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Pseudovirions in the Study of Papilloma- and Polyomaviruses

Helena Faust

Doctoral Thesis



LUND
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Lund University, Medical Faculty, Laboratory Medicine, Medical Microbiology,
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papillomavirus type 32 pseudovirions by Helena Faust

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SUMMARY

The papilloma- and polyomaviruses are small DNA viruses that infect humans. Some members of these virus families can cause cancer in experimental animals and some are also established as carcinogenic to humans. Detection of specific antibodies to these viruses allows tracking past and present infections to elucidate natural history and association of infection with subsequent disease. As there are more than 150 types of Human papillomaviruses (HPV) and at least 10 Human polyomaviruses (HPyV), the study of antibodies to these virus families require high-throughput methods. Although it is difficult to produce infectious virus stocks of these viruses, so-called pseudovirions that morphologically and immunologically resemble native virions but lack the viral genome can be produced *in vitro*. In the present thesis, we produced such pseudovirions and used them to i) delineate the importance of hypervariable surface loops for the antigenicity and biological function of the HPV particle ii) developed and validate serological assays for measuring specific antibodies to HPV and Merkel cell polyomavirus (MCPyV) and iii) perform prospective seroepidemiological studies to evaluate whether infection with MCPyV was associated with an increased risk for Merkel cell carcinoma.

Site-directed mutagenesis of the surface loops of the HPV capsid found that these loops were essential for the incorporation of the minor capsid protein L2, the genome encapsidation and proper immunogenicity of the particle.

Pseudovirion-based methods were correlated to presence of viral DNA. The pseudovirion neutralization assays and multiplexed assays using pseudovirions bound to heparin-coated fluorescent beads for 21 HPV and 2 HPyV types were correlated with viral DNA for 16 HPV types and MCPyV. MCPyV specific antibody levels in serum were found to be strongly correlated to the MCPyV viral load in skin. Finally, biobank-based seroepidemiological studies found that MCPyV infection was associated with an increased risk for Merkel cell carcinoma (MCC), in particular among females.

LIST OF PAPERS

This thesis is based on the following six papers:

I Mutations in HPV-16 L1 hypervariable surface-exposed loops affect L2 binding and DNA encapsidation

Helena Faust and Joakim Dillner (Submitted)

II Validation of multiplexed Human papillomavirus serology using pseudovirions bound to heparin-coated beads

Helena Faust, Paul Knekt, Ola Forslund and Joakim Dillner

Journal of General Virology (2010), 91, 1840-1848

III Comparison of three serological methods for Human papillomaviruses (HPV) in relation to presence of HPV in skin

Helena Faust, Kristin Andersson, Ola Forslund and Joakim Dillner (Submitted)

IV Serum antibodies to Human papillomavirus (HPV) pseudovirions correlate with natural infection for 13 genital HPV types

Helena Faust, Mateja Jelen, Mario Poljak, Irena Klavs, Veronika Učakar and Joakim Dillner (Submitted)

V Antibodies to Merkel cell polyomavirus correlate to presence of viral DNA in skin

Helena Faust, Diana Pastrana, Christopher Buck, Joakim Dillner, Johanna Ekström

Journal of Infectious Diseases (2011), 203, 1096-1100

VI Prospective study of Merkel cell polyomavirus and risk of Merkel cell carcinoma

Helena Faust, Kristin Andersson, Johanna Ekström, Maria Hortlund, Trude Eid Robsahm and Joakim Dillner (Submitted)

ABBREVIATIONS

Aa	Amino acid
Ab	Antibody
AK	Actinic keratosis
BCC	Basal cell carcinoma
bp	base pair
CI	Confidence intervals
cLIA	Competitive Luminex immunoassay
CTL	Cytotoxic T lymphocyte
ds	Double stranded
ELISA	Enzyme– linked immunoabsorbent assay
EV	<i>Epidermodysplasia verruciformis</i>
GST	Glutathion-S-transferase
HPV	Human papillomavirus
HPyV	Human polyomavirus
HR	High risk
Ig	Immunoglobulin
KIPyV	Karolinska Institute polyomavirus
LCR	Long control region
LR	Low risk
LT	Large T antigen
mAb	Monoclonal antibody
MCC	Merkel cell carcinoma
MCPyV	Merkel cell polyomavirus
MFI	Mean fluorescence intensity unit
MPyV	Murine polyomavirus
MWPyV	Malawi polyomavirus
OR	Odds ratios
PCR	Polyomerase chain reaction
PE	Phycoerythrin
PML	Multifocal leukoencephalopathy
PsV	Pseudovirion
SCC	Squamous cell carcinoma
SEAP	Secreted alkaline phosphatase
ST	Small T antigen
SV40	Simian vacuolating virus 40
TS	<i>Trichodysplasia spinulosa</i>

VLP	Virus like particle
WT	Wild type
WUPyV	Washington University polyomavirus

INTRODUCTION

HISTORY OF THE SMALL DNA TUMOUR VIRUSES

Polyomaviruses and papillomaviruses are both non-enveloped double stranded DNA (dsDNA) viruses that were discovered about 60 years ago. Previously, they were classified into the *Papovaviridae* family. As their sequences are not related, they were separated into two distinct families (*Papillomaviridae* and *Polyomaviridae*) in 2001 (1). Recorded papillomavirus research began in the late nineteenth century with a description of experimental transmission of warts between humans. By the early twentieth century the viral nature of the transmissible agent was established (reviewed in (2)). The history of DNA tumour viruses started with a discovery that the Cottontail rabbit papillomavirus could induce malignant tumours (3). Further studies with animal models expanded the knowledge of the basic biology of papillomaviruses until the 1950s, when papillomavirus research froze in technical struggle. It became clear that papillomaviruses could not be propagated in standard monolayer cell culture system and mice-specific papillomaviruses were not yet discovered. Also, papillomaviruses in humans were believed not to have any medical importance, hence research interest was focused towards the recently discovered polyomaviruses (4). Murine polyomavirus became the archetypal member of the polyomavirus family as it is easy to propagate *in vitro* and causes cancer in small rodent system (5). In 1960, the Simian vacuolating virus 40 (SV40) was discovered in rhesus monkey kidney cells used for the production of polio vaccines (6). As SV40 is able to cause tumours in experimental animals, it became a public health concern. This led to extensive studies of SV40 biology and epidemiology that has overall not found SV40 to be associated with human cancer (reviewed in (7)).

The renaissance for papillomavirus research started in the 1970s, when recombinant DNA technology was born. Early sequencing techniques revealed the existence of multiple HPV types. Soon, the medical importance of HPV was discovered as HPV was found in malignant lesions of *epidermodysplasia verruciformis* patients (8). Also, a sexually transmitted agent as a cause for uterine cervix cancer was proven, when zur Hausen and colleagues uncovered the existence of HPV-16 and -18 in cervical tumours (9). Their work was recognized with a Nobel Prize in Medicine in 2008 (reviewed in (10)) and lead to the extensive studies of HPV as an aetiological factor

for human cancers and to the construction of HPV preventive vaccines and HPV-based screening methods. Recently, a possible importance of Human polyomaviruses in human cancers was identified when Merkel cell polyomavirus (MCPyV or MCV) was found in most of biopsies of a rare neuroendocrine skin cancer called Merkel cell carcinoma (MCC) (11). With the emergence of modern sequencing technology, both the papilloma- and polyomavirus families have been expanding rapidly. High throughput technologies are needed to help elucidate the epidemiology of these viruses and their possible role in human diseases.

PAPILLOMAVIRUSES

Classification

Papillomaviruses are icosahedral non-enveloped circular dsDNA viruses that infect a wide range of vertebrate hosts. They are host specific and infect stratified squamous and cutaneous epithelia. Papillomaviruses are grouped into genus, species, types, subtypes and variants based on the DNA-sequence similarity of the major capsid protein, encoded by the L1 gene (12). HPVs are currently divided into 159 distinct types and the list of new types continues to expand (13) (Fig. 1). They are divided into two groups depending on the target (the mucosa or the skin) of the infection. Mucosal types belong mainly to the genus *alpha* whereas skin infecting cutaneous types can be found mainly in the genera *beta*, *gamma*, *mu*, *nu* and some also in *alpha* (Fig. 1). The mucosal types are grouped depending on their ability to induce tumours as high risk (HR) and low risk (LR) HPV types (14). Most likely the HR types have a common ancestor as they cluster together if their early genes are used to create a phylogenetic tree (15). Carcinogenicity of HR HPV is at least partly explainable with functional differences of the oncoproteins E6 and E7 among HR and LR types (reviewed in (16)).

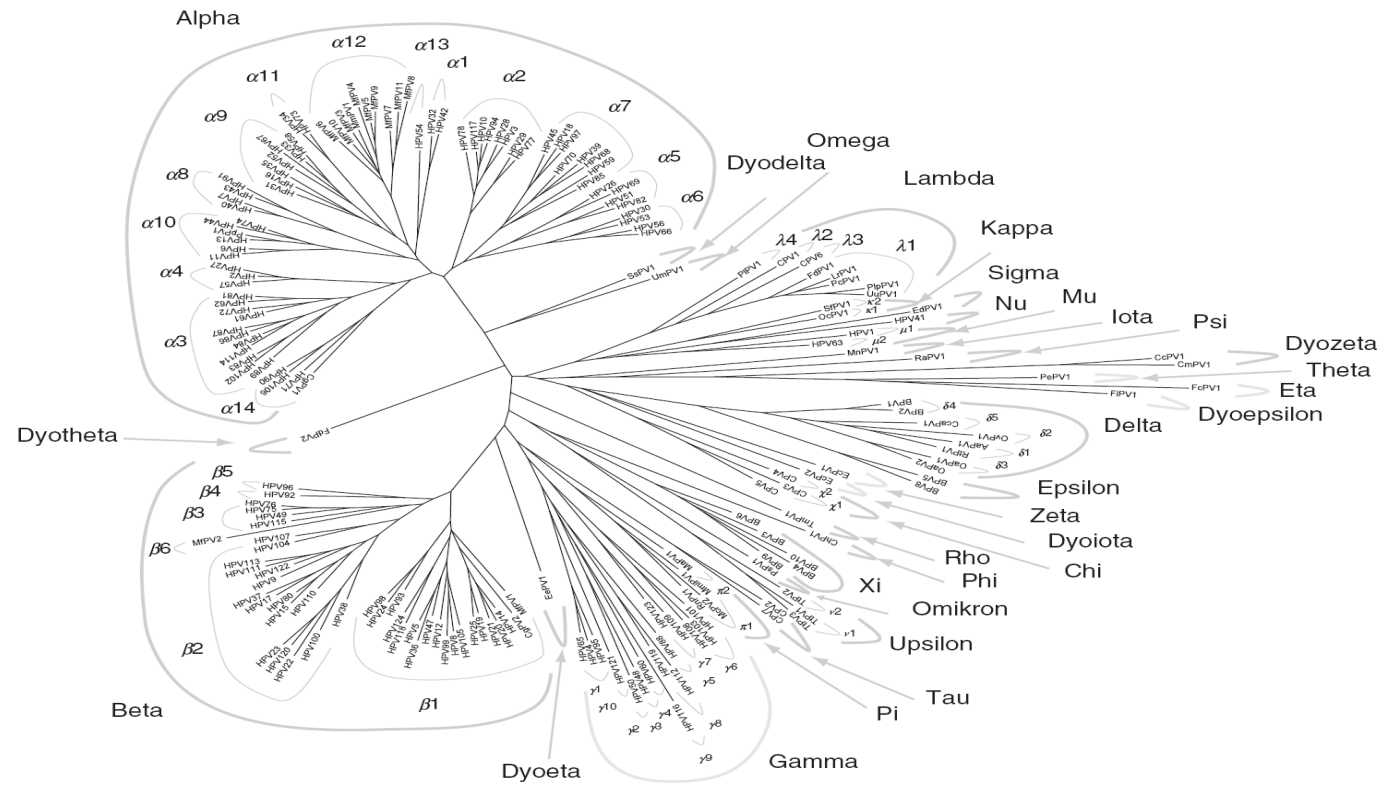


Figure 1. Current *Papillomaviridae* phylogenetic tree by Van Doorslaer et al. Evolution of Human papillomavirus carcinogenicity, in *Advances of Virus Research* 2010;77:41-62 , with permission from Elsevier. *Alpha* 7 and 9 contain HR, *alpha* 10 LR and *beta* 1,2,3 studied cutaneous HPV.

