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Evaluation of Human Papillomavirus and Chlymdia Tachomatis Infections in the Irish Female Population: Determination of Prevalence in Adults U ndergoing Opportunistic Cervical Screening

Helen Keegan
Technological University Dublin, helen.keegan@tudublin.ie

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Evaluation of Human Papillomavirus and *Chlamydia trachomatis* infections in the Irish female population: Determination of prevalence in adults undergoing opportunistic cervical screening

A thesis submitted for the degree of Doctor of Philosophy

by

Helen Keegan

Dublin Institute of Technology

2005

Evaluation of Human Papillomavirus and *Chlamydia trachomatis* infections in the Irish female population: Determination of prevalence in adults undergoing opportunistic cervical screening

A thesis submitted for the degree of Doctor of Philosophy

by

Helen Keegan, BA Mod Microbiology

Supervisors: Dr Helen Lambkin and Dr Fergus Ryan

School of Biological Sciences
Dublin Institute of Technology

October 2005

ABSTRACT

Human papillomaviruses and *Chlamydia trachomatis* are two of the most common sexually transmitted infections worldwide. They can be detected using urine, swab and cervical scrapings from the anogenital region. Liquid-based cervical cytology is fast becoming the method of choice for the preparation of cervical smears, with the major advantage of extra cellular material from which nucleic acids can be extracted and molecular tests performed. In this project, nucleic acid based methods for the detection of HPV and *C. trachomatis* and the quantitation and typing of HPV from PreservCyt cervical material were established and applied in an epidemiological study of the prevalence of these organisms in the female Irish population.

The study was divided into three parts A:- Optimisation of nucleic acid extraction from PreservCyt cervical samples, B:- Development of multiplex PCR for the simultaneous detection of HPV and *C. trachomatis* and the determination of the prevalence of these organisms and C:- HPV genotyping, quantitation and evaluation of the relationship of these correlates with dcervical neoplasia and patient factors including age and smoking status.

In the first paper published from the study, three methods for DNA extraction from PreservCyt cervical samples were compared by the downstream PCR amplification of *C. trachomatis* using the CTP plasmid (201 bp) and MOMP gene (540 bp) primers (Keegan *et al*, 2005a). *C. trachomatis* bacterial load was calculated by real-time *LightCycler* PCR for the amplification of the *hsp60* gene (650 bp). The Proteinase K-chelex digestion method and QIAamp method liberated similar bacterial copy numbers however the commercial QIAamp DNA extraction kit was the most efficient method for the preparation of DNA for PCR amplification regardless of amplicon size.

The second paper published from the study details optimisation and evaluation of a multiplex PCR for the simultaneous detection of HPV and *C. trachomatis* from PreservCyt cervical samples (Keegan *et al*, 2005b). This multiplex was then applied to a cohort of 997 PreservCyt cervical samples from Irish women undergoing opportunistic cervical screening. The prevalence of HPV and *C. trachomatis* were 20% and 5% respectively. Prevalence was highest for both organisms in the under 25 years age group and decreased with age ($P<0.0001$). A coinfection rate of 1% was established for HPV and *C. trachomatis*. HPV was associated with abnormal cytological smear results and HPV detection was 100% sensitive for the detection of high-grade cervical intraepithelial lesions. *C. trachomatis* infection was not associated with abnormal cytology.

In the final part of this study (paper in preparation), HPV infections were investigated further in terms of viral load and genotype. HPV viral load was determined by real-time quantitative *LightCycler* PCR. Viral load was higher in women with borderline cytology or CIN lesions than in women with normal smears ($P<0.001$), however viral load did not increase with grade of CIN. Genotyping was performed by sequencing of the MY09/11 L1 gene PCR product. High-risk HPV types predominated in all grades of cytology including normal smears. HPV16 and 18 were the most common followed by high-risk types 66, 53 and 33 and low-risk types 6, 61 and 70. HPV16 and 18 were the most common in all grades of cytology except in borderline cases where HPV66 was the most common. Cigarette smoking was more common in women with abnormal cytology ($P<0.001$) and also in women with evidence of HPV ($P=0.008$) or *C. trachomatis* infection ($P<0.001$).

The main findings of this study were the overall prevalence of HPV and *C. trachomatis* in an opportunistically screened Irish female population; the strong association between HPV infection and high grade premalignant neoplasia; that HPV viral load was not significantly different in HPV positive cases with mild and high-grade cervical abnormalities; the commonest high-risk HPV types were 16, 18, 66, 53 and 33 and low-risk types 6, 61 and 70. Cigarette smoking was strongly associated with abnormal cytology smear results and HPV, *C. trachomatis* and abnormal cytology smear results were more common in women under 35 years of age. The results of this study are similar to findings in other European countries.

DECLARATION

I certify that the thesis which I now submit for examination for the award of PhD, is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for an award in any other Institute or University.

The work reported on in this thesis conforms to the principles and requirements of the Institute's guidelines for ethics in research.

The Institute has permission to keep, to lend or to copy this thesis in whole or in part, on condition that any such use of the material of the thesis be duly acknowledged.

Signature Helen Keegan **Date** 29-6-06

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My friends, especially Niamh, Aiden, Louise, Nora, Tanya, Ruth, Sarah and Susan

To the memory of Anne Keegan

ABBREVIATIONS

Full Term	Abbreviation
16S subunit of ribosomal ribonucleic acid	(16SrRNA)
23S subunit of ribosomal ribonucleic acid	(23SrRNA)
Acquired immunodeficiency syndrome	(AIDS)
Adenine	(A)
Analysis of variance	(ANOVA)
Atypical glandular cells of undetermined significance	(AGUS)
Atypical squamous cells of undetermined significance and low-grade squamous intraepithelial lesions HPV triage study	(ALTS)
Aytpical squamous cells of undetermined significance	(ASCUS)
Base pair	(bp)
Cell cycle protein 107	(p107)
Cell cycle protein 130	(p130)
Cell cycle protein 21	(p21)
Cell cycle protein 27	(p27)
Centers for Disease Control and Prevention	(CDC)
Cervical intraepithelial neoplasia	(CIN)
Chromosomal deoxyribonucleic acid	(cDNA)
Cohort number	(n)
Cyclin dependant kinase	(CDK)
Cytomegalovirus	(CMV)
Cytosine	(C)
Deoxynucleotidetriphosphates	(dNTPs)
Deoxyribonucleic acid	(DNA)
Elementary body	(EB)
Enzyme-immunoassay	(EIA)
Elongation factor 2	(E2F)
Epstein-BarVirus	(EBV)
Food and Drugs Administration	(FDA)
General Practitioner	(GP)

Genitourinary medicine	(GUM)
Guanine	(G)
Health Protection Surveillance Centre	(HPSC)
Heat-shock protein	(Hsp)
Herpes-simplex virus	(HSV)
High-grade squamous intraepithelial lesions	(HSIL)
Hour	(h)
HPV in addition to routine testing	(HART)
Human herpesvirus	(HHV)
Human immunodeficiency virus	(HIV)
Human leukocyte antigen	(HLA)
Human Papillomavirus	(HPV)
Hybrid capture II	(HCII)
Hybrid capture III	(HCIII)
Identification test	(ID)
Immunoglobulin	(Ig)
In situ hybridisation	(ISH)
In vitro fertilisation	(IVF)
Interleuken	(IL)
International Agency for Research on Cancer	(IARC)
Invasive cervical carcinoma	(ICC)
Kilobase	(kb)
Kilodalton	(kDa)
Leokocyte esterase	(LE)
Line-probe assay	(LiPa)
Lipopolysaccharide	(LPS)
Low-grade squamous intraepithelial lesions	(LSIL)
Lymphogranuloma venereum	(LGV)
Major outer membrane protein	(MOMP)
Megabase	(mb)
Melt-temperature	(Tm)
Microimmunofluorescence	(MIF)
Micromolar	(µM)
Millimolar	(mM)
Minute	(min)

Mitogen-activating protein kinase	(MAP-Kinase)
Molecular weight	(M_r)
Mouse toxicity prevention test	(MTPT)
Nanometers	(nm)
National Disease Surveillance Centre	(NDSC)
Nested-multiplex polymerase chain reaction	(NMPCR)
Nucleic acid amplification tests	(NAATs)
Pelvic inflammatory disease	(PID)
Phase one of growth in cell cycle/ DNA synthesis checkpoint	(G1/S)
Picomole	(pmol)
Polymerase chain reaction	(PCR)
Proliferating cellular nuclear antigen	(PCNA)
Proteinase K	(PK)
Relative light units/cut-off value ratio	(RLU/CO)
Reticulate body	(RB)
Retinoblastoma protein	(pRB)
Reverse-transcription polymerase chain reaction	(RT-PCR)
Revolutions per minute	(rpm)
Ribonucleic acid	(RNA)
Ribosomal ribonucleic acid	(rRNA)
Second	(s)
Sexually transmitted infection	(STI)
Statistical Package for the Social Sciences	(SPSS)
Tris-EDTA	(TE)
T-helper	(Th)
Threonine	(T)
Touchdown-enzyme time release polymerase chain reaction	(TETR-PCR)
Tumour suppressor protein 53	(p53)
Unit	(U)
Upstream regulatory region	(URR)
World Health Organisation	(WHO)
Year	(yr)

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CHAPTER 1

General Introduction

1.1 Foreword

In recent decades, the number of cases of sexually transmitted infections (STIs) has risen globally (WHO, 1995). STIs are a significant cause of morbidity and mortality worldwide, particularly in women. The Acquired Immunodeficiency Syndrome (AIDS) pandemic has generated much interest in other STIs most notably because of their contributory role in the pathology of this disease. While the majority of cases of STI are at the acute or subacute level, adverse disease outcomes such as carcinogenesis and debilitating reproductive disorders may be associated with a minority of cases, in particular infections with Human Papillomavirus (HPV) and *Chlamydia trachomatis.*

Cervical cancer is one of the most common cancers in women worldwide causing over 200,000 deaths annually (Wick, 2000). Early epidemiological studies of cervical neoplasia suggested a direct causal relationship with sexual activity (Munoz and Bosch, 1996). Viruses directly contribute to approximately 15% of all human cancers, with hepatocellular cancer and cancer of the cervix making up about 80% of all virus-associated cancers. After cigarette smoking, viral infections are the second most important risk factor for the development of cancer (zur Hausen, 1991). Over the last 20 years a number of sexually transmitted viruses including HPV, Epstein-Barr virus (EBV), Cytomegelovirus (CMV) and Human Herpes Viruses (HHV) and the components of semen were proposed in the quest for the identification of possible venereally transmitted carcinogens, however only HPV was found to have any significant association with cervical cancer. A German virologist, Harold zur Hausen, first demonstrated the link between genital HPV infections and cervical cancer in the 1980's. There is now compelling evidence, both molecular (Southern and Herrington, 1998) and epidemiological (Munoz, 2000) that persistent HPV infection is a necessary cause of cervical cancer worldwide. In 1995, the International Agency for Research on

Cancer (IARC) concluded that there was sufficient evidence to classify HPV types 16 and 18 as definitive human carcinogens (Vainio *et al*, 1995). Results of an IARC study on the prevalence of HPV in invasive cervical cancer found that overall, 99.7% of 1,000 cases of histologically confirmed invasive cervical cancers were HPV DNA positive (Walboomers *et al*, 1999). *C trachomatis* infections are a leading cause of infertility and ectopic pregnancy worldwide (Centers for Disease Control and Prevention (CDC), 2005) and are now believed to act as a cofactor in the development of invasive cervical cancer (Smith *et al*, 2004).

1.2 Human papillomavirus biology

1.2.1 *Classification*

Papillomaviruses are members of the recently declared *Papillomaviridae* family (de Villiers *et al*, 2004) They are ubiquitous and have been identified in a large variety of animals including humans and are specific for their respective hosts. More than 200 types have been identified that infect humans with over 85 types well characterised. One hundred and twenty isolates have been characterised as potential new genotypes based on their DNA sequences (zur Hausen, 1999). HPVs infect the basal epithelial layers of the skin or the mucosal surfaces and as such can be classified as either cutaneous or mucosal. Cutaneous types are epitheliotrophic and commonly infect the skin of the hands or feet, while mucosal types infect the lining of the mouth, throat, respiratory tract or anogenital epithelium. HPV can be classified into high-risk and low-risk types based on their association with cervical cancer. High-risk types include types 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 70, while low-risk types include types 6, 11, 42, 43, and 44 (de Villiers *et al*, 2004). High-risk types have recently been implicated in cancers other than cervical cancer including prostate and nose and throat cancers (Carozzi *et al*, 2004; Syrjanen, 2005).

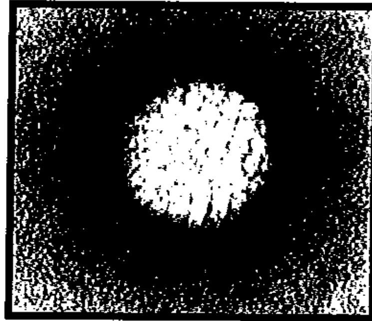


Figure 1.1. Electron micrograph of an HPV virion particle

1.2.2 *HPV structure*

HPVs are small non-enveloped double-stranded DNA viruses with an icosahedral structure composed of 72 capsomers. Each capsomer is a pentamer of the major capsid protein L1 (M_r 53,000-59,000), (Baker *et al*, 1991), and each virion contains approximately 12 copies of the minor capsid protein L2 (M_r 70,000), (Sapp *et al*, 1995). Each virion is approximately 55 nm in diameter and they are said to somewhat resemble a golf ball on using electron microscopy (Figure 1.1).

1.2.3 *HPV genome*

The HPV genome is approximately 7.9 kb in size and is organised into a single molecule of circular double stranded DNA with its associated histones (Favre *et al*, 1975) (Figure 1.2). The genome is functionally divided into three major regions. The first of these is the upstream regulatory region (URR). This is a highly-variable non-coding region of 400 to 1000 bases containing the p97 core promoter and enhancer and silencing sequences that are necessary for regulation of expression. The coding region of the genome is divided into two major parts: the early region and the late region. The early region contains genes E1, E2, E4, E5 E6 and E7. The E1 and E2 genes are

involved in viral replication and maintenance. E1 encodes a helicase which is involved in the unwinding of the duplex DNA and in bringing the DNA polymerase to the origin of replication where E1 complexes with E2 to initiate replication (Desaintes and Demeret, 1996). E2 also acts as a transcriptional repressor of E6. The E4 protein functions as a fusion protein with E1 later in the HPV life cycle. It is found localised in the upper layers of the epithelium prior to release of viral particles from the epithelial surface. In HPV16 transformed cell lines the E1^E4 fusion protein has been associated with disruption of the cytokeratin intermediate filament network, possibly to allow virion particles exit the cell (Wang *et al*, 2004). The E5 gene product interacts with cell membrane growth factors and is thought to play a role in the transformation process (Straight *et al*, 1995). The E6 and E7 proteins are the major transforming proteins (Herrington, 1994). The late region is expressed in later stages of the life cycle and encodes the L1 and L2 structural proteins, which make up the viral capsid (Doorbar, 2005).

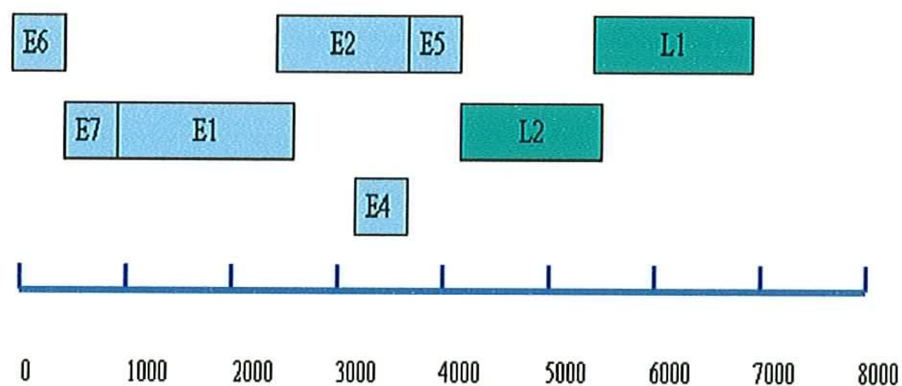


Figure 1.2. Molecular organisation of 7.9 kb HPV genome. E represents the early genes and L represents the late genes

1.2.4 *HPV life cycle*

As HPVs are epitheliotrophic their life cycle is closely linked to squamous cell differentiation (Bedell *et al*, 1991). In the cervix initial infection occurs through small abrasions in the tissue or during metaplasia of the transformation zone allowing the virions to access the basal epithelial cells. Entry of HPV6 is thought to occur via the cellular receptor alpha-6 integrin present on the surface of the basal epithelium (Evander *et al*, 1997). HPV16 and HPV33, like many other viruses attach to host cells via cell surface heparan sulphate, possibly stabilised by another cell surface receptor or proteoglycans (Giroglu *et al*, 2001). The viral genome remains at low copy numbers replicating its DNA, on average once per cell cycle, in the basal epithelium until the infected cell reaches the upper layers where vegetative replication occurs (Flores and Lambert, 1997). Events culminating in the release of viral particles in the late phase are not well understood but are thought to involve the differential-dependant regulation of expression from the viral promoters (Ozbun and Meyers, 1998). The cytological effects of this type of infection are koilocytosis, nuclear enlargement, multinucleation and/or dyskaryosis. This type of infection manifests itself clinically as anogenital warts or as low-grade lesions. Such lesions may regress, persist or progress to cancerous lesions.

Non-permissive transformable infections occur when viral replication and release of progeny do not occur. Viral DNA can persist extra-chromosomally or by integration into the host genome at fragile sites (Popescu and DiPaolo, 1989). The site of integration into the host genome is not consistent but the breakpoint in the viral genome often occurs within the E2 ORF and sometimes within the E1 gene. This causes loss of function of these genes, in turn removing the transcriptional repression of E6/E7 causing upregulation. E6 and E7 gene products cause immortalisation of cells *in vitro* and during prolonged passage of these cells further genetic changes occur which are necessary for progression to the malignant state (Munger *et al*, 1989; Solinas-Toldo *et*

al, 1997). Because of their small genome size, HPVs do not encode essential replication proteins and so must subvert host regulatory factors such as cyclins and cyclin-dependant kinases, cyclin inhibitors and cell cycle associated proteins, resulting in altered cell cycle control. Cells that have left the cell cycle are restimulated and cells containing damaged DNA are allowed to progress through the cell cycle resulting in the accumulation of genetic abnormalities.

1.3 Epidemiology and pathogenesis of HPV infections

1.3.1 *Clinical manifestations of HPV infections*

The clinical outcome of a HPV infection is largely dependant on the infecting HPV type involved. Sexually transmitted HPV infection leads to one of three possible results.

(i) The first is the benign condition of anogenital warts. These are generally associated with types 6 and 11 and are found in both men and women. These are often asymptomatic and may regress over a period of 3-4 months, increase in size and number or remain the same. Anogenital warts can be treated by clinical procedures such as ablation or excision or by the application of chemical agents such as 0.5% podophyllin or 0.5% imiquimod.

(ii) The second result is latent or inactive infection. This type of infection is often asymptomatic with the infected area remaining cytologically normal. It is estimated that high-risk HPV16 or 18 is present in approximately 10% of women with cytologically negative Pap smears (Arora *et al*, 2005).

(iii) The third possible clinical outcome is active infection. This is associated with high-risk HPV types and is the type of infection involved in the production of penile, urethral, bladder, prostate, vaginal, vulvar and cervical neoplasia. The risk of progression of disease is greater for HPV types 16 and 18 than for other high-risk HPV types (Khan *et al*, 2005).

1.3.2 *Epidemiology of HPV infections*

HPV is considered to be an ubiquitous presenting organism (Helmerhorst and Meijer, 2002) and estimates of the prevalence of the organism in the general population range from 2% to 44% worldwide (Bosch and de Sanjose, 2003) depending on the age of the population studied and on the diagnostic test used. A recent study conducted in Scotland, found an overall HPV prevalence of 20.5% in women with a mean age of 36.6 years attending routine cervical cytological screening and a prevalence of 15.7% for high-risk infection only (Cuscheiri *et al*, 2004). The prevalence of HPV infections is highest among young women and falls with age (Schiffman, 1992). Some studies have reported a second peak in prevalence in women over the age of 35 (Herrero *et al*, 2000; Giuliano *et al*, 2005; Rassu *et al*, 2005). Possible reasons for this may be reactivation of latent virus, inability to clear new infections or they may represent persistent infections. Oncogenic HPV types are more common than non-oncogenic types (Giuliano *et al*, 2002; Richardson *et al*, 2003). A worldwide study by Munoz *et al* (2003) on HPV prevalence in nine countries found that HPV16 was the most commonly infecting type followed by HPV18, 31, 45, 35, 58 and HPV6.

The prevalence of HPV is so high that the chances of a HPV infection are estimated at 80-85% over a lifetime (Jenkins *et al*, 1996). The cumulative incidence of HPV infection among women aged 15-19 years in England was found to be 44% over a 3 year period increasing to 60% over 5 years (Woodman *et al*, 2001). HPV infections may be transmitted by direct skin-to-skin contact or by transmission via contaminated fomites, as HPV virions are resistant to heat and desiccation. Vertical transmission has also been reported (Rintala *et al*, 2005).

1.3.3 *HPV proteins and oncogenesis*

Active viral replication is commonly seen in low-grade lesions and in the benign wart-like lesions of the condition condylomata acuminata. During active replication the virus remains episomal in basal nuclei and vegetative viral reproduction occurs only in the squamous epithelium undergoing terminal differentiation. Low levels of expression of viral mRNA are seen in the basal layers with increased levels of E6 and E7 mRNAs being produced in the differentiated layers (Durst *et al*, 1992; Stoler *et al*, 1992). Replication of the HPV genome begins with the interaction of host cell factors with the upstream regulatory region (URR) of the HPV genome. Integration of the viral genome into the host genome is seen in most invasive cancers and in high-grade lesions (Klaes *et al*, 1999). Deregulation of critical cellular genes is thought to be as a result of insertional mutagenesis, although HPV integration sites are randomly distributed over the host genome (Wentzensen *et al*, 2004). Integration of the HPV genome also results in an interruption of the E2, frequently of the E1 and partial deletion of the E4, E5 and L1 and L2 genes. The E6 and E7 proteins subvert cell growth regulatory pathways. It has been shown that disruption of intergenomic regulation leads to dysregulation of viral E6/E7 transcription and increases the immortalisation capacity of high-risk viral types. As the E6 and E7 gene products are produced, tumour suppressor proteins, cell cyclins and cyclin-dependant kinases become deregulated (Southern and Herrington, 2000). Both E6 and E7 are needed for induction and maintenance of the transformed phenotype, particularly by interference with apoptosis and cell cycle control. The normal cell cycle is governed largely by two major proteins the tumour suppressor protein p53 and the retinoblastoma gene product, pRB. The normal functions of p53 include governing of G1 arrest, apoptosis and DNA repair. The high-risk HPV E6 protein complexes via a cellular protein, E6-associating protein (E6-AP) and binds to and targets p53 for rapid degradation by the ubiquitin-ligase mechanism (Thomas *et al*,

1999). Thus the normal functions of p53 are abrogated. Low-risk HPV types do not bind p53 at detectable levels and have no effect on p53 stability *in vitro*. E6-mediated interferences with p53 functions together with inactivation of pro-apoptotic protein Bak to prevent cells from undergoing apoptosis, resulting in genetic instability and the accumulation of genetic abnormalities (Thomas and Banks, 1999)

The HPV E7 gene product binds to the hypophosphorylated form of the RB family of proteins including pRB, p107 and p130 (Dyson *et al*, 1989; Davies *et al*, 1993) thereby interfering with their control over the G1/S transition of the cell cycle. Preferential binding of the E7 oncoprotein to pRB causes the release of E2F transcription factor, cell cycle progression and the loss of regulation of E2F responsive genes (Chellappan *et al*, 1992). Inactivation of pRB by E7 can be identified by a permanent upregulation of its inhibitor $p16^{ink4a}$. As $p16^{ink4a}$ expression is regulated by a negative feedback loop, continuous inactivation of pRB results in elevated levels of $p16^{ink4a}$ expression, as seen in cervical intraepithelial neoplasia and cervical carcinomas (Klaes *et al*, 2001). The outcome is cellular DNA synthesis and cell proliferation. The E7 protein from low risk HPV types binds with decreased affinity to pRB. High-risk E7 proteins also bind to cellular proteins independent of the RB family. Increased levels of cyclin E and cyclin E associated kinases are seen in E7 expressing cells, these are thought to phosphorylate cyclin E regulated targets (Duronio *et al*, 1996). The E7 protein binds cyclin A, but the mechanisms involved are unknown (Arroyo *et al*, 1993). The E7 protein also binds the cyclin dependant kinase inhibitor proteins p21 and p27 (Demers *et al*, 1996; Zerfass-Thome *et al*, 1996). HPV E7 prevents p21 from inhibiting both proliferating cellular nuclear antigen (PCNA)-dependant DNA replication and cyclin E/CDK2 activity (Funk *et al*, 1997; Jones *et al*, 1997).

The E5 protein causes increased mitogen activated protein kinase (MAP-kinase) activity (Crusius *et al*, 2000), which enhances cellular response to growth and stimulating factors. As a result of uncontrolled cellular proliferation the host cell accumulates more and more damaged DNA that cannot be repaired. Eventually mutations accumulate that lead to fully transformed cells.

1.4 Risk factors for the development of cervical cancer

1.4.1 *Sexual behaviour and age*

The most important risk factor for the acquisition of high-risk HPV infection is the number of sexual partners (Deacon *et al*, 2000; Kjaer *et al*, 2001). Condom usage may not adequately protect an individual as HPV infections can be transmitted by contact with infected labial, scrotal and anal tissues that are not protected.

Young age at first sexual intercourse is also an important factor in determining risk of HPV infection (Burk *et al* 1996a; Adam *et al*, 2000). A large proportion of women and men are infected by the age of 30 years (Kjaer *et al*, 2000). Most cervical cancers arise at the squamocolumnar junction of the cervix. This is an area of high metaplastic activity where the columnar epithelium of the endocervix meets the squamous epithelium of the ectocervix. The greatest risk of HPV infection coincides with greatest metaplastic change. Greatest metaplastic activity occurs at puberty and during pregnancy in particular the first one, and declines after the menopause. HPV infections are commonly seen in women between the ages of 18 and 30 and there is a sharp decrease in the prevalence after 30 years of age. However, cervical cancer is most commonly seen in the 35 plus age bracket suggesting persistent high risk HPV infection at a young age with slow progression to cancer.

1.4.2 *HPV status and other sexually transmitted infections*

The attributable fraction of high-risk HPV infection to cervical cancer is 95% (Bosch *et al*, 2001), however only 20% of high-risk HPV infections cause morphological changes in the epithelium of the cervix indicative of premalignant lesions with a smaller fraction still resulting in cervical cancer. Regression rates of mild and moderate dysplasia are estimated at 50% over three years, whereas the regression rate of more severe dyskaryosis with persistent high-risk HPV infection is much lower (Nobbenhuis *et al*, 2001). A variety of other factors must therefore act in conjunction with high risk HPV infection to determine whether a woman will develop cervical cancer.

Sexually transmitted infections may act as cofactors in the development of cervical cancer. CMV, herpes simplex virus (HSV)1 and 2, HHV6 and 7 have all been detected in the cervix (Lanham *et al*, 2001). A role for HSV2 has been postulated in the initiation of cervical cancer (zur Hausen, 1982) and putative transforming oncogenes have been proposed for CMV and HHV6 (Kashanchi *et al*, 1997; Doniger *et al*, 1999). However, a recent study has found that these viruses are only bystanders in the development of cervical cancer (Chan *et al*, 2001b). *C trachomatis* has also been proposed as a further risk factor in the development of cervical cancer (Smith *et al*, 2004). Another important factor in the development of cervical cancer is the emergence of HPV variants exhibiting different virulence patterns. HPV viral load has also been linked to increased risk for progression of disease. Studies using type specific quantitative PCR for estimation of HPV load have found that HPV16 can reach much higher viral loads than other types and that only for HPV16 does an increased viral load correlate with increased severity of disease (Swan *et al*, 1999; Zerbini *et al*, 2001).

