is bivalent (HPV16 and 18) Cervarix® (produced by GlaxoSmithKline). In these prophylactic vaccines HPV major capsid protein, L1 are presented in the form of virus like particles (VLPs) expressed either in Saccharomyces (for Gardasil®) or in insect cells, SF9 cells (for Cervarix®). VLPs do not contain
oncogenic viral DNA, thus they are noninfectious and non-oncogenic. They resemble virions and elicit virus-neutralizing antibodies (Kirnbauer et al., 1992), the prerequisite for effective prophylactic vaccines against most other viruses (Zinkernagel, 2003). These two vaccines are recommended for vaccinating young adolescent women at or before onset of puberty. Both these vaccines are administered by intramuscular route, in three doses (0, 1 or 2 and 6 months). The clinical trial data of both the vaccines showed near 100% seroconversion in the different populations. The Peak geometric mean antibody titers (GMTs) elicited by these vaccine were almost 50 - 100 fold higher than that of the natural antibody titers. (Villa et al., 2006).

In addition, a reduction in the incidence of the genital warts is observed in quadrivalent vaccines and a reduction in laryngeal papillomatosis also predicted among their children (Arbyn & Dillner, 2007). As a result, it is anticipated that a reduction in morbidity and mortality from HPV-related anogenital diseases may occur in populations who receive the available prophylactic vaccines. These VLP based HPV vaccines are obviously not therapeutic but the studies in animals and clinical trial data shows the induction of cell mediated immune response to L1. It therefore requires further investigations to verify the ability of these vaccines in the regression of HPV infection or genital lesions. The therapeutic nature of Cervarix was examined in an interim analysis of
the Costa Rican trial (Hildesheim et al., 2007) and trial indicates that the vaccine did not induce the clearance of genital infections of other HPV types. In the Future II trial, therapeutic activity of Gardasil was examined, no significant difference in the rate of CIN+ cases was observed in the vaccine vs. placebo control arm (11.1% vs. 11.9%; Ault, 2007). These studies confirm that HPV VLPs based vaccines does not show therapeutic activity.

The benefits of prophylactic vaccines in a broad public health perspective will be accomplished if these vaccines can reach to the women where the effective screening programs are absent.

2.14.2.2. Therapeutic vaccines

Therapeutic vaccines are administered to reduce or eradicate existing disease or infections by targeting cells expressing tumour-associated or tumour-specific antigens on their surface (Steller, 2002). Protein-based strategies for disease prevention require APC engulfment of exogenous proteins and the presentation of processed peptide fragments to T cells in a MHC-restricted manner (Steller, 2002). Endocytosis of denatured protein by APCs (consisting of a heterogeneous population of leukocytes including Langerhan cells, macrophages, B cells and dendritic cells) effectively induces CD8+ CTLs (Martinez-Kinader et al., 1995; Schirmbeck et al., 1995). The use of full-length E6 and E7 proteins for
therapeutic vaccines has the advantage of including all the putative immunogenic epitopes for every MHC haplotype, whereas peptides are attractive vaccine candidates because they can be synthesized in large quantities and are largely non-toxic. A variety of strategies have been used to design and test potential therapeutic vaccines: these include oncogenic protein and peptide-based vaccines, virus vector based vaccines, DNA vaccines and cell-based vaccines. Vaccination with the HPV16 E7 protein administered together with an adjuvant, PROVAX, protected mice against E7-transfected K1735 metastatic melanoma cell line C3 and elicited an E7-specific cytotoxic T lymphocyte response in the vaccinated mice. The effect of vaccination was abolished by depletion of CD8+ and CD4+ cells (Hariharan et al., 1998). A fusion protein vaccine comprising *Mycobacterium bovis* hsp65/HPV-16 E7 induced regression of E7 positive TC-1 tumours, which were obtained after co-transfection of murine lung cells with E6/E7 HPV-16 and activated Ha-ras DNA. This fusion protein conferred protection against tumour re-challenge and allowed long-term survival of the vaccinated mice (Chu et al., 2000). Vaccination with HPV-16 L2, E6 and E7 as a single fusion protein has been shown to elicit HPV-16-specific cytotoxic T lymphocytes, T-helper cells, and antibodies in a mouse model (van der Burg et al., 2001). Fernando et al. (1999) demonstrated that vaccination with HPV-16 E7
fused to glutathione-S-transferase protects mice against subsequent lethal challenge with an HPV16 E7 DNA-transfected C3 cell line.

Human cytotoxic T lymphocytes induced by using the HPV-16 E7 peptides 11-20 or 86-93 are capable of recognizing and lysing CaSki cells that contain integrated HPV-16 DNA (Alexander et al., 1996; Ressing et al., 2000; Steller, 2002).