Morphology and genomic organization

The HPV virion has a diameter of about 55 nm and consists of 72 L1 capsomeres, which are arranged on a T=7 icosahedral lattice (17). The minor protein L2 is positioned inside of every capsomere (18). Over-expression of L1 in artificial expression systems leads to spontaneous formation of HPV virus-like particle (VLP) (19-22). These L1-containing VLPs can to some extent encapsidate DNA and infect cells (23), but both of these processes are significantly increased when L2 is also present in the virus particle (24). L1 and L2 together can co-assemble spontaneously and take up DNA without any help of cellular chaperons (reviewed in (2)). On the virion surface both linear and conformational epitopes have been identified (25). L1 loops extending towards the outer surface of the capsomere contain type specific epitopes, where neutralizing antibodies can bind (26, 27). Predicted 3D model of the L1:L2 interactions indicates that L1 surface loops interact with L2 proline-rich regions (28).

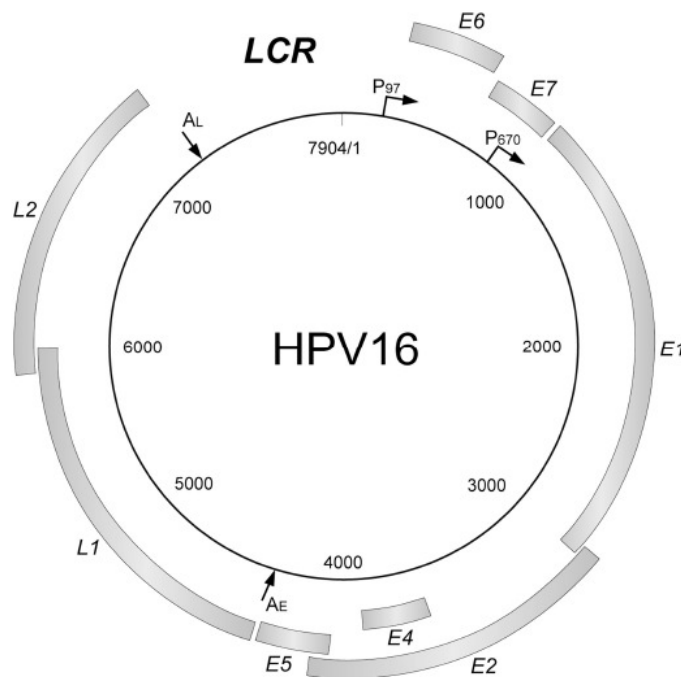


Figure 2. HPV-16 genomic organization by Kajitani et al. Productive Lifecycle of Human papillomaviruses that Depends Upon Squamous Epithelial Differentiation, in *Frontiers in Microbiology* 2012;3:152, open access. HPV genome is circular dsDNA. The viral genes are transcribed clockwise. There are genes coding non-structural proteins (E) and structural proteins (L). LCR contains a DNA replication origin. The major promoters (p) and polyadenylation signals (A) are indicated.

HPV virions contain circular dsDNA of about 7900 bp genome which is associated to cellular histones to form a chromatin like structure (29, 30). The genome codes for eight proteins (E1, E2, E4, E5, E6, E7, L1 and L2) on the same sense strand and carries an upstream regulatory region, also known as the long control region (LCR) (Fig. 2). The genes are named based on their expression during epithelial differentiation (early (E) or late (L)). LCR contains the origin of replication and enhancer elements for gene expression regulation (reviewed in (31)).

Viral proteins

The functions of each viral protein and their known cellular partners are summarized in Table 1(32). In short, helicase E1 and E2 are cooperatively involved in the initiation of viral DNA replication and episomal maintenance. E2 helps E1 to bind to LCR, which recruits host replication factors to the viral replication origin (31). E2 also functions as a transcriptional transactivator (33). E6 and E7 modulate the cell cycle control of host cells and induce cell cycle re-entry to replicate viral DNA (34). This could immortalize cells and uncontrolled cell cycle progression could lead to cancer development. Therefore E6 and E7 are called oncoproteins. E6 and E7 contribute also to viral genome maintenance (35). Though E4 and E5 are speculated to modulate the productive phase of the HPV life cycle, their biological roles remain unclear (36-38). L1 is called the major capsid protein and L2 the minor one as the ratio between these in mature virion is about 5:1. L2 also seems to have a role as an agent to encapsidate viral DNA into virion and taking part in the infectious events (2).

Table 1. Summary of HPV protein functions by Kaijtani et al. Productive Lifecycle of Human papillomaviruses that Depends Upon Squamous Epithelial Differentiation, in *Frontiers in Microbiology* 2012;3:152, open access, with added information about L1 and L2 functions by the thesis author.

Function in viral lifecycle	Activities	Target factor
E1		
Replication of viral genome	DNA-binding activity, helicase activity, ATPase	RPA, topoisomerase, polymerase alpha-primase
E2		
Transcription of viral genes		
Replication of viral genome	Transactivation/transrepression, DNA-binding activity, DNA segregation in mitotic cell	Brd4, ChIR1
Maintenance of viral genome		
E6		
Reactivation of cellular replication mechanisms		
Proliferation, immortalization, inhibition of apoptosis	Interaction with various cellular proteins	p53, ADA3, p300/CBP, E6AP, SP1, c-Myc, NFX1-91, TERT, FAK, FADD, Caspase 8, BAX, BAK, IRF3, PDZ domain proteins
Maintenance of viral genome		
E7		
Reactivation of cellular replication mechanisms		
Proliferation, genomic instability, inhibition of apoptosis	Interaction with various cellular proteins	RB, p107, p130, HDAC, E2F6, p21, p27, CDK/cyclin, ATM, ATR, gamma-tubulin
Maintenance of viral genome		
E4		
Unknown	Destruction of keratin network, induction of G ₂ M arrest of cell cycle	Cytokeratin 8/18
E5		
Possibly involved in proliferation and/or inhibition of apoptosis	Affection of cellular signaling pathway	EGFR, PDGFR, V-ATPase, MHC1, TRAIL receptor, FAS receptor
L1		
Major capsid protein	Shell for virus genome	
L2		
Minor capsid protein	DNA encapsidation, infectious delivery of virus genome	

Virus life cycle

The life cycle of HPV is tightly regulated by the differentiation program of the host cells (Fig. 3). Papillomaviruses have evolved to exploit the biology of keratinocytes, which form the surface of the epithelium. It is thought that the entire keratinocyte differentiation pathway takes about 2 weeks. The viral life cycle can be broadly divided into three stages: (1) virion penetration of the epithelial surface and infection of basal cells by delivering viral DNA into nucleus; (2) establishment and maintenance of the viral episome; (3) production and release of virions (2).

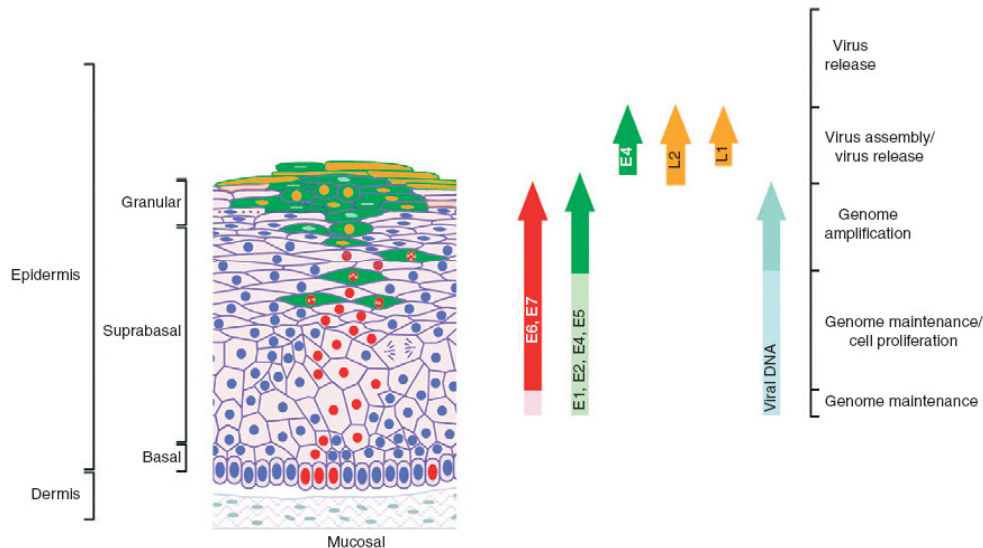


Figure 3. HPV life cycle in epithelium by Van Doorslaer et al. Evolution of Human papillomavirus carcinogenicity, in *Advances of Virus Research* 2010;77:41-62, with permission from Elsevier. The arrows indicate protein's expression, while the colors represent the amounts expressed. Cells with red nuclei are progressing through the cell cycle. Green cells with red nuclei represent viral genome replication. Cells containing amplified viral DNA (yellow) begin expressing structural proteins. The green/yellow cells contain infectious virus particles ready to be shed to environment from naturally dying cells.

It is generally believed, that disruption (micro wound) of the epithelium is needed before papillomavirus can reach the basal cells. According to the current knowledge, papillomavirus attaches to the basal membrane underlying the basal cells after physical damage of the epithelium (39) and binds to heparan sulfate proteoglycans by major protein L1 (39-41). This induces conformational changes exposing a site on L2 susceptible to proprotein convertase (furine) cleavage (39, 42). After L2 cleavage, an L2 neutralizing epitope is exposed and a previously unexposed region of L1 binds to an unidentified secondary receptor on the basal keratinocytes. Then the virus enters the cell via an endocytic pathway and within 4 hours localizes in endosome. By 12 hours, the virus uncoats within endosome and viral genome in complex with L2 is released. The L2-genome complex traffics through the cytoplasm and enters the nucleus by 24 hours. After the nucleus entry, RNA transcription begins (reviewed by (43)).

Following viral entry into host cell, HPV genome is maintained at a low-copy number in the basal cells (50-100 copies per cell) (44). As HPV does not encode DNA polymerase activity for viral genome replication, the host DNA replication machinery is required. The incoming viral genome expresses very low levels of viral early genes (45, 46). Collectively, these viral proteins (E1, E2, E5, E6, and E7) drive the basal keratinocytes to divide and stimulate low levels of replication. It is believed

that papillomavirus can persist in basal cells of the host as expression of only trace amount of the viral proteins does not induce immune-mediated clearance (2).

Keratinocyte differentiation triggers a shift in the behaviour of the virus. The p97 promoter directs expression of E6 and E7 genes necessary for cell cycle S-phase entry (47). In the middle layer of the epidermis, the viral genome is amplified to much higher copy numbers (thousands of viral genomes per cell) (44) and expression of early genes is accelerated. After the viral genome amplification has been completed, two structural proteins are expressed (48). Synthesis of L2 is followed by L1 in E4 expressing cells (49). Infectious particles are assembled in the upper layers of the epithelium (50). As the expression of the highly immunogenic viral capsid proteins L1 and L2 occurs only in the outermost layers of the epidermis, the virus is shielded from immune surveillance and thus gets the chance to reproduce. Assembled virions are shed into the environment through the normal process of desquamation without triggering the pro-inflammatory signals (2).

Immune response to HPV

The exclusively intra-epithelial life cycle of HPVs is central to understanding the host response. Viral life cycle is linked to differentiation programme of the keratinocytes and virus particles are released in naturally dying cells, which do not alert the innate immunity. Therefore adaptive immune responses to HPV are prevalent. Adaptive immune response consists of cell mediated and humoral immunity (51) (Fig. 4).

Adaptive immune responses

Cell mediated immunity (Th1)

Th1 CD4⁺ T cells help:

- Killer T cells CD8⁺ and CD4⁺
- Activated macrophages*
- Cytokines*
- Innate lymphocytes*
 - iNKT cells, NK cells, $\gamma\delta$ T cells

Humoral immunity (Th2)

Th2 CD4⁺ T cells help B cells

- make antibodies
- make antibody
 - IgM, IgG, IgA, IgE

Figure 4. Adaptive immune response to HPV by Stanley et al. Potential mechanisms for HPV vaccine-induced long-term protection, in *Gynecologic Oncology* 2010;118:S2-7, with permission from Elsevier. * - Innate effectors enhanced and activated by adaptive responses.