1.4.3 *Immune status*

Host immune status is a principal determinant of persistent HPV infection (Bontkes *et al*, 1998b). The primary immune response to HPV infection is cell mediated. Conditions which impair cell-mediated immunity, such as renal transplantation or human immunodeficiency virus infection significantly increase the risk of progression of HPV (Torrisi *et al*, 2000; Calore *et al*, 2001). Genetic predisposition was found to be an even greater component of risk for tumour development in cervical cancer than in many other cancers such as lung, colorectal and melanoma (Magnusson *et al*, 2000). In the development of cervical cancer, heritability could influence many factors such as susceptibility to infection, ability to clear infection, persistence rates and the time in staging of development of dyskaryosis and invasive cancer.

1.4.4 *Oral contraceptive usage and smoking*

Long-term use of oral contraceptives is considered a significant risk factor in the development of high-grade disease (Moreno *et al*, 2002), as is parity. The URR of the HPV genome contain sequences similar to the glucocorticoid-receptor elements that are inducible by steroid hormones such as progesterone, the active component of oral contraceptives (Bromberg-White and Meyers, 2003). Current smoking has been found to be more associated with higher grades of dyskaryosis than lower grades and is the most important cofactor after HPV infection in high-grade disease (Adam *et al*, 2000). Smoking may act by local suppression of the immune system or via the mutagenic components of cigarette smoke. Components of cigarette smoke have been found in genital mucosal secretions (Prokopczyk *et al*, 1997).

1.5 Detection of cervical neoplasia

1.5.1 *Conventional cytology*

The primary diagnostic tools for the detection of cervical cancer have been cytology and histopathology to detect neoplastic alterations in the cervical epithelium. Lesions mainly arise in the stratified squamous epithelium and are classified by the British as CIN (cervical intraepithelial neoplasms), and by the USA as SIL (squamous intraepithelial lesions), which may be high-grade (HSIL) or low-grade (LSIL). The natural history of cervical cancer is a progression from mild cervical intraepithelial neoplasia (CINI) to moderate and more severe degrees of neoplasia (CINII and CINIII), which may take 10-15 years to develop. The risk of progression of CINI to CINIII is only 1% per year, while the risk of progression of CINII to CINIII is 16% over 3 years and 25% over 5 years. However, it is agreed that early detection of neoplasia can significantly reduce the risk of progression to cancer (Spitzer, 1998).

The Pap test has been the standard diagnostic test for pre-cancerous cervical lesions since the 1940s. It was initially described by George Papanicolau in 1928 and was later instituted as a screening method for the detection of cervical carcinoma (Wick, 2000). The Pap smear has been credited with reducing the incidence of cervical cancer in the USA from 44 per 100,000 women in 1947 to 5 to 8 per 100,000 in 2000 and it has reduced the mortality rate of cervical cancer by more than 70% (Wick, 2000). However, the reported estimates of false negatives diagnoses ranged from 1.5% to 55% (Hutchinson *et al*, 1994). Many false negative cases are the result of poor sample transfer and poor quality slide preparation. Investigators have observed that as much as 80% of cellular material remains on the collecting device, which is discarded, after a conventional smear is prepared (Malle *et al*, 2003). For the traditional Pap smear method only a small proportion of the cells collected become deposited on the slide, meaning this small sub sample is not necessarily representative of the whole sample.

Moreover, the traditional smearing technique produces slides that vary in cellular density and morphological quality, making interpretation and quality control difficult.

1.5.2 *Introduction of liquid based cytology*

In recent years there has been increasing pressure to improve the standard of Pap preparations to reduce the occurrence of false negative results with their associated morbidity and mortality for the women involved and the ensuing legal consequences for health professionals. A number of smear preparation technologies both manual and automated have been developed: ThinPrep (U.S.A. Food and Drug Administration (FDA) approved; Cytyc Corp., Boxborough, Massachusetts, U.S.A.), AutoCyte PREP (TriPath Imaging, Inc., Burlington, North Carolina, U.S.A.), DNACITOLIQ (Digene Brazil, Sao Paulo, Brazil) and SurePath™ (TriPath Imaging®, Inc., Burlington, North Carolina, U.S.A.). Of these the ThinPrep and AutoCyte PREP systems have been evaluated most extensively.

1.5.3 *Advantages of liquid based cytology*

Unlike conventional cytology, where the cellular material is removed from the cervix and spread onto a glass slide, in liquid-based cytology, it is rinsed into a vial of alcohol derived liquid transport medium eg. PreservCyt (ThinPrep system) and sent to the laboratory as a cell suspension. In the laboratory, the sample is homogenised, and a representative fraction is collected on a single-use filter, transferred to a glass slide and stained in the usual fashion. The result is a reduced area of evenly spread, well preserved cellular morphology with reduced mucus, blood and inflammatory content. Slides can be screened in about half the time of conventional smears increasing work output per screener by 25-40% (Mc Googan, 2001). Only a proportion of the PreservCyt

material is used to prepare the slide, facilitating the preparation of a second slide from the same sample if required.

The effectiveness of thin-layer liquid-based technologies in cervical screening is reflected in numerous reports of increased detection of significant cervical abnormalities, reductions in the numbers of samples with indeterminate diagnosis such as atypical squamous cells of undetermined significance (ASCUS) or atypical glandular cells of undetermined significance (AGUS) and in improvements in specimen adequacy. (Roberts *et al*, 1997; Monsonego *et al*, 2001; Malle *et al*, 2003; Tibbs *et al*, 2003). A study by Roberts *et al* (1997), which evaluated the ThinPrep Pap test as an adjunct to the conventional Pap test on 35,560 paired split sample slides, found that the addition of the ThinPrep Pap test improved detection rates of cervical abnormalities by 12% and reduced the numbers of unsatisfactory samples by 94%. Using biopsy as the gold standard in an unscreened high-risk population, it was found that the ThinPrep Pap test had a sensitivity of 94% for CINII and a specificity of 78%, a sensitivity of 98% and 100% for CINIII and for cancer respectively (Belinson *et al*, 2001).

1.5.4 *Molecular analysis of liquid based cytology specimens*

A major advantage of liquid based cytology is that there is a considerable amount of residual sample cellular material available from which nucleic acids can be extracted for ancillary molecular analyses. Numerous studies have demonstrated the diagnostic value of HPV testing as an adjunct to cytology in the detection of neoplasia (Sherman *et al*, 1997; Yarkin *et al*, 2003) and in the resolution of atypical diagnoses (Layfield and Qureshi, 2005). HPV DNA testing by PCR based methods in liquid-based cytological preservatives has been suggested as a useful adjunct to cytological screening, especially in the management of patients with ASCUS (Yarkin *et al*, 2003).

A study designed to assess the long-term stability of DNA in PreservCyt specimens stored over 8 years found that HPV DNA as detected by the hybrid capture assay (HCII, Digene Corp., U.S.A.) was unaffected by storage time, however human DNA was significantly degraded as detected by the inability to amplify multiple beta-globin fragments by PCR (Castle *et al*, 2003c). The FDA has approved the HCII assay for the detection of high-risk HPV DNA in PreservCyt specimens stored for up to 21 days (Sailors *et al*, 2005). Studies into the preservation of RNA have shown that PreservCyt can yield RNA of suitable quality for microarray analysis (Habis *et al*, 2004) and HPV reverse transcription-PCR (Tarkowski *et al*, 2001) and that good quality RNA can be detected for at least 14 days after sample collection (Cuschieri *et al*, 2005).

Liquid-based cytology specimens may also be used for additional STI testing (Boardman *et al*, 2005). PreservCyt samples have successfully been used for the detection of genital HSV infections (Fiel-Gan *et al*, 1999). Many studies have demonstrated the feasibility of *Chlamydia* screening of PreservCyt samples (Koumans *et al*, 2003; Hopwood *et al*, 2004). In this research project, the aim was to establish sensitive, specific molecular methods for the detection of *C. trachomatis* and HPV in cervical PreservCyt samples.

1.6 Molecular methods for HPV detection

1.6.1 *Traditional detection methods*

Unlike other mammalian viruses, HPV cannot be grown in conventional cell cultures. Accurate diagnosis of a HPV infection relies upon identification of the presence of HPV DNA. Serological assays fail to distinguish between past and current infection, as antibodies to the HPV L1 protein remain detectable after many years (Dillner, 1999).

HPV DNA can be detected in cervical smears and biopsies by various methods. *In-situ* hybridisation (ISH) involves the application of labelled generic HPV probes that bind to HPV DNA. Although ISH has limited sensitivity it has the advantage of localising HPV infection in the sample material with the possibility of co-localisation with other markers e.g. the cyclin dependant kinase inhibitor $p16^{ink4a}$, a marker of dyskaryosis (Sato *et al*, 1998). HPV typing by ISH involves the application of type-specific probes in multiple experiments and is time-consuming and expensive.

Other hybridisation methods include the Southern-blot and the dot-blot. HPV Southern-blot analysis involves restriction enzyme digestion of isolated HPV DNA, separation of the digested fragments by gel electrophoresis and immobilisation onto a nitrocellulose membrane followed by probing of the restricted DNA by radio-labelled HPV specific probes. Southern-blotting, although highly labour intensive, is useful for HPV typing and played a fundamental historical role in the discovery and identification of different HPV types (Matsukura and Sugase, 1990). However, a fundamental requirement for the Southern-blot is the presence of high quality, high concentration DNA. Another disadvantage is in the interpretation of uncharacterised HPV types. The Southern-blot is relatively insensitive and unsuitable for high-throughput screening (Kuypers *et al*, 1993; Melchers *et al*, 1989). The dot-blot method, like the Southern-blot method also involves the use of a solid phase membrane and the detection of a signal by autoradiography. Unlike the Southern-blot, the dot-blot does not rely on probing of digested fragments but of PCR amplified fragments with the result of increased sensitivity.

1.6.2 *Commercial hybridisation systems for HPV detection*

The hybrid capture system (HCII, Digene Corp., U.S.A.) offers a non-radioactive alternative hybridisation method for HPV detection. It involves the hybridisation of

HPV DNA to labelled RNA probes in solution, followed by signal amplification and detection (Bozzetti *et al*, 2000; Lorincz, 1996). Two probe cocktails may be used, one for 5 low-risk HPV types 6, 11, 42, 43, and 44 and the other for 13 high-risk types, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. This FDA approved test has been used in numerous large-scale HPV screening studies (Castle *et al*, 2004; Cubie *et al*, 2005) however, it cannot distinguish particular HPV types. It is less sensitive than PCR and has a detection limit of approximately 5,000 HPV genome copies (Kucera *et al*, 2001). Cross-reactivity with HPV types not included in the probe-cocktails has also been reported by some studies (Castle *et al*, 2002; Poljak *et al*, 2002).

An alternative to the hybrid capture method is the recently developed Roche Molecular Systems Amplicor HPV Multiwell Plate Assay. Unlike the hybrid capture method a biotinylated PCR product of 170 bp is generated prior to the hybridisation step. The biotinylated PCR product is denatured and captured onto a streptavidin coated multiwell plate. The wells of the plate are then probed with high-risk HPV oligonucleotide probes and hybrids are detected following a binding of conjugate reaction. Using this method, 13 high-risk types can be detected. The reported sensitivity of this reaction is similar to that of the hybrid capture method (Monsonego *et al*, 2005).

Reverse hybridisation methods involve the capture of multiple oligonucleotide probes onto a solid phase and probing with a PCR product in the liquid phase. Hybridisation is followed by a colorimetric detection stage. Reverse hybridisation methods have been useful in the development of type-specific hybridisation methods as they allow the simultaneous hybridisation of a PCR product to multiple oligo-probes. The most commonly used reverse hybridisation assay is the line-probe assay (Li-Pa, Roche Molecular Systems). This involves binding of a consensus HPV PCR product amplified

from the L1 gene by the SPF10 primers (Kleter *et al*, 1999), to type-specific HPV probes immobilised onto a membrane strip. The PCR product is generated using biotinylated primers, and is then captured by the type specific probe. Uncaptured product is washed off and a signal is generated by the addition of a streptavidin conjugate. The signal can be read visually. This method allows multiple type detection in a single step and requires only a small amount of PCR product (Kleter *et al*, 1999; Melchers *et al*, 1999). Alternatives to the LiPa assay have been developed using different primers targeted to the L1 gene e.g. the PGMY primers (Coutlee *et al*, 2002), and the GP5+/6+ primers (van den Brule *et al*, 2002). The LiPa assay is currently undergoing clinical evaluation (Kornegay *et al*, 2003; Tabrizi *et al*, 2005) and has not yet been used in large-scale screening studies.

1.6.3 *Nucleic acid amplification tests (NAATs)*

PCR is the most widely used HPV DNA amplification technique. PCR involves specific targeting of a region of DNA of interest by oligonucleotide primers for amplification in a thermocycling process by a thermostable DNA polymerase. There are two major approaches to HPV detection by PCR: - consensus PCR for the identification of a wide number of HPV types and type-specific PCR for the amplification of an exclusive HPV type.

As the HPV L1 gene is the most conserved region of the HPV genome multiple consensus PCR primer sets have been designed to target this area (Husnjak *et al*, 2000). The most commonly used primer sets targeting the L1 region are the MY09/11 primer set (Manos *et al*, 1989) and the Gp5+/6+ primer set (de Roda Husman *et al*, 1995). The MY09/11 primers are degenerate primers with varied base substitutions at specific sites of sequence variation called degenerate sites. The degenerate primer set is comprised a

mixture of 24 oligonucleotide sequences with the ability to amplify over 30 genital HPV (Bernard *et al*, 1994). Since then various modifications have been made to the original MY09/11 primer set including the addition of another primer HMB01 for amplpification of HPV type 51 (Hildesheim *et al*, 1994). A further modification has been the creation of inosine modified pools of oligonucleotides comprising a set of 5 upstream oligonucleotides PGMY11 and a set of 13 downstream oligonucleotides, PGMY09 (Gravitt *et al*, 2000), with the added advantage of improved quality control in production of primer stocks over the original MY09/11. The GP5+/6+ primers were developed from the original general primers GP5/6 by the elongation of the primer sequences at their 3' end to reduce background amplification and to improve specificity and sensitivity (de Roda Husman *et al*, 1995). The GP5+/6+ primers amplify a 150 bp region within the 450 bp MY09/11 product. The GP5+/6+ primers are designed to fully complement only a few HPV genotypes. To compensate for this annealing is performed at a slightly lower temperature to allow less stringent binding of the primer at mismatched sites. Other less widely used primers sets targeting the L1 gene include the SPF10 (Kleter *et al*, 1998) and the LIC1/2 primer sets (Yoshikawa *et al*, 1991). Much debate has been generated over the choice of primers. In general the efficiency of a PCR decreases with increase in amplimer size. However, this is only a limiting factor where the quality of template DNA is in question, thus choice of DNA extraction procedure should be considered alongside choice of primer set (Keegan *et al*, 2005a). In addition to primer selection, debate exists over the combined use of multiple primer sets. Husnjak et al (2000), compared the performance of 5 different primer sets and found that the MY09/11 and LIC1/2 primer sets demonstrated equal sensitivity for HPV detection when used separately but increased sensitivity when used sequentially (Husnjak *et al*, 2000). Similarly, improved sensitivity of HPV detection was seen when

MY09/11 and GP5+/6+ primers were used in a nested PCR format (Husjnak *et al*, 2000; Strauss *et al*, 2000).

A further consideration to be taken into account when choosing primers for HPV amplification is that on integration of some HPVs into the host sequences, the L1 gene and E1 genes may be deleted (Matsukura T *et al*, 1989; Wagatsuma *et al*, 1990). Integration often disrupts the integrity of the E1 and E2 genes. However, the E6 and E7 genes, which are retained in all tumours, are too variable in sequence for the design of general primer sets. The CPI/II primers (Tieben *et al*, 1993) directed to the E1 gene are less sensitive than either the MY09/11 or GP5+/6+ primers when used separately (Karlsen *et al*, 1996). However, the sensitivity of detection of HPV by a combination of the three primer sets is greater than detection by separate type specific PCRs for types 11, 16, 18, 31, 33 and 35 (Karlsen *et al*, 1996).

A recent development in the area of HPV primer design, has been the design of multiplex nested PCR assay for the detection of 15 high-risk HPV types by amplification of sequences in the early region of the genome so as to ensure the amplification of integrated virus in the absence of episomal forms (Brestovac *et al*, 2005a). A further advantage of this assay is the ability to genotype infecting HPVs based on the difference in size of the amplified fragments (Brestovac *et al*, 2005a).

Type-specific PCR using primers designed to amplify an exclusive HPV type may also be performed (Depuydt *et al*, 2003; Kraus *et al*, 2004). Primers are commonly designed to amplify in the highly variable E6-E7 region, however successive rounds of amplification must be performed with each primer set separately making it labour intensive and expensive. A newer approach to typing by PCR had been the development

of a nested multiplex PCR (NMPCR) directed to the E6 gene. The NMPCR method has shown similar sensitivity to the MY09/11 and GP5+/6+ with the ability to detect multiple infections (Sotlar *et al*, 2004).

A novel method for HPV detection and typing was developed by Szuhai *et al* (2001) involving detection by the CPI/II primers in a SYBR Green™ detection assay followed by typing using type-specific molecular beacons. Other groups have developed real-time quantitative assays applicable to PreservCyt samples for a limited number of types in a multiplex format (Tucker *et al*, 2001). Detection, typing and quantitation are possible using scorpion probe technology (Hart *et al*, 2001). Real-time PCR has also been used to estimate the physical status of HPV by comparison of E2 and E6 gene copy numbers (Nagao *et al*, 2002; Peitsaro *et al*, 2002). Numerous studies have involved the use of Taq-Man® technology in HPV quantitation (Swan *et al*, 1999; van Duin *et al*, 2002; Gravitt *et al*, 2003b). Real-time methods based on reverse-transcription PCR have been developed to estimate the levels of E6 and E7 expression (Lamarcq *et al*, 2002; Wang-Johanning *et al*, 2002).

While numerous in-house PCR methods have been developed for the amplification and or typing and quantitation of HPV, none of these have received FDA approval and lack external quality assessment making interlaboratory comparisons and large-scale evaluation difficult. Currently there is one commercially available HPV RNA transcription assay the PreTect HPV Proofer (Norchip, Norway).

1.6.4 *HPV testing in cervical screening*

The realisation that HPV is a cause of cervical cancer worldwide has led to much debate on the incorporation of HPV testing into cervical screening programmes. Three main

strategies have been proposed. The first is that HPV testing be used either as a stand alone test or as an adjunct to cytology in primary screening, the second is that HPV testing be used in the management and triage of women with LSIL or mild epithelial abnormalities and the third is that HPV testing be used as a test of cure after treatment of high-grade disease.

HPV testing alone

The sensitivity of HPV testing in the identification of high-grade neoplasia cases is very high and greater than that of cytology in the identification of true disease (Cuzick *et al*, 1999b; Schneider *et al*, 2000). However the specificity of a single HPV test is lower than that of cytology (Schneider *et al*, 2000; Clavel *et al*, 2001; Lee *et al*, 2004). This is because the vast majority of HPV infections are permissive and clear without any deleterious effects (Ho *et al*, 1998). This is particularly true in the case of young sexually active women who show the highest rates of prevalence with a peak in incidence during their early twenties (Burk *et al*, 1996b). It has been reported that 70%-90% of infections in young women clear within 12-24 months (Moscicki, 2003). HPV testing alone, would therefore, only be useful in the over 30 years age group. One of the objectives of this research project was to stratify the incidence of HPV infections according to age, in Irish women attending routine cervical screening.

HPV and cytology combined testing

The major cost involved in running cervical cancer screening programmes is in the taking and processing of the sample. As the development of cervical cancer is staged over a number of years, the average woman will undergo a number of screening rounds in her lifetime. Any attempt to improve the cost-effectiveness of screening should aim to increase the safe-limit of screening intervals. With this in mind numerous studies

have looked at performing the more sensitive HPV testing as an adjunct to the more specific cytology.

A recent Dutch study by Van den Akker-van Marie *et al* (2003), found that HPV testing could be used to decrease the screening interval from 5 years to 7-10 year intervals. In a recent French study by Clavel *et al* (2004), which followed 4,400 women for 12-72 months it was found that a double negative cytology and HPV test result had a negative predictive value of 99.9% for cervical neoplasia which increased to 100% if a follow up HPV test 2 years later was also negative (Clavel *et al*, 2004).

In the U.K., the HART (HPV in Addition to Routine Testing), study which involved HPV testing and conventional cytological screening of 11,000 women aged 30-60 years with randomisation to repeat HPV, repeat cytology and colposcopy at 12 months, or immediate colposcopy, concluded that HPV testing could be used for primary screening of women over 30 years of age using cytology as a triage (Cuzick *et al*, 2003).

In the U.S.A. the high negative predictive value of a HPV test has led to the FDA approval for HPV DNA testing in women over 30 in combination with cytology for primary screening with a 2-3 year recall period for double negative women (Wright *et al*, 2004). Other studies have shown that HPV DNA testing could be used to reduce the age of exit from screening programmes from the currently recommended 65-75 years to 50 years (Cruickshank *et al*, 2002; Baay *et al*, 2004).

HPV testing in the management of women with LSIL or borderline abnormalities.

Existing management of patients with low-grade disease or repeat borderline abnormalities is either immediate colposcopy, or follow-up smears with colposcopy.

This contributes greatly to the workload of any screening programme, particularly considering that the majority of low-grade lesions will regress over time. It is estimated however that 10-15% of low-grade lesions contain high-grade abnormalities that may put the patient at risk in the absence of close follow-up (Kinney *et al*, 1998). It has been argued that due to its high positive predictive value in detecting high-grade disease outcome, HPV testing could be an effective triage of these cases. An investigation was conducted by the National Cancer Institute of America to compare three management strategies for women with LSIL or ASCUS diagnoses, namely HPV triage with referral if positive, immediate colposcopy or repeat cytology with referral to colposcopy if HSIL or worse was detected. The results of this ASCUS-LSIL Triage Study (ALTS) indicated that HPV testing was effective in the case of AGUS and ASCUS diagnosis (ALTS Group, 2003b) but not for LSIL as the HPV positivity rate was greater than 80% (ALTS Group, 2003a).

HPV as a test of cure following treatment of high-grade disease

Due to the very low incidence of HPV negative CINII or III cases, HPV testing has been proposed to detect women treated for CINII or III who have residual disease. Treatment failure of 10% has been reported and most commonly occurs in the first 2 years (Baldauf *et al*, 1998). A study performed by Zielinski *et al* (2003), where women were tested by cytology at 3, 6, 12 and 24 months post treatment and HPV at 3 months and thereafter if positive, found that those who tested double negative were at no more increased risk of development of CIN as women who tested negative in routine screening.

1.6.5 *HPV and cervical cancer screening in Ireland*

In Ireland, deaths from cancer account for 23.4% of all deaths. Of these, cancer related deaths, 2.1% are from cancer of the cervix (Comber and Gavin, 2005). The lifetime risk of cervical cancer in Irish women is estimated at 1 in 80 (Irish Cervical Screening Programme (ICSP), 2005). In 1997, a ministerial decision announced that a National Cervical Screening Programme would commence in the Mid-Western Health Board (MWHB). In October 2000, the ICSP was established with the main aim to develop a national strategy to tackle cervical screening through the development of an organised screening approach. The main focus of the cervical screening programme is the development of a secure national registry containing the contact details and test results of women eligible for cervical screening. At present the ICSP is operational in the mid-western health board region only. Free cervical screening is offered to women between the ages of 25 and 60 years with a screening interval of 5 years and women are invited to attend for screening by mail. For the rest of the country, screening is opportunistic, and takes place among those attending general practitioners, postnatal examinations, sexually transmitted disease and family planning clinics and by self-referral. It is well known that cervical cancer is linked to HPV infection and at present there is no routine HPV testing performed, although a commercial service has recently become available in Dublin. Features of HPV infection are routinely reported in cervical smear test results.

1.7 *Chlamydia trachomatis* biology

1.7.1 *Classification*

Chlamydiae are small, Gram-negative intracellular eubacteria. Due to their unique developmental cycle, chlamydiae are recognised as their own order *Chlamydiales*. The word "*chlamys*" is Greek for "cloak draped around the shoulder". This describes how

intracytoplasmic inclusions containing the organism are draped around the infected cell nucleus, (Figure 1.3).

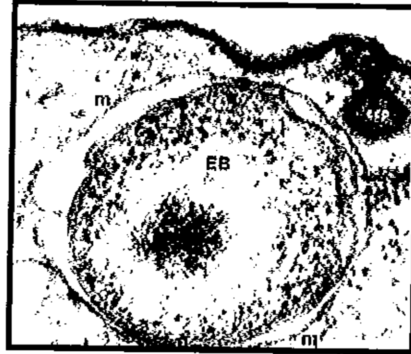


Figure 1.3. Electron micrograph of *C. trachomatis* elementary body (EB) contained within a membraneous vacuole (M)

This order consists of one family Chlamydiaceae, and two genera *Chlamydia and Chlamydophila* with nine characterised species (Everett, 2000). The species *C. trachomatis* is further divided into two biovars: the first of these is the trachoma biovar consisting of serotypes A, B, Ba, C-K with serotypes A-C commonly causing trachoma and serotypes D-K causing genital infections (Everett, 2000). The second of these is the lymphogranuloma venereum biovar with serotypes L1-L3 (Schachter and Meyer, 1969). The classification of *C. trachomatis* into serotypes was originally done using the mouse toxicity prevention test (MTPT) (Wang and Grayston, 1963) and later using the microimmunofluorescence (MIF) test (Wang and Grayston, 1970). Modernly, genotyping based on differences in the sequences of the variable regions of the major outer membrane proteins is used for type identification (Millman *et al*, 2004; Osterlund *et al*, 2005).

1.7.2 *C. trachomatis structure*

C. trachomatis has a unique biphasic life cycle in which there are two morphologcially and functionally distinct cell types: the infectious elementary body (EB) and the reproductive reticulate body (RB). The EBs are ~ 0.3μm in diameter and are adapted to extracellular survival, whereas the RBs are ~ 1μm in diameter, exist intracellularly and are metabolically active (Hammerschlag, 2002). A cell envelope similar to the Gram-negative cell envelope structure surrounds both cell types. The envelope consists of two trilaminar membranes, an outer and an inner membrane, however there is no peptidoglycan in the periplasmic space (Yen *et al*, 2005), a factor that contributes to the integrity of other Gram-negative envelope systems. This was shown by the inability to detect muramic acid by anti-muramic acid antibodies (Hatch, 1996). The outer membrane of *Chlamydia* contains a major outer membrane protein (MOMP) with a molecular weight of ~ 40 kDa. MOMP accounts for approximately 60% of the dry weight of the outer membrane of the EB and is the most antigenic protein of *C. trachomatis* (Gomes *et al*, 2004). It is analogous to porin proteins found in other Gram-negatives. Disulphide bridging is responsible for crosslinking of the MOMP maintaining the integrity of the cell structure (Hatch, 1996). The MOMP is predominantly in a monomeric form in the RB, however, it forms multimonomeric complexes in EBs and is responsible for resistance to mechanical and osmotic stress (Raulston, 1995.) Cysteine-rich proteins in the outer membrane may be the functional equivalent to peptidoglycan, potentially forming disulfide-linked complexes with other membrane proteins (Yen *et al*, 2005). Chlamydial specific lipopolysaccharide (LPS) is present on the outer membrane of the organism at all stages in the developmental cycle. In superinfected cells LPS has been isolated from the host cell membrane. The incorporation of LPS into the membrane would reduce membrane fluidity and make the infected host less susceptible to cytotoxic T-cell attack (Raulston, 1995).

Chlamydiae possess unique hemispheric surface projections that are specialisations of the plasma membrane (Gregory *et al*, 1979). These protrusions are clustered hexagonally. They range from 45-90 nm in length and consist of a helical arrangement of subunits of average diameter 6 nm. The EB contains ~ 18-22 of these projections in an ordered array, while in the RB these structures are much less ordered, are often found in close contact with the inclusion membrane and may serve in the acquisition of nutrients from the host cytoplasm (Raulston, 1995). These projections may also act in conjunction with the MOMP and cysteine-rich proteins in promoting attachment and entry to the host cell.