2.14.2.3. Chimeric Vaccines

Chimeric vaccines are prepared by fusing the HPV early-gene polypeptides and C-terminal of either L1 or L2 proteins (Greenstone et al., 1998; Muller et al., 1997). Chimeric VLPs elicited significant amount of neutralizing antibodies and T cell response. In mice, single dose of chimeric VLPs without adjuvant protected them from challenge with E7-expressing syngeneic tumors (TC-1). However, effective CMI responses were not boosted in mice (Da Silva et al., 2001; Liu, 2003). As an alternative, combination of L1 VLPs with a successful therapeutic vaccine may be tried in future studies. VLP binding activates innate immune responses in several types of dendritic cells, B cells and monocytes (Lenz et al., 2001; Yang et al., 2005). Whether these responses would facilitate CMI responses against the therapeutic component of the vaccine need to be verified in future studies.
2.14.2.4. Second – Generation Vaccines

The Hallmark of the second-generation vaccine is to develop products that will be more suitable for low resource countries by producing affordable vaccine which is having long lasting immunity and could incorporate other HPV types (Aires et al., 2006; Sasagawa et al., 2005; Munoz et al., 2004). Development of a needle free vaccine with less number of shots may increase its availability to the lower section of women. Roden et al. (1997) demonstrated that the HPV16 pseudovirions are resistant to desiccation and this nature may be help to stabilize VLPs in powder formulation. Also, VLPs delivery through nasal route in aqueous solution is shown to be immunogenic in women (Nardelli-Haefliger et al., 2005).

HPVL2-based vaccines have the advantage of inducing cross neutralizing antibodies and L2 protein can be generated as polypeptides in E. coli (Pastrana et al., 2005). In theory, a single polypeptide immunogens might protect against all genital types and cutaneous types as well. However, to date the titers of type-specific antibodies induced by L2 immunogens have not approached the titers induced by VLP-based vaccines, both in several mouse studies (Kawana et al., 2001; Pastrana et al., 2005; Roden et al., 2000) and in one clinical trial (Kawana et al., 2003).
2.15. Production of HPV VLPs in different heterologous expression systems

2.15.1. Live viral vectors

Live recombinant vaccinia viruses have been used to express PV major (L1) and minor (L2) capsid proteins which form VLPs in mammalian cells (Volpers et al., 1994; Hagensee et al., 1993; Zhou et al., 1993) and also for delivery of therapeutic antigens (Davidson et al., 2001; Lamikanra et al., 2001). Recombinant vaccinia virus expressing modified E6 and E7 genes protected against subsequent tumour challenge in a variety of experimental systems (Boursnell et al., 1996; Gao et al., 1994).

The vaccinia virus system has been successfully used for large scale (1000L) production of different proteins such as HIV-1 rgp160 and human pro-thrombin by (Wurm & Bernard, 1999). The advantages of vaccinia virus systems are the wide host range of mammalian cells that can be infected, the high expression levels, as well as the ease of virus stock production, however, requires biosafety level 2 for production (Carroll & Moss, 1997). Trasfection of Vero cells with replication-competent recombinant Mahoney poliovirus DNA expressing HPV-16 L1 gene resulted in expression and assembly of the HPV-16 major capsid protein into VLPs (van Kuppeveld et al., 2002). Unfortunately immunization of mice with the recombinant virus induced a very weak
anti-HPV-16 L1 immune response. Recombinant polioviruses have used to express a variety of viral antigens, including HBV surface antigen and SIV proteins (Crotty et al., 2001; Yim et al., 1996).

Despite the numerous studies, including clinical trials, conducted using live recombinant viral vectors, no such vaccine candidate has progressed beyond the phase II trial, since profiles of the immune responses elicited were not considered ideal (Liljeqvist & Stahl, 1999).

2.15.2. E. coli based expression of HPV L1 protein

Expression of HPV L1 protein in E. coli has generally produced modest quantities of assembly-competent pentamers (Banks et al., 1987). Yields have been enhanced by using GST fusions and His6 tag expression. The purified HPV L1 protein was subsequently assembled into VLP using suitable buffers under proper ionic strengths (Chen et al., 2000).

2.15.3. Live bacterial vectors

HPV16 L1 expressed in attenuated Salmonella typhi strain (Ty21a), that has been proven to prevent Salmonella-induced intestinal disease and the mice studies were encouraging (Baud et al., 2004). Salmonella based approach may be explored as an attractive HPV vaccine. Oral delivery of a live recombinant bacterial vaccine could address the cost of the vaccine and ease of the delivery.
2.15.4. Plant based expression system

HPV 16 L1 and HPV 11 L1 proteins were expressed in tobacco and potato through the nuclear genome (Bimelt *et al.*, 2003; Varsani *et al.*, 2003). There are studies demonstrating that transplastomic expression of heterologous proteins achieve high levels of recombinant antigen compare to the transgenic plant expression (Lenzi *et al.*, 2008 and Fernandez- San Millan *et al.*, 2008). Plant expressed proteins are less likely to be contaminated with human pathogenic microbes and these studies show plant as an affordable alternate expression system. The limitation with plant expressed proteins is they are less immunogenic compare to other expression system produced proteins (Mason *et al.*, 1992).