Cell mediated immunity (Th1)

There are large amounts of CD4+ and CD8+ T cells and macrophages in regressing genital warts (52) and they express pro-inflammatory cytokines (53). CD4+ response directed to E2 and E6 is a hallmark of effective immune control over HPV infection (54) as it appears in regressing cervical disease and is not seen in persistent infections (55-57). CD8+ cellular immune responses to E7 are found in patients with cervical cancer, though these are unable to clear the infection (58). Cell mediated cytotoxicity is the most important effector mechanism for the control and clearance of viral infections. It implements antigen specific cytotoxic T lymphocytes (CTL) and “innate lymphocytes” like natural killer cells enhanced and activated by adaptive responses. HPV specific CTLs (both CD4+ and CD8+) (59) are mainly targeted to E6 (60), but also to L1 (61).

Humoral immune responses

The most immunogenic component of papillomaviruses is the major capsid protein L1. The infection with HPV elicits antibodies against the viral capsid. The major isotypes of serum antibodies against HPV capsids are IgG1 and IgA (62). IgA is a marker for recent or active infection as it appears for short time when HPV DNA is also detected (62). IgG1 is stable over a long time period even after clearance of the virus (63). IgG seroconversion takes more than 6 months and some individuals never respond with antibodies at detectable level (63-67). IgGs directed to the capsid is likely a measure of past or present persisting HPV infection, as a persisting HPV infection elicits these more effectively than transient infections (66, 67). The minor capsid protein L2 may also elicit some antibodies during natural infection (68, 69), but detection of these antibodies are difficult as L2 is displayed only after virion binding to the cell surface (70). For early proteins, the most consistent antibody response is seen in cervical cancer patients and is directed to E7 (71). Elevated levels of E1, E2 and E7 specific antibodies are also detected in HPV-16 positive head-and neck cancer patients (72).

A controversial issue is whether naturally acquired low-levels of anti-L1 antibodies protect against re-infection with the same HPV type. At least in some animal systems, HPV is not cleared when lesions regress, but may persist in apparently healthy skin (73). The new detection of HPV DNA of the same type in sero-positive individual may therefore reflect increased viral load of an already present virus rather than re-infection (reviewed in (74)).

Vaccine induced immunity

Two prophylactic HPV vaccines based on L1 VLPs are currently licensed: the quadrivalent (HPV-16, -18, -6, -11) Gardasil® and the bivalent (HPV-16, -18) Cervarix™. Both vaccines provide durable protection against infection with the incorporated HPV types and associated disease (75, 76). Mathematical modelling of

antibody decay suggests that detectable antibody could persist up to 50 years (75). VLP-s are highly immunogenic: peak antibody levels are up to 100-fold higher than natural infection elicit and after 1.5 years, the mean levels remain up to 16-fold higher than the typical levels seen after natural infection (77). This difference in antibody levels is explained by accessibility of the immune system to the antigen. VLP vaccines are delivered by injection via intramuscular route, with immediate access to the vasculature and lymphatic system. Vaccines encounter stromal dendritic cells which are strongly activated to mature. These cells migrate to the draining lymph nodes and initiate T-cell responses (78). VLP-s bound to local immunocytes are shuttled into lymph node to encounter and prime naïve B cells (79).

HPV VLP vaccines elicit neutralizing antibodies and these protect against new HPV infection (51). Virus neutralizing antibodies most likely bind to the capsid and prevent binding to the cells or disturb viral entry (80). These neutralizing antibodies are mainly IgG, as IgG enzyme-linked immunoabsorbent assay (ELISA) and neutralization assays agree well (81). Immunization with HPV VLPs predominantly produces type-specific virus neutralizing antibodies, with only low levels of cross-reactive antibodies against related types being seen. In spite of this, cross-protection against these other, related HPV types is observed. The phenomenon suggests that only low levels of antibodies are needed for protection (82).

HPV associated diseases

Mucosal HPV infections

Infection with HPV is the most common sexually transmitted infection worldwide (83). Most of the HPV infections are transient, as viral DNA is detectable only in short time, and are usually acquired just a few years after sexual debut (84, 85). Persistence of HR HPV over long time periods is the most important risk factor for development of malignancies (51, 85). Approximately 10% of the HPV infected women fail to clear the infection (86). HR HPV infections are causally related to cancer and pre-cancerous lesions of the cervix, vagina, vulva, anal canal, penis and oropharynx (87). HPV is detectable in virtually 100% of cervical cancer cases (88). HPV-16 is the most common type and combined with HPV-18 account for over 70% of all cases of cervical cancer (89). Cervical cancer is the third most common cancer in women worldwide and the second most common in developing regions (90). The early discovery of the disease ensures better survival rates. Randomised controlled trials have shown that HPV-based screening is more effective than cytology-based cervical screening (Pap smear) (91). LR HPV types HPV-6 and HPV-11 are responsible for development of genital warts and recurrent respiratory papillomatosis (92). HPV prophylactic vaccines will be the main solution to stop appearance of HPV-related diseases.

Cutaneous HPV infections

Common warts are usually caused by HPV types -1, -2 or -4 while also other genotypes are found among immunosuppressed patients (93). Warts are very common among primary school children, 33% being affected (94), commonly located on hands and sole of foot and regress spontaneously (93). Plane warts are also common among children, but can occasionally occur among adult women. These are caused mainly by HPV-3 and -10 and typically located on face, dorsum of the hands and the shins (93).

Epidermodysplasia verruciformis (EV) is a chronic genetically inherited skin condition that arises when susceptible individuals are infected with certain specific HPV types (95). Individuals with primary immunodeficiency caused by mutations in the long arm of chromosome 17 are susceptible for infection with HPVs (96). In these patients, HPV-5 and -8 infections often lead to development of skin malignancies like invasive squamous cell carcinoma (SCC) in sun-exposed skin (96, 97).

Non-melanoma skin cancer tissue commonly contains cutaneous HPV infections (98, 99). A common “smoking gun” trait of cancers with a well-documented viral etiology is their increased incidence in immunocompromised individuals (100). This is attributable to impaired immunological control over otherwise harmless infections. Risk of non-melanoma skin cancers, including cutaneous SCC, is increased after immunosuppression, with a more than 50-fold increase in incidence among recipients of solid-organ transplants. It has therefore long been suspected that SCC may be caused by an infectious agent and research has been focused on cutaneous HPVs (98). However, transcriptome sequencing has not detected HPV RNAs in cutaneous SCC (101). Serology studies have demonstrated a slight, but statistically significant association between HPV and cutaneous SCC, but these studies are all carried out with serology based on glutathione-S-transferase fused L1 protein (GST-L1) Luminex, a method which does not correlate well with natural infection (102-104) and is further discussed in the following chapter.

HPV serology methods

HPV serology is used for both seroepidemiology and vaccinology studies. While seroepidemiology focuses on natural infections and on elucidating biology, natural history and disease associations of infections (105), vaccinology focuses on immunogenicity testing in relation to vaccination (81). Therefore, the methods applied are dependent on the purpose of the testing. Seroepidemiology studies follow the footprints of the natural infection as antibodies are hallmarks for past and present infections. Seroepidemiology needs sensitive methods with coverage of multiple HPV types. Immunogenicity testing before and after vaccination focuses on vaccine included and related, cross-reactive HPV types and is particularly interested in the

levels of protective (neutralizing) antibodies. Antibodies are induced after the immunization and different methods can detect these (Fig. 5).

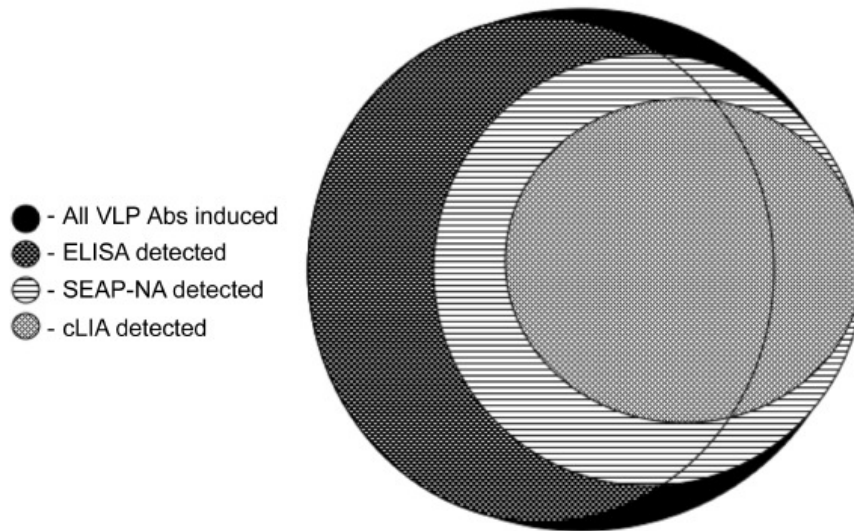


Figure 5. Relationship among VLP antibodies detected by different serology methods as these can detect subgroups out of all induced antibodies. SEAP-NA- Secreted alkaline phosphatase neutralization assay, cLIA- competitive Luminex immunoassay. Reprinted from Schiller et al. Immunogenicity testing in Human papillomavirus virus-like-particle vaccine trials in *Journal of Infectious diseases* 2009;200:166-71, with permission from Oxford University Press.

Enzyme-linked immunosorbent assay (ELISA)

ELISA is historically the oldest method applied in HPV serology and most seroepidemiological studies have used it (106) making it a “golden standard” method. Also GlaxoSmithKline has used VLP-based ELISA as the principal assay of immunogenicity in its bivalent vaccine Cervarix™ trials (77). ELISA measures antibodies that bind to antigen fixed to a solid surface. Most widely used antigens are L1 VLPs or L1/L2 VLPs (106), but also GST-L1 fusion protein capsomeres have been used (107). To ensure that only correctly folded VLPs bind to the solid surface, the surface can be coated with heparin first (108) as interaction with heparin requires intact conformation of VLPs (40, 109). The bound antibodies are detected by the addition of a secondary antibody that recognizes the constant region of a human antibody of a specific class (e.g., IgG). The secondary antibody has an enzyme (e.g., alkaline phosphatase) conjugated to it, and the enzyme’s activity is monitored by a change in the substrate (e.g., a colour change that can be measured in a spectrophotometer) (81). VLP ELISA is sensitive, rapid, and reproducible; therefore it is suitable also in low-resource settings and can be automated. Correlations across

laboratories can be increased by reporting results to the responses to type-specific standard antisera (110). The drawback of ELISA is that it is laborious and requires large amount of resources (serum and VLP) if antibodies to multiple HPV in multiple samples are intended to be tested. Also, ELISA specificity is dependent on the quality of the antigen used and if low-quality VLPs are used also cross-reactive antibodies between related HPVs may be detected.

High-throughput serology assays

Classical ELISA can be transformed to a high-throughput method using multiplexing and a Luminex analyzer. The purpose of this is to measure antibodies to multiple HPV types simultaneously. This saves both time and material if large numbers of serum samples need to be tested. The multiplex method uses beads with distinct fluorescent properties as solid surface to carry one type of antigen on it. Up to hundreds of different beads with distinct wavelength coated with different antigens can be used at the same time in the same assay-well. The Luminex system is the most widely used system. This is a cytometer-like analyzer with two lasers, one detecting the identity of the bead and the other one detecting the intensity of fluorescent secondary antibody (Fig. 6).

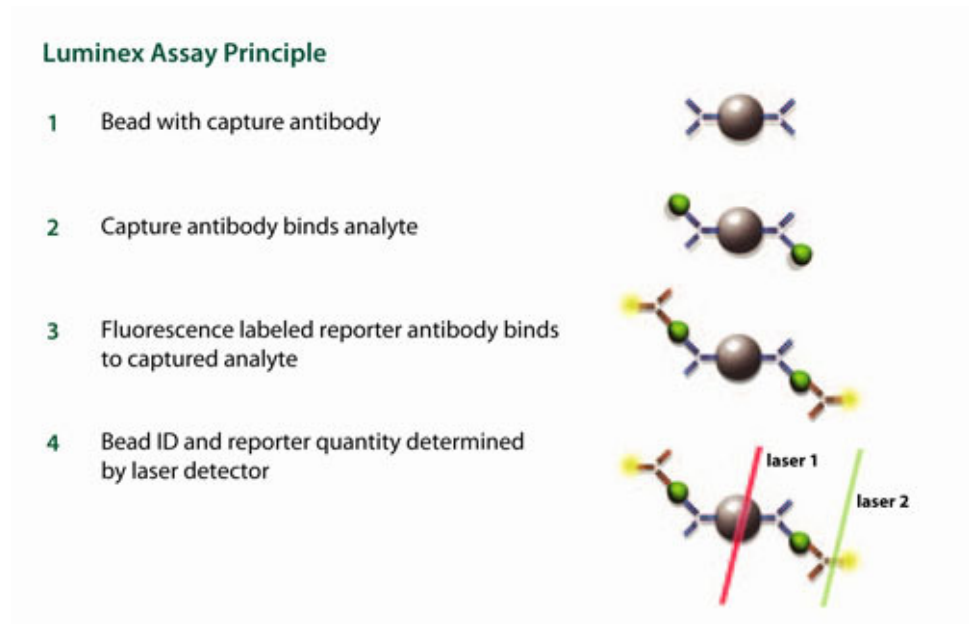


Figure 6. The Luminex assay principle.