1.7.3 *C. trachomatis genome*

The chlamydial chromosome consists of 1,045 kb divided into 894 open reading frames between 135 and 5,358 nucleotides long with a median length of 867 nucleotides and arranged as double stranded circular DNA (Brunelle *et al*, 2004). The acquisition of exogenous DNA is thought to have played a limited role in the evolution of the species since adaptation to the intracellular environment over a billion years ago (Stephens, 2002). This lack of genetic transformation systems has made the study of their molecular biology difficult in the past, however genome sequencing has greatly advanced existing knowledge on the biology of these organisms (Vandahl *et al*, 2004).

Sequencing of various strains of *C. trachomatis* has revealed a high level of genomic conservation and synteny, suggesting that minor differences account for the wide-ranging tissue tropisms and virulence levels (Carlson *et al*, 2004). These differences have been localised to a polymorphic region of the genome termed the plasticity zone. Common polymorphisms include mutations in the tryptophan synthase operon, which

allow genital strains to persist among mixed microbial infections and in the cytotoxin locus, which confer increased virulence (Carlson *et al*, 2004).

A 7.5 kb cryptic plasmid is a common and perhaps essential component of the *C. trachomatis* genome. DNA sequences homologous to the *C. trachomatis* plasmid are not found in eukaryotic DNA nor in other chlamydial species, thus the origin of this plasmid is unknown (Palmer and Falkow, 1986). The *C. trachomatis* plasmid is present at 6-10 copies per organism. The plasmid is temporally expressed during growth in eukaryotic cells. Plasmid specific RNA molecules are detected from 24-48 h post infection, thus it does not function in the early stages of the life cycle. Instead the synthesis of plasmid RNA correlates to the growth of reticulate bodies and their differentiation into elementary bodies (EBs) (Palmer and Falkow, 1986). The *omp1* gene, which codes for the major outer membrane protein (MOMP) consists of five constant and four variable regions. The variable regions form the basis for genotyping by sequencing of the *omp1* gene. Variable sequence regions I, II, and IV are surface exposed and are involved in specific antibody interactions.

1.7.4 *C. trachomatis life cycle*

Infection of host cells is initiated by the attachment of EBs to host cells. As is the case for many bacteria, it is likely that multiple mechanisms of attachment exist. A heparin sulphate ligand on the surface of the host cell has been suggested to be involved in a trimolecular interaction with both host cell and chlamydial receptors (Raulston, 1995). Other experiments implicate proteinaceous components in the attachment process as infectivity of EBs is adversely affected by an increase in temperature (Raulston, 1995).

Once attached, the EB enters the cell by receptor-mediated endocytosis involving both microfilament and clathrin-dependant mechanisms with associated tyrosine kinase

activation in a cascade of cytoskeletal rearrangements modulated at certain stages by intracellular calcium (Raulston, 1995). After attachment and ingestion the EB enters the cell contained in an enlarged endocytic vacuole called an inclusion body. In *C. trachomatis* infected cells these inclusions eventually fuse to form one inclusion body per cell whereas those containing *C. psittacci* develop independently resulting in multiple inclusion bodies per cell.

Once the EB has entered the cell it reorganises itself into the RB. The RB is the metabolically active replicating form. It divides by binary fission with a doubling time of ~2 h, enlarging the endosome while genus specific antigen becomes associated with the host cell surface (Raulston, 1995). EBs, RBs and intermediate forms may be found in the same inclusion (Gupta *et al*, 1979). After approximately 24 h the DNA condenses within the RBs to form infectious EBs. MOMPs are crosslinked by disulphide bonding. The infectious EBs are released 48 h after initial infection by exocytosis. The sequelae of genital chlamydial infection often involve a persistent form of the organism. In persistent infection RB-like forms remain in an aberrant metabolic and mechanical state in cytoplasmic inclusions (Gerard *et al*, 2002).

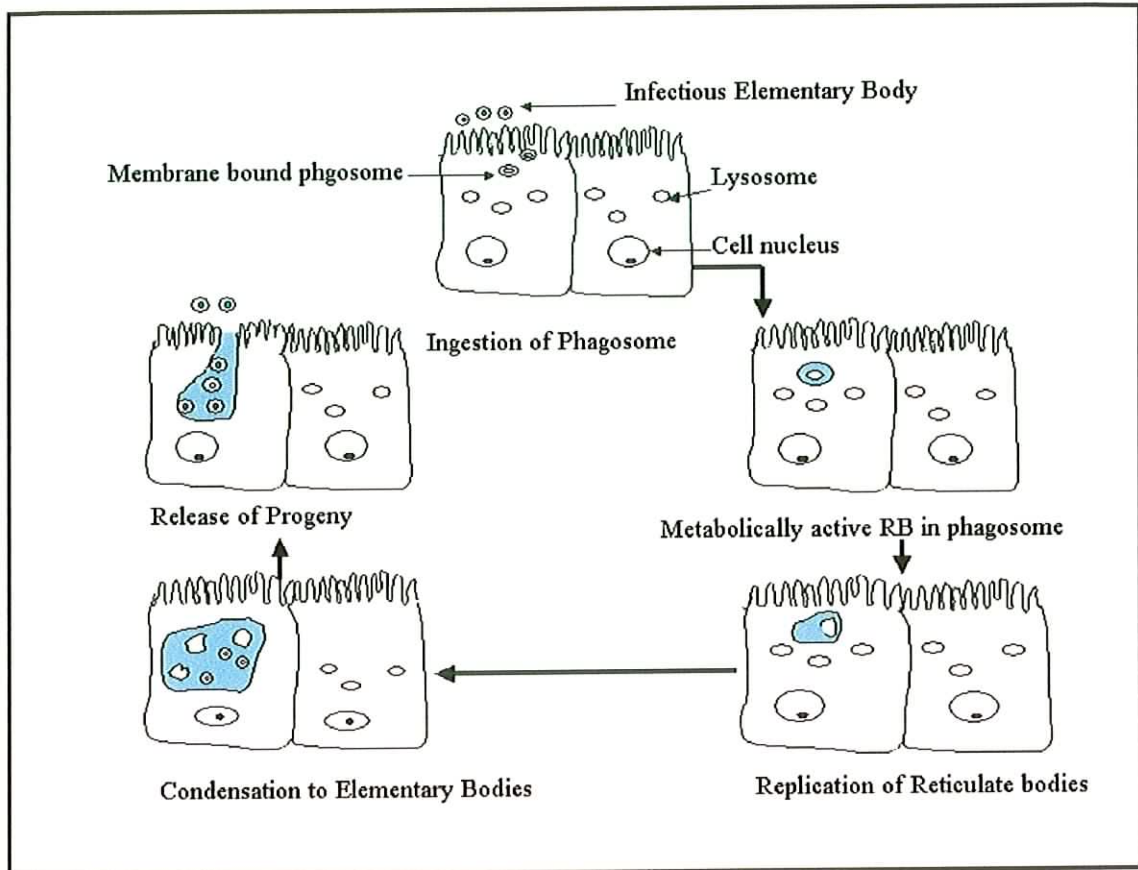


Figure 1.4. Bi-phasic lifecycle of *C. trachomatis*. Adapted from DeMets, 2005

1.8 *C. trachomatis* epidemiology and pathogenesis

1.8.1 *Clinical manifestations of C. trachomatis infections*

Chlamydial species cause a wide range of infections in humans. Respiratory infection with *Chlamydophila pneumoniae* occurs in the majority of people over the course of their lifetime (Wang and Grayston, 1990). *C. pneumoniae* causes an average of 10% of community-acquired pneumonia cases and 5% of all sinusitis and bronchitis cases (Kuo *et al*, 1993). *C. pneumoniae* DNA has also been detected in atherosclerotic lesions (Kravenbuehl *et al*, 2005) with over 50% of lesions examined positive by nucleic acid amplification techniques (Danesh *et al*, 1997). Avian strains of *Chlamydophila psittaci* are a well-known cause of psittacosis in humans (Saito *et al*, 2005). Recently, it has been suggested that persistent *Chlamydia* infections are associated with the

development of schizophrenia and other mental disorders (Fellerhoff *et al*, 2005) including Alzheimer's disease (Itzhaki *et al*, 2004).

C. trachomatis species cause a wide spectrum of human disease depending on the serotype involved. Serotypes A-C cause conjunctivitis particularly following vertical transmission through the birth canal. Persistent infections with these serotypes are a major cause of trachoma and blindness in developing countries worldwide. The World Health Organisation (WHO) estimates that 146 million people have trachoma due to ocular *C. trachomatis* infections and that 4.9 million of these are totally blind (Whitcher *et al*, 2001).

Serotypes L1-3 are sexually transmitted and cause the systemic disease Lymphogranuloma Venereum which is characterised by inflammation and drainage of the inguinal and lower abdominal lymph nodes and destruction and scarring of the surrounding tissue (Mabey and Peeling, 2002). Serotypes D-K are the main genital types. These commonly cause infections in both men and women with more serious consequences for women. In men, *C. trachomatis* infections cause a range of symptoms such as urethritis, epididymitis, proctitis, uveitis and the pathogen has also been implicated in Reiter's Syndrome, an inflammatory disease that can affect multiple sites in the body.

In women, *C. trachomatis* is a common cause of urethritits (Stamm *et al*, 1980) and cervicitis (Brunham *et al*, 1984). The symptoms of chlamydial cervicitis include endocervical mucopus, erythema, oedema and induced mucosal bleeding (Brunham *et al*, 1984). In women, most cases of chlamydial cervicitis are asymptomatic showing no specific clinical signs. Approximately half of all *C. trachomatis* infected women have

the organism present at both the cervix and the urethra, one third at the cervix only and 15%-25% at the urethra only (Paavonen and Eggert-Kruse, 1999). Histopathological findings associated with chlamydial cervicitis include plasma cell infiltrations of the cervical stroma, intraepithelial and intraluminal inflammation and well-formed lymphoid follicles comprising transformed lymphocytes (Paavonen and Eggert-Kruse, 1999). While the majority of chlamydial cervicitis cases are subacute, persistent chronic genital *C. trachomatis* infections can have serious consequences for the reproductive health of the women. Complications associated with ascending intraluminal spread of the organisms include pelvic inflammatory disease (PID), infertility, ectopic pregnancy, premature rupturing of the membranes, chorioamnionitis and preterm delivery.

PID refers to infection of the uterus fallopian tubes and adjacent pelvic structures that is not associated with surgery or surgical procedures (McCormack *et al*, 1979). An increasing proportion of cases of PID are atypical or silent (Paavonen, 1985). The clinical spectrum of PID ranges from subclinical endometritis to overt salpingitis, tubo-ovarian abscess, peritonitis, periappendicitis and perihepatitis. Chlamydial PID is the most important preventable cause of infertility and adverse pregnancy outcome. After a single episode of PID the relative risk of tubal infertility is 10% with each incident doubling the risk (Westrom, 1994). The link between past chlamydial infection and tubal infertility was first proposed in 1979 (Punnonen *et al*, 1979). Worldwide, huge amounts of money are spent on *in vitro* fertilisation (IVF) as a result of post-PID tubal infertility. Women with a history of PID are 7-10 times more likely to have ectopic pregnancies compared with women with no history of PID (Westrom *et al*, 1998). Ectopic pregnancy is the main cause of maternal death in the first trimester of pregnancy in developing countries and accounts for 9% of all pregnancy related deaths in the U.S.A.

1.8.2 *Epidemiology of C. trachomatis infections*

C. trachomatis infection is the most common bacterial sexually transmitted disease worldwide, with an estimated 89 million cases occurring worldwide (WHO, 1995). It is estimated that 4 million new cases of the disease occur in the US alone each year with an annual cost of 2.4 billion dollars. *C. trachomatis* infections increase the risk of transmission of the HIV virus, thus treatment and prevention of these infections would influence the spread of HIV, particularly in high risk groups. The major risk factors associated with *C. trachomatis* infections are young age, number of sexual partners, a new sexual partner, lack of use of barrier contraceptive devices, and a history of other STIs. Although the major disease burden of *C. trachomatis* infections is born by women, men and children can also be infected. Up to 80% of infected women and 50% of infected men are asymptomatic resulting in a large reservoir of unrecognised infected individuals capable of transmitting the disease to others. Immunity to the organism is type specific therefore recurrent infections are common. The prevalence of *C. trachomatis* in sexually active young women can range from 10%-40% depending on the setting and population studied and is found to decrease with increasing age (van den Hoek *et al*, 1999). A recent pilot study of opportunistic screening in England found that the prevalence among 16-24 year old women in Portsmouth and Wirral was 9.8% and 11.2% respectively (La Montagne *et al*, 2005) with the highest burden of infection seen in the <20 years age bracket (Pimenta *et al*, 2003). The Biomed Concerted Action Group conducted a systematic review of prevalence studies of *C. trachomatis* in asymptomatic women in Europe in 2002. The prevalence of *C. trachomatis* ranged from 1.7% to 17% depending on the setting context and country (Wilson *et al*, 2002). Genital serotypes D-K are the main infecting serotypes globally while infection with the trachoma and LGV serotypes are more commonly found in developing countries. Infection with LGV is most commonly found in Africa, Southeast Asia, Central and

South American and Caribbean countries (Mabey and Peeling, 2002). However, in recent years there has been an increase in prevalence in industrialised countries in particular in male homosexuals (Spaargaren *et al*, 2005; Niewenhuis *et al*, 2004).

C. trachomatis infection is one of the 11 notifiable sexually transmitted infections in Ireland (Health Protection Surveillance Centre (HPSC), 2005). Since 1995 there has been an increasing number of cases reported each year reaching 1,649 in 2001. Notified cases increased by 573.1% during these years. Between 2003 and 2004 the numbers of reported *C. trachomatis* cases rose by 24.1% increasing from 2,258 to 2,803 during these years (HPSC, 2005). This increase in *C. trachomatis* prevalence is thought to be associated with an increase in unsafe sexual behaviour although the rise in numbers of cases may reflect increased testing rates and the greater use of nucleic acid based detection systems (HPSC, 2005). One of the aims of this research project was to establish the prevalence of *C. trachomatis* in the Irish female urban population, attending their general practitioner (GP) for routine cervical screening, as currently there are no figures available for *C. trachomatis* prevalence in the general female population.

1.8.3 *Immunopathogenesis of C. trachomatis infections*

The understanding of the immunopathological pathways involved in *C. trachomatis* infection and reproductive sequelae remains incomplete. Studies in macaques have shown that single episodes of *C. trachomatis* infection are usually resolved but repeated infections eventually produce tubal scarring. These and other findings, suggest that acquired immune responses are important in the pathology of *C. trachomatis* infections (Patton *et al*, 1997).

C. trachomatis stimulates both humoral and cell-mediated immune responses. The outcome of an infection depends on the balance of cytokines secreted by activated lymphocytes. Interferon γ a product of T-helper (Th1) cells is considered the single most important factor in the defence against chlamydial infection, while susceptibility has been associated with enhanced expression of IL-10, a marker of T helper (Th2) cell activation (Beatty *et al*, 1993).

One current hypothesis is that the chronic sequelae of *C. trachomatis* infection are caused by a delayed hypersensitivity reaction to an increase in expression of the chlamydial heat-shock protein (Hsp60), (Hogan *et al*, 2004). The increase in expression of the *hsp* genes facilitates immune evasion and allows the bacterium to persist in otherwise uninhabitable environments. As chlamydial growth becomes inhibited by acquired immune responses autoimmunity may sustain inflammation and tissue damage (Cohen and Brunham, 1999). Numerous studies have established the link between antibodies to chlamydial Hsps and adverse outcome of infection (Cortinas *et al*, 2004; Karinen *et al*, 2004; Di Felice *et al*, 2005).

Significant evidence exists for the role of host immunogenetic determinants in the outcome of *C. trachomatis* infections, specifically the role of human leukocyte antigen (HLA) genotypes in the manifestation of adverse consequences. (Holland *et al*, 1996; Geisler *et al*, 2004; Ness *et al*, 2004).

What determines the immunopathological outcome of an infection depends on a number of factors including HLA determinants and cytokine profile. Predicting those who may develop serious disease may contribute to the overall management of infected women at early stages and assist in prevention.

1.8.4 *C. trachomatis and the link with cervical cancer*

Over the last decade, many investigations have been conducted into the role that sexually transmitted infections other than HPV may have in the development of cervical cancer. Numerous longitudinal studies with cervical cancer as an end-point have identified an association with *C. trachomatis* and increased risk for the development of cervical cancer (Hakama *et al*, 1993; Koskela *et al*, 2000; Anttila *et al*, 2001; Wallin *et al*, 2002). Cross-sectional case-control studies identifying *C. trachomatis* as a possible co-factor in cervical cancer development were conducted as far back as 1975 (Schachter *et al*, 1975).

In 2004, the IARC, Lyon, France, conducted a large multi-centre hospital-based case-control study to determine whether *C. trachomatis* is consistently associated with an increased risk of invasive cervical carcinoma. The study comprised 1,238 cases of invasive cervical carcinoma (ICC) and 1,100 control women from 7 countries. *C. trachomatis* serum antibody was detected by means of the MIF assay. After adjustment for age, oral contraceptive usage, centre, history of Pap smears, number of full-term pregnancies and HSV2 positivity, the risk of squamous cell carcinoma was elevated in *C. trachomatis* seropositive women. The contributory factor of seropositivity to risk of development of ICC increased with increasing *C. trachomatis* antibody titres and was higher in women under 55 years of age. Moreover, an association of *C. trachomatis* was found in all cases and controls regardless of HPV infection status (Smith *et al*, 2004).

When the relationship of specific genotypes of *C. trachomatis* to the development of cervical squamous cell carcinoma was investigated, it was found that type G was most strongly associated followed by serotypes I and D and that the presence of multiple types conferred increased risk (Anttila *et al*, 2001). Other studies have postulated that *C.*

trachomatis infection may simply be an indicator of sexual activity and hence a surrogate marker of oncogenic HPV infection (Lanham *et al*, 2001).

The molecular mechanisms for the association of *C. trachomatis* and cervical cancer have not yet been fully elucidated. However, a number of suggestions have been proposed, most founded on the induction and maintenance of chronic inflammation at the cervix by *C. trachomatis* during persistent infection. The mechanism of epithelial cell proliferation is thought to be different to that of HPV. Upon macrophage recognition of chlamydial antigen, inflammatory cytokines; tumour necrosis factor alpha (TNF-α) and interleuken (IL)-1a are produced causing neighbouring fibroblasts to release IL-6, which causes upregulation of tumour growth factor alpha (TGF-α) and amfiregulin. The release of these stimulatory cytokines causes proliferation of keratocytes (Fischer, 2002). It was found that infection with *C. trachomatis* caused a simultaneous increase in the expression of TGF-α and in the expression of epithelial growth factor receptor (Fischer, 2002). In this same study it was found that *C. trachomatis* infection causes an increase in expression of HPV16 in CINI cases.

C. trachomatis induced proliferation may act synergistically with HPV in the promotion of dysplasia in the cervix. This notion of *C. trachomatis* as a cofactor in carcinogenesis may be corroborated by a study by Woodworth *et al* (1995), which demonstrated an association between upregulation of IL-1 and TNF-α production and increased proliferation of HPV16 and 18, albeit in HPV immortalised cell lines (Woodworth *et al*, 1995). In this way *C. trachomatis* may act as a cofactor to HPV infection by conferring a selective growth advantage on cells previously immortalised by HPV.

C. trachomatis may also be an independant cofactor in cervical carcinogenesis, mainly through the induction of an anti-apoptotic environment at the cervix. During chronic *C. trachomatis* infection upregulation of *hsp60* is necessary to maintain persistence. It has been hypothesised that the accumulation of exogenous Hsp60 in the cytoplasm of actively replicating eukaryotic cells may cause aberrant regulation of the apoptotic pathway (Di Felice *et al*, 2005). More specifically antibodies to Hsp60-1 (one of the three isotypes of chlamydial Hsp60) have been associated with increased risk of development of cervical cancer (Paavonen *et al*, 2003). Recently *C. trachomatis* L2 strain was demonstrated to cause proteolytic degradation of Bmf, Noxa and tBid, members of the proapoptotic Bcl-2 protein family (Ying *et al*, 2005). A recent cDNA microarray experiment on *C. trachomatis* infected and uninfected non-replicating cell lines found a 2-fold decrease in expression of the tumour suppressor gene caveolin-1 and an increase in expression of the oncogene *c-myc*, a promoter of cervical carcinogenesis (Schlott *et al*, 2005). Further cDNA microarray experiments would prove useful in the elucidation of possible mechanisms by which *C. trachomatis* may contribute to neoplastic changes in the transformation zone of the uterine cervix.

1.9 Clinical diagnosis of *C. trachomatis* infection

1.9.1 *Traditional methods*

For many years, the "gold-standard" for detection of *C. trachomatis* was cell culture. Cell culture has, in the past, been the reference method for assessing new diagnostic techniques due to its high specificity. Recently the CDC declared that a sample should be considered positive if two or more nucleic acid amplification tests are positive. While highly specific, culture is relatively insensitive (70-80% in the hands of experts), when compared to nucleic-acid based techniques (Loeffelholz *et al*, 1992). Cell culture is also very labour intensive and time-consuming requiring 3-7 days for results. Cell

culture is the only method that is based on the detection of live organisms. Stringent requirements for cold-chain transport of the specimens and various aspects of the cell culture technique can compromise the viability of the organism, thus it is not particularly suited to routine screening. In brief, collected organisms are inoculated onto McCoy cell monolayers and cultured for 48-72 h. Inclusions are then detected by staining with monoclonal antibodies to the MOMP.

Differential Fluorescence antibody tests (DFA) involve the application of fluorescein-conjugated antibody directed to the common determinant of MOMP or to the LPS directly to the sample material on the slide. The sensitivity of DFA is 80-90% and the specificity is 90-100% relative to culture (Chernesky *et al*, 1986; Quinn *et al*, 1987; Smith *et al*, 1987). Although the DFA test is rapid and applicable to a wide range of sample types, it involves microscopic evaluation and requires specially trained personnel and, therefore, is not suited to screening in a low prevalence population. The DFA test has lost favour as a primary diagnostic test and is used mostly as a confirmative test for presumptive positive results of other non-culture tests in light of its high specificity (Singh *et al*, 2002). DFA could prove useful in a point of care same day diagnosis setting in a high-risk population (Swain *et al*, 2004).

Enzyme Immunosorbent Assays (EIAs) involve the application of enzyme linked conjugated antibodies directed to the LPS of the organism. The total processing time of the assay is 3-4 h, but antibodies to *C. trachomatis* LPS may also cross-react with other chlamydial species and with the LPS of other Gram-negative organisms giving false positive results. Manufacturers (MicroTrac, Chlamydiazyme) have developed a blocking antibody test to verify *C. trachomatis* positive results reducing false positives. Both DFAs and EIAs are commonly used in the detection of anti-chlamydial IgG in the

sera of women with infertility, pelvic inflammatory disease, and ectopic pregnancy (Mouton *et al*, 2002; Jones *et al*, 2003).

In 1979 Gupta described changes in Pap smears that might be attributable to *Chlamydia trachomatis* infection and suggested the possibility of detecting the organism through routine cervical cytology (Gupta *et al*, 1979). Giemsa staining can be used to visualise intracytoplasmic inclusion bodies with some degree of sensitivity (>90%), (Schachter *et al*, 1978) in cases of chlamydial conjunctivitis, however when applied to genital infections this sensitivity drops considerably to an average of 27% (Bernal *et al*, 1989). Direct cytology is associated with large numbers of false positives and false negatives and is not recommended for laboratories that receive specimens only occasionally (Black, 1997). More recently the use of endocervical Gram stain results together with clinical information have been proposed in the identification of women at high-risk for chlamydial infection (Myziuk *et al*, 2001).

Paler *et al*, (2000) reported that the presence of inflammatory cells in Pap smears as a marker for *C. trachomatis* infection had a positive predictive value of 65% and a sensitivity of 83% (Paler *et al*, 2000). *C. trachomatis* has been reported in 20% of women who have an inflammatory Pap smear (Burke and Hickey, 2004).

The leukocyte esterase test involves the detection of enzymes produced by leukocytes containing the bacteria in urine. It is a quick dipstick based test and results are immediate. The dipstick holds an absorbent patch containing indoxyl carbonate ester that forms a purple colour when hydrolysed by leukocyte esterase. The sensitivity of this test for *C. trachomatis* infection varies from 31%-100% and specificity ranges from 83%-100% (Black, 1997). When compared to NAAT for the detection of *C.*

trachomatis the sensitivity and specificity of this test in an asymptomatic male population were 57.9% and 78.3% respectively (Blake *et al*, 2005). Leukocyte esterase may be produced on infection with any number of organisms, thus a positive test requires an additional organism specific test to determine the cause of the urethritis, but it is a useful point of care test.

1.9.2 *Hybridisation based tests*

The development of nucleic acid based tests has resulted in significant improvements in the sensitivity of detection of *C. trachomatis.* A number of probe-based tests have been developed for the detection of *C. trachomatis.* The PACE 2 Test (Gen-probe, San Diego, California, U.S.A.) is the most commonly used *C. trachomatis* test in public laboratories in the U.S.A. The PACE 2 test involves the hybridisation of a chemiluminescent DNA probe to the species-specific sequence of the chalmydial 16S rRNA. Once the DNA-RNA hybrid is formed it becomes immobilised onto a paramagnetic bead and the chemiluminescence response is measured using a luminometer (Black, 1997). In a five-centre study using a new gold standard of positivity by two or more tests, it was concluded that ligase chain chain reaction (LCx; Abbott Laboratories) and PCR performed better than the PACE 2 assay when performed on endocervical swabs and urine (Black *et al*, 2002). In a comparison study by Young *et al*, (2000) on the relative sensitivities and specificities of PACE 2, LCx and culture of endocervical specimens, PACE 2 was less sensitive (52.4%) than both culture (71.4%) and LCx (100%) but just as specific.

The hybrid capture test (HCII CT/NG), (Digene, Maryland, U.S.A.) is a combination test designed to detect *C. trachomatis* and *Neisseria gonorrhoeae* in a single specimen. If the specimen tests positive, a further *C. trachomatis* identification (ID) test needs to be performed. This test operates on the same principle as the PACE 2 test and involves

the formation of DNA-RNA hybrids. When compared to culture its sensitivity and specificity was 97.7% and 98.2% respectively for the detection of *C. trachomatis* in 1,370 cervical specimens (Schacter *et al*, 1999). In a study by Girdner *et al*, (1999) the performance characteristics of the HCII CT-ID test were compared to those of culture and Amplicor *Chlamydia trachomatis* PCR (Roche Molecular Systems, Branchburg, New Jersey, U.S.A.) for the detection of *C. trachomatis* in 587 cervcial samples. While the sensitivities of both the HCII and PCR were much higher than cell culture (81.5%), the HCII method was more sensitive than the PCR method (95.4% vs 90.8%), however their specificities were the same at 99% (Girdner *et al*, 1999). In a study by Modarress *et al*, (1999), the HCII test was found to be more sensitive than the Gen-probe PACE 2 test in the detection of *C. trachomatis* when Gen-probe transport medium was used in the collection of specimens for the two tests.

A specifically designed rapid capture system has recently been developed by Digene which integrates all liquid handling, incubation, and shaking steps of the assay in an attempt to increase throughput of the system. The performance of this system, evaluated on 1,241 endocervical specimens, was comparable to that of the manual system (Van der Pol *et al*, 2002). Thus experimental evidence suggests that the HCII method is more sensitive than culture, PCR, PACE 2 and LCx while its specificity is equal to that of PCR.

1.9.3 ***Nucleic acid amplification tests for C. trachomatis***

Commonly used primers for *C. trachomatis* PCR target sequences on the cryptic plasmid, the *omp1* gene, and 16S rRNA gene sequences, although recently primers have been designed for use in real-time PCR targeting the heat-shock genes *hsp60* and *hsp70* (Wood *et al*, 2002). The plasmid primers and the MOMP primers remain the most

popular with the Amplicor PCR test involving the amplification of a 207 bp fragment of the plasmid. As the plasmid is present in *C. trachomatis* strains at a copy number of 7-10 per genome, plasmid directed PCR is more sensitive than the MOMP primers (1 copy per genome equivalent). However, amplification of the *omp1* gene can be used to genotype the organism by sequencing of the PCR product (Pannekoek *et al*, 2000). Although strains of *C. trachomatis* have been discovered which lack the plasmid (Farencena *et al*, 1997) independent laboratories have not demonstrated the absence of the plasmid consistently. Thus the clinical significance of such strains is controversial.