2.15.5. Insect cell expression system

Recombinant baculovirus have been used extensively used for expressing HPV capsid proteins (Christensen *et al.*, 1994; Kirnbauer *et al.*, 1993; Rose *et al.*, 1993). Despite the high level of expression of recombinant proteins, the baculovirus system is relatively expensive. Baculovirus produced bivalent HPV16 and 18 (Cervarix®) prophylactic vaccine has successfully been introduced into the market.
2.15.6. HPV VLPs expression in *Saccharomyces cerevisiae*

HPV VLPs were expressed in the classic brewer’s yeast *S. cerevisiae* (Patel *et al.*, 2009). At present the quadrivalent HPV vaccine ‘Gardasil’ (HPV 16/18/6/11) expressed in *S. cerevisiae* successfully introduced into the market. The first yeast selected for production of heterologous eukaryotic proteins was *Saccharomyces cerevisiae*. The advantage of using *S. cerevisiae* as an expression system is ease of scaling up and affordable media components. They also generally provide an appropriate environment for eukaryotic post-translational processing and secretion, resulting in a product that is often identical or very similar to the native protein (Sudbery, 1996).

2.15.7. Expression of HPV VLPs in *Pichia pastoris* system

The first methyotrophic yeast was discovered in the 1969 and it was used first time in industrial production of single cell protein at Phillips Petroleum Company in 1970s. A strain of *Pichia pastoris* was selected based on very high cell mass yield, high protein content and stable fermentation characteristics (Hagenson, 1991). Being methyotrophic yeast, *P. pastoris* can grow on methanol as a sole carbon and energy source, and it has a highly inducible methanol utilization pathway. Alcohol oxidase accounts up to 35% of the total protein in cells grown on methanol, whereas it is very much reduced in cells grown on glucose,
glycerol or ethanol (Sreekrishna et al., 1997). Strain GS115 (containing \textit{his4} allele) is often used as expression host. This strain is mutant of Histidinol dehydrogenase gene that prevents from synthesizing Histidine and does not grow in minimal media in the absence of Histidine. Two types of vectors have been developed for heterologous protein expression in \textit{P. pastoris} and these are integrative and autonomous. Autonomous vectors replicate independently as episome, but they are generally of low copy number and unstable upon successive cultivation. Integrative vectors, which are generally stable and best suited for manufacturing scale operations. These vectors integrate into the host chromosomal DNA. Integrations can be site directed at the alcohol oxidase (AOX1) locus. When AOX 1 is knocked out due to integration of the vector AOX 2 gene controls the growth of \textit{P. pastoris} in the presence of methanol. AOX1 gene deleted clones show a slower growth phenotype ( Mut$^S$ Methanol utilization slow) on minimal methanol medium compared to AOX1 intact clones which have phenotypically normal growth characteristics ( Mut$^+$) (Sreekrishna \textit{et al.}, 1997).

\textbf{2.16. Codon bias on heterologous protein expression}

Codon optimization is a genetic tool used to achieve optimum expression of a heterologous gene in a host’s cell system is achieved by replacing existing codons with a set of more suitable host codons (Sandhu \textit{et al.}, 2008). Certain organisms are biased towards using certain sets of
codons. When inserting a gene into an organism, it is possible that the specific bias of the organism does not correlate with the gene or specific aminoacyl t-RNA genes may be absent altogether. This may have a significant impact on heterologous protein expression (Gustafsson et al., 2004).

A codon adaptation index (CAI) indicates the relationship or correlation between the codon usage of a gene and its expression level. A high CAI towards a certain codon does not necessarily means a gene will be expressed well in organism. Preferred codons correlate well with profusion of transfer (tRNA) in the cell content and this in turn optimizes the translational capacity of the organism. Alternatively, heterologous expression can be improved by supplying the host with extra copies of rare tRNA genes (Carstens, 2003). Leder et al. demonstrated that codon optimizing the HPV-16 L1 gene for expression in mammalian cells resulted in an increased efficacy of the L1 DNA vaccines (Leder et al. 2001). Therefore, codon optimizing the L1 or L2 gene towards Pichia codons may enhance the expression of HPVL1 or L2 proteins in P. pastoris.

It is clear from the review of literature that cervical cancer in Indian women is a significant public health concern. Amongst various approaches to manage the disease burden, use of VLP based prophylactic vaccine may be an attractive approach to consider.
Vaccinating large number of people requires high quality vaccine made available at a reasonable cost such that public health funding may be able to support such vaccination program.

We embarked on a study to establish proof of concept that may be translated to industrial process for producing VLP based prophylactic vaccine expressed in *Pichia pastoris*.

**The objectives of this study were as follows:**

1. Codon optimization of HPV 16 L1/L2 genes for *Pichia pastoris*

2. Cloning and expression of HPV 16 L1/L2 genes in *Pichia pastoris*

3. Purification and characterization of expressed proteins from *Pichia pastoris*. 