Competitive Luminex immunoassay (cLIA)

This method was developed to measure immunogenicity after vaccination with the quadrivalent HPV vaccine Gardasil® in clinical trials (111). Serum samples from the vaccines are evaluated for their ability to prevent VLP binding by a type-specific neutralizing monoclonal antibody that has a phycoerythrin fluorescent tag (Fig. 7). Therefore, the strength of the antibody response is inversely proportional to the detection of the monoclonal antibody binding signal. This method is suitable for vaccinology since it is based on non cross-reacting monoclonal antibodies and is highly type-specific. The major disadvantage of the assay is that it measures only the subset of neutralizing antibodies that compete with the specific monoclonal antibody for VLP surface binding. Thus, this assay can under represent the potentially protective antibody responses induced by vaccine (81). Therefore Merck later developed a direct binding IgG assay using Luminex and VLPs of HPV types -6, -11, -16, -18, -31, -33, -45, -52 and -58 (112) for use in clinical trials of a nonavalent vaccine.

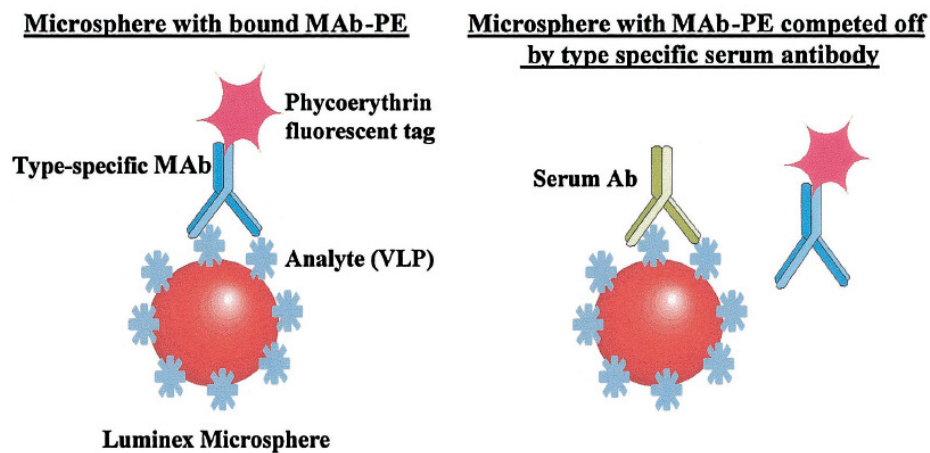


Figure 7. Competitive Luminex assay principle. Reprint from Opalka et al. Simultaneous quantitation of antibodies to neutralizing epitopes on virus-like particles for human papillomavirus types 6, 11, 16, and 18 by a multiplexed luminex assay, in *Clinical and Diagnostic Laboratory Immunology* 2003;10:108-15, with permission from American Society of Microbiology

GST-L1 fusion protein Luminex

This method was developed Dr. Michael Pawlita's laboratory (German Cancer Research Centre, Heidelberg) to cover as many HPV types as possible to include in seroepidemiology studies. It uses *in situ* affinity purified glutathione S-transferase (GST) fusion proteins produced in bacteria as antigens (Fig. 8A) (113). The production and purification of such proteins is not laborious. To date, the method

includes L1 proteins of 37 cutaneous HPV (114), but it has been developed also to detect mucosal HPVs (107, 113, 115). This method has been widely applied for investigating associations between cutaneous HPV and non-melanoma skin cancer (116). Unfortunately, several studies have been showing limited concordance between HPV DNA presence and antibodies to the same type detected with this method (102-104). Possibly, the antibodies binding to GST-L1 fusion protein are not exactly the same that would bind to the correctly folded virus capsid. Because of limited data on whether antibodies detected by this method do reflect HPV infection, interpreting results obtained with this method is difficult.

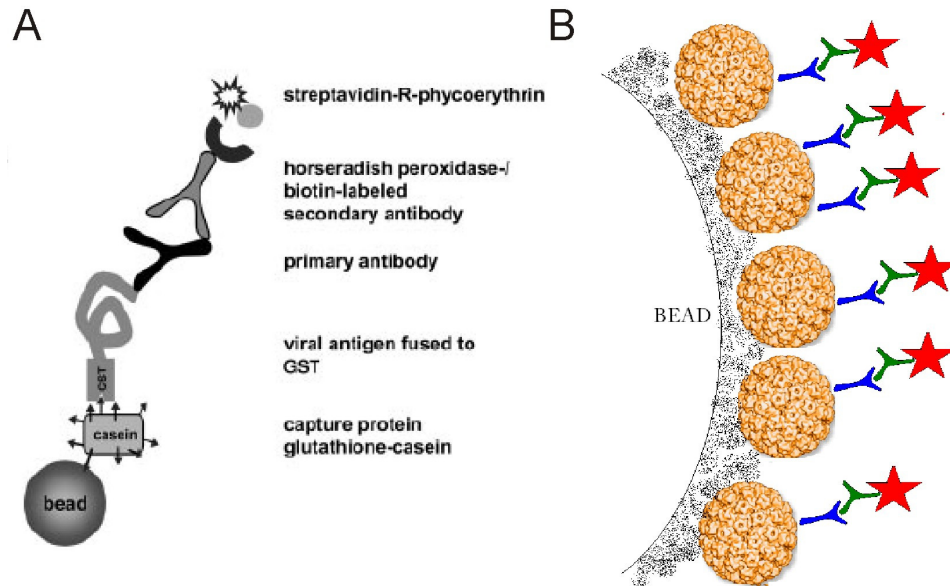


Figure 8. Principles of two high-throughput serology methods. A. GST-L1 Luminex by Waterboer et al. Multiplex Human papillomavirus serology based on in situ-purified glutathione s-transferase fusion proteins, in *Clinical Chemistry* 2005;51:1845-53., with permission from American Association of Clinical Chemistry. B. heparin-pseudovirion Luminex by Helena Faust. Shaded area- heparin, light brown ball- pseudovirion, blue- serum antibody, green- anti-human IgG, red- secondary antibody conjugated with streptavidin-R-phycoerythrin.

Heparin-pseudovirion Luminex

This thesis has developed a non-commercial high-throughput HPV serology method. The aim was to elevate both sensitivity and specificity of the HPV serology assay. At the same time it should be high-throughput, cover multiple HPV types (both mucosal and cutaneous) and should be validated for correlation with natural infection. This method is based on high quality antigen (mammalian derived pseudovirions), which mimic the real virus particle as they contain both L1 and L2 proteins (Fig. 8B). To ensure that only the correctly folded capsids bind to the

Luminex beads, the beads are first coated with heparin. Naturally, heparin is secreted by mast cells and interferes with HPV-pseudo-infection and has been shown to efficiently bind VLPs (40, 41). The interaction with heparin requires intact conformation of VLPs (40, 109). The results from the initial studies presented in this thesis are promising, demonstrating the validity of the assay (117). The drawback of the method is the complicated re-design of L1 and L2 genes. As L1 and L2 expression in the nature is strictly confined to differentiated epithelial cells and the expression is very limited using natural DNA sequences of these genes in cell culture system. Therefore codon-modifications which do not change the primary amino acid sequence of the proteins, but remove negative regulatory elements from the DNA sequence are essential to ensure a large increase in capsid protein production in cell culture (118). Re-designing genes is time consuming and chemical gene synthesis is expensive. Therefore collaboration between study groups interested in new types of pseudovirions is preferred, as was done in the current thesis.

Neutralization assay

This is an *in vitro* cell culture assay, where (pseudo) virions are mixed together with serum and incubated to enable binding of serum antibodies to the virions. Thereafter the virion-serum mixture is seeded to cell culture. If the serum contained neutralizing antibodies, these antibodies block the virion entry into cells or prevent uncoating. Neutralization is monitored by a signal expressed from the pseudogenome of the pseudovirion (Fig. 9). The first reproducible quantitative neutralization assay that is not limited by availability of infectious capsid is widely used now (119). It involves the cell culture production of high-titer infectious L1/L2 pseudovirions that have encapsidated a gene whose activity easily can be measured as a marker of infection. In the most widely employed variant, the pseudovirus carries the gene for secreted alkaline phosphatase (SEAP). The major disadvantage of the neutralization assay is that it is the most laborious method compared with ELISA or Luminex assays. However, the results of the neutralization assay are the most likely to correlate with protection of HPV challenge, because it presumably measures all neutralizing antibodies regardless of immunoglobulin class and only detects potentially protective antibodies (81). Strong correlation between individual ELISA and neutralizing-antibody titers has been observed in trials of the bivalent vaccine (120). Nevertheless, the ELISA may somewhat overestimate the protective antibody response (generate false positive results) if an individual predominantly generates a response to non neutralizing epitopes (81). Cross-neutralization detected in the neutralization assays appears to correlate with biologically significant cross-protective response (121).

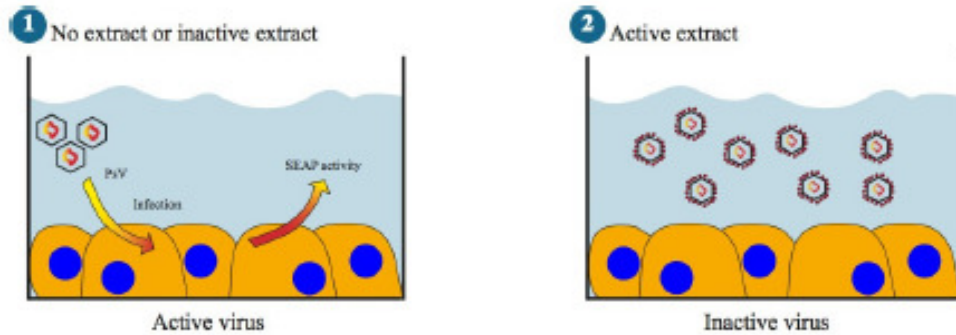


Figure 9. Principles of the neutralization assay. Reprinted from Biodiscovery@UQ by Ian Frazer with permission from Robert J. Capon. On the left slide, there was no neutralizing antibodies to block viral entry into cells and secreted alkaline phosphatase is secreted into cell-culture medium. On the right panel, the pseudovirions are blocked by neutralizing antibodies.

POLYOMAVIRUSES

Classification

The family name *Polyomaviridae* derives from the observation that the first discovered member of this family (Murine polyomavirus) could induce multiple (latin *poly*) tumours (latin *oma*) in mice. The polyomaviruses infect vertebrate hosts and have been found in mammals and birds. To date, genome sequences of 30 polyomaviruses have been deposited in GenBank, including 10 species infecting humans (Fig. 10) (122). The first Human polyomaviruses (HPyV) BK and JC, named by the patients initials, were isolated four decades ago from the urine of a kidney transplant recipient and from the brain of a Hodgkin lymphoma patient, respectively (123, 124). These viruses are now abbreviated as BKPyV and JCPyV according to the recommendations of the International Committee on Taxonomy of Viruses (125). Multiple new viruses were found following advances in DNA sequencing techniques. In 2007, the Karolinska Institute (KI) and Washington University (WU) polyomaviruses (KIPyV and WUPyV) were found in nasopharyngeal aspirates and named by the initials of the universities where they were first detected (126, 127). In 2008, the Merkel cell polyomavirus (MCPyV) was discovered in Merkel cell carcinoma transcriptomes (11). HPyV-6 and HPyV-7 were found in skin swabs of healthy individuals in 2010 (128). In the same year, *trichodysplasia spinulosa* (TS) associated polyomavirus (TSPyV) was found in a boy suffering from the rare skin disease TS (129). To date, the latest described HPyVs are HPyV-9, and HPyV-10 which were found in serum from a kidney transplant patient (130) and in skin specimens of a patient with a rare genetic disorder known as myelokathexis syndrome, respectively (131). One more possible

Human polyomavirus was identified in human stool samples and named Malawi polyomavirus (MWPyV) as it originated from a child from Malawi (132). HPyV-10 and MWPyV might be the same virus according to Buck *et al.* as sequence similarity of these is 95-99 % (131).