False negative PCR results pose a greater problem than false positives. False positives are most often a result of contamination. False negatives often arise due to the presence of PCR inhibitors in human-derived clinical samples. A nested PCR using the plasmid primers followed by the MOMP primers is often used to solve discrepant results (Pannekoek *et al*, 2000). In a study by Singh *et al* (2002), the sensitivity, specificity, positive predictive value and negative predictive value of plasmid PCR were 80%, 75%, 66.6% and 85.7% respectively when DFA was considered true positive.

Madico *et al*, (2000) have developed a touchdown enzyme time release PCR (TETR)-PCR for the detection of chlamydial species *C. trachomatis*, *C. pneumoniae* and *C. psittaci*. The assay amplifies sequences in the 16S and 16-23S spacer rRNA genes and distinguishes between species based on the size of the product. It employs a touchdown PCR to improve the annealing specificity of the primers. It uses a time-released hot start polymerase to avoid artefacts of non-specific amplification and allow amplification over 60 cycles, improving the sensitivity of the test. However, it is not yet commercially available. It has a sensitivity (96.7%) and specificity (99.6%) comparable to that of AMPLICOR PCR.

The commercially available BDProbeTEC™ET system, (Becton Dickenson Microbiology Systems) for the detection of both *C. trachomatis* and *N. gonorrhoeae* is an isothermal real-time assay using strand-displacement amplification. Unlike Taqman PCR the BDProbeTEC™ET uses an exonuclease–free polymerase (*Bst* DNA polymerase). The BDProbeTEC™ET was found to have sensitivity and specificity comparable to that of the Amplicor PCR (Chan *et al*, 2000).

The most commonly used commercial PCR-based test for the detection of *C. trachomatis* is the Amplicor CT/NG test (Roche Molecular Systems). This test has the potential to amplify both *C. trachomatis* and *N. gonorrhoeae* and is available in two formats, semi-automated and fully automated. In the semi-automated version amplified products are detected by an EIA on microwell plates while in the fully automated version (COBAS AMPLICOR Analyser) products are amplified and detected in the same unit. The test also includes an internal control to check for the presence of inhibitors in the sample. In a recent study by Bianchi *et al*, (2002) it was determined that PreservCyt is a suitable transport medium for the detection of *C. trachomatis* using the AMPLICOR assay. In a multicentre evaluation of the COBAS AMPLICOR and the Amplicor CT/NG on 2,192 matched endocervical swab and urine specimens obtained from women and for 1,981 matched urethral swab and urine specimens obtained from men the sensitivity off the COBAS AMPLICOR for the detection of *C. trachomatis* ranged from 92.1% to 98.8% depending on the specimen type, while the specificity was 99%. The two assays gave concordant results in 98.1% of the specimens (Van der Pol *et al*, 2000).

Another commercial nucleic-acid amplification technology for the detection of *C. trachomatis* is the Ligase Chain Reaction (LCx, Abbott Laboratories). In the LCx test

four sets of probes anneal to the target site on the cryptic plasmid thus the sensitivity and specificity of LCx are at least comparable to that of PCR. LCx may be performed on first-void urine or cervical swab samples in women.

The presence of a large number of erythrocytes in a sample has been associated with decrease in sensitivity of the assay, however studies have indicated that the presence of one endocervical cell is sufficient for *C. trachomatis* detection (Loeffelholz *et al*, 2001). A recent study by Castriciano *et al*, (2002) questioned the accuracy and reproducibility of the LCx on clinical urine samples with positive or near cut-off results. Twenty of 102 possible positive specimens showed discordant results by two repeat LCx assays (Castriciano *et al*, 2002). In February 2001, Abbott Laboratories issued a device correction letter stating that the specificity of some on-market lots of the test kit had fallen to 92%, but that the test sensitivity remained within the same range as before (Castriciano *et al*, 2002). Since then the LCx assay has been withdrawn from the market. Abbot laboratories are currently conducting research into the development of an improved LCx assay for the detection of *C. trachomatis* and *N gonorrhoeae* (www.abbott.com).

The Gen-Probe APTIMA™ assay (Gen-Probe Inc, San Diego, U.S.A.) for *C. trachomatis* uses transcription mediated amplification to detect sequences in the 23S rRNA, which is present in hundreds of copies of each organism. A study which compared Abbott LCx (LCx), BD ProbeTec ET (ProbeTec), and Gen-Probe APTIMA Combo 2 (AC2) on 506 urine specimens found that AC2 was the most sensitive (100%) with a specificity of 98.8% (Gaydos *et al*, 2004). The Gen-Probe APTIMA™ assay has just recently received clearance from the FDA for use in the detection of *C. trachomatis* and *N. gonnorhoeae* from PreservCyt samples (www.gen-probe.com).

An assay based on the amplification of 16S rRNA using the nucleic acid sequence-based amplification technique (NASBA; NucliSens Basic kit, Organon Teknika, Boxtel, The Netherlands) has been developed, (Mahony *et al*, 2001). Real-time PCR assays are currently being developed for *C. trachomatis* detection due to their quick turnover time (Koenig *et al*, 2004), and at present there is one commercially available real-time PCR assay for the detection of the *omp1* gene (RealArt *C. trachomatis* PCR Abbott Laboratories), (Eickhoff *et al*, 2003). However, their usefulness in quantitation of *C. trachomatis* infections has been less well explored (Burton *et al*, 2005). In this research project real-time PCR was employed to compare the *C. trachomatis* load in samples extracted by three different DNA extraction procedures.

1.9.4 ***Screening for C. trachomatis***

The importance of screening for *C. trachomatis* in routine pelvic examinations is highlighted by the fact that most infections are asymptomatic and if left untreated the infected person becomes a reservoir for further infection. Prevalence rates and the success of any screening programme are dependant on the positive predictive value of the diagnostic method employed. There is much debate over which diagnostic method gives the best possible results, however it is generally accepted that DNA based tests offer greater sensitivity and are less time consuming than more traditional methods. The CDC has highlighted the possible need for confirmatory testing when using NAATs in populations where the prevalence is less than 2% (Kohl *et al*, 2003).

Numerous studies have evaluated and advocated opportunistic screening approaches in health care settings (Harris, 2005; Tayal *et al*, 2005). In the UK, an opportunistic screening pilot study for *C. trachomatis* was conducted in Portsmouth and the Wirral between 1999 and 2000. Sexually active women aged 16-24 were offered a urine test

for *C. trachomatis* whenever they attended a healthcare setting. Currently in the U.S.A., the CDC recommends that women under the age of 25, women with multiple sexual partners, women having had a change in partner, women who have symptoms suggestive of chlamydial infection and those who have had a previous STI are screened at regular intervals. These recommendations have been translated into active screening programmes across all states with well documented evidence of a reduction in prevalence in areas where intervention has been in place for a number of years (CDC, 2001). Similarly in Sweden, a national *Chlamydia* screening programme implemented in the 1980s has been associated with a dramatic reduction in incidence of *Chlamydia* and its adverse sequelae (Kamwendo *et al*, 1998).

Screening for *C. trachomatis* may contribute to the prevention of pelvic inflammatory disease and impact on the crippling cost of reproductive health problems to the state and individuals. In a systematic literature review of cost effectiveness studies conducted between 1990-2000, screening for *C. trachomatis* was cost effective at prevalences of 3.1%-10% and cost saving at prevalences of >1.1% if age was used as a selection factor and DNA based tests on urine used (Honey *et al*, 2002). In Ireland, there is limited testing for *C. trachomatis* and there is potential for extended testing of the female and male population.

1.10 *Project aims*

The overall aim of the project was to develop rapid inexpensive molecular based methods for the detection of both HPV and *C. trachomatis* from PreservCyt cervical samples. A secondary aim was to apply these methods to determine the prevalence and characterise the nature of these infections in an urban Irish population undergoing routine cytological cervical screening.

Chapter 2 details experiments conducted to determine the optimal method of DNA extraction from exfoliated cervical cells in PreservCyt medium for the detection of *C. trachomatis*.

Chapter 3 introduces the notion of screening for both *C. trachomatis* and HPV from residual PreservCyt material by the development of a multiplex PCR assay. The sensitivity and specificity of the multiplex PCR were determined by comparison with single PCR for both HPV and *C. trachomatis* and by comparison with the commercial HCII for HPV and LCx assay for *C. trachomatis*. The multiplex PCR was then used to screen 997 cervical PreservCyt samples to determine the age related prevalence of HPV and *C. trachomatis* in the Irish female urban population presenting for cervical screening.

The aim of *Chapter 4* is to further characterise the nature of HPV infections in the population by comparison of HPV status and cytology, by estimation of HPV viral load using real-time PCR, and by HPV genotyping.

CHAPTER 2

Comparison of DNA extraction from cervical cells collected in PreservCyt solution for the amplification of *C. trachomatis*

2.1 Introduction

2.1.1 *Aim*

PreservCyt has recently received FDA approval for the amplification of HPV as an aid to cytological diagnoses. A single cervical sample could be used to screen for a number of diseases of the female genital tract including *C. trachomatis* infection (Anquenot *et al*, 2001; Hopwood *et al*, 2004). Few studies to date have investigated *C. trachomatis* detection from PreservCyt specimens (Bianchi *et al*, 2002; Zhang *et al*, 2002; Koumans *et al*, 2003). These studies have employed mostly commercial lysis buffer methods for DNA extraction (Bianchi *et al*, 2002; Koumans *et al*, 2003). In this study, three different DNA extraction methods from PreservCyt were investigated for the amplification of *C. trachomatis* DNA.

2.1.2 *DNA extraction from cervical cells*

Conventional approaches to DNA extraction from cervical cells commonly involve proteinase K extraction in a lysis buffer, with or without a further purification step (Gopalkrishna *et al*, 1992; Jacobs *et al*, 2000). While these techniques are effective and liberate large quantities of DNA, they are time-consuming, often involve dangerous solvents, have a high risk of contamination and are not applicable to a diagnostic setting. Commercial microbial testing kits have their own specimen collection procedures, transport media and DNA extraction methods. This creates difficulties for comparison studies; therefore, there is a need for simple rapid methods of DNA extraction from cervical cells on which tests for a number of organisms can be performed.

Numerous rapid in-house methods have been described for the preparation of cervical cells for PCR. Lou *et al*, (1993) developed simple microwave and thermal cycler boiling methods yielding satisfactory results for the detection of HPV DNA from cervicovaginal lavage specimens while Gopalkrishna *et al* (1992) developed an extended proteinase K digestion method at a higher temperature of 65°C for the detection of HPV from cervical scrapes. Both of these methods used only one step without further purification, reducing the potential for contamination. Modifications of commercial DNA extraction methods have, in some instances, been found to increase the sensitivity of commercial nucleic acid amplification techniques (Pasternack *et al*, 1998; Niederhauser and Kaempf, 2003).

Recently many solvent-based methods for DNA purification have been replaced by chelex purification and spin column methods (Sweet *et al*, 1996; Chan *et al*, 2001a). In this study two DNA extraction methods were developed (boiling in Tris-EDTA buffer and proteinase K digestion in a lysis buffer, both with chelex purification steps) and compared with a commercial DNA extraction method (QIAamp Mini-Kit, Qiagen Ltd) for the extraction of *C. trachomatis* DNA from PreservCyt. The quality of DNA extracted was evaluated by comparison of sensitivity of detection of *C. trachomatis* by PCR for three distinct target DNA sequences, namely the *C. trachomatis* plasmid (CTP primers), the major outer membrane protein gene, (MOMP), (MOMP primers) and the 60 kDa heat-shock protein gene (Hsp60 primers). These were compared to a gold-standard of routine *C. trachomatis* testing by LCx assay (Abbott Laboratories).

2.1.3 *Real-time PCR for the detection and quantitation of C. trachomatis*

In this study real-time *LightCycler*® (Roche Molecular Biosystems) SYBR Green PCR was performed to determine the concentration of *C. trachomatis* in samples extracted by

the three different methods and thus determine the relative efficiency of each method. Real-time PCR differs to conventional PCR in that the generation of products can be monitored over time (Wittwer *et al*, 1990). The analysis of products during amplification has become known as "real-time" PCR. The *LightCycler* offers the possibility of "Rapid Cycle Real Time PCR". Real-time PCR allows quantitation of PCR products generated by comparison to a standard curve of known copy number PCR product (Figures 2.1. and 2.2).

Few studies have explored the potential of real-time PCR for the detection or quantitation of *C. trachomatis* (Eickhoff *et al*, 2003; Koenig *et al*, 2004). Where real-time PCR for *C. trachomatis* has been performed it has mostly been in studies investigating the pathogenesis of chlamydial disease e.g. in the validation of microarray experiments to estimate generation time (Wilson *et al*, 2004) or in the evaluation of genes involved in the maintenance of persistence and the regulation of expression of membrane proteins (Gerard *et al*, 2001; Gomes *et al*, 2005).

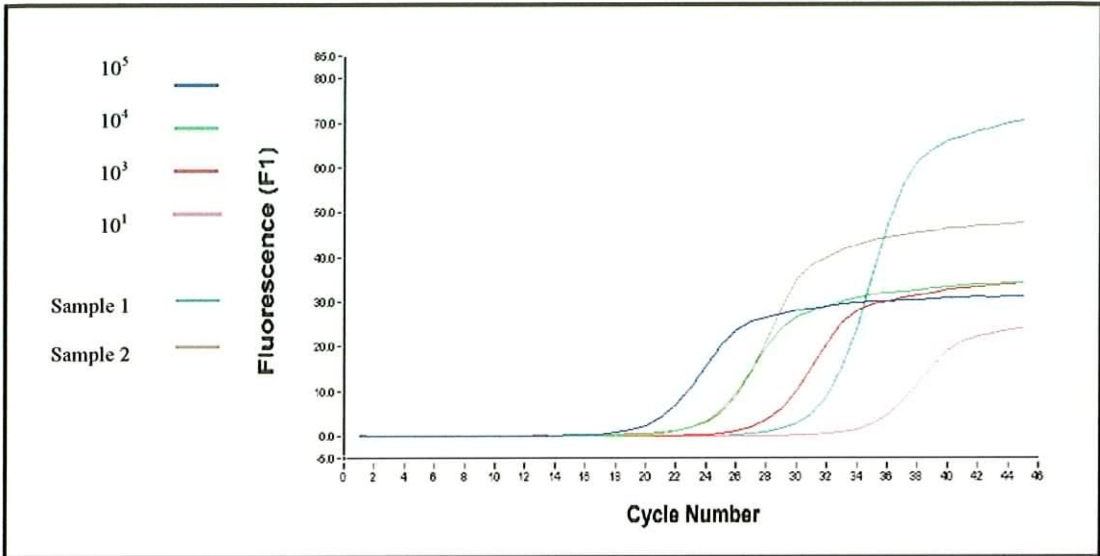


Figure 2.1. *LightCycler* PCR amplification curve of cloned *C. trachomatis* standards. pBSII*hsp 60* standards ranging from 10^5 copies of *C. trachomatis hsp60* to 10 copies per µL of extracted DNA and *C. trachomatis* positive samples 1 and 2

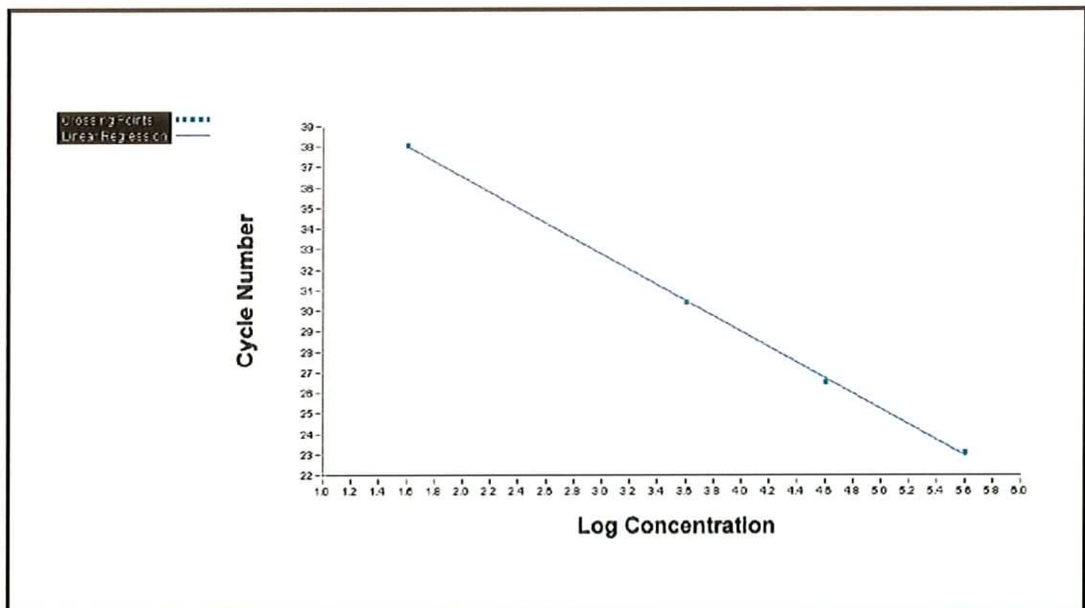


Figure 2.2. Standard curve plot of Hsp60 standards for quantitation of *C. trachomatis* copy numbers by *LightCycler* real-time PCR

2.2 Methods

2.2.1 *Study population and clinical specimens*

Cervical samples were taken from women attending a genitourinary medicine clinic, at St. James' Hospital, Dublin, Ireland and placed in LCx transport medium (LCx; Abbott Laboratories, Chicago, Ill., U.S.A.). These were tested for *C. trachomatis* using the Ligase Chain Reaction assay (LCx), (LCx; Abbott Laboratories, Chicago, Ill., U.S.A.). A second cervical sample was taken on the same visit and placed in a vial of PreservCyt medium and transported to the cytology laboratory of the hospital where a Pap smear was prepared. Specimens were then kept at room temperature and the DNA was extracted within 6 weeks. The sample population in this study consisted of 38 women who tested positive for *C. trachomatis* by the LCx.

2.2.2 *DNA extraction from PreservCyt cervical cells*

PreservCyt specimen (12 mL) was vortexed briefly and divided into three 4 mL aliquots. These were then centrifuged at 3,000 rpm and the pellet was washed twice with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and resuspended in a TE buffer (200 µL). In extraction method A (TE-Chelex), the cell suspension was boiled for 10 min with 0.1% Chelex solution (Sigma–Aldrich). In method B (Proteinase K-Chelex), the cells were resuspended in 200 µL of cell lysis buffer (10 mM Tris-HCl, 400 mM NaCl , 2 mM Na_2EDTA, pH 8.2), with 20 µL of proteinase K (20 mg/mL) and 0.5% sodium dodecyl sulphate and incubated at 65°C for 2.5 h. This was then boiled for 20 min with 0.1% Chelex-100 solution. In method C (QIAamp), the QIAamp DNA Mini Kit (Qiagen Ltd., U.K.) was used according to the manufacturer's instructions (Appendix A). DNA was extracted from a single sample using the three extraction

methods within the same 48 h period. Following DNA extraction samples were stored at -20°C, until required for PCR.

2.2.3 *PCR amplification of a region of the C. trachomatis plasmid and omp gene*

The following primer sets were used for detection of *C. trachomatis*: a plasmid primer set (CTP), (Griffais and Thibon, 1989), and a primer set (MOMP) for the major outer membrane protein gene, (Mittal, 1998), (Table 2.1). The positive control was DNA extracted from a urine sample, which was *C. trachomatis* positive by culture. PCR was performed in 20 μL reaction volume, containing 2 μL PCR buffer (Invitrogen Ltd, UK), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 25 pmol of each primer set, 1 U of *Taq* DNA polymerase (Invitrogen Ltd.) and 5 μL of DNA sample (Appendix B). The PCR reaction mixture was performed in a Hybaid Omni-E thermal cycler, with an initial denaturation of 95°C for 5 min followed by 40 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 2 min. After 40 cycles, a further elongation step was carried out at 72°C for 5 min. The products were run on a 1% agarose gel containing 0.5 μg/μL ethidium bromide (Figure 2.3).

Table 2.1. DNA oligonucleotides used for polymerase chain reaction

Gene target	Primer	Sequence	Amplicon size (bp)
plasmid	CTP1	5'-TAGTAACTGCCACTTCATCA-3'	201
	CTP2	5'-TTCCCCTTGTAATTCGTTGC-3'	
momp	MOMPA	5'-TATACAAAAATGGCTCTCTGCTT-3'	540
	MOMPB	5'-CCCATTTGGAATTCTTTATTCACATC-3'	
hsp60	Hsp60F	5'-GATGGTGTTACCGTTGCGA-3'	650
	Hsp60R	5'-CCTCCACGAATTCTGTTCAC-3'	

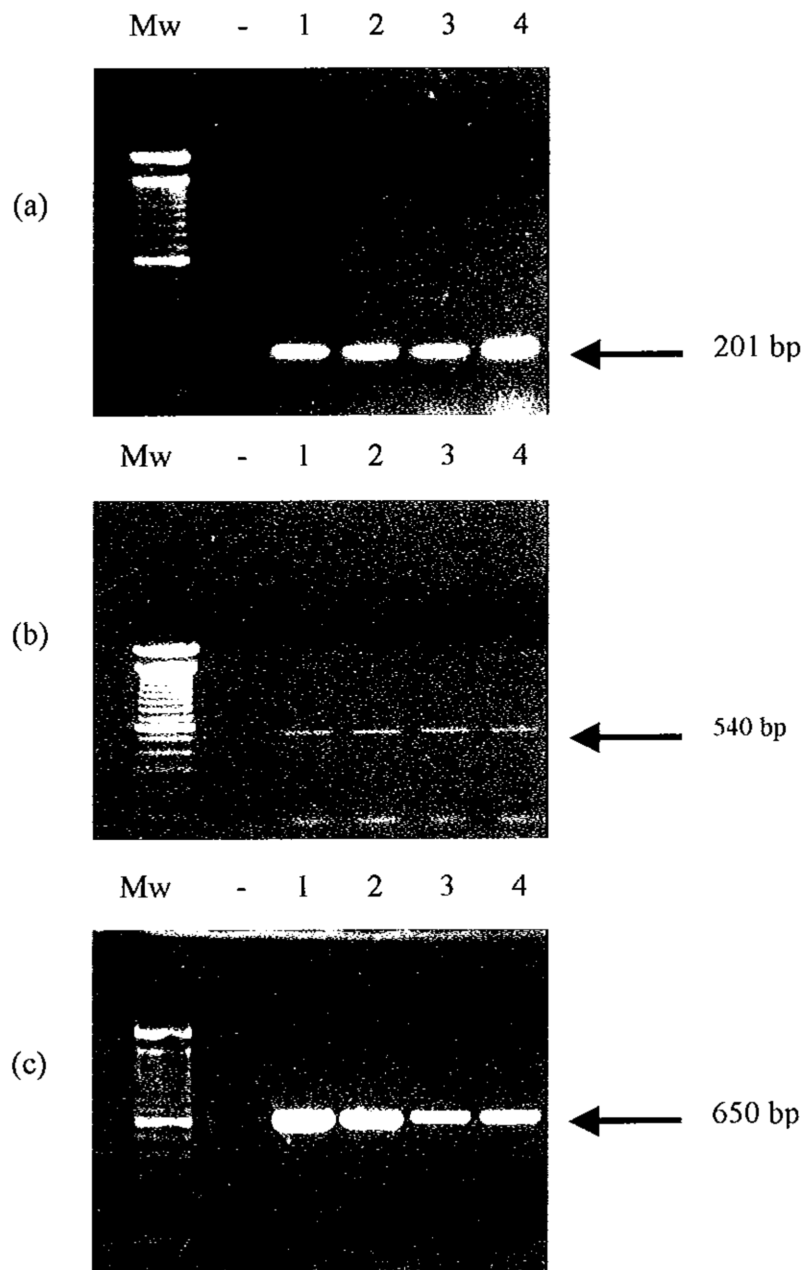


Figure 2.3. PCR amplification of *C. trachomatis*. (a) PCR amplification of a region of *C. trachomatis* plasmid (201 bp) using the CTP primer set. (b) PCR amplification of *C. trachomatis* major outer membrane protein gene (540 bp) using the MOMP primer set. (c) Real-time PCR amplification of the *C. trachomatis* heat-shock protein 60 gene (650 bp) using the Hsp60 primer set. M=100 bp DNA marker; -: negative H_2Ocontrol; 1: positive control; 2-4: *C. trachomatis* positive samples

2.2.4 *Real-time PCR detection and quantitation of C. trachomatis DNA*

Real-time PCR for detection and quantitation of *C. trachomatis* was performed on all DNA samples using the Hsp60 primer set (Wood *et al*, 2002), specific for the heat-shock protein 60 gene (Table 2.1). An aliquot (1 µL) of each sample was added to a reaction mixture (9 µL) containing 3 mM $MgCl_2$, 0.5 µM primers and 1 µL of LC DNA FastSTART Master SYBR Green I enzyme mix, (Roche Biochemicals), (Appendix B). Samples were amplified on a *LightCycler* under the following cycle conditions: an initial 10 min at 95°C for activation of the FastSTART *Taq* DNA polymerase, followed by 40 cycles of 5 s of denaturation at 95°C, 10 s of annealing at 68°C, decreasing to 65°C at a rate of 1°C/cycle and 30 s of extension at 72°C. Data were obtained after the extension period in the single acquisition mode. The hsp60 PCR product was cloned into a pBSII vector and serially diluted cloned copies were used to create a standard curve (10^5 to 10^2 copies) for quantitation of chlamydial copy numbers. These standards were run with each *LightCycler* run and the machine made a reading of *C. trachomatis* copy numbers during the exponential phase of amplification. A melt curve step was included to confirm the amplification (Figure 2.4). Samples which gave copy numbers outside the range of the standards or whose melt-temperature (Tm) was outside those of the standards had their real-time PCR product analysed by agarose gel electrophoresis. A sample was positive if amplification of the 650 bp product occurred during the amplification programme of 40 cycles.

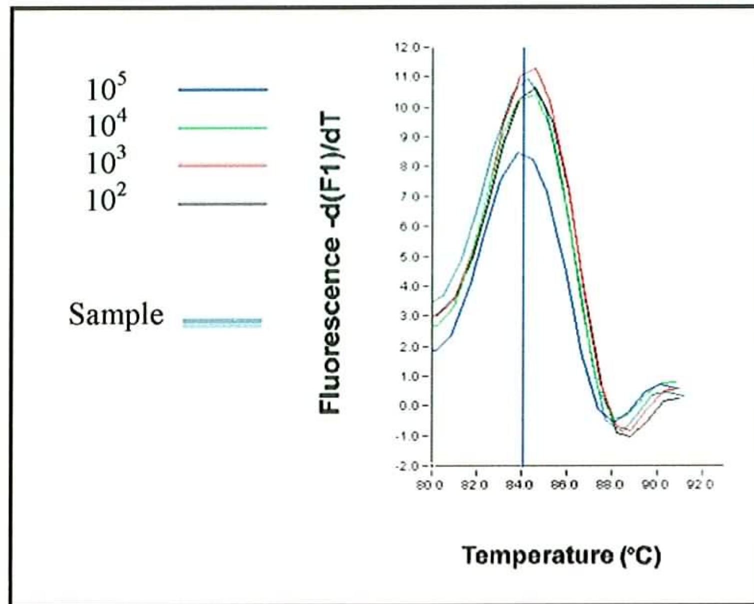


Figure 2.4. Melt curve analysis of *C. trachomatis* Hsp60 standards (10^5 copies – 10^2 copies/ μL) and sample

2.3 Results

DNA isolated from the 38 LCx positive samples by the three extraction protocols was analysed for *C. trachomatis* amplification by PCR and quantified using real-time PCR. A positive result for either the CTP or the MOMP primer set was determined by the presence of 201 bp product for the CTP primer set and a 540 bp product for the MOMP primer set on agarose gel electrophoresis. The Hsp60 real-time PCR assay was positive if quantified *C. trachomatis* copy numbers were inside the range of the standards or if running of the product on an agarose gel gave the required 650 bp product size. Five TE-Chelex, 5 Proteinase K-Chelex, and 2 QIAamp samples had copy numbers below the range of the standards (<100 copies/μL DNA) but were positive on gel electrophoresis of the PCR product.

2.3.1 *Detection of C. trachomatis by plasmid PCR, MOMP PCR and Hsp60 real-time quantitative PCR*

The majority, 95% (36/38) of samples extracted using either the TE-Chelex method or the Proteinase K-Chelex method were positive for *C. trachomatis* by either of the three PCRs. The QIAamp samples gave a slightly higher overall positivity rate of 97% (37/38) with a sensitivity of 95% for detection using plasmid PCR, 90% by MOMP PCR and 95% by Hsp60 real-time PCR (Table 2.2). Of the QIAamp samples 90% (34/38) amplified for each of the three PCRs as opposed to 71% (27/38) of the Proteinase K-Chelex samples and only 50% (19/38) of the TE-Chelex samples (Table 2.2). The plasmid primers were the most successful for the amplification of *C. trachomatis* DNA extracted by each of the three methods, followed by the Hsp60 primer set and then the MOMP primer set (Table 2.2).

Table 2.2. Comparison of positive PCR results for three DNA extraction methods from LCx *C. trachomatis* positive samples (n=38) using the CTP, MOMP primers in conventional PCR and Hsp60 primers for real-time PCR detection

Primer Set	No. (%) of samples positive for each extraction method.		
	TE-Chelex[a]	PK-Chelex[b]	QIAamp[c]
CTP	34 (90%)	35 (92%)	36 (95%)
MOMP	23 (61%)	28 (74%)	34 (90%)
Hsp60	28 (74%)	32 (84%)	36 (95%)

[a]Boiling in Tris-EDTA buffer with Chelex purification
[b]Proteinase K digestion followed by Chelex purification
[c] QIAamp DNA extraction kit

2.3.2 *Real-time quantitative PCR analyses of C. trachomatis DNA*

Real-time quantitation of *C. trachomatis* copy numbers in samples was performed to determine if reduced sensitivity of detection by PCR was associated with low copy numbers of the organism and to determine the relative yield of *C. trachomatis* copy numbers for each extraction method (Figure 2.2).

The QIAamp and the Proteinase K-Chelex samples most commonly gave copy numbers of 10^4/ µL of extracted DNA as opposed to the TE-Chelex samples which gave a lower yield of 10^2/ µL of extracted DNA (Table 2.3). Failure of amplification for the plasmid and the MOMP gene were frequently associated with low bacterial load by real-time quantitative PCR. Of the four TE-Chelex extracted samples negative by CTP PCR, three were below the detection level of 100 copies of *C. trachomatis*/ µL of extracted

DNA and of the 15 MOMP-negative samples three samples had <1000 copies and 12 had <100 copies of *C. trachomatis* by quantitative PCR. Of the 4 QIAamp extracted samples, which did not amplify for all three genes, one sample was positive by Hsp60 real-time PCR only, with a copy number of 10^2. Two others amplified for the plasmid but not the *momp* gene; the real-time PCR result was negative for one and copy numbers <100/ µL DNA for the other).

Table 2.3. Real-time Hsp60 quantitative PCR for *C. trachomatis* copy number determination (n=38 LCx *C. trachomatis* positive samples)

Chlamydial copy/ µL	No. of samples		
	TE-Chelex[a]	PK-Chelex[b]	QIAamp[c]
Negative	10	6	2
<100	5	5	2
$10^2 \leq x < 10^3$	14	6	8
$10^3 \leq x < 10^4$	6	6	11
$10^4 \leq x < 10^5$	2	8	13
$10^5 \leq x < 10^6$	1	6	2
$10^6 \leq x$	0	1	0
Total	38	38	38

[a]Boiling in Tris-EDTA buffer with Chelex purification

[b]Proteinase K digestion followed by Chelex purification

[c] QIAamp DNA extraction kit

2.4 Discussion

The advent of liquid based cervical cytology may enable gynaecologists to screen for both cervical preneoplastic lesions and sexually transmitted infectious agents from the same sample. This is based on the ability of liquid fixed cells to yield nucleic acids suitable for molecular based assays (Tarkowski *et al*, 2001; Habis *et al*, 2004). There are a growing number of publications using PreservCyt specimens for molecular detection of organisms including HPV, Herpes Simplex Viruses, *Trichomonas vaginalis* and *C. trachomatis* (Fiel-Gan *et al*, 1999; Lanham *et al*, 2001). As DNA extracted from PreservCyt specimens may be used to screen for multiple organisms there is a need for the development of simple rapid inexpensive DNA extraction methods, which can readily be applied in the diagnostic setting.

2.4.1 *DNA extraction from cervical cells in PreservCyt medium*

Numerous DNA extraction studies have been performed on cervical cells from a variety of specimen types including paraffin wax embedded archival tissue (Sepp *et al*, 1994) and cervical swab specimens (Hording *et al*, 1994; Dalesio *et al*, 2004). However, few studies have compared cell preparation methods from liquid-based cytological media such as PreservCyt (Cuschieri *et al*, 2003). While numerous studies have demonstrated that PreservCyt is a suitable medium for the extraction of both DNA and RNA suitable for molecular analysis (Peyton *et al*, 1998; Cuschieri *et al*, 2005; Sailors *et al*, 2005) studies into the efficiency of DNA extraction from PreservCyt are warranted especially given that the medium was designed for the preservation of cellular morphology rather than ease of cell lysis. In a study by Cuschieri *et al*, (2003) which evaluated the Qiagen BioRobot 9604 (Qiagen Ltd., U.K.) for the automated extraction of HPV DNA from PreservCyt samples it was found that considerable optimisation of the protocol was

required for PreservCyt samples as compared to whole blood or plasma (Hertogs *et al*, 1998; Hoffmeyer *et al*, 2000). This may be as a result of dehydration of the cell membrane by the methanol in the PreservCyt reducing the efficiency of ionic lysis by detergents commonly contained in DNA extraction buffers. The main aim of this study therefore, was to compare the efficiency of two inexpensive in-house DNA extraction procedures and one kit based method for the detection of *C. trachomatis* from PreservCyt by PCR.

The results of this study indicate that the commercial QIAamp extraction kit was the most successful extraction method for amplification of the three different target genes, but that the Proteinase K-Chelex method had a similar success rate for *C. trachomatis* amplification when the plasmid primers were used. However, amplification of larger PCR products was less successful from Proteinase K-Chelex than with the commercial extraction kit. This would have implications for the application of other molecular methods to these DNA samples, particularly restriction enzyme digestion for restriction fragment length polymorphism analysis.

In this study there was 90%, 92% and 95% sensitivity of *C. trachomatis* detection by plasmid PCR amplification by the TE-Chelex, Proteinase K-Chelex and QIAamp. Koumans *et al*, (2003) analysed PreservCyt samples for *C. trachomatis* detection using a commercial extraction and detection system and reported a similar sensitivity of 97% for *C. trachomatis* detection. Berg *et al*, (2003) evaluated three DNA extraction kits for the isolation of *C. pneumoniae* DNA from vascular tissue and found that the QIAamp kit was the most successful.

2.4.2 *PCR amplification of C. trachomatis plasmid, momp and hsp60 genes*

Most non-commercial in-house PCR assays for *C. trachomatis* detection amplify a region of either the *C. trachomatis* plasmid (Molano *et al*, 2005), the major outer membrane protein gene *momp* (Molano *et al*, 2004) or the rRNA (Claas *et al*, 1991). The 7.5 kb plasmid is found in practically all *C. trachomatis* isolates at varied copy numbers. It has been suggested that during the replicative phase the number of plasmids per organism increases (Pickett *et al*, 2005). The high-sensitivity of plasmid PCR has been well documented (Roosendaal *et al*, 1993; Mahony *et al*, 1993). Plasmid primers are more sensitive than either the MOMP or rRNA primers for *C. trachomatis* detection (Roosendaal *et al*, 1993). In a study by Mahony *et al*, (1993) it was found that plasmid primers were 10-100 times more sensitive than chromosome-based assays. In this study amplification for the plasmid gene was the most successful method for detection of *C. trachomatis* and showed the greatest concordance for the three extraction methods.

Some studies have reported lack of reproducibility of *C. trachomatis* detection, even with commercial systems, and recommend duplicate samples or the application of two NAAT detection methods (Castriciano *et al*, 2002). PCR using the MOMP primer set is commonly performed to verify plasmid positive samples (Loeffelholz *et al*, 1992; Shattock *et al*, 1998). Koenig *et al*, (2004) detected *C. trachomatis* by BD ProbeTec ET followed by in-house *LightCycler* PCR to improve the sensitivity of screening (Koenig *et al*, 2004). In this study, all samples were amplified for three different genes to increase the specificity of detection. This study recommends the combined use of the plasmid PCR and real-time heat shock protein 60 PCR for the detection of *C. trachomatis*.

2.4.3 *Real-time PCR quantitation of C. trachomatis from PreservCyt*

Real-time PCR is a fast and effective method for the detection and quantitation of bacterial load (Tondella *et al*, 2002) in clinical samples and for validation of DNA extraction methods. Real-time quantitation of *C. trachomatis* load has been performed previously to determine the effect of mass antibiotic treatment (Burton *et al*, 2005) and in the correlation of bacterial load with disease severity (Soloman *et al*, 2003). Real-time PCR quantitation was used in this study to show that a drop off in sensitivity of detection, particularly for the larger PCR products, was associated with low copy numbers of *C. trachomatis* following DNA extraction from PreservCyt.

This study shows that a single PreservCyt cervical specimen can be used as a source of high quality DNA for testing for sexually transmitted infections. Optimisation of the method of DNA extraction from PreservCyt is essential to avoid false negatives and ensure adequate sensitivity of detection. Careful selection of genes to be amplified and the PCR product size for the detection of *C. trachomatis* is recommended. Real-time PCR quantitation is a valuable method for validation of the sensitivity of PCR detection methods

2.4.4 *Summary*

Summary

- QIAamp DNA extraction method was the most successful for *C. trachomatis* DNA extraction followed by the Proteinase K-Chelex and TE-Chelex DNA extraction methods.
- PCR amplification using the CTP plasmid primers was the most sensitive for *C. trachomatis* detection followed by Hsp60 PCR and then MOMP PCR.
- DNA extraction by the QIAamp DNA method followed by CTP plasmid PCR amplification exhibited the highest sensitivity of detection of *C. trachomatis*.
- Failure of amplification during PCR was associated with low bacterial load as determined by real-time quantitative PCR for the *hsp60* gene.
- *C. trachomatis* was detected with similar sensitivity when CTP plasmid PCR was performed on QIAmp and Proteinase K-chelex extracted DNA but product size may be a limiting factor in the success of *momp* gene amplification.
- Real-time quantitative PCR is a useful tool in the determination of the copy number sensitivity of conventional PCR methods.

CHAPTER 3

Prevalence of HPV and *C. trachomatis* infections in Irish women undergoing routine cervical screening

3.1 Introduction

3.1.1 *Sexually transmitted infections in Ireland*

In keeping with the current trend across Europe, STIs continue to rise in Ireland year after year. STIs have been increasing each year since 1994, with an increase of 173.8% seen between 1994 and 2003. In 2003, 11,153 cases of STIs were reported in Ireland (HPSC, 2004). This represents an increase of 6.5% compared to figures for 2002. Between 2003 and 2004 the number of reported STIs rose by 12.1% (HPSC, 2005). HPV and *C. trachomatis* infections are among the most common cases of STI reported in Ireland. In 2004, the three most commonly reported STIs were ano-genital warts (n=4,174), *C. trachomatis* (n=2,803) and non-specific urethritis (n=2,746) (HPSC, 2005). In Ireland, 11 sexually transmitted diseases are currently legally notifiable. STI surveillance in Ireland is mostly genitourinary medicine clinic based, with few incidence reports made from primary care settings (HPSC, 2005). As such reported figures indicate trends in infection but are an underestimate of the true incidence due to the asymptomatic nature of some of the infections. The main aim of this study was to generate population based prevalence data on HPV and *C. trachomatis* infections in an urban Irish population presenting for routine cervical testing.

3.1.2 *C. trachomatis and HPV infections in Ireland*

Notified cases of *C. trachomatis* (n=2,803) increased by 24.1% in 2004 compared to 2003 (n=2,258) (HPSC, 2005). While these figures represent the largest increase in prevalence rates of all reported STIs with the exception of *N. gonorrhoeae* during these years, they may also be the result of improvements in disease notification, testing methods and the increased social acceptability of testing for STIs. Numerous advertising campaigns including the highly successful "Convenience Advertising

Campaign" were launched in response to the National Health Promotion Strategy 2000-2005. At present there is no organised *C. trachomatis* screening programme in Ireland. The STI subcommittee of the Scientific Advisory Committee of HPSC is currently assessing the need for a *C. trachomatis* screening programme. Our study will provide some basic information on the demographics of *C. trachomatis* infection in Ireland which may be useful in the decision making process.

Ano-genital warts (n=4,174) were the most commonly notified STI in Ireland in 2004 increasing by 4.8% compared to 2003 (HPSC, 2005). These figures represent a very small proportion of HPV infections in the community as many people harbor HPV infections that remain inactive or latent. Furthermore, most anogenital warts are HPV type restricted and contain mostly benign HPV types 6 or 11. Molecular methods used in this study enable pan-HPV detection and so provide a true estimate of the burden of HPV infections in the urban female Irish population.

Few studies have investigated the prevalence of HPV or *C. trachomatis* in the Republic of Ireland. In 1984, a study was conducted by Mohammed *et al*, on the incidence of *C. trachomatis* infection in patients attending a Dublin venereal disease clinic (Mohammed *et al*, 1984). This was followed in 1992, by a study conducted on 32 teenage girls attending the sexually transmitted disease clinic in the Mater Misericordiae Hospital in Dublin (Fitzpatrick *et al*, 1992). The most common diagnosis was of ano-genital warts (18.8%) followed by *C. trachomatis* and then *N. gonorrhoeae* (Fitzpatrick *et al*, 1992). In 2004, a study was conducted in the mid-western region to determine the prevalence of *C. trachomatis* in men and to determine the risk factors for its acquisition. Of 562 men attending orthopaedic clinics and university sports facilities, 5.9% were positive for *C. trachomatis* (Powell *et al*, 2004). Few studies have been performed on HPV

infections in Ireland (O'Leary *et al*, 1997; Skyldberg *et al*, 1999; Brennan *et al*, 2001) and none have investigated HPV prevalence in the normal population.

3.1.3 *Multiplex PCR*

Multiplex PCR is the simultaneous amplification of two or more DNA targets in a single reaction (Mahony, 1996). It is a rapid and cost-effective method for screening for two or more microorganisms simultaneously. In this study, a novel multiplex PCR was developed for the amplification of HPV and *C. trachomatis* from a single PreservCyt sample. Numerous studies have employed multiplex PCR for the detection and typing of HPV using either consensus or type-specific primers (Lukaszuk *et al*, 2003; Ho *et al*, 2003). A limited number of studies have looked at the detection of *C. trachomatis* in liquid-based cervical samples using molecular detection methods (Lentrichia *et al*, 1998; Bianchi *et al*, 2002). A few other studies have used multiplex PCR on cervical samples for the detection of organisms other than HPV and *C. trachomatis* (Bassiri *et al*, 1997; Mgone *et al*, 2002). Mahony *et al*, (1996) used multiplex PCR for the detection of numerous sexually transmitted infections in cervical samples including HPV, *C. trachomatis* and *N. gonnorrhoeae*, however, this assay was not performed on ThinPrep samples. Very few studies have applied multiplex PCR for HPV and *C. trachomatis* detection despite the fact that they are the most likely candidate organisms to screen for in the female population. In addition to its application to screening, multiplex PCR may help to determine co-infection rates for various STIs in populations and has also been suggested for use in the provision of syndromic management (Ballard *et al*, 2002). It can readily be automated to facilitate large-scale testing for microorganisms, which could be incorporated into a screening programme. In this study, a multiplex PCR was optimised and applied to a sample cohort of 997

opportunistically screened women to determine the prevalence of HPV and *C. trachomatis*.

3.1.4 *Hybrid capture detection of HPV*

The hybrid capture II test (HCII) is the only FDA approved commercially available HPV detection assay. In this study, the sensitivity and specificity of the multiplex for the detection of HPV was compared to detection by the combined probe HPV HCII assay for the detection of high and low-risk HPV. The HCII assay has been employed in large population based studies and has become the HPV detection standard in many countries despite its inability to specifically type or quantify infecting HPVs (Soloman *et al*, 2001; Cuzick *et al*, 2000). Since the development of cervical cancer is dependant on persistent high-risk HPV infection, the ability to specifically type an HPV infection following detection would provide additional information on the potential oncogenic risk of a HPV infection. Retrospective analysis of HPV type in women with adequate follow-up would also be useful in the evaluation of the natural history of the disease.

Kulmala *et al*, (2003) evaluated the sensitivity of HCII and PCR for the detection of significant cervical lesions using DNA extracted from the transport medium specifically designed for the HCII assay (Kulmala *et al*, 2004). Concordance was achieved between the two assays for 85% of samples. A possible reason cited for discordant results was that the DNA extracted by the Digene denaturing method was mostly in the single stranded form and that some DNA may have been lost during the extraction procedure (Kulmala *et al*, 2004). A number of studies have assessed the feasibility of using stored HCII denatured specimens for HPV typing and PCR assays following additional DNA extraction (Poljak *et al*, 2002; Rabelo-Santos *et al*, 2005). In this study, the feasibility of performing the HCII assay on an aliquot of DNA extracted using the QIAamp method

from PreservCyt was investigated and compared to the standard extraction protocol used for the HCII method on PreservCyt samples.

3.1.5 *Aims*

The main focus of this research study was the development and evaluation of a novel multiplex PCR assay for the simultaneous detection of both HPV and *C. trachomatis* in liquid based cervical samples. The sensitivity and specificity of the multiplex assay were determined by comparison with single PCRs. The copy number sensitivity of the multiplex PCR assay was determined by amplification of cloned PCR products. The assay was further evaluated by comparison with the commercially available HCII assay for HPV detection and the LCx assay for *C. trachomatis*. HCII detection of HPV was also compared following DNA extraction using the QIAamp procedure and the standard Digene preparation method. The multiplex assay was then used to determine the age-related prevalence of HPV and *C. trachomatis* infections in an Irish female population (n=997) presenting for routine cervical screening.

3.2 Methods

3.2.1 *Design of multiplex PCR for the detection of C. trachomatis and HPV*

A multiplex PCR was developed for the simultaneous detection of HPV and *C. trachomatis* from PreservCyt solution. The MY09/11 primers (Manos *et al*, 1989) were used for HPV and plasmid primers (Griffais and Thibon, 1989) were used for *C. trachomatis* detection. These generated fragments of 450 bp and 201 bp respectively (Figure 3.1). Primers for the human ß-globin gene (Saiki *et al*, 1992) were included in the multiplex as an internal control of DNA integrity, generating a 110 bp product (Figure 3.1.). Primer and $MgCl_2$ concentrations in the reaction were titrated so that the optimized reaction contained 5 pmol of each of the forward and reverse primers of the MY09/11 and CTP1/2 primers and 10 pmol of each of the forward and reverse primers of the PCO3/4 primer set, 200 µM deoxynucleoside triphosphates, 10X PCR buffer (containing 10 mM Tris HCl [pH 8.3], 50 mM KCl), 2 mM $MgCl_2$ and 1 U of Platinum *Taq* DNA polymerase (Invitrogen Ltd.) in a final volume of 20 µL, (Appendix B). The annealing temperature of the PCR reaction was optimised following calculation of the Tm of each primer set (MY09/11:Tm=51°C, CTP1/2:Tm=49°C, PCO3/4:Tm=51°C) using MeltCalc™ by increasing the annealing temperature in a step-wise fashion from 49°C until no non-specific PCR products were produced. The PCR was initiated by a 10 min denaturation and enzyme activation step at 95°C and completed by a 10 min extension step at 72°C. The temperature cycles were as follows: 40 cycles of 30 s at 95°C, 1 min at 57°C, and 1 min at 72°C.

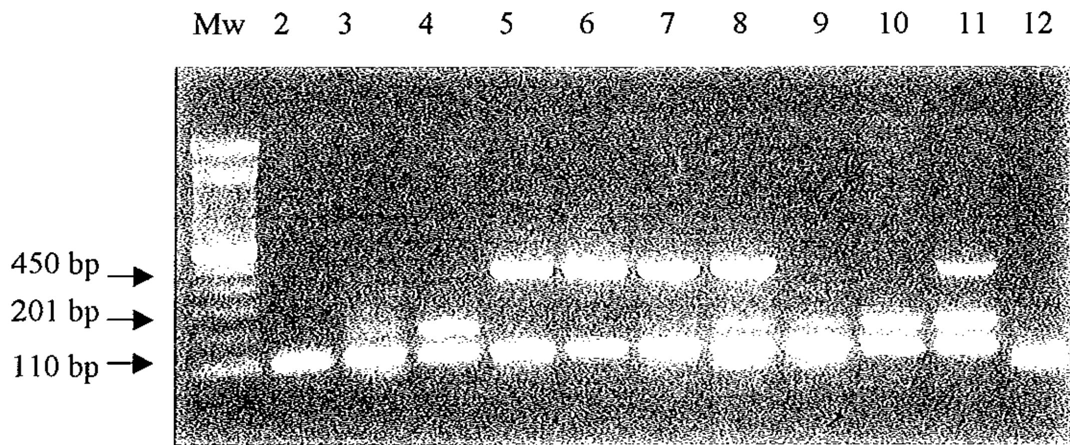


Figure 3.1. Multiplex PCR amplification of *C. trachomatis* and HPV DNA in various samples. Human ß-globin control: 110 bp, *C. trachomatis* PCR product: 201 bp, HPV PCR product: 450 bp. Mw: 100 bp DNA marker. Lane 2: sample negative for both HPV and *C. trachomatis*. Lanes 3, 4, 9 and 10: samples positive for *C. trachomatis* only. Lanes 5, and 6: samples positive for HPV only. Lanes 7 and 8: samples positive for HPV and *C. trachomatis*. Lane 11: positive sample control for HPV and *C. trachomatis*. Lane 12: negative H_2O control.

3.2.2 *Validation of multiplex assay*

Multiplex validation by pilot study on 100 samples

As a pilot study, the multiplex PCR was performed on DNA extracted from 100 cervical PreservCyt fluid specimens collected from consenting women attending their general practitioner for routine cervical screening. To evaluate the sensitivity and specificity (Appendix C) of the multiplex for the detection of each organism in the pilot study population (n=100), single PCRs for the detection of HPV was performed on samples using the GP5+/6+ primers (de Roda Husman *et al*, 1995) generating a 150 bp product and for the detection of *C. trachomatis*, using the Hsp60R2 primers specific to the *hsp60* gene of *C. trachomatis*, generating a truncated version (309 bp) of a previously described product (Wood *et al*, 2002), (sense, 5' GAT GGT GTT ACC GTT GCG A) and (antisense, 5' TAA TAA TCG TCT TTA ACA ACG T), (Appendix B). PCRs were also performed using the MY09/11 and CTP1/2 primer sets singly, (Appendix B). All PCRs were performed on DNA extracted using the QIAamp DNA extraction method (section 2.2.2) using the QIAamp DNA extraction kit (Qiagen, UK) as per manufacturer's instructions, (Appendix A).

Estimation of sensitivity for C. trachomatis detection

To estimate the sensitivity of the multiplex assay for the detection of *C. trachomatis*, multiplex PCR was performed on 34 PreservCyt fluid specimens from women attending a genitourinary medicine (GUM) outpatient clinic, who had tested positive for *C. trachomatis* by the LCx (Abbott laboratories).

Estimation of sensitivity for HPV detection

To estimate the diagnostic sensitivity of the multiplex assay for the detection of HPV a random selection of 20 HPV positive and 20 HPV negative samples as determined by multiplex PCR results on the pilot study population were tested for HPV by the HCII

assay (Digene UK), (Figure 3.2). DNA was extracted from a second aliquot of each sample for HCII analysis using the Digene Sample Conversion Kit (5127-1220) as per manufacturer's instructions, (Appendix D). Briefly, DNA was extracted from 4 mL of PreservCyt sample by alkali denaturation at 65°C for 1 h. The HCII HPV DNA Test (Product description 5196-1330) was carried out using high and low-risk probe cocktails for the detection of HPV on all 40 samples, (Appendix D). A cut-off point of 1.0 relative light units/ cut-off value (RLU/CO) was set as per manufacturer's instructions. Samples that had RLU/CO of 1.0+/- 0.3 were retested. HCII testing was also carried out on an equivalent volume of genomic DNA extracted by the QIAamp method for comparison of extraction method (Figure 3.2), (Appendix D).

Estimation of copy number sensitivity

The copy number sensitivity of the multiplex was evaluated by performing the PCR on serial dilutions of cloned standards of the HPV MY09/11 and the *C. trachomatis* CTP PCR product in the pCR®2.1-TOPO® (Invitrogen Ltd), ranging from 10^8 to 10^1 copies/ µL. Cloning was performed using the TOPO-TA Cloning kit (Invitrogen Ltd), as per kit manual.

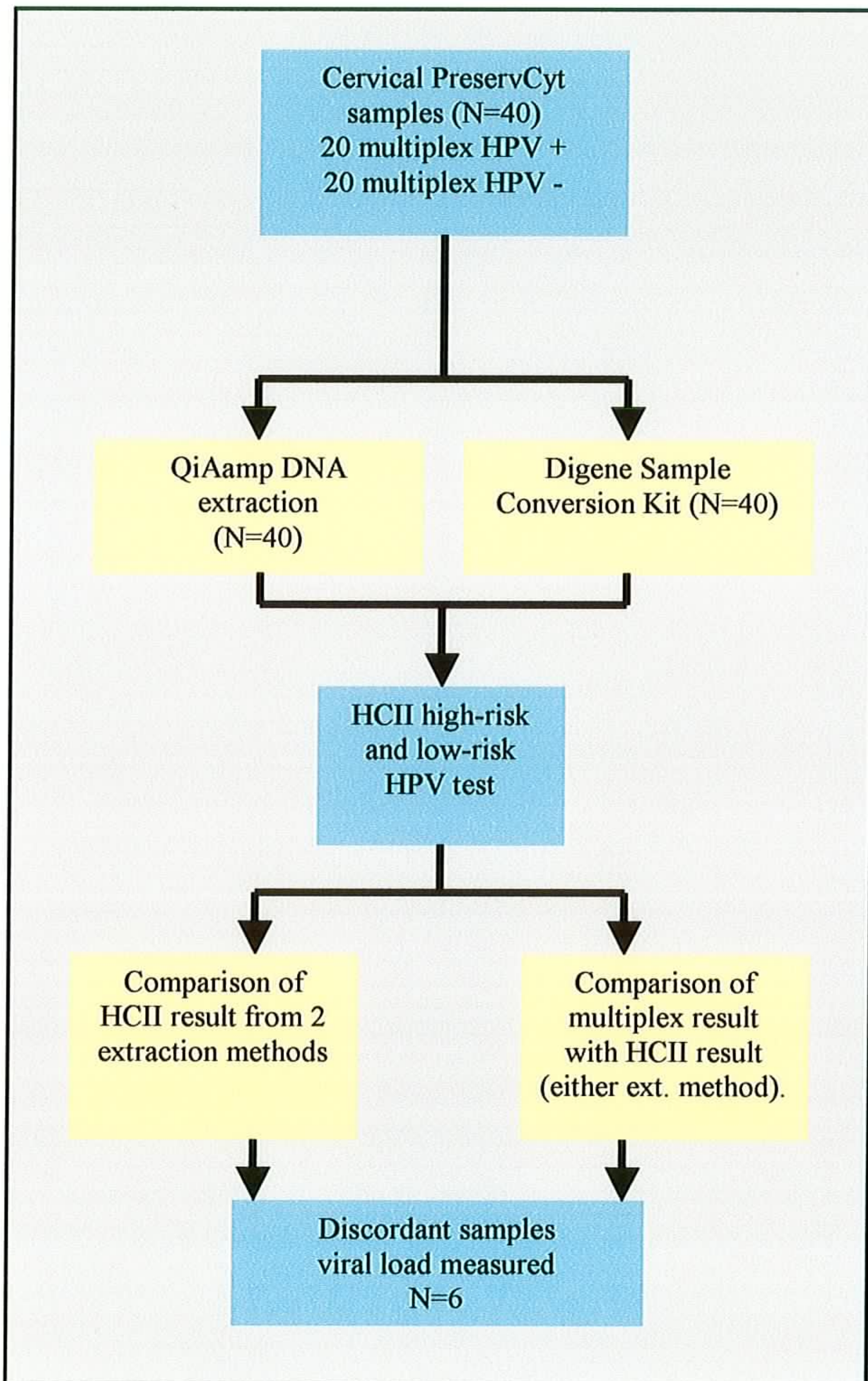


Figure 3.2. Hybrid capture (HCII) study algorithm

3.2.3 *Study population and ethical approval*

Ethical approval was obtained for the epidemiological study from the St. James' Ethics Committee Review Board in August 2003, (Appendix E). The study population consisted of women attending their local GP in the Dublin, Meath and Kildare areas for routine cervical screening by pelvic examination and cervical smear. The study was anonymised and no patient identification was recorded. Patient consent was sought for all samples, (Appendix E).