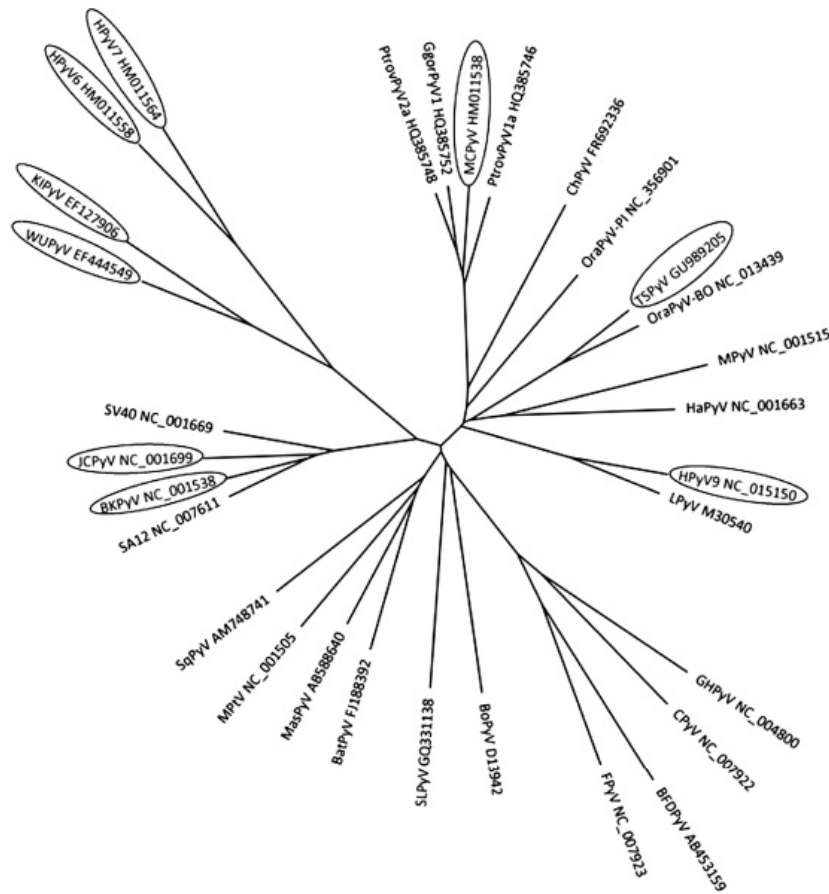


Figure 10. *Polyomaviridae* phylogenetic tree including 9 HPyVs (highlighted). Reprinted from Van Ghelue et al. Genome analysis of the new Human polyomaviruses, in *Reviews in Medical Virology* 2012, with permission from Wiley. The phylogenetic tree was constructed before the discovery of HPyV-10 and MWPyV.

Morphology, genomic organization and viral proteins

The genomes of polyomaviruses are around 5 kb. They exist as circular dsDNA closely associated with cellular histones, and are packaged into chromatins, resembling cellular genomes (minichromosomes), within nonenveloped 40-45 nm

icosahedral capsids composed of the structural proteins VP1-3. The viral capsid is composed of 72 pentamers of the major capsid protein VP1 that contacts 72 copies of the minor capsid proteins VP2/3. The genome is divided almost evenly into an early and a late region encoded on opposite strands. These two regions are separated by a noncoding regulatory region containing origin of replication and transcriptional control elements (Fig. 11). Early region encodes at least two proteins Large T (LT) and Small T (ST) proteins. LT is a multifunctional nuclear phosphoprotein with genetically separable functions in promoting viral DNA replication by forming a complex with the cellular replication proteins, unwinding the dsDNA as having helicase activity and stimulating cell-cycle progression into S- phase. LT-induced cell-cycle progression is the main contributor to oncogenic transformation. ST stimulates viral DNA replication. Without ST, polyomaviruses are viable, but grow slowly and are less productive. The late region encodes 3 to 4 capsid proteins VP (7).

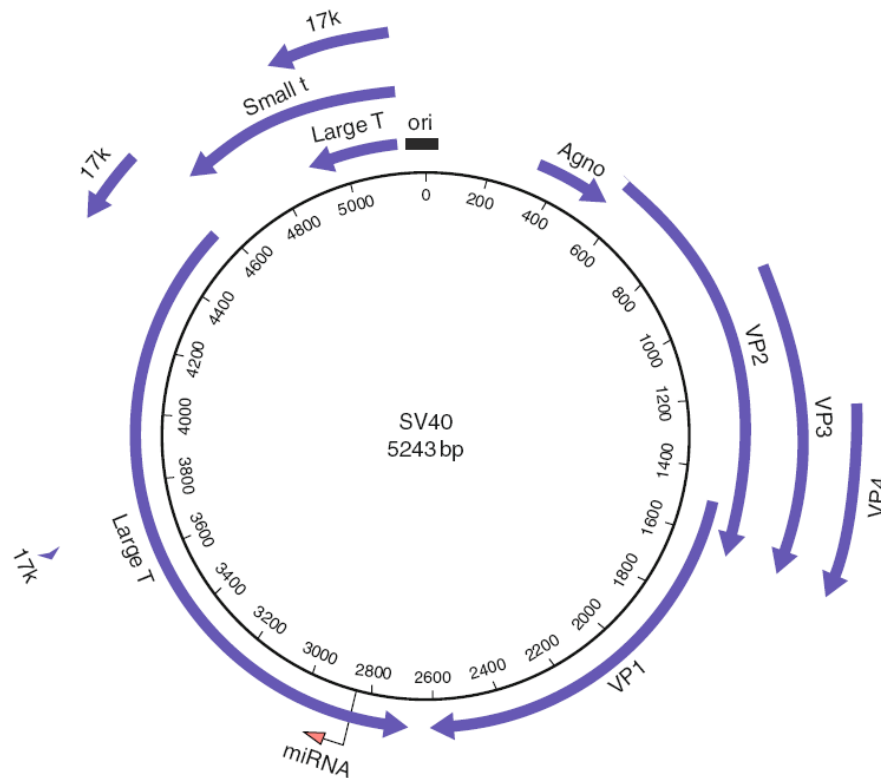


Figure 11. Genomic organisation of SV40 as an example of a well studied polyomavirus. Reprinted from Gjoerup et al. Update on Human polyomaviruses and cancer, in *Advances in Cancer Research* 2010;106:1-51, with permission from Elsevier. The early region of the viral genome (the left half) encodes LT, ST and 17k differential splicing. The late region (the right half) encodes agnoprotein and structural proteins (VP). Gene products are generated by differential splicing and internal translation.

Virus life cycle

In human specific species, only the life cycles of JCPyV and BKPyV are well described so far. In the lytic cycle, virus attaches onto the cell surface via receptors like gangliosids and the serotonin receptor (133-135). Virus then traffics through caveolae and the endoplasmatic reticulum to the nucleus, where it is uncoated and the early message is transcribed. In the case of JCPyV, clathrin-dependent endocytosis precedes localization in cavesomes (136). After translation, LT initiates DNA replication of the viral genome. The shift to late viral protein expression is not fully clear, but likely involves LT transcriptional activation of the late and repression of the early promoter (7). Capsid proteins self assemble around the replicated genome to form new mature virions, which are released by a mechanism involving cell lysis (137).

Cell-transforming properties are well studied on the model polyomaviruses SV40 and murine polyomavirus (MPyV), which can induce tumours in rodents. Transformation is a characteristic of a failed lytic infection, either because the host cell is not permissive for replication or because the infecting virus is defective in replicative functions. Viral DNA integrates into the host genome randomly and free MPyV and SV40 viruses cannot be isolated from tumour material (137).

Natural infection of HPyV and associated diseases

All described HPyV are highly prevalent in the human population and infection starts during childhood. Seroprevalences are measured by IgG antibodies specific to major capsid protein V1 and peak at 92% for BKPyV, 80% for JCPyV, 67% for KIPyV, 98% for WUPyV, 69% for HPyV-6, 35% for HPyV-7, more than 60% for MCPyV, around 70% for TSPyV (138-144) and 47% for HPyV-9 (145) in the general population. Faecal-oral, oral and respiratory routes of transmission have been proposed (7). Stable JCPyV and BKPyV can be found in sewage samples and rivers. DNA of HPyVs has been detected in tonsillar tissue, a possible point of entry. It is likely that hematolymphoid cells can carry or harbour polyomaviruses as during host immunosuppression, prevalence of these viruses is increased in blood and lymphoid tissues (7).

The exact mode of potentially carcinogenic Merkel cell polyomavirus (MCPyV) transmission is also unclear. Intrauterine transmission does not appear to occur, as no MCPyV DNA was detectable in miscarried or aborted fetuses (146). However, it is clear that MCPyV infection occurs already in early childhood (140, 147, 148). Kean et al reported a 20.5% MCPyV seroprevalence among 1-5 year old children (140), Tolstov et al a 43% MCPyV seroprevalence among children 2-5 years (148) whereas we have found a 32% MCPyV seroprevalence among Swedish children ≤ 12 years (Faust *et al*, Paper IV). Early school age seems to be an age for of MCPyV antibodies

to appear (seroconversion). Chen et al. found that while children 1-4 years of age had a seroprevalence of 9%, the seroprevalence was 35% among children age 4-13 years of age. Also, 33% of MCPyV seronegative children at the age up to 3 years were found to have seroconverted when tested again 5-8 years later (147). Rates of acquisition appear to be substantial also among adults. In a cohort of 117 MCPyV-seronegative males, 31 seroconverted over a 4-year follow-up period, corresponding to a 6.6% annual seroconversion rate. Once seroconversion has taken place, the MCPyV IgG levels remain detectable up to 25 years after the exposure. No signs, symptoms, or routine diagnostic test results were associated with MCV infection, indicating that the primary infection is mostly asymptomatic (149).

MCPyV DNA is common on the human skin (128, 150) and frequent presence of MCPyV DNA on environmental surfaces that has been in contact with human skin suggest that virus may be shed from the surface of infected skin. In a study of 60 environmental surface samples, 45 (75.0%) were positive for MCPyV DNA and in a few of these samples the viral DNA was even protected from DNase degradation, suggesting that it represented viral DNA encapsidated inside infectious virus particles (151). Apart from the skin, MCPyV DNA has also been found at high levels in the upper aerodigestive tract, in the digestive system and in saliva, but was less frequently found in lung and genitourinary system samples (152). However, MCPyV has been found in the lower respiratory tract (153) as well as on the tonsils (147). Presence of MCPyV DNA appears to accumulate with age, being more frequent in adults than in children (154). MCPyV can also persist in inflammatory monocytes and spread along the monocyte migration routes (155). MCPyV has also been found in the lymphatic system (156).

BKPyV is considered as nephrotropic and is associated with urinary tract pathologies in transplant patients (157). BKPyV-associated nephropathy occurs in 2-5% of renal transplant patients with graft loss in nearly half of these cases. Also, with hematopoietic stem cell transplantation hemorrhagic cystitis, hematuria and renal impairment are seen. Viral reactivation during these complications is robust and can be monitored (158, 159). JCPyV is neurotropic and is the etiological agent of progressive multifocal leukoencephalopathy (PML) in immunosuppressed patients (157). PML is an acquired demyelinating disease caused by JCPyV reactivation in the central nervous system and JCPyV can be detected in the cerebral spinal fluid (160). Lately, JCPyV was suggested to be associated with male infertility as infertile male semen contained JCPyV DNA in 25% of the cases compared to 11% in controls (161). Both JCPyV and BKPyV can act as transforming viruses in rodents and *in vitro* cell culture models, but no consistent association with human cancers has been found (7). KIPyV and WUPyV have not yet been found to be associated with human disease, although both of these viruses were originally detected in respiratory samples of symptomatic children (162). Lately, high prevalence and high viral load of TSPyV DNA has been found in TS lesions, implying a tight relation between TSPyV

infection and TS disease (163). HPyVs- 6, -7, -9, -10 and MWPyV have not been associated with any human disease so far.

Merkel cell polyomavirus association with Merkel cell carcinoma

Merkel cell carcinoma (MCC) is a rare but highly aggressive neuroendocrine skin malignancy that affects elderly and immunosuppressed individuals. The incidence of primary MCC in the United States is 3.4 per million person-years (164). The number of MCC cases has increased about 3- fold during the past 20 years, due to the aging population (165, 166). In Denmark, the MCC incidence between 1995 and 2006 was 2.2 cases per million person-years (167) and in Japan it was 1.45 per million (168). The incidence rate of MCC in Denmark has increased 5.4 fold over the 18 year period from 1986 until 2003 (169). There is a strong association between MCC and white/fair-skinned individuals, advanced age and sun exposure. At least in Scandinavia, MCC occurs more often among females than males (167, 170). MCC incidence is increased in both AIDS and organ transplant populations. The disease tends to develop at a younger age in these populations (164). The prognosis is poor and dependent on stage at diagnosis, with 5-year survival rates of patients with localized, regional and distant disease reported to be 64%, 39% and 18% respectively (171). Clinically, MCC typically presents as a fast growing, nontender, red to violet papule or nodule on sun-exposed areas of the skin, especially in the head and neck region. As also other skin malignancies display such features, histological examination of biopsy specimens is required for the diagnosis (172). MCC is not only a skin malignancy, but can appear also in numerous other sites such as lymph nodes, oral cavity, breast, vaginal walls, and salivary glands. Also extracutaneous MCCs harbour polyomavirus DNA (173).

MCPyV was described first time in 2008 by Feng et al who found a previously unidentified Human polyomavirus in 8 of 10 of MCC tumours (11). The discovery of MCPyV was made using digital transcriptome subtraction, i.e. bioinformatics subtraction of transcripts found in control tissue from transcripts found in case tissue (174). Moreover, MCPyV was demonstrated to be monoclonally integrated in the human genome in MCC (11). A carcinogenic pathway of MCC in which MCPyV undergoes a 2-step process has been proposed: random viral integration into the host genome and then a mutation in the LT region that eliminates viral replication capacity but retains Rb-binding capacity (Fig. 12) (175, 176). MCPyV is widespread around the world and its genome is stable as the presence of the virus in MCC has been confirmed by several independent research groups (reviewed in (175)). On the other hand, MCPyV is also very commonly found on healthy human skin all over the body (128) although viral loads outside of the MCC tumor are more than 60 times lower (152). Different studies have attempted to link MCPyV also to other human diseases, with suggestive evidence implying that it may be involved also in a small subset of chronic lymphocytic leukemia (177).

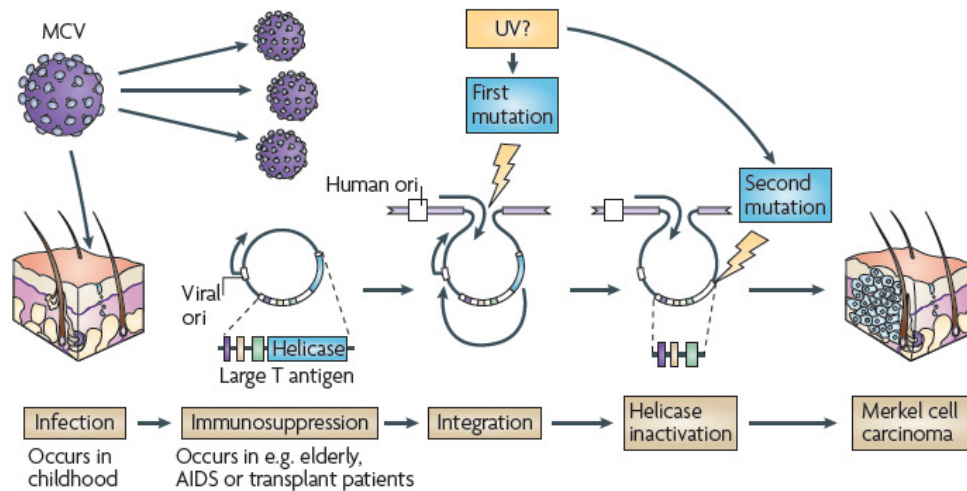


Figure 12. Development of MCPyV-MCC. Reprinted from Moore et al. Why do viruses cause cancer? Highlights of the first century of human tumour virology, in *Nature Rev Cancer* 2012;10:878-89, with permission from Nature Publishing Group.

Although a majority of the population has been infected with MCPyV, the incidences of MCC remains very rare, implying that other events in addition to infection with MCPyV (e.g. UV-exposure or immunosuppression) may be essential for the development of MCC. Patients with MCPyV-positive MCC generally have higher levels of serum IgG antibodies specific for the MCPyV virion than do healthy individuals (148, 178, 179) and elevated MCPyV virion antibodies are detectable many years before the MCC tumor is diagnosed (Faust *et al*, Paper VI). Presence of high MCPyV specific antibody levels is strongly associated with high MCPyV viral loads (139, 180), suggesting that a persistent presence of a high viral load MCPyV infection is an important step on the carcinogenic pathway (148, 181). Serum IgG levels against the MCPyV T antigens are elevated in 40.5% of patients with MCC, but the levels of antibodies to the T antigen appear to follow the extent of the growing tumour (182). This implies that antibodies to the T antigen are more an effect of the tumor than a marker of tumor etiology, but MCPyV T antigen antibodies could represent a clinically useful tumor marker for predicting disease status. In contrast, the MCPyV-neutralizing antibodies to the virion are, especially in females, elevated already decades before the tumor arises, indicating that the neutralizing antibodies are markers of an etiologic factor on the causal pathway to the MCC tumor (Faust *et al*, Paper VI).

It is clear that not all MCC cases contain MCPyV DNA, suggesting that MCC may exist as 2 etiologically distinct subtypes: virus-related and virus-unrelated (175). MCPyV -negative MCC have more irregular nuclei ($P < .001$) and more abundant cytoplasm ($P = .001$) than MCPyV-positive MCC, which have uniform, round nuclei and scant cytoplasm (183). MCPyV -positive and -negative MCCs may also have

different prognosis. MCC patients with high viral load MCPyV DNA have a better prognosis than MCC tumors with little or no viral DNA (181, 184, 185). However, two reports did not find any association between MCPyV status and prognosis of MCC (186, 187).

Therapeutics against MCPyV associated cancer and prophylactic vaccines are under development. MCPyV infection activates the *BIRC5* gene, which encodes the survivin oncoprotein, and is highly upregulated by LT sequestration of Rb. A small molecule survivin inhibitor (YM155) has been tried as therapy for MCPyV-MCC and found to prolong survival of mice bearing MCC tumours (188). A candidate prophylactic vaccine based on MCPyV virus-like particles (VLPs) has been found to, in mice, elicit antibody responses that robustly neutralize MCPyV reporter vectors *in vitro*, suggesting that a VLP-based vaccine could be effective for preventing MCPyV infection (178). There is also a candidate therapeutic DNA vaccine containing the MCPyV LTgene (aa1-258; pcDNA3-LT) that has been found to generate antitumor effects against a transplantable MCPyV LT carrying murine melanoma cell line in vaccinated C57BL/6 mice (189).

In just five years since the discovery of MCPyV, intensive research has established a basic knowledge regarding the epidemiology, immunology and pathogenesis of the virus and has clarified that this virus has an etiological role in a majority of MCC cases. The list of infections known to cause cancer in man has been continuously expanding and the MCPyV demonstrates that modern research technology now can enable discovery, validation and basic characterization in just a few years.

PRESENT STUDIES

AIMS

Paper I

To investigate the importance of hypervariable residues positioned in major capsid protein L1 surface-exposed loops for the immunogenicity of HPV-16 and for the L1 interaction with minor capsid protein L2.

Paper II

To develop and validate a high-throughput Human papillomavirus (HPV) serology based on Luminex technology, using pseudovirions of eight mucosal and two cutaneous HPV types bound to heparin-coated beads.

Paper III

To compare seroreactivity to HPV antigens measured with two different high-throughput assays (GST- L1 fusion protein Luminex and heparin-pseudovirion Luminex) and neutralization assay and the correlation of these with HPV infection in skin.

Paper IV

To expand the HPV heparin-pseudovirion Luminex assay to cover 21 HPV types and validated the method by correlating the HPV antibodies with the presence of HPV DNA in cervical samples from a population-based cohort in Slovenia.

Paper V

To apply the heparin-pseudovirion Luminex method to Human polyomaviruses Merkel cell polyomavirus (MCPyV) and JCPyV and validate whether MCPyV serology correlates with MCPyV infection in skin.

Paper VI

To perform a prospective study on the risk of future Merkel cell carcinoma in relation to baseline seropositivity to MCPyV.

MATERIALS AND METHODS

Pseudovirions (PsV) (190) were used in all studies included in the present thesis. The pseudovirion production method is based on transfection of a 293TT cell line originating from human embryonic kidney, which is engineered to express high levels of SV40 T antigen. The cells are co-transfected with codon-modified capsid genes with or without pseudogenome plasmid containing the SV40 origin of replication. Because expression of papillomavirus capsid genes L1 and L2 is normally very low in cultured mammalian cells, these genes must be modified extensively to overcome negative regulatory features of the wild-type open reading frames. These codon changes do not change the primary amino acid sequence of the proteins but lead to a large increase in capsid protein production. The genes must be chemically synthesized and cloned into an expression vector. Efficient purification of the PsVs is achieved by Optiprep (iodixanol) density gradient ultracentrifugation. The majority of the pseudovirion expression constructs utilized in the present thesis originates from collaborators (n=15), but an important part (n=7) were designed in-house by the author. Mainly, the pseudovirions were used as antigens in high-throughput pseudovirion-heparin Luminex serology assays or in ELISA, but also utilized in neutralization assays and as models to investigate the effects of the mutations in L1 and used as immunogens in animal experiments.

The present thesis describes development, expansion and validation of the pseudovirion-heparin Luminex serology method. The most important method validation step was the comparison of antibody detection with natural viral infections to ensure reliability of the test. Method development started with 10 HPV types (Paper II), then was extended also for Human polyomaviruses (V) and expanded to cover 16 HPV types (III) and finally (IV) detected antibodies to 21 HPV types (HPV -3, -5, -6, -11, -15, -16, -18, -31, -32, -33, -35, -38, -39, -45, -52, -56, -58, -59, -68, -73, -76) and 2 HPyV types (MCPyV and JCPyV). Results obtained with pseudovirion-heparin Luminex were also compared with results obtained with other serological methods (ELISA, GST-L1 Luminex and neutralization assay).

Paper I

Ten amino acid substitutions were made in HPV-16 L1 gene. The mutated L1 DNA sequence (hereafter called V5⁻) was chemically synthesized and cloned into wild type (WT) HPV-16 pseudovirion expression construct. Three different HPV-16 capsids variants (WTL1L2, V5⁻L1WTL2 and V5⁻L1 without L2) were produced with and without reporter plasmid. Reduced capsid samples were analysed on SDS-Page and Western blot assay using anti-HPV-16 L1 and L2 monospecific antipeptide antibodies and the density of the protein bands quantified. DNA was extracted from pseudovirions using ProteinaseK treatment and reporter gene specific PCR was performed to investigate the DNA encapsidation ability of the pseudovirions. ELISA with monoclonal antibodies and antipeptide antibodies was performed to investigate

the conformational changes in the mutated pseudovirions compared to the wild type ones.

To investigate immunogenicity of the pseudovirions, mice were injected with pseudovirions with adjuvant and after 2 immunizations, blood was collected. Immunogenicity testing was performed using ELISA and pseudovirion neutralization assays. Serums from immunized mice were diluted serially up to 10^5 fold in the ELISA and up to 10^4 in the neutralization assay. Pseudovirions with encapsidated secreted alkaline phosphatase (SEAP) expressing reporter plasmid as pseudogenome were utilized in the neutralization assay as described by Pastrana *et al.* (119). Spleens of immunized mice were collected after termination for cytotoxic T lymphocyte (CTL) response testing. Lymphocytes were isolated from spleens, pooled and co-cultured with stimulator cells (RMA-S). T-lymphocytes were separated and specific cytolytic activity was determined by CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturers' recommendations using C3 cells (containing HPV-16 genome) as target cells. Lactate dehydrogenase content in cell culture medium of co-cultured target/effector cells (C3/T-lymphocytes) was measured as absorbance at 490 nm. The level of cytotoxicity was calculated following the manufacturer's formula.