3.2.4 *Prevalence of HPV and C. trachomatis in the study population*

DNA was extracted from 997 residual PreservCyt samples using the QIAamp method (see Section 2.2.2.) as per manufacturer's instructions, (Appendix A). Multiplex PCR was optimised in a multiwell plate system for the simultaneous detection of HPV and *C. trachomatis* (Appendix B). Controls included a previously characterised HPV positive sample, a *C. trachomatis* PCR and LCx positive sample, a sample positive for both organisms and a negative H_2O contamination control. A sensitivity of detection control containing human genomic DNA spiked with 10^2 copies of cloned MY09/11 and CTP PCR products was also included. PCR amplicons were analysed by ethidium bromide gel electrophoresis in a 1.5% agarose gel.

3.2.5 *Statistical analyses*

Statistical significance was determined for the association of HPV and *C. trachomatis* infections with age using the Pearson Chi-Square test calculated using Statistical Package for the Social Sciences (SPSS) software version 11.0. All statistical analyses were carried out with the assistance of Dr. John Kearney, resident statistician and epidemiologist at the Dublin Institute of Technology.

3.3 Results

3.3.1 *Multiplex validation by pilot study on 100 samples*

In the pilot study population, 21% (21/100) of samples were positive for HPV by the multiplex PCR (Table 3.1). Using the MY09/11 primers in single PCR another 3 samples were identified as HPV positive that were not detected by the multiplex assay (Table 3.1). Either the MY09/11 or the GP5+/6+ primers confirmed all 21 of the samples positive by the multiplex assay. Two samples were positive for *C. trachomatis* in the multiplex assay (Table 3.1). These were confirmed positive by the CTP1/2 and Hsp60R2 primers in single PCRs and no additional positive samples were detected. A positive sample was defined as positive by either the multiplex or any of the single PCRs for that organism. The sensitivity and specificity of the multiplex with respect to single PCR for the detection of HPV in the pilot study samples were 89% and 100%, (Table 3.1). The multiplex assay was 100% specific and sensitive for the detection of *C. trachomatis* with respect to single PCR in the pilot study population (Table 3.1).

Table 3.1. Comparison of multiplex and single PCR for the detection of HPV and *C. trachomatis* in PreservCyt cervical samples

Sample Cohort	Multiplex positive		Single PCR positive			
	MY09/11[a]	CTP1/2[b]	MY09/11	GP5+/6+[a]	CTP1/2	Hsp60R2[b]
GP[c] clinic (n=100)	21	2	24	14	2	2
GUM clinic (n=34)	10	34	10	10	34	34

[a] Primers for the detection of HPV

[b] Primers for the detection of *C. trachomatis*

[c] Women who attended general practitioner (GP) clinics for routine cervical screening

3.3.2 *Estimation of sensitivity for C. trachomatis detection*

The sensitivity of the multiplex for the detection of *C. trachomatis* in the GUM clinic population was 100% with respect to the commercial LCx assay (Table 3.1). Ten of the 34 samples (29.4%) were also positive for HPV by the multiplex assay.

3.3.3 *Comparison of multiplex PCR to HCII for HPV detection*

A total of 40 samples were tested for both high-risk and low-risk HPV. 20 of these were HPV positive and 20 were HPV negative by the multiplex PCR. A positive result for HCII was defined as a sample positive for either high or low-risk HPV following DNA extraction by either the Digene Conversion method or the QIAamp method. The sensitivity of the multiplex with respect to the HCII method for the detection of HPV was 86% and the specificity was 100%. Concordant results were obtained in 34/40 (85%) of the samples. All multiplex positive samples were high-risk positive and three were also low-risk positive yielding a co-infection rate of high and low-risk HPV types of 15% (3/20).

HCII assay was performed on 40 samples with DNA prepared by two methods: - the QIAamp method and the Digene Conversion method. Two multiplex PCR HPV positive samples were negative by the Digene Conversion DNA preparation method but positive by the QIAamp DNA extraction method (Table 3.2). For the multiplex negative samples, two samples were HCII positive by both the Digene and QIAamp method with a further positive sample detected for each method, but not by both (Table 3.2). The RLU/CO values obtained ranged overall from 1,564.5 to 1.08 with an average 261.97 for HCII positive samples. Lower than average RLU/CO values were obtained for samples with HPV result discordant from the multiplex result (Table 3.3). The viral loads (see Section 4.2) of these samples were analysed retrospectively. The viral load of

each of these samples was lower than the median viral load (1.5 x 10^6 HPV/ μg DNA) of all HPV positive samples (n=194) quantified.

Table 3.2. Comparison of HPV detection by Digene hybrid capture (HCII) assay using two DNA preparation methods

	HCII positive	HCII negative
HPV positive by multiplex PCR		
Digene DNA	18	2
QIAamp DNA	20	0
HPV negative by multiplex PCR		
Digene DNA	3[a]	17
QIAamp DNA	3[b]	17

[a] 2 of these samples were HCII positive using QIAamp extracted DNA

[b] 2 of these samples were HCII positive using Digene extracted DNA

Table 3.3. RLU/CO luminescence value, viral load of samples with discordant results between multiplex PCR and HCII

M-PCR[a]	RLU/CO[b] Digene	RLU/CO[b] QIAamp	Viral load[c]
+	0.44 (-)	1.18 (+)	1.9 x 10^5
+	0.15 (-)	1.22 (+)	9.7 x 10^5
-	0.15 (-)	21.69 (+)	4.7 x 10^4
-	11.66 (+)	3.89 (+)	1.2 x 10^5
-	262.87(+)	51.83 (+)	8.6 x 10^4
-	3.37(+)	0.72 (-)	3.0 x 10^5

[a] multiplex PCR

[b] relative light unit/cut-off value

[c] HPV copy/ μg genomic DNA

3.3.4 *Estimation of copy number sensitivity*

The multiplex assay could detect as few as 100 copies of *C. trachomatis* plasmid (Figure 3.3) and 100 copies of the HPV genome per µL of extracted DNA (Figure 3.4).

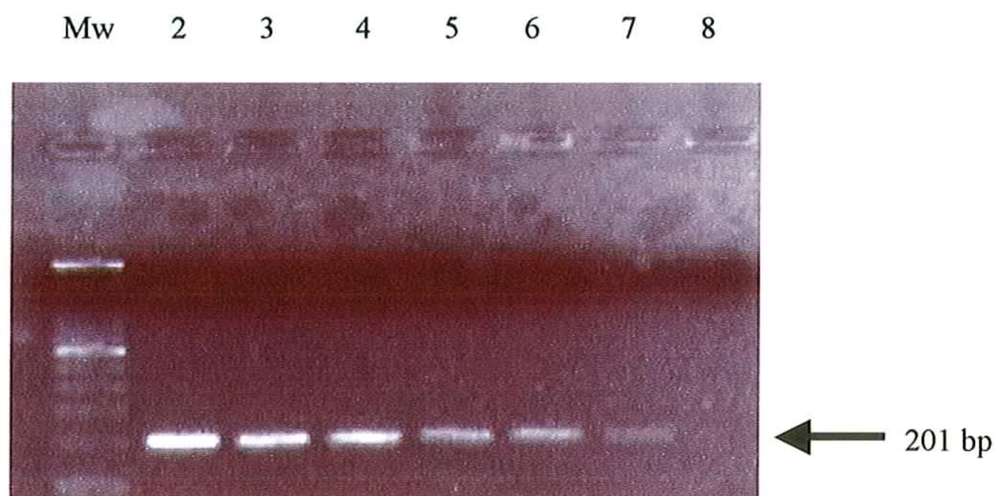


Figure 3.3. Copy number sensitivity of multiplex for *C. trachomatis* plasmid detection. MW: 100 bp ladder, lanes 2-7 diluted standard curve of 10^7-10^2 copies of cloned CTP plasmid PCR product (201 bp), lane 8 negative H_2O control

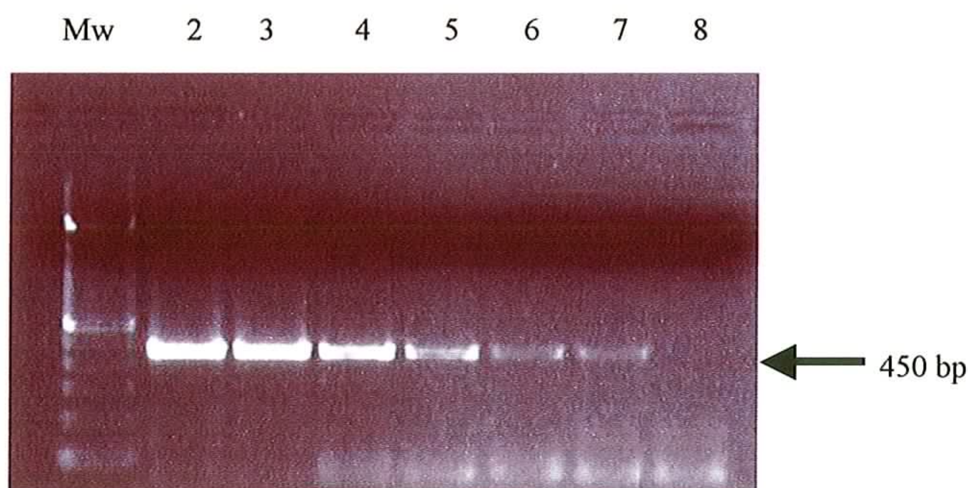


Figure 3.4. Copy number sensitivity of multiplex for HPV MY09/11 PCR product detection. MW: 100 bp ladder, lanes 2-7 diluted standard curve of 10^7-10^2 copies of cloned HPV MY09/11 PCR product (450 bp), lane 8 negative H_2O control

3.3.5 *Age characteristics of the study population*

The age of the study population ranged from 16-72 yr. The average age of women presenting for routine cervical screening was 35 yr. Age data was obtained for 996/997 HPV positive cases. 187/996 (19%) of the population studied were < 25 yr, 401/996 (40%) were between the ages of 25 yr and 35 yr, and 408/996 (41%) > 35 yr (Figure 3.5).

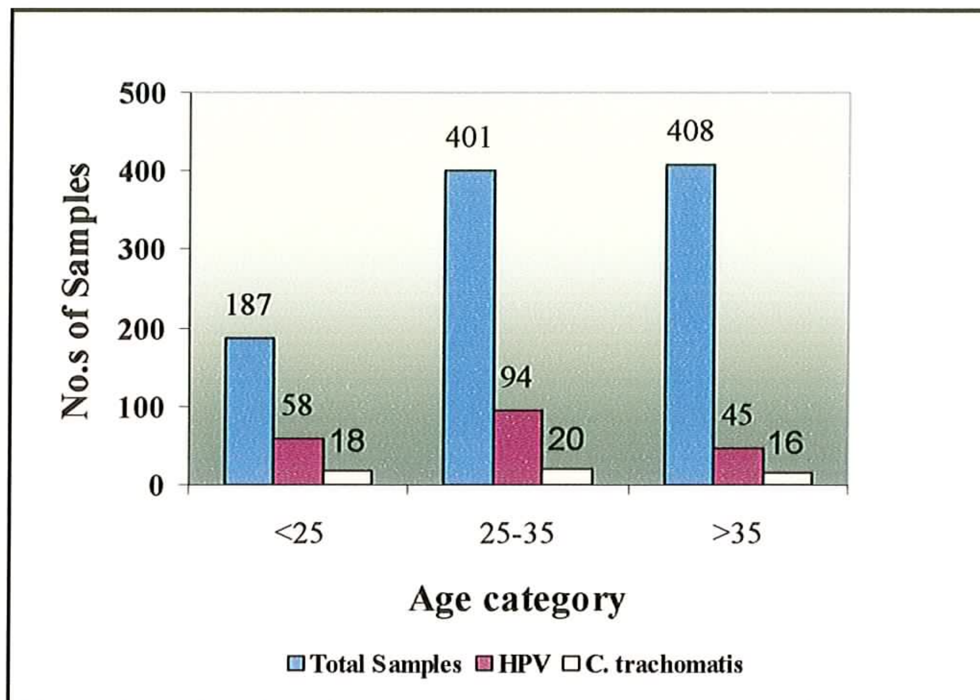


Figure 3.5. Number of HPV and *C. trachomatis* infections detected by the multiplex PCR method in the female population by age (n=996)

3.3.6 *Prevalence of HPV and C. trachomatis in the study population*

All samples amplified for ß-globin using the PCO3/4 primers in the multiplex. HPV was detected in 197/997 (19.8%) of samples and *C. trachomatis* in 54/997 (5.4%) of samples (Figure 3.6). 11/997 (1.1%) of samples were infected with both HPV and *C. trachomatis* (Figure 3.6).

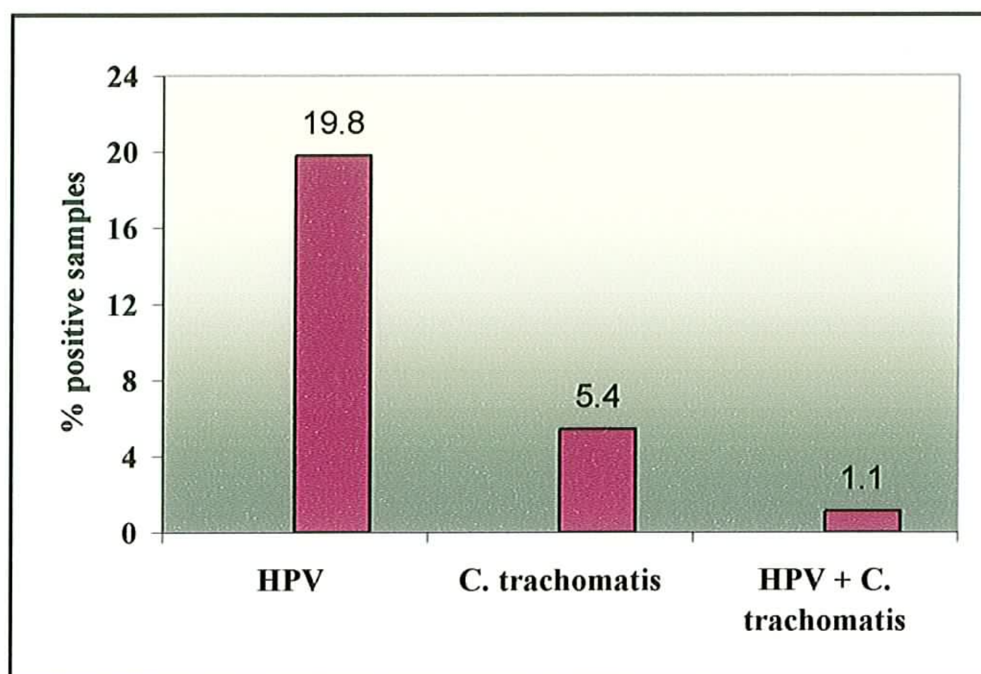


Figure 3.6. Prevalence of HPV and *C. trachomatis* in the Irish female population presenting for routine cervical screening (n=996)

In the < 25 yr age group, 58/187 (31%) and 18/187 (10%) women were infected with HPV and *C. trachomatis* respectively. In the 25-35 yr age group 94/401 (23%) and 20/401 (5%) were infected with HPV and *C. trachomatis* respectively. In the > 35 yr age group 45/408 (11%) and 16/408 (4%) were infected with HPV and *C. trachomatis* respectively (Figure 3.5).

Of cervical HPV infections, 29% (58/197) were in the < 25 yr age group, 48% (94/197) were in the 25-35 yr age group and 23% (45/197) were in the > 35 y age group (Figure 3.7). Cumulatively 77% (152/197) of HPV infections occurred in the < 35 yr age group. The trend of decreasing HPV prevalence with age was highly significant ($P<0.0001$). In the > 35 yr age group, 19% (38/197) occurred in women aged between 36 and 49 yr inclusive and 4% (7/197) in women over the age of 50 yr. For *C. trachomatis* 33% (18/54) of infections were in the < 25 yr age group, 37% (20/54) in the 25-35 yr age group and 30% (16/54) in the > 35 yr age group (Figure 3.8). Cumulatively 70% (38/54)

of *C. trachomatis* infections occurred in the < 35 yr age group. The trend of decreasing *C. trachomatis* prevalence with age was highly significant ($P<0.0001$). The average age of women infected with both organisms was 31 yr, with 5/11 concomitant infections occurring in the <25 yr age group, 3/11 in the 25-35 yr age group and the remaining 3/11 in the >35 yr age group.

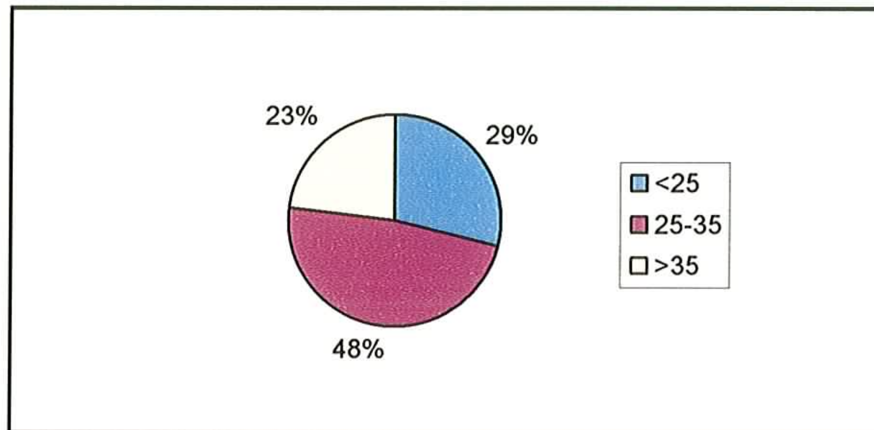


Figure 3.7. Proportion of total HPV infections (N=197) in each age category, < 25 yr = 58 cases, 25-35 yr = 94 cases, >35 yr = 45 cases

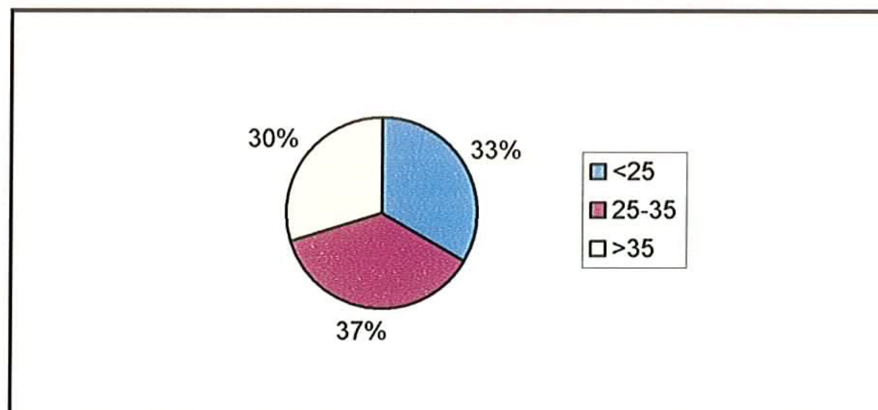


Figure 3.8. Proportion of total *C. trachomatis* infections (N=54) in each age category, < 25 yr = 18 cases, 25-35 yr = 20 cases, >35 yr = 16 cases

3.4 Discussion

The ThinPrep Pap Test is fast becoming the method of choice for cervical cytology and permits further studies on residual cellular material. The aim of this study was to evaluate the potential for screening for both HPV and *C. trachomatis* from a single ThinPrep sample by a rapid cost-effective multiwell multiplex PCR and to apply this technology to estimate the prevalence of *C. trachomatis* and HPV in the Irish female population attending for routine cervical screening.

3.4.1 *Design of multiplex for HPV and C. trachomatis detection*

The multiplex was designed for the detection of HPV by amplification of a region of the L1 gene using the MY09/11 consensus primers, and for the detection of *C. trachmatis* by amplification of a region of the cryptic plasmid using the CTP primers. The consensus MY09/11 primers were chosen for HPV amplification as they amplify over 30 genital types of HPV (Gravitt *et al*, 2000) and sequences amplified using these primers are commonly used for downstream typing methods (Feoli-Fonseca *et al*, 2001; Richardson *et al*, 2003). Moreover, use of the MY09/11 primers facilitated optimisation of the annealing temperature of the multiplex PCR to allow co-amplification of the CTP and β-globin PCR products unlike the GP5+/6+ primers, which rely on a less stringent annealing temperature of 40°C to allow amplification of a similar range of HPV types (de Roda Husman *et al*, 1995; Jacobs *et al*, 1997). A recent study, which compared biases in HPV genotype prevalence assessment associated with the commonly used consensus primers MY09/11, PGMY09/11 and GP5+/6+, reported that GP5+/6+ exhibited poor sensitivity for HPV52 detection (Chan *et al*, 2005). In the diagnostic setting PCR for *C. trachomatis* is commonly performed using primers targeted to the cryptic plasmid (Singh *et al*, 2003; Molano *et al*, 2005). As the plasmid is present in

multiple copies per organism amplification is highly sensitive when compared to chromosomal targeted PCR assays (Mahony *et al*, 1993; Lehmann *et al*, 1999). The multiplex PCR was optimised for use on a multiwell plate and the choice of primers used in combination with each other, created sufficient differences in amplicon size (Figure 3.1) to allow analysis of PCR products on 1% agarose gels. In this way, processing of many samples was achieved with minimum cost.

3.4.2 *Estimation of the sensitivity and specificity of the multiplex PCR for HPV detection*

To evaluate the sensitivity and specificity of the multiplex for the detection of *C. trachomatis* and HPV, a pilot study was carried out on 100 samples. The pilot study also allowed testing of the sample collection process.

Initial testing of the multiplex on 100 samples revealed a HPV prevalence of 21%, which is similar to the findings of other population based HPV studies, and is within the range of worldwide prevalences estimated by IARC (Franceschi, 2005). The sensitivity of the multiplex for the detection of HPV with respect to single PCR was 89% and the specificity was 100% as calculated on the 100 pilot study samples. The multiplex was slightly less sensitive than single PCR. This is to be expected, as greater competition for reagents exists when PCR is performed in a multiplex format. Other studies have reported that the MY09/11 primers are more sensitive than the GP5+/6+ primers when used separately (Husnjak *et al*, 2000; Brennan *et al*, 2001), however when used in an nested PCR format with the MY09/11 primers, the GP5+/6+ PCR confers increased sensitivity of detection as the positioning of the annealing sites for the GP5+/6+ primers are within the MY09/11 PCR product (Remmerbach *et al*, 2004). Performance of a nested PCR for HPV detection, although it would be ideal, would require an additional

amplification step and would not be feasible for high-throughput population based screening studies.

The multiplex could detect 100 copies of the MY09/11 PCR product. This compares favourably to the limits of detection of the HCII assay, which can detect 5,000 viral copies (Lorincz, 1996).

The multiplex was compared to the HCII assay for the detection of HPV in 20 HPV positive and 20 HPV negative samples (Table 3.2). Sensitivity and specificity values of 86% and 100 % were obtained. Concordant results were obtained between the multiplex and HCII for the detection of HPV in 87.5 % of samples. Other studies have detected similar concordance rates of 85% (Kulmala *et al*, 2004) and 76.5% (Nonogaki *et al*, 2004) for HPV detection between HCII and PCR.

False negative multiplex PCR results may be explained by the fact that the HCII analysis was carried out on a second aliquot of 4 mL from the original PreservCyt sample, which may not have contained equal numbers of HPV infected cells. Similarly, other studies have reported false negative samples when using just one set of consensus primers for the detection of HPV (Husnjak *et al*, 2000; Remmerbach *et al*, 2004).

Classification of HPV risk by HCII revealed a predominance of high-risk infections, with low-risk infections occurring only in cases of concomitant high-risk infection. Other studies have shown that the majority of HPV infections are indeed high-risk (Beby-Defaux *et al*, 2004; Thomas *et al*, 2004). Recently, concerns have been raised over the misclassification of HPV risk resulting from cross-reactivity of some low risk probes with high-risk HPV types (Poljak *et al*, 2002). The newer hybrid capture (HCIII)

assay aims to address this problem. In this assay a biotinylated capture DNA oligonucleotide specific for selected HPV DNA sequences is used to capture DNA:RNA hybrids onto streptavidin coated wells instead of an immobilised antibody as in the HCII system. This could reduce the possibility of non-specific DNA:RNA hybrid capture and thus reduce cross-reactivity and false positives (Castle *et al*, 2003b).

3.4.3 *Estimation of the sensitivity and specificity of the multiplex PCR for C. trachomatis detection*

Initial screening of 100 pilot study samples revealed a *C. trachomatis* prevalence of 2%. The multiplex was both highly sensitive (100%) and specific (100%) for *C. trachomatis* detection as no additional positive samples were detected by single PCR using either the CTP or the Hsp60R2 primers on the 100 pilot study samples. Other studies performed using the CTP primers for PCR detection of *C. trachomatis* have shown similar sensitivity rates (Shattock *et al*, 1998; George *et al*, 2003).

The multiplex could detect as few as 100 copies of the CTP plasmid. As the plasmid is present at between 6 and 10 copies per organism this translates to a sensitivity of less than 100 organisms. Recent reports have shown that the plasmid copy number increases during the replicative phase of the lifecycle indicating that the sensitivity of detection may be increased further depending on stage of the developmental cycle (Pickett *et al*, 2005).

The sensitivity of the multiplex for *C. trachomatis* detection was further evaluated on 34 GUM clinic samples positive for *C. trachomatis* by the commercial LCx assay. The LCx assay is an amplification assay, which also targets the *C. trachomatis* plasmid

(Gaydos *et al*, 2004). The multiplex was 100% sensitive and specific by comparison to the LCx assay for the detection of *C. trachomatis* in this higher risk GUM clinic control population. Such high levels of sensitivity are comparable to those seen for commercial nucleic acid based tests such as the COBAS Amplicor CT/NG test for the detection of *C. trachomatis* (van der Pol *et al*, 2000).

Few studies have looked at HPV status with respect to infections with other STIs (Lehmann *et al*, 1999; Golijow *et al*, 2005). In this study 29.4% of *C. trachomatis* positive samples were also HPV positive. These results highlight the need for an integrated approach to STI screening and women's health initiatives.

3.4.4 *Comparison of two DNA extraction procedures for HCII analysis*

Four samples yielded discrepant HCII results on comparison of the two DNA extraction methods (Table 3.3). Real-time PCR quantitation of these samples indicated that negativity by either extraction method might be as a result of low viral load (Table 3.3) of these samples. Further evaluation of convenient DNA extraction procedures for use in HCII analysis is needed on a greater number of samples with particular consideration given to viral load.

3.4.5 *Prevalence of C. trachomatis in the female population*

One of the aims of this study was to establish the prevalence of *C. trachomatis* infection in cervical PreservCyt samples taken from Irish women presenting to GPs for routine cervical screening in an urban setting. The prevalence of *C. trachomatis* in the population studied was 5.4%. The asymptomatic nature of *C. trachomatis* facilitates its spread and promotes a reservoir of infection. Prevalence data may provide an indication of the size of this reservoir in the Irish female population.

The prevalence of *C. trachomatis* depends upon the setting, context and country studied. In 2002, the European Union BioMed Concerted Action Group undertook a systematic review of the prevalence of *C. trachomatis* among European women. From an analysis of >300 papers it was found that only 14 studies met their strict inclusion criteria. The prevalence was found to range from 1.7% to 17%. The mode was 6% for women seeking contraception and 4% for women having cervical smears (Wilson et al, 2002). Our finding of 5.4% in women having cervical smears correlates well with the European average. More recent studies have shown a prevalence of 3.5% in Greece (Levidiotou *et al*, 2005), 8.2% along the US-Mexico border (Baldwin *et al*, 2004) and 1.1% in India (Joyee *et al*, 2004).

It has been demonstrated previously that young sexually active females are at highest risk for Chlamydia infection and for repeat infections, which further increase their risk of sequelae. A study by Shrier *et al*, (2004) using nucleic acid amplification based tests on a variety of samples demonstrated a prevalence of 22% in the 16-25 yr age group. Previous studies have demonstrated a decrease in prevalence with age (van den Hoek *et al*, 1999; Adams *et al*, 2004). A similar trend was observed in our study with incidence reducing from 10% in the < 25 yr to 5% in the 25-35 yr group and 4% in the > 35 yr group (Figure 3.5).

A review of *C. trachomatis* prevalence studies in the U.K. reported that the most influential variables on prevalence were age and setting of the population studied (Adams *et al*, 2004). Using the criteria set down by the British Chief Medical Officer's expert advisory group on *C. trachomatis* screening, on Belgian women it was found that age was not the most influential factor in prevalence but rather risky sexual behaviour (Verhoeven *et al*, 2003). Our study found a high prevalence among young women

attending GP clinics for cervical screening with 70% of all infections occurring in the < 35 y age group (Figure 3.8) and indicated that screening of this group would identify the majority of infections. The incidence of *C. trachomatis* infections was highest in the < 25 yr age group, however this group is not commonly targeted for cervical screening. Further studies to determine additional risk factors for *C. trachomatis* infection in the Irish population are warranted.

3.4.6 *Prevalence of HPV in the female population*

In this study the HPV prevalence was 19.8%. Great variations in prevalence figures for total HPV infection have been reported worldwide. A recent IARC study which surveyed 10 population-based studies on approximately 15,000 samples from 4 continents showed that the prevalence of HPV infection varies greatly from 2%-30% (Franceschi, 2005). Recently conducted studies show prevalences of 26.3% in Nigeria (Thomas *et al*, 2004), 14.32% in women with no history of abnormalities in France (Boulanger *et al*, 2004), 17.7% in sexually active women in Argentina (Matos *et al*, 2003), 27.5% in married women 30-50 years of age in China, 10.9% in South Vietnam (Pham *et al*, 2003), 28% in Honduras (Tabora *et al*, 2005), 27% in Western Australia (Brestovac *et al*, 2005b) and 26.5% in Costa Rica (Herrero *et al*, 2005).

A recent IARC worldwide analysis of the epidemiology of HPV infections has shown that the age distribution varies worldwide (Franceschi, 2005). Numerous studies have demonstrated the highest HPV prevalences in young women with a decrease in prevalence with age (Matos *et al*, 2003; Beby-Defaux *et al*, 2004). In our study, prevalence decreased with age from 31% in the <25 y group to 23% in the 25-35 y group and 11% in the >35 y group. Some studies have shown a bimodal pattern of infection with prevalences highest in the youngest and oldest age categories (Castle *et*

al, 2005; Herrero *et al*, 2005) suggesting acquisition of the virus at young age with persistent infections more evident with age. In our population it was found that 23% of all HPV infections occurred in the >35 y age group and that within this age group prevalence decreased with age. Screening for HPV in this age group may identify persistent infections and identify women at greatest risk for developing cervical cancer. This approach has been adopted as part of the screening algorithm in countries such as the U.S.A. and France.

3.4.7 *Coinfection by HPV and C. trachomatis*

In our study 29.4% of *C. trachomatis* infections in the high risk GUM population also contained HPV. In the epidemiological study 1% of the general population were found to harbour both organisms. A recent study in Argentina found that *C. trachomatis* prevalence was higher in HPV positive women than in HPV negative women (Golijow *et al*, 2005). *C. trachomatis* is now considered an independent risk factor for the development of cervical cancer (Franceschi, 2005). A recent study on colposcopy patients reporting a prevalence of 3.4%, suggested that routine screening for *C. trachomatis* be carried out in colposcopy clinics (Tayal *et al*, 2005). *C. trachomatis* infected cohorts may overlap with cohorts harbouring other STIs and those at risk of development of cervical neplasias. Comprehensive STI prevention programmes that involve a range of STI service providers are needed to successfully reduce the STI-related health burden in the population.

3.4.8 *Population based screening for C. trachomatis and HPV*

Population based screening for HPV and *C. trachomatis* has been reviewed extensively and is highly recommended by a number of studies under particular conditions (CDC, 1993; Cuzick *et al*, 2003). Combined screening for both *C. trachomatis* and HPV

infections is justifiable since they are highly prevalent, easily diagnosed and there is now substantial evidence for the contributory effect of *C. trachomatis* infections in the development of cervical intraepithelial neoplasias. This is the first study looking at the detection of both HPV and *C. trachomatis* from a single ThinPrep sample using multiplex PCR. The ideal situation would be to screen for the presence of a number of organisms by multiplex PCR from a single fluid based sample. This simple multiplex is cost effective, rapid and could be used to screen cervical ThinPrep samples for both HPV and *C. trachomatis*, especially in a high-risk population.

3.4.9 *Summary*

Summary

- Multiplex PCR is 100% sensitive and specific for *C. trachomatis* detection and 89% sensitive and 100% specific for HPV detection when compared to single PCRs.
- There is 100% agreement between LCx and multiplex PCR for *C. trachomatis* detection and 88% between HCII and multiplex PCR for HPV detection.
- 5.4% prevalence of *C. trachomatis* in the female Irish population justifies a screening programme.
- 19.8% prevalence of HPV in the Irish female population- this is the first study in Ireland to generate HPV population prevalence data.
- 29.4% of *C. trachomatis* positive GUM clinic attendees had HPV infections.
- 1% of the Irish female population harbour both *C. trachomatis* and HPV
- The prevalence of both infections decreased with age.

CHAPTER 4

Characterisation of HPV infections in Irish women undergoing opportunistic cervical screening

4.1 Introduction

4.1.1 *Aims*

Since the prevalence of human papillomaviruses (HPV) in the general population is so high, HPV testing as a primary screening tool for cervical abnormalities is not appropriate. HPV data that may have greater predictive value include HPV genotype identification and HPV viral load, as high load of certain HPV types is associated with neoplasia. The aim of this research was to gain further information on the nature of HPV infections in the Irish population, in particular HPV type and viral load and to investigate these parameters in relation to cytological abnormalities of the cervix.

4.1.2 *HPV prevalence and cytology*

While cervical screening programmes are credited with dramatically reducing the incidence of mortality worldwide due to cervical cancer, approximately 50% of cases of invasive cervical cancer can be attributed to the inherent limitations of cytological screening (Cuzick, 1998). The sensitivity of cytology in the detection of high-grade cervical lesions is estimated at only 50-80% (Cuzick, 1998). Since the 1980s there has been growing support for the introduction of adjunctive HPV testing into cervical screening programmes to improve the accuracy of diagnosis, especially in the distinction between high-grade and low-grade disease. The presence of HPV DNA has been proposed as a diagnostic marker considering that 99.9% of all cervical cancers contain HPV DNA (Munoz, 2000). Those positive for HPV DNA have a 15-20 times higher risk of developing cervical cancer than those without HPV DNA (Lo *et al*, 2002). The presence of high-risk HPV DNA has been consistently associated with all grades of cervical lesions (Kulasingam *et al*, 2002). One of the aims of this research was to establish the baseline prevalence of HPV DNA in all grades of pre-neoplastic disease and in normal samples from an Irish population, and secondly to establish the rate of

high-risk HPV DNA presentation in these samples. Recently, *C. trachomatis* infection and smoking have been classified as contributory risk factors in the development of neoplasia (Smith *et al*, 2004; Matos *et al*, 2005). A further aim of this study was to determine if there was any relationship between *C. trachomatis* infection and smoking status and the presence of cervical disease.

4.1.3 *HPV load as a prognostic or diagnostic indicator*

The presence of HPV alone is not a sufficient indicator of disease potential. Apart from the type of HPV, other characteristics of HPV infections are currently being explored such as the viral load.

Viral load could function as an alternative method for assessing the risk of cancer development because high viral load could result from an active viral replication, which may support viral persistence (Ylitalo *et al*, 2000). HPV viral load has also been used after surgical excision of cervical lesions as a test of cure procedure (Lillo *et al*, 2005) and also to determine the likelihood of lesional regression and viral clearance in abnormal cytology (van Duin *et al*, 2002). High viral load has been shown to increase the chance of development of cervical intraepithelial neoplasia grade three (CINIII) by 60-fold (Josefsson *et al*, 2000), however the use of viral load as a prognostic indicator remains controversial. When hybrid capture (HCII) was used to estimate viral load, an association was determined between HPV viral load and presence or grade of squamous intraepithelial lesions in some, but not all, cross-sectional studies (Clavel *et al*, 1998b; Nindl *et al*, 1998). In a study by Szoke *et al*, (2003) which followed the disease course of 455 patients who had had a routine HCII test due to cervical squamous abnormalities, it was found that viral load did not predict oncogenic progression. However, viral load estimation by HCII is only considered semi-quantitative.

It has also been suggested that viral load may also be used to determine the grade of disease. While studies have suggested a relationship between grade of disease severity and viral load (Cuzick *et al*, 1992; Josefsson *et al*, 2000), this relationship is not yet clear.

Viral load significance has been particularly associated with HPV16 (Swan *et al*, 1999). Studies have demonstrated that cervical disease is consistently associated with high HPV16 viral load (Ylitalo *et al*, 2000; Lo *et al*, 2005). Moreover, HPV16 viral load has also been associated with prostate cancer (Serth *et al*, 1999). HPV16 viral load has been used to predict the risk of cervical carcinoma before the development of squamous intraepithelial lesions (Josefsson *et al*, 2000). A study by Lo *et al*, (2005) showed that for HPV16 the viral load was higher in high-grade and cervical cancer cases, lower in low-grade cases and the lowest in normal cases. In a recent study which looked at the relationship between stage of cervical carcinoma and HPV viral load it was reported that HPV viral load did not increase with increased severity of disease (Biedermann *et al*, 2004). It was also suggested that high HPV16 viral load may favour prognosis by switching the host's immune status from a silent state induced by the immunosuppressant quality of tumour cells to an active state induced by infection (Biedermann *et al*, 2004). Ikenberg *et al*, (1994) also found a better survival trend in patients with higher copy number HPV infections. One of the aims of our study was to investigate if viral load was associated with disease as opposed to normal samples and to assess whether the viral load varied according to grade of precancerous disease.

4.1.4 *Real-time quantitation of HPV*

The presentation of data in the literature with respect to technical and theoretical aspects of HPV viral load is incredibly heterogeneous. A gold-standard method for HPV

quantitation has not yet been proposed. Early studies categorized samples as low viral load if HPV quantity was only detected by PCR and high viral load if the less sensitive southern blot could be used (Morrison *et al*, 1992). Following on from that, HPV was quantified by measuring the signal intensity of gel bands or hybridization signals following standard PCR assays (Flannelly *et al*, 1995; Forslund *et al*, 1997; Bavin *et al*, 1993; Swan *et al*, 1999). Numerous studies have used HCII to estimate viral load semi-quantitatively (Peyton *et al*, 1998; Lorincz *et al*, 2002; Gravitt *et al*, 2003a) however, the accuracy of HCII for quantitation has been questioned, particularly because of the lack of standardisation for input genomic DNA. The HCII assay is reported to be quantitative between approximately 5×10^4 and 5×10^7 viral copies, but this does not cover the full range observed in clinical samples (about 10^2 to 10^9 copies), (Cavuslu *et al*, 1996). Good correlation between HPV viral load determined by real-time PCR as compared to HCII has been reported in other studies (Pretet *et al*, 2004).

Recently real-time PCR assays have been developed (Josefsson *et al*, 1999; Szuhai *et al*, 2001; Tucker *et al*, 2001). It is generally accepted that real-time PCR assays targeting type-specific DNA with normalization for input of human DNA provide the best measurement of HPV viral load (Gravitt *et al*, 2003a). Real-time PCR has the advantage of high specificity, high sensitivity and with a capacity to quantify over a large linear range (Tucker *et al*, 2001). Real-time PCR has also been used to simultaneously determine the integration status and viral load of HPV infections (Nagao *et al*, 2002; Peitsaro *et al*, 2002).

Real-time PCR has been adapted extensively for HPV quantitation; however, there is no consensus on the choice of format. The most notable difference between assays is the choice between a consensus HPV amplification approach or type-specific amplification.

Recent studies have indicated that cervical cancer may not be exclusively a monoclonal disease brought about by one oncogenic HPV type but may result from the interplay of potential oncogenic HPV types within the same local environment (Huang *et al*, 2004; Munoz *et al*, 2004). With this in mind real-time assays have been designed for consensus PCR amplification (Lillo *et al*, 2005) and type-specific amplification (Hart *et al*, 2001; Tucker *et al*, 2001; Josefsson *et al*, 1999).

Numerous real-time chemistries have been explored including Scorpion probes (Hart *et al*, 2001), SYBR Green™ E1 gene PCR (Szuhai *et al*, 2001), 5' exonuclease probes (Josefsson *et al*, 1999; Tucker *et al*, 2001). In this study real-time SYBR Green™ PCR employing consensus MY09/11 primers was used to determine viral load in all HPV positive samples with respect to an imported external standard curve of cloned HPV16 MY09/11 consensus PCR product. It was important to develop a consensus PCR as the full range and frequencies of different genotypes of HPV in the general Irish population were unknown.

4.1.5 *HPV genotyping*

Testing for high-risk HPV types dramatically increases the positive predictive value of an HPV test for the detection of neoplasia. It is therefore important in any HPV screening situation to select HPV types for screening according to local prevalence. At present there is no information on the prevalence of the various HPV genotypes in the normal Irish female population presenting for opportunistic cervical screening. Even within other European countries, few population-based opportunistic HPV typing studies have been performed (Johnson *et al*, 2003; Cuschieri *et al*, 2004; Speich *et al*, 2004). The aim of this research was to determine the genotype of HPV infections in Irish women presenting for opportunistic cervical screening.

The prevalence of HPV genotypes varies according to geographical location. Most studies assessing HPV genotype have focused on cervical cancer cases and HPV genotype in preneoplastic lesions exclusively. A study conducted by the International Agency for Research on Cancer (IARC) in 2003 on HPV types in cervical cancer cases based in 9 countries worldwide found that after HPV16 and 18 the prevalence of other oncogenic types varies considerably from country to country (Munoz *et al*, 2003). HPV16 was the most common type in all countries (overall prevalence 58.9%), ranging from 43.9% in the Philippines to 72.4% in Morocco. HPV18 was the second most common type, followed by HPV45. Types 31 and 35 were more common in countries of Latin-America and the prevalence of HPV52 was highest in Peru (8.6%).

A more recent study conducted by IARC on 10,058 cervical cancer cases worldwide found that the most common HPV types found in cervical cancer cases were, in order of decreasing prevalence, HPV16, 18, 45, 31, 33, 58, 52, 35, 59, 56, 6, 51, 68, 39, 82, 73, 66, and 70 (Franceschi, 2005). HPV16 (51%) or 18 (16.2%) were associated with over two-thirds of cervical cancer cases worldwide (Franceschi, 2005). Cohort effects in type distribution, particularly of high-risk types other than HPV16, have been observed within countries (Touze *et al*, 2001).

Even within the high-risk group of HPV types, oncogenic potential differs. In Europe the most prevalent type is HPV16 (Bosch *et al*, 1995) but there are several reports that HPV18 infections may lead to a more aggressive form of the disease (Arends *et al*, 1993; Nobbenhuis *et al*, 1999). HPV16 has been shown to induce malignant transformation without integrating into the host genome in contrast to HPV18, 31 and 35, which always seem to be present in an integrated state in malignant lesions (Pirami *et al*, 1997; Badaracco *et al*, 2002; Hudelist *et al*, 2004).

HPV types are distinguished on the basis of their nucleic acid sequences. By definition the sequence of a new type should be no more than 90% similar to existing types in the L1 gene sequence for it to be classified as a new type (Torrisi *et al*, 2000). HPV types are numbered in sequence according to their historic description. The Virus Reference Centre at the German Cancer Research Centre in Heidelberg regulates this numbering process. HPVs have been further classified into subtypes within a type if they show between 90% and 98% homology to the corresponding type. In addition to this HPV can be further classified into intratypes or type variants when there are differences in homology of no more than 2% (de Villiers *et al*, 2004). HPV variants differ in their biological and chemical properties and pathogenicity. Based on sequence variation in the L1, L2 and LCR regions, five naturally occurring geographical clusters of HPV16 have been identified: European (E), Asian (As), Asian-American (AA), African-1 (Af1) and African-2 (Af2). Intratypic sequence variation has also been found in the E2, E4, E5, E6 and E7 genes of these variants (Giannoudis and Herrington 2001).

4.1.6 ***HPV typing methods***

Traditional methods for HPV typing included the southern blot (Matsukura and Sugase, 1990), restriction fragment length polymorphism (RFLP) (Tucker *et al*, 1993; Hwang *et al*, 2003) and *in situ* hybridisation (Cooper *et al*, 1991). Type-specific PCRs have also been employed for the identification of specific types (O'Leary *et al*, 1997; Chow *et al*, 2000). However, the choice of types to be amplified relies upon some previous knowledge of the expected occurrence of each type in the population tested. Type-specific PCR also requires successive rounds of amplification with each type-specific primer and is labour intensive and costly. Recently developed methods include the HPV DNA chip (Biomedlab Co., Seoul, Korea), (Lee *et al*, 2003) and the line-probe assay

(LiPa, Roche Molecular Biosystems), (Gravitt *et al*, 1998). Both of these are now commercially available and allow identification of multiple HPV types in a single test.

Sequencing of the L1 gene is considered the gold-standard method for the identification of HPV types (de Villiers *et al*, 2004). Sequencing of PCR products does not allow for multiple type identification, however, the predominant HPV type present in the sample is preferentially amplified during the PCR reaction. An advantage of sequencing is that it allows identification of persistent infection with the same HPV type over time. Genotyping by PCR of the L1 gene followed by sequencing is the common method performed (de Francesco *et al*, 2005). In this project, genotyping was performed by sequencing of the MY09/11 PCR product. The MY09/11 primers were chosen as they have the ability to amplify a wide range of HPV types and also have the potential to bind to sequences of unknown HPV type due to the incorporation of degenerate bases into the primer design.

4.2 Methods

4.2.1 *Correlation of multiplex HPV result with cytology*

Detection of HPV by the multiplex PCR was evaluated with respect to cytology result. For the purpose of analysis results were classified as either normal, borderline (borderline nuclear changes including all cases of atypical squamous cells of unknown significance (ASCUS) and glandular cells of undetermined significance (AGUS)), CINI, CINII or CINIII. For statistical analyses CINII and III were combined.

4.2.2 *Quantification of HPV using real-time PCR*

MY09/11 LightCycler PCR for HPV quantitation

Real-time PCR for quantitation of HPV was performed on HPV positive samples using the MY09/11 primers. An aliquot (5 μL) of each DNA sample was added to a reaction mixture (15 μL) containing 2 mM $MgCl_2$, 200μM deoxynucleotide triphosphates (Invitrogen Ltd., U.K.), 10 pmol primers (Sigma-Aldrich, U.K.), 1 U platinum *Taq* polymerase (Invitrogen Ltd., U.K.), 1 μL of 100X bovine serum albumin, (New England Biolabs, U.S.A.), and 2 μL of (1:30000) dilution of SYBR Green™, (Sigma-Aldrich, U.K.), (Appendix B). Samples were amplified on a *LightCycler* under the following cycle conditions: an initial 5 min at 95°C for activation of the platinum *Taq* DNA polymerase, followed by 45 cycles of 5 s of denaturation at 95°C, 1 min of annealing at 60°C, decreasing to 55°C at a rate of 1°C/cycle and 1 min of extension at 72°C. Data were obtained in the single acquisition mode after raising the temperature to 81°C. The MY09/11 PCR product amplified from HPV16 DNA extracted from CaSki cell lines (American Type Culture Collection) was cloned into a pCR®2.1-TOPO® (Invitrogen Ltd.) using the TOPO-TA Cloning kit (Invitrogen Ltd.) as per instruction manual, and serially diluted cloned copies were used to create a standard curve (10^9 to

10^3 copies) for quantitation of HPV copy numbers (Figure 4.1). These standards were used to create a standard curve, which was imported into each assay and calibrated using a sample of known copy number (Figure 4.2). The machine took a calculation of MY09/11 copy numbers at the crossing point of each sample, during the exponential phase of amplification. A melt curve step was included to confirm the amplification of the 450 bp product. All PCR products were run on a 1% agarose gel stained with ethidium bromide to confirm positivity.

Normalisation of results by quantitation of concentration of sample DNA

Real-time PCR results were normalised with respect to total DNA concentration of each sample. The DNA concentration of each sample was estimated using the Hoechst 33258 DNA quantitation kit (Bio-Rad Laboratories, U.S.A.) as per instruction manual. Briefly, Hoechst 33258 is an excitable fluorescent dye, which binds to the minor groove of DNA with a preference for AT sequences (Labarca *et al*, 1980). Upon binding to double stranded DNA the wavelength of the fluorescence output changes. These fluorescence changes can be measured using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Known concentrations of calf thymus DNA are used to construct a standard curve from which the concentration of unknown samples can be read. MY09/11 copy number for each sample was calculated per μg of total DNA to control for possible differences in the cellularity of each sample.

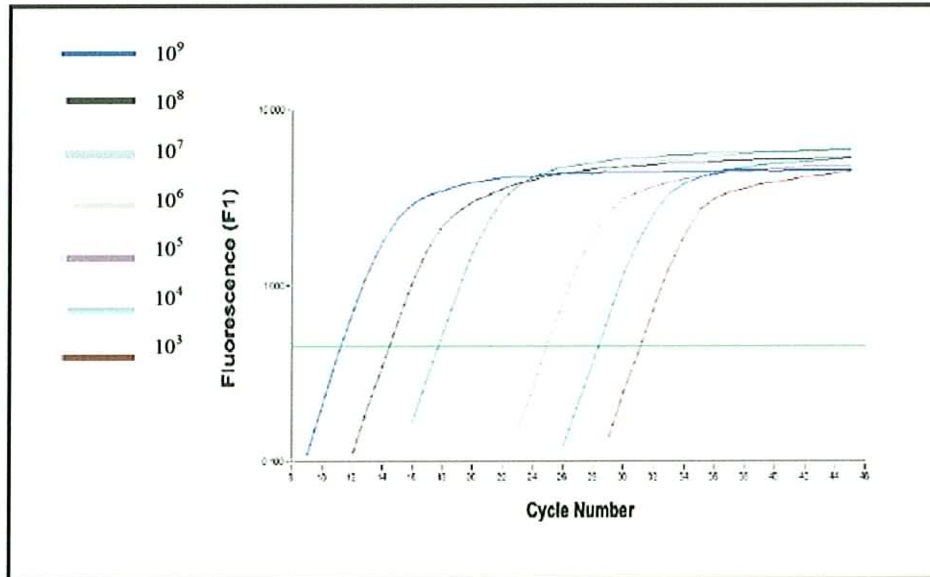


Figure 4.1. ***LightCycler*** **PCR amplification curve of cloned** ***HPV standards*** **standards.** pCR®2.1-TOPO®*MY09/11* standards ranging from 10^9 copies of HPV MY09/11 PCR product to 10^3 copies per µL of extracted DNA

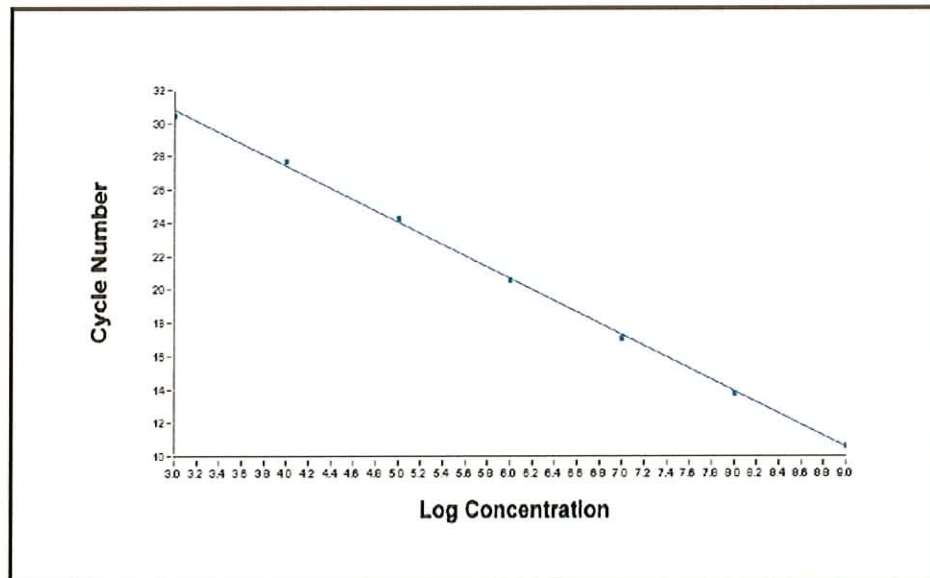


Figure 4.2. Standard curve plot of MY09/11 standards for quantitation of HPV copy numbers by ***LightCycler*** **real-time PCR**

4.2.3 ***Hybrid capture (HCII, Digene, UK) analysis***

DNA was extracted from each HPV positive sample as determined by the multiplex PCR for HCII analysis. DNA was prepared using the Digene Sample Conversion Kit as per manufacturer's instructions, (Appendix D). Briefly, DNA was extracted from 4 mL of PreservCyt sample by alkali denaturation at 65°C for 1 h. HCII was carried out using high-risk probe cocktail for the detection of high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) on 144/197 HPV positive samples (Appendix D). A cut-off point of 1.0 relative light units/ cut-off value (RLU/CO) was set as per manufacturer's instructions. Samples that had RLU/CO of 1.0+/- 0.3 were retested. Samples that were high-risk negative were tested for low-risk HPV using the low-risk probe cocktail which tests for low-risk HPV types (6, 11, 42, 43, and 44).

4.2.4 ***Genotyping of HPV positive samples by sequencing***

HPV positive samples were genotyped by PCR-direct sequencing. Cloning of positive PCR products was not performed due to the large numbers of samples to be processed. A unidirectional sequencing approach was adopted due to resource limitations, however bi-directional sequencing would be ideal and would provide a method for verification of the sequences obtained.

MY09/11 PCR

Cervical samples detected positive for HPV in the multiplex were amplified for the MY09/11 450 bp product. PCR was optimized so that the reaction mix contained 12.5 μL DNA, 10 pmol of each of the forward and reverse primers of the MY09/11, 200 μM deoxynucleoside triphosphates, 1X PCR buffer (containing 10 mM Tris HCl [pH 8.3], 50 mM KCl), 2 mM $MgCl_2$ and 1 U of Platinum *Taq* DNA polymerase (Invitrogen Ltd.) in a final volume of 50 μL (Appendix B). The PCR was initiated by a 5 min

denaturation and enzyme activation step at 95°C and completed by a 5 min extension step at 72°C. The temperature cycles were as follows: 35 cycles of 1 min at 95°C, 1 min at 57°C, and 1 min at 72°C. PCR was performed in a multiwell plate. PCR products were visualised by electrophoresis of 2 µL on 1% agarose gel. Samples that gave weakly positive results were re-amplified from original DNA stocks and the PCR products from the two reactions were pooled together.

Purification of PCR products

PCR products were purified to remove excess primers, nucleotides, *Taq* polymerase and salts using the GenElute™ PCR Clean-Up kit (Sigma-Aldrich) (Appendix F). Briefly, DNA becomes bound to a silica membrane in a spin-column format; this is then washed in an ethanol-based solution and eluted in a Tris-HCl based buffer. Purified PCR product (2 µL) was analysed by 1% agarose gel electrophoresis to ensure that the PCR product remained after purification.