Paper II

Expression constructs for HPV-5, -6, -11, -16, -18, -31, -45, -52 and -58 pseudovirions were received from Dr. John Schiller's laboratory (National Cancer Institute, Bethesda, United States of America). Construct for HPV-38 was designed successfully in-house and HPV-38 pseudovirions did not differ in size or shape from the other HPV pseudovirions. The most suitable conditions for pseudovirion-heparin Luminex were established by titration of heparin and pseudovirion concentrations for each type.

Monoclonal antibodies and serum samples from subjects with known infection status were used to validate the established method. Monoclonal antibodies were used to ensure monospecific reactions with pseudovirions. Validation panels of serum samples came from five previously published studies. Positive control serum samples came from 130 cervical cancer patients from Latvia (191) and 124 cervical cancer patients from Mozambique (192) who all had PCR-verified HPV DNA status. The control serum panel for the cutaneous types HPV-5 and -38 were from 434 patients with known HPV DNA status in skin biopsies in a skin cancer study in Sweden and Austria (193). Sixty- nine samples from middle-aged Swedish women with self-reported *condylomata acuminata* history (194) were used as a positive control serum panel for HPV-6 and -11. A serum panel containing serum from 63 children with an average age of one and a half year (195) were analyzed as negative controls. In addition, samples from 71 middle age adult women with up to 1 lifetime sexual partner were analyzed as negative controls (196). After the method was established and validated, 208 serum samples from a biobank-based, nested case-control study of

cervical cancer (197) were analyzed. The study population comprised 18 814 women from the Finnish Mobile Clinic study and were followed for up to 23 years. During that time period 71 eligible cervical cancer cases occurred. Altogether 137 controls, individually matched for age and municipality, were selected (198). This study had previously been studied for antibodies to HPV-16 by a standard ELISA method (197) and these results were used to validate a cut-off for the heparin-pseudovirion Luminex method. Relative risks of cervical carcinoma were estimated by conditional logistic regression for matched sets of case-control triplets (one case, two controls) using SAS, version 9.2. Agreement between assays beyond the agreement expected by chance was quantified using *kappa* statistics.

Paper III

The heparin-pseudovirion Luminex serology was expanded by design and production of pseudovirions of HPV types -3, -15, -32, -33, -68 and -76. The extended method detected antibodies to 16 HPV types (HPV -3, -5, -6, -11, -15, -16, -18, -31, -32, -33, -38, -45, -52, -58, -68 and -76). The method was applied to dermatology clinic patients as the main interest in this study was to validate and compare serology for cutaneous HPV types.

Overall, 434 immunocompetent patients attending dermatology clinics in Sweden (n=400) or Austria (n=34) were included in this study (193). Seventy-two patients had squamous cell carcinoma (SCC) (mean age 80 years, range 50-94), 160 patients had basal cell carcinoma (BCC) (mean age 73 years, range 34-93) and 81 subjects actinic keratosis (AK, mean age 75 years, range 53-95). As hospital-based controls (N=121, mean age 71 years, range 29-97), we enrolled patients attending the same clinics who had non-malignant skin lesions, the most common being seborrhoeic keratosis (SK, N=62). All subjects donated a serum sample and two skin biopsies, one 2 mm biopsy from the lesion and another from healthy skin of the same patient, approximately 10-15 cm from the lesion. Before taking the biopsy, the skin was anesthetized and stripped with tape to avoid surface contaminations (199), as distinguishing true skin infection from skin surface contamination of virus merely deposited on the skin may otherwise be difficult. DNA was extracted as described (200).

HPV DNA status in skin biopsies and the data on HPV specific antibodies measured with GST-L1 Luminex method originated from a previous study (102). For six of the HPV types (HPV -5, -6, -15, -16, -32 and -38) comparison with the present heparin-pseudovirion Luminex data and GST-L1 method was possible. Information about HPV DNA status was available for 427 subjects and the most commonly detected HPV types were the *beta* types -5, -15, -38, and -76.

The pseudovirion neutralization assay for HPV -5, -38 and -76 was performed for all HPV DNA biopsy positive subjects (N=84) and for 120 randomly selected HPV DNA negative subjects. Neutralization assay protocol by Pastrana *et al* (119) was followed, using the final serum dilution of 1:50. Agreement of categorical values

between assays was quantified calculating Kappa values using GraphPad QuickCalcs online calculator. R^2 was calculated using Microsoft Excel to compare continuous data of GST-L1 and PsV Luminex assays.

Paper IV

The heparin-pseudovirion Luminex were further expanded by adding PsVs of HPV types -35, -39, -56, -59 and -73 to cover altogether 21 HPV types (-3, -5, -6, -11, -15, -16, -18, -31, -32, -33, -35, -38, -39, -45, -52, -56, -58, -59, -68, -73, -76) and 2 HPyVs (MCPyV, JCPyV). The method was scaled up to enable testing thousands of serum samples. As we intended to validate serology for genital HPV types, two negative control serum panels were included in the study: serum samples from 133 Swedish children aged up to 12 years with an average age of 6 (195) and 71 serum samples from Swedish women with reported up to 1 sexual partner during their life, with a mean age of 43 years (117, 196).

The study population samples originated from 3,321 women aged 20-64 years who participated in the ongoing routine National Cervical Cancer Screening Program ZORA in Slovenia (201, 202). Cervical samples were tested in parallel with Hybrid Capture 2 HPV DNA Test (hc2) and RealTime High Risk HPV Test. All samples with positive RealTime/hc2 results and 1,000 randomly selected samples were further analyzed with the Linear Array HPV Genotyping Test to detect 37 HPV types. Serum samples were tested with extended heparin-pseudovirion Luminex assay. To define seropositivity, cut-off values were calculated independently for each HPV type by analysing the mean fluorescence intensity (MFI) values obtained from 133 childrens' sera (under age of 12 years). The World Health Organization (WHO) HPV LabNet recommended algorithm (203) was applied: mean MFI value of negative Lab control serum panel plus 3 standard deviations. If the calculated cut-off value was less than 400 MFI, the 400 MFI cut-off was used. The new technological approach (freezing the beads and antigen in the same vial) improved the method's sensitivity and the minimum cut-off was raised from 250 MFI (117) to 400 MFI.

The odds ratios (OR) and 95 % confidence intervals (95 % CI) were estimated by single two-by-two tables using Statcalc of Epi-info version 3.5.1.

Paper V

Expression construct for Merkel cell polyomavirus pseudovirion was received from collaborator Dr. Christopher Buck (National Cancer Institute, Bethesda, United States of America). MCPyV-based reporter vector (pseudovirion) stocks were generated by transfection of 293TT cells with MCPyV VP1/2 expression plasmids pWm and ph2m, respectively, together with or without (for VLP production) a reporter plasmid, phGluc, as described elsewhere (178). JCPyV VLPs derived from *Saccharomyces cerevisiae* were a kind gift from K. Sasnauskas. These VLPs consist of the major capsid protein VP1 (204). For serology testing, MCPyV reporter vector stock carrying an encapsidated plasmid encoding a *Gaussia* luciferase reporter gene was used

to perform neutralization assays, as described (178) and both polyomaviruses were used as antigens in heparin-pseudovirion Luminex.

The study population was the same as described in Paper III (dermatology clinic patients from Sweden and Austria). Skin biopsies were tested for MCPyV DNA content and quantity. Primers and probes for qPCR were as described (205). Standard curves used serial dilutions from 100,000 copies to one copy of the purified MCPyV DNA plasmid pCR.MCV. The number of copies of cellular DNA in the sample was determined by qPCR for the β -globin gene (206). Epi-info version 3.5.1 estimated odds ratios (OR) and 95% confidence intervals (95% CI).

Paper VI

Two large biobanks (the Southern Sweden Microbiology Biobank and the Janus Biobank in Norway) that together contain samples from more than 850 000 donors were linked to the Cancer Registries in Sweden and Norway to identify Merkel cell carcinoma (MCC) cases. The case had to have donated serum samples to the biobanks prior to diagnosis.

Twenty-two MCC cases (9 males and 13 females) occurring up to 30 years after donation of serum samples and four matched controls were identified. Controls had to be free of skin cancer at time of the case diagnosis and were matched for age, sex, number of sampling occasions, and length of follow up and in the Janus biobank also for county. Mean age at MCC diagnosis was 70 years (range 47 to 91) and the oldest samples available for testing were obtained on average 12 years (range 1 to 26) before the cancer diagnosis. In total, time of follow up for cases was 247 years.

Serum samples (n=144) were analysed with MCPyV neutralization assay as described in Paper V and with high-throughput heparin-pseudovirion Luminex assay for IgG antibodies to MCPyV, using JCPyV and HPV types -3, -5, -6, -11, -15, -16, -18, -31, -32, -33, -38, -45, -52, -58, -68 and -76 as control antigens. Fourteen paraffin blocks with MCC tissue sample were retrieved for MCPyV DNA testing. Twelve out of these had histopathology confirmation that the sections made for DNA testing contained MCC tissue (6 epithelial MCC, 4 lymphatic MCC and 2 mixed tumour type). The tumor tissue sections were de-paraffinized with xylene and digested with ProteinaseK and were tested with quantitative PCR to estimate MCPyV viral loads as described in Paper V.

Prism 5 Software (Graphpad) was used to investigate agreement between the two serological methods. Two-tailed Spearman rank correlation coefficients and R square were obtained, along with the associated *P* value. Exact conditional logistic regression models were used to assess the relationship between virus antibodies and the risk of future MCC, using LogXact version 6 (Cytel Software). *P* values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Paper I

In this study the effects of amino acid (aa) substitutions in HPV-16 L1 surface loops to capsid formation and immunogenicity was examined. We found that intact L1 surface loops are important for proper L2 binding as both SDS-Page and Western Blot analysis showed that WT capsids contained about twice as many L2 molecules than the mutant V5·L1WTL2 did. Also, intact L1 surface loops are essential for genome encapsidation, perhaps through correct binding to L2, as mutated pseudovirions did not contain pseudogenomes at all. The 10 amino acid substitutions in HPV-16 L1 surface loops had effects also on the HPV capsid conformation as most reactions of studied mAbs and antisera with mutated capsids differed compared to their reaction with WT capsids. These results were L2 independent. Antigenicity of all L1 loops was affected in the mutant capsids, including the EF-loop that had no inserted mutation. L2 specific antisera always reacted weaker with mutated capsids compared to WT capsids, due to lower L2 content in mutated capsids. Also, L2 specific antisera reacted stronger with WT disrupted capsids than with intact ones, which is in an agreement with the fact that L2 protein is mainly hidden behind L1 molecules in intact capsids.

Despite the effect of mutations on the capsid conformation, these mutations did not destroy the major neutralizing epitope completely as H16.V5 and H16.E70 still reacted with mutated capsids, although in a reduced manner (OD₄₀₅= WT 2.8 for both mAbs, V5·L1WTL2 OD₄₀₅=1.1 and 1.8 respectively). Mutated capsids were still able to induce sufficient amount of neutralizing antibodies as serum from immunized mice neutralized WT HPV-16 pseudovirions up to a serum dilution of 1:10³ for V5·L1 and 1:10² for V5·L1WTL2. Cytotoxic T lymphocyte response was strongly reduced for the mutated capsids. For the Effector/ Target cell ratio 10/1 WT capsids induced 100% cytotoxicity, while for the mutated capsids CTL response was less than 10 %. The reason for impaired L2 binding into mutated capsids might be an overall conformational change in the capsids rather than a direct binding between the L1 surface-loops and L2. As we studied 10 aa substitutions at the same time, we can not determine which residues were most important. As we did not mutate the L1 DNA-binding domain, the defect to encapsidate pseudogenome is most likely attributable to the reduced L2 content in the mutated capsids.

Paper II

The reactivity with monoclonal antibodies (mAbs) indicated adequate type-specificity and antigenic reactivity of heparin-pseudovirion Luminex serology, except for HPV-5, -38 and -45 where no mAbs were available. The HPV-16 PsVs data was in substantial agreement ($kappa= 0.77$) with standard ELISA data. The specificity evaluation using serum samples from children found similar results as in previous

studies using VLP ELISA (195), where seropositivity for sexually transmitted HPV types were found to be uncommon in children. Also seroprevalence of cutaneous HPV among children was lower than in grown-ups, which was expected. Analysis of the serum samples from middle-aged women with up to 1 lifetime sexual partner showed modest amount of seropositives for sexually transmitted HPV types (4% of HPV-16, 3% of HPV-6 and -45, 1% of HPV-18, -31 and -58) but higher seroprevalences for cutaneous HPV types (11% for HPV-5 and 13% for HPV-38).

For the HPV positive serum panels, serum samples from Africa had a 3-fold higher background signal and therefore lower agreement with natural infection than serum samples from Europe. The expected proportion of seropositive samples out of DNA positives is around 50% and in the present study this applied to HPV-5,-6,-16,-18,-31,-38 and -58 using serum samples from Europe. Seroprevalences of HPV-6 and -11 correlated with self-reported genital condylomata. For unknown reasons, validity of established HPV-45 and -52 tests did not meet the expectations. Our results from the prospective cervical cancer study demonstrated association between later cervical cancer and prevalence of antibody response at baseline to HPV-16 OR= 7.7 (95% CI 2.6-23) and HPV-31 OR=4.1 (95% CI 1.6-10.8) demonstrating that the established serological method does reflect a biologically relevant, cancer-causing HPV infection.

Paper III

In this study heparin-pseudovirion Luminex serology (117) was expanded by adding pseudovirions of HPV types -3, -15, -32, -33, -68 and -76. We observed no significant differences in seroprevalences between patients with SCC, BCC, AK and benign skin lesions when detected with heparin-pseudovirion Luminex. We observed increased sensitivity of the *beta* papillomavirus specific antibody detection with the new method compared to the commonly used GST-L1 fusion protein method (average of 40%, maximum 63%) compared to the GST-L1 method (average of 20%, maximum of 25%).

Agreement of the three serological methods varied from poor to moderate and showed limited consistency, which might indicate that these serology methods detect different sub-populations of antibodies. The continuous data agreement between the two Luminex based methods was best for HPV-16 ($R^2 = 0.44$) and worst for HPV-6 ($R^2 = 0.07$) and for the other types R^2 was around 0.2. The categorical data had an agreement that ranged from “poor” (HPV-6, $kappa = -0.06$), “slight” (HPV-32, $kappa = 0.17$), “fair” (HPV-15, $kappa = 0.38$) to at best “moderate” (HPV-38, $kappa = 0.55$, HPV-16, $kappa = 0.45$ and HPV-5, $kappa = 0.45$). The heparin-pseudovirion Luminex detected more positive samples compared to the GST-L1 method for HPV-5 (74 vs. 12), HPV-15 (56 vs. 23), HPV-32 (22 vs. 4) and HPV-38 (51 vs. 17) but fewer positives for HPV-6 (23 vs. 135) and HPV-16 (11 vs. 20). The agreement between the high-throughput Luminex methods and the neutralization assay varied from “fair” to “moderate”. Heparin-pseudovirion Luminex and Neutralization assay comparison $kappa$ values were 0.36 (fair) for HPV-5, 0.49 for

HPV-38, 0.4 for HPV-76 (both “moderate”). The GST-L1 Luminex and the neutralization assay agreed at a “moderate” level for HPV-5 ($kappa= 0.44$) and “fair” for HPV-38 ($kappa= 0.24$).

Paper IV

Heparin-pseudovirion Luminex method was successfully expanded by adding to the antigen panel HPV-35,-39,-56,-59 and -73 and MCPyV pseudovirions produced by synthetic genes designed by collaborators. JCPyV was received as ready VLPs from K. Sasnauskas. As expected, seroprevalence of all studied viruses was lower in children compared to grown-ups. In the control group of 133 children, the HPV antibody prevalence did not exceed 4% for any studied HPV type. In the groups of adult women, HPV-16 had the highest seroprevalence (14.1 % in the control group of 71 women with ≤ 1 lifetime sexual partner and 25.2 % in the study population of 3,291 women). A majority of women with ≤ 1 sexual partner who were seropositive for antibodies to genital HPV types were seropositive to a single HPV type (29.6 % singles vs. 15.5 % multiple), whereas in the general population seropositivity to multiple genital HPV was more common (21.5 % singles vs. 44.3 % multiple). Among a group of 3,291 women from the general population the overall seropositivity to any HPV type reached 76.6 %, whereas seropositivity to any genital type reached 65.8 %. Seropositivity for cutaneous HPV types was mostly found as seropositivity for only a single HPV type, in all studied populations.

Among women DNA positive to a specific HPV type, antibodies to the same HPV type could be detected with heparin-pseudovirion Luminex method with an average sensitivity of 51 % (range 15-100 %). Strong correlations with presence of HPV DNA were found for 13/15 genital HPV types, with only one assay (HPV-52) failing in the validation and one infection (HPV-11) being too rare in cervix to allow conclusions. The sensitivity of serology tended to be better among women with only a single HPV infection in their cervical sample (median OR=10.5, (CI 95 %=2.4-48.4)) than among women with multiple HPV infections (median OR=4.6, (CI 95 %=1.8-11.7)) suggesting that multiple HPV infections tend to impair the development of the type-specific antibody response. Among women with a single genital HPV infection at the time of screening, antibodies to the specific HPV type and to other genital HPV types were more common than to the specific HPV type only (46 % vs. 8 % respectively).

Paper V

The MCPyV heparin-pseudovirion Luminex method was found to produce results similar to the previously established MCPyV reporter vector-based neutralization assay ($R=0.72$, $p<0.0001$). The proportion of MCPyV DNA positive subjects showed little variation between the patient groups (average 18%). The viral copy numbers were on average 0.07 copies per cell among the study subjects.

The MCPyV antibody levels did not differ between patient groups (average 18%). There was a very high correlation between presence of MCPyV DNA load and MCPyV seropositivity measured with heparin-pseudovirion Luminex [OR= 27.85 (95%CI 6.6-166.5) and no correlation between MCPyV DNA antibodies to JCPyV [OR= 1.39 (95%CI; 0.8-2.43)]. Overall, seroprevalences were similar (~65%) for the 2 studied Human polyomaviruses. The average MCPyV antibody level was about 9 times higher among MCPyV DNA positive subjects compared to the MCPyV DNA-negative group when determined by the heparin-pseudovirion Luminex assay (8.3 versus 0.9 units, respectively).

A striking finding was that average MCPyV antibody levels were connected to the viral load (2.9 units in the lowest copy number quartile up to 21.2 units in the highest copy number quartile). The neutralizing activity was increasing also together with increasing viral load. There was a strong trend for increasing neutralizing activity and increased antibody level by viral load (both $p < 0.01$). This data demonstrates that high levels of MCPyV antibodies correlate with higher burdens of MCPyV DNA.

Paper VI

The two MCPyV serological methods (pseudovirion neutralization and Luminex) were found to correlate well ($R^2 = 0.83$). The risk for future MCC was associated both with baseline presence of MCPyV-neutralizing antibodies (OR 5.3, 95% CI 1.3-32.3) and with presence of high levels of antibodies against MCPyV (OR 4.4, 95% CI 1.3-17.4). Baseline presence of any level of antibodies binding MCPyV tended to associate with MCC risk, but not significantly. To investigate whether acquisition of MCPyV antibodies after the baseline serum donation would affect MCC risk, we tested all prediagnostic serum samples available and estimated the MCC risk in relation to seropositivity in any one of the prediagnostic samples. The risks were similar to those estimated using only the baseline samples: OR for MCC associated with high levels of MCPyV antibodies in any prediagnostic sample: 3.6, 95% CI 1.1-12.7; OR for MCC associated with MCPyV neutralizing activity: 4.8, 95% CI 1.3-21.8). For none of the studied control virus antigens (JC polyomavirus and six HPV types that infect skin) was presence of antibodies associated with future risk for MCC.

In sex-stratified analysis, we did not observe any significant association of MCPyV antibodies with MCC risk among males, whereas the future MCC risk among females was associated with MCPyV antibodies, in particular the presence of baseline neutralizing antibodies (OR=14.3 (95%CI=1.7-677)). High MCPyV antibody levels in the first prediagnostic sample was significantly more common among female cases than males cases ($P = 0.025$).

For 14 cases, formalin- fixed paraffin embedded tumour blocks could be retrieved. Four 5µm sections were used to extract DNA and two sections taken before and after the DNA sections were haematoxylin-eosin stained for histopathological re-review. Only 12 blocks contained MCC tumor tissue in both sections (6 epithelial MCC, 4

lymphatic MCC and 2 mixed tumour type). Among the 12 cases where a histopathology block with MCC tumour tissue was available, 7 (58%) were positive for MCPyV DNA with a viral load varying from 0.1 to 56 copies per cell. The 7 MCPyV DNA positive cases were all MCPyV seropositive

CONCLUDING REMARKS

In the present thesis, pseudovirions were used to study small dsDNA tumor viruses. The pseudovirions mimic natural viruses and are essential for studies on viruses, which are not possible to harvest from the natural host or can not be cultured as natural virus in cell culture systems. Pseudovirions of 21 HPV types and 2 HPyV types were validated to be used as antigens in a high-throughput serology assay named heparin-pseudovirion Luminex. It detects IgG antibodies specific to the native viruses and this assay was validated using presence of the viral DNA in tissue as a marker for natural infection. The seroreactivity measured with heparin-pseudovirion Luminex correlated well with natural infection for 16 HPV types (-5, -6, -16, -18, -31, -33, -35, -38, -39, -45, -56, -58, -59, -68, -73, -76) and MCPyV. For HPV types -3, -11, -32 and JCPyV, correlation of the antibodies with infection could not be validated because we did not have access to serum samples from subjects with known infection status and for HPV-15 and -52 no correlation was found. The results obtained with heparin-pseudovirion Luminex were compared to other common serological methods and better sensitivity and specificity was found. This method was also applied for seroepidemiology to study the viral causality of cancer, providing the first prospective evidence implicating HPV31 in cervical cancer and MCPyV in Merkel cell carcinoma. In general, this method was found to be a reliable tool as it associates with natural viral infection and gives results comparable with results obtained with “golden standard” serology methods. The pseudovirion expression system was also successfully utilized to study effect of the mutations in virus capsids on immunogenicity and biological function of the virus particle.

The main findings of the present thesis besides the development of improved virus serology are the following:

Paper I- Intact HPV-16 L1 hypervariable surface loops were essential for successful L2 binding and genome encapsidation. Amino acid replacements in the surface-loops also affected capsid conformation in non-mutated sites. Both humoral and cellular immune response were reduced against mutated pseudovirions compared to WT one, but the main neutralizing epitope was not completely removed.

Paper II- Serum antibodies of 6 HPV types correlated with the presence of HPV DNA of the same type. Serum samples from Africa gave high background signal of antibodies compared with the ones from Europeans and this caused worse correlation

with natural HPV infection. Antibodies to HPV-16 and HPV-31 were both associated with increased risk of future cervical cancer in a prospective study.

Paper III- No significant difference of HPV seroprevalences among dermatology patient groups was detected. The studied HPV serology methods (GST-L1 and heparin-pseudovirion Luminex and neutralization assay) did not agree well with each other. Antibodies detected with heparin-pseudovirion Luminex correlated well with HPV DNA in skin of the same type for 3 cutaneous HPV types.

Paper IV- Seroprevalences of 21 HPV types and 2 HPyV types was described among Slovenian women and control populations. Serum antibodies correlated with presence of same HPV type for 13 genital HPV types. Multiple HPV infections tended to impair the development of the specific antibody response. Most of the women with single HPV detected in cervix had antibodies against this particular HPV and also to some another genital HPV types.

Paper V- We developed a high-throughput MCPyV serology method that correlated well with the “golden standard” neutralization assay and with the presence of MCPyV DNA in skin. MCPyV specific antibody levels in serum were correlated with the MCPyV viral load in skin.

Paper VI- In a prospective biobank-based study, high levels of MCPyV specific antibodies were associated with future MCC, especially in females. This provides important evidence regarding the direction of causality of the association between MCPyV and MCC, as it implies that MCPyV is a causal agent of MCC development, not just a passenger virus that is reactivated by the growing tumor.

The future direction of heparin-pseudovirion Luminex technology is to expand it even more widely, as there exist 159 types of HPV and 10 HPyV and these numbers are now rapidly increasing. Except for the most common HPVs and HPyVs, very little is known about the natural history of most viruses in these families and their associations with human diseases. Efficient seroepidemiological methods are required to enable a rapid elucidation of the natural history and role in human diseases for all these new viruses. The use of pseudovirions as antigens in multiplexed format could enable the study of the seroepidemiology of these novel viruses in an efficient way. Pseudovirions also enable fine-mapping of the biological, immunological and structural importance of different sites in the virus particles. With the examples provided in the present thesis, we have expanded the application of pseudovirions as a versatile research tool for studying essential research questions in tumor virology.

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