Sequencing of PCR product

Purified PCR product (approximately 25 µL) was airdried with centrifugation in 1.5 mL eppendorfs. Each sample was labelled using the prepaid barcode labels from MWG-Biotech and their details entered on the online ordering system. MY11 primer (5 µL) was enclosed at a concentration of 60 pmol for each sample. Samples were sent to MWG-Biotech, Germany for sequencing using the "Value Read" sequencing service. Samples were sequenced in one direction. Results were obtained using the E-com system and specific HPV types were determined using the basic local alignment sequences tool (BLAST) available on the National Centre for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov).

4.2.5 *Statistical analyses*

For statistical analyses of correlates of cytology, HPV, *C. trachomatis*, HPV and *C. trachomatis* coinfection and smoking status, the Pearson Chi-Square test was performed. All cases of CINII and CINIII were grouped together for statistical analyses. The association of HPV viral load with cytology was determined by analysis of variance between groups (ANOVA). The median viral load and interquartile range for each cytological category was calculated on log-transformed data. Differences in viral load for each cytological category were examined for significance using the least square difference test for multiple comparisons on log-transformed data. All statistical analyses were performed using SPSS software version 11.0.

4.3 Results

4.3.1 *Overall cytological findings*

Of the 997 cervical samples collected, 887 (88.9%) had normal cytology, 41 (4.1%) had borderline cytology, 49 (4.9%) had CINI, 11 (1.1%) had CINII and 9 (0.9%) had CINIII lesions respectively. Of the 996 women for whom age data was recorded, 187 were < 25 yrs, 401 women were between 25 and 35 yrs and 408 women were > 35 yrs. Borderline cytology and CIN lesions were more common in women <25 yrs and were less common in older women ($P<0.0001$), (Table 4.1). 21/41 (51%) of all women presenting with borderline cytology were in the 25 yr to 35 y age group as opposed to 12/41 (29%) in the < 25 yr group and 8/41 (20%) in the > 35 yr age group. Of women presenting with CIN lesions, 26/69 (38%) were < 25 yrs, 31/69 (45%) were between 25 and 35 yrs and 12/69 (17%) were > 35 yrs (Table 4.1).

Table 4.1. Cytology result and age (n=996)

Age Category	Cytology			Total
	Normal	Borderline[a]	CIN[b]	
< 25	149 (80%)	12 (6%)	26 (14%)	187
25 to 35	349 (87%)	21 (5%)	31 (8%)	401
> 35	388 (95%)	8 (2%)	12 (3%)	408
Total	886	41	69	996

[a] borderline nuclear changes, including atypical glandular and squamous cells of undetermined significance

[b] cervical intraepithelial neoplasia

4.3.2 *HPV status in relation to cytology result and age*

Of the 197 HPV positive samples, 101 had normal cytology (51.3%), 35 (17.8%) had borderline cytology, 41 (20.8%) had CINI, 11 (5.6%) had CINII and 9 (4.6%) had CINIII lesions. Of the 101 HPV positive cases with normal cytology, 75 (74%) were in women under 35 years of age (Table 4.2). Borderline cytology and CINI lesions were more common in HPV negative women under the age of 35 years than their older counterparts (Table 4.2). Of the 197 HPV positive cases, the 25-35 years age group had the highest number of borderline cytology and CINI cytology reports (Table 4.2). Of the 800 HPV negative samples, 786 (98.25%) had normal cytology, 6 (0.75%) had borderline cytology, 8 (1%) had CINI, and no samples had CINII or III lesions (Table 4.3).

Of the total samples collected in each cytological category, 101/887 (11.4%) of samples with normal cytology, 35/41 (85.4%) of samples with borderline cytology, 41/49 (83.7%) of samples with CINI, 11/11 (100%) of samples with CINII and 9/9 (100%) of samples with CINIII were HPV positive (Table 4.3). HPV infection was strongly associated with abnormal cytology ($P<0.0001$). HPV detection was 100% sensitive for CINII and III lesions. HPV infection was associated with borderline cytology and CIN lesions respectively ($P<0.0001$ and $P< 0.0001$).

4.3.3 *Chlamydia trachomatis positivity in relation to cytology*

Of the 54 *C. trachomatis* positive samples, 50 (92.6%) had normal cytology, 2 (3.7%) had borderline cytology and 2 (3.7%) had CINI lesions. *C. trachomatis* infection was not statistically associated with abnormal cytology ($P=0.84$). 11 women were coinfected with HPV and *C. trachomatis*. 10/11 women coinfected with HPV and *C. trachomatis*

had normal cytology, 1/11 had borderline cytology. Coinfection with HPV and *C. trachomatis* was not associated with abnormal cytology (P=0.84).

Table 4.2. HPV status, age and cytology result (n=996)

HPV Status	Age	Cytology (No. of samples)					Total
		Normal	Borderline[a]	CINI[b]	CINII	CINIII	
HPV positive	<25	27	10	13	6	2	58
	25-35	48	17	22	3	4	94
	>35	26	8	6	2	3	45
HPV negative	<25	122	2	5	0	0	129
	25-35	301	4	2	0	0	307
	>35	362	0	1	0	0	363
Total		886	41	49	11	9	996

[a] borderline nuclear changes, including atypical glandular and squamous cells of undetermined significance

[b] cervical intraepithelial neoplasia

Table 4.3. HPV status and cytology result (n=997)

HPV Status	Cervical Cytology					
	Normal	Abnormal[a]	Borderline[b]	CINI[c]	CINII	CINIII
Positive (n=197)	101 (51.3%)	96 (48.7%)	35 (17.8%)	41 (20.8%)	11 (5.6%)	9 (4.6%)
Negative (n =800)	786 (98.3%)	14 (1.7%)	6 (0.8%)	8 (1.0%)	0 (0%)	0 (0%)
Total (n=997)	887	110	41	49	11	9

[a] classified as having either borderline cytology or CIN

[b] borderline nuclear changes, including atypical glandular and squamous cells of undetermined significance

[c] cervical intraepithelial neoplasia

4.3.4 *Relationship of smoking status to cytology result, HPV and C. trachomatis status*

Details of tobacco smoking were obtained for 706 of the 997 women in the study. Overall, 191/706 (27.1%) of individuals smoked on a regular basis. 19.4% of the 191 smokers had some degree of abnormal cytology ie. evidence of either borderline cytology or CIN lesions versus 7.4% of non-smokers (Table 4.4). Smoking was strongly associated with abnormal cytology (P<0.0001).

The percentage of women within each category of abnormal cytology was higher for the smokers than the non-smokers. Of the women who smoked 154/191 (80.6%) had normal cytology, 16/191 (8.4%) had borderline cytology, 15/191 (7.8%) had CINI, 3/191 (1.6%) had CINII and 3/191 (1.6%) had CINIII lesions. Of the non-smokers, 477/515 (92.6%) had normal cytology, 15/515 (2.9%) had borderline cytology, 16/515 (3.1%) had CINI, 6/515 (1.2%) had CINII and 1/515 (0.2%) had CINIII lesions respectively (Table 4.4).

Of the women who smoked 46/191 (24%) had HPV infections and 15/191 (8%) had *C. trachomatis* infections. Of the non-smokers, 80/515 (16%) had HPV infections and 23/515 (4%) had *C. trachomatis* infections (Table 4.5). Smoking was associated with both HPV and *C. trachomatis* infections (P=0.008 and P< 0.001). Four of 191 (2.1%) smokers were coinfected with HPV and *C. trachomatis* versus 2/515 (0.4%) non-smokers (Table 4.5).

Table 4.4. Smoking status and cytology result (n=706)

Smoking Status		Cervical Cytology					
		Normal	Abnormal[a]	Borderline[b]	CINI [c]	CINII	CINIII
Smoker	(n=191)	154 (80.6%)	37 (19.4%)	16 (8.4%)	15 (7.8%)	3 (1.6%)	3 (1.6%)
Non-smoker	(n=515)	477 (92.6%)	38 (7.4%)	15 (2.9%)	16 (3.1%)	6 (1.2%)	1 (0.2%)
Total	(n=706)	631	75	31	31	9	4

[a] classified as having either borderline cytology or CIN

[b] borderline nuclear changes, including atypical glandular and squamous cells of undetermined significance

[c] cervical intraepithelial neoplasia

Table 4.5. Smoking status, HPV and *C. trachomatis* infection and coinfection (n=706)

Smoking Status		HPV		*C. trachomatis*		Coinfection	
		Negative	Positive	Negative	Positive	Negative	Positive
Smoker	(n=191)	145 (76%)	46 (24%)	176 (92%)	15 (8%)	187 (98%)	4 (2%)
Non-smoker	(n=515)	435 (84%)	80 (16%)	492 (96%)	23 (4%)	513 (99.6%)	2 (0.4%)
Totals	(n=706)	580	126	668	38	700	6

4.3.5 *Real-time PCR quantitation of HPV viral load*

Real-time quantitative PCR was performed on 194/197 HPV positive samples. Individual results for each cytological category and for normal versus abnormal cytology were analysed visually by scatterplot (Figures 4.3 and 4.4). The overall median viral load was 1.5×10^6 HPV/ μg total DNA. HPV load was higher in samples with abnormal cytology as opposed to normal cytology (Table 4.6), (Figure 4.5), ($P<0.001$). Samples with borderline cytology had significantly higher viral load than samples with normal cytology ($P=0.001$). The median viral load of samples with CINI and CINII/III lesions was higher than samples with borderline cytology, however this was not statistically significant ($P=0.15$ and $P=0.9$), (Figure 4.5). Viral load did not increase with grade of CIN. The median viral load of CINI lesions was higher than CINII/III lesions (Table 4.6), (Figure 4.5). Viral load was not associated with grade of CIN ($P=0.66$).

Table 4.6. HPV viral load and cytology result

Cytology	25th Percentile [a]	75th Percentile [a]	Median [a]
Normal (n=98)	6.2×10^4	2.7×10^6	5.2×10^5
Borderline (n=36)	2.5×10^5	3.5×10^7	2.2×10^6
CIN I (n=40)	2.7×10^6	7.8×10^7	1.8×10^7
CIN II/III (n=20)	7.1×10^5	5.4×10^3	8.8×10^6

[a] HPV copies/ μg total DNA

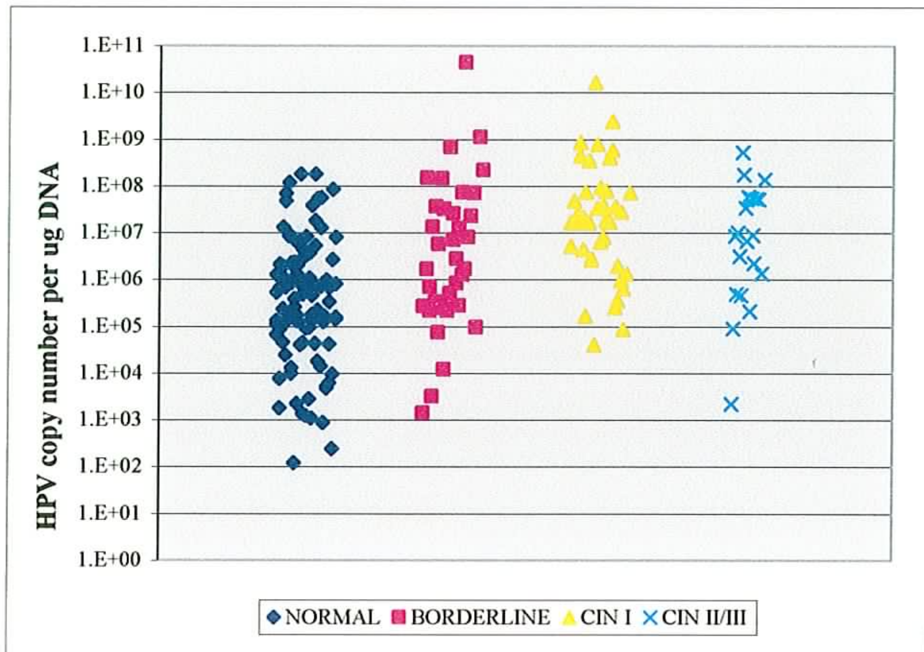


Figure 4.3. HPV copy number and cytology

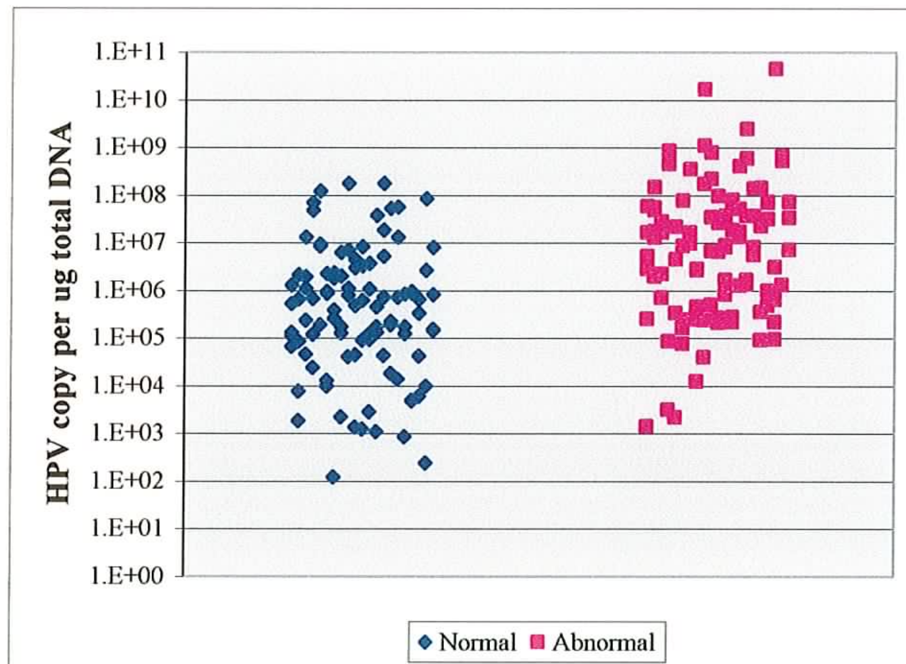


Figure 4.4 HPV copy number in samples with normal versus abnormal (borderline, CINI, CINII or CINIII lesions) cytology

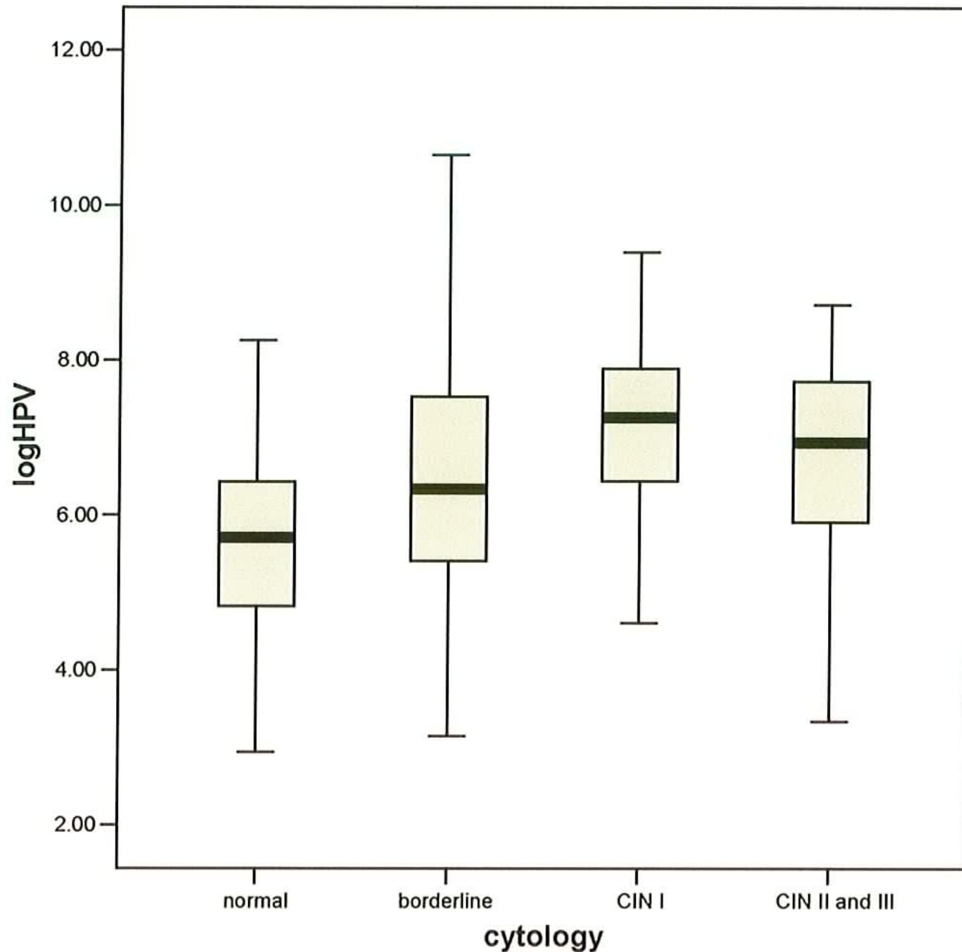


Figure 4.5 Median and interquartile range of HPV viral load for samples with normal or borderline cytology and CINI and CINII/III lesions respectively

4.3.6 *HPV genotype prevalence*

132/197 HPV positive samples were genotyped by sequencing. 20 HPV genotypes were identified (Appendix G). The most common genotypes in descending order were 16, 18, 66, (53 and 33), 6, 61, (70, 31 and 58), 83, (81, 62, and 68), (59 and 54), and (73, 52, 11, and 84) (Table 4.7, Figure 4.6). High-risk HPV genotypes predominated. 97/132 (74%) typed samples contained high-risk HPV types and 35/132 (26%) contained low-risk HPV types. 41/66 (70%) of HPV positive samples with normal cytology and 18/23 (78%) of HPV positive samples with borderline cytology were high-risk positive (Table 4.7). 27/31 (87%) of HPV samples with CINI lesions and 10/12 samples (83%) of

samples with CINII and III lesions were high-risk positive (Table 4.7). The most common HPV type in each cytological category was HPV16 except for the borderline smear category where HPV66 was the most dominant.

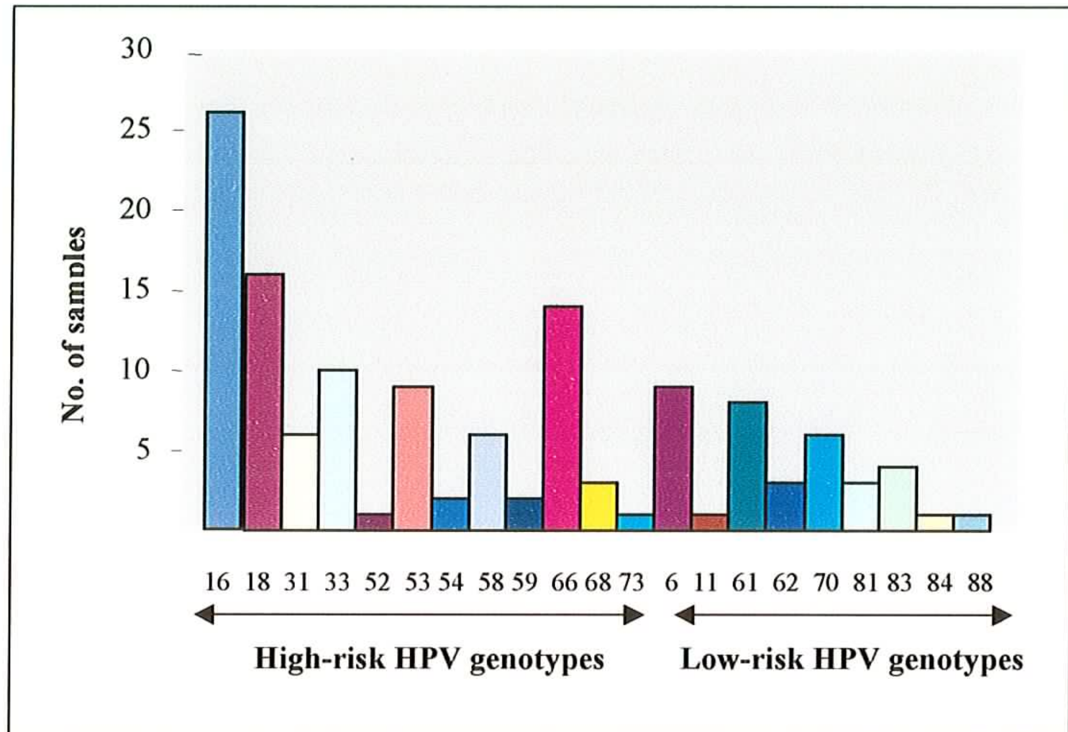


Figure 4.6. Genotype distribution of high and low risk genital HPV types in an Irish female population

Table 4.7. HPV genotype prevalence according to cytology

HPV type	Risk[a]	Total No. of samples	Overall Frequency (%)	Cytology			
				Normal	Borderline	CINI	CINII/III
16	high	26	20	10	2	9	5
18	high	16	12	10	2	3	1
66	high	14	11	3	6	3	2
33	high	10	8	3	3	3	1
53	high	9	7	5	2	2	0
6	low	9	7	6	1	2	0
61	low	8	6	6	0	1	1
70	low	6	5	5	1	0	0
31	high	6	5	3	2	1	0
58	high	6	5	4	0	1	1
83	low	4	3	3	0	0	1
81	low	3	2	2	1	0	0
62	low	3	2	2	1	0	0
68	high	3	2	1	0	2	0
59	high	2	2	0	1	1	0
54	high	2	1	2	0	0	0
73	high	1	1	0	0	1	0
52	high	1	1	0	0	1	0
11	low	1	1	1	0	0	0
84	low	1	1	0	1	0	0
88	low[b]	1	1	0	0	1	0
total		132		66	23	31	12

[a] Classified as high or low-risk according to Munoz *et al*, 2003 [b] Classified as low-risk according to de Villiers *et al*, 2004

4.3.7 *Hybrid capture (HCII, Digene, UK) analysis*

144/197 HPV positive samples were tested using the HCII assay. 107/144 (74%) samples tested positive for HPV using the HCII assay. 95/144 (66%) of the HPV positive samples tested were positive for high-risk HPV. When tested using the low-risk probe, a further 12 samples were positive for low-risk HPV. Of 100 samples tested by HCII that were also genotyped, HCII detected 19/21 (91%) HPV16 samples and (5/12) 42% of HPV18 samples (Table 4.8).

34 samples, which were HPV positive using multiplex PCR, were neither high nor low-risk positive by HCII. 20 of these 34 HCII negative samples were genotyped. Twelve samples contained types outside of the range detected by the HCII assay. These genotypes were 53, 54, 61, 62, 70, 81, 83, 84. Thirty-three of these 34 samples were quantified using real-time PCR. The range of HPV load was between 1.2×10^2 and 2.1×10^8 and the median was 2×10^5 HPV/µg of total DNA. The HCII probe cocktail mix cross-reacted with some but not all samples containing high-risk types 53, 54 and low-risk types 61, 70, 83, 81, 62, and 84 which are not tested for in either the high or low risk probe cocktail mix (Table 4.8). All HPV positive samples genotyped as HPV66 were detected by HCII even though these types are not tested for in either of the HCII probe cocktail mixes (Table 4.8).

Table 4.8. HPV genotype and detection by hybrid capture (HCII)

HPV type	HCII[a] test probe	Risk[b]	No. of HCII samples tested	No. of samples detected by HCII
16	HR	high	21	19
18	HR	high	12	5
31	HR	high	4	4
33	HR	high	8	8
58	HR	high	5	5
68	HR	high	2	2
6	LR	low	7	7
11	LR	low	1	1
53	ND	high	8	6
54	ND	high	1	0
61	ND	low	5	4
62	ND	low	3	2
66	ND	high	12	12
70	ND	low	4	3
81	ND	low	3	1
83	ND	low	3	1
84	ND	low	1	0
Total			100	80

[a] Included for testing using the HCII high-risk probe (HR), low-risk probe (LR) or not tested for by either probe (ND)

[b] Classified as high or low-risk according to Munoz *et al*, 2003

[c] Classified as low-risk according to de Villiers *et al*, 2004

4.4. Discussion

In this chapter the HPV infections in an Irish female population were further characterised. The relationship of HPV to cytological abnormalities and risk factors for cervical disease such as smoking, HPV viral load and genotype was explored. HPV and *C. trachomatis* infection were examined as predictors of abnormal cervical cytology.

4.4.1 *Overall cytological findings*

The overall cytological findings in 997 cervical liquid-based samples were assessed in terms of age and grade of abnormality. This is the first Irish study of such magnitude to relate cytology results to age, HPV status and cigarette smoking in a healthy asymptomatic opportunistically screened population. The majority of women presenting for screening were over the age of 25 years which is representative of the child-bearing population in Ireland and the majority (90%) of cervical smears were cytologically normal, followed by borderline diagnoses (4%), CINI lesions (5%), CINII (1%) and CINIII lesions (1%). In the U.K., Cuschieri *et al*, (2004) also reported that 90% of all cervical smears were cytologically normal. The prevalence of cervical abnormalities was more common in the under 25 yrs age group and prevalence decreased with age. This trend has been reported previously (Lawson *et al*, 1998). Cervical abnormalities, particularly high-grade lesions, have been reported to be more prevalent in the less than 25 yrs age group in Ireland previously and are thought to be on the increase (Treanor *et al*, 2002). The highest incidence of CIN lesions detected was in the 25-35 yrs age group, followed by the under 25 yrs group indicating that women 25-35 yrs should be more closely monitored for the development of CIN lesions than their older counterparts. It has been suggested previously that the best opportunity for preventing invasive squamous cervical carcinomas lies in screening women aged 20-39 yrs when the

incidence of CINIII is highest (Herbert and Smith, 1999). Results of our study support this.

4.4.2 *HPV and C. trachomatis as predictors of cervical abnormalities*

In this study it was determined that the value of pan-HPV testing using consensus primers would be as a negative predictor of neoplasia since 98% of HPV negative samples had normal cytology and no samples with high-grade epithelial lesions were detected that were HPV negative. Pan-HPV testing was 100% sensitive for both CINII and CINIII lesions respectively. HPV testing has been demonstrated previously to have a high sensitivity for the detection of high-grade lesions (Bigras and de Marval, 2005) and most studies have demonstrated a high negative predictive value of HPV testing in primary screening (Cuzick *et al*, 1999a; Cuzick *et al*, 2000; Rozendaal *et al*, 2000).

A number of studies have reported on the positive predictive value of pan-HPV testing by consensus PCR in the detection of cervical disease (Cuschieri *et al*, 2004; Kalantari *et al*, 1997). In a recent study, which used PCR for HPV detection using the MY09/11 primers, the prevalence of HPV was significantly higher in women with evidence of squamous intraepithelial lesions than in normal cytology (Beby-Defaux *et al*, 2004). In another similar population based HPV prevalence study using consensus primers for HPV detection, HPV was found in 12.7% of negative cytology cases, 85% of borderline cytology, 96% of mild abnormalities and 94% of high-grade disease (Cuschieri *et al*, 2004). We reported similar prevalences in these cytological categories of 85% HPV prevalence in borderline cytology cases, 84% in CINI lesions (mild cytological abnormalities) and 100% in CINII and III lesions (high-grade disease).

Approximately half of all HPV positive samples in this study had normal cytology reported. Numerous studies have reported high prevalence of HPV in normal smears. A study by Golijow *et al*, (2005) demonstrated a HPV prevalence of 30% in normal smears. Kalantari *et al*, (1997) showed that 69% of cytologically normal cases were HPV positive by MY09/11 PCR but that the majority of these were HPV6 genotype (Kalantari *et al*, 1997). In our study, genotyping of a representative fraction of cytologically normal HPV positive cases showed that 62% of these were high-risk infections. While the prevalence of HPV6 was greatest in the cytologically normal group as opposed to other cytological categories the most likely explanation for such high prevalence of HPV in the cytological normal samples was young age (Table 4.2). HPV DNA detection in normal smears is more common in younger women than in older women, therefore HPV testing as a primary screening tool has been recommended only in the screening of women >30 years of age (Herrington, 2001). From our study, it can be concluded that the absence of HPV DNA, as determined by consensus PCR amplification may be more clinically useful than its presence in the prediction of development of cervical disease.

In our study neither *C. trachomatis* infection alone nor HPV and *C. trachomatis* coinfection were statistically associated with abnormal cytology. 93% of *C. trachomatis* infected samples and 91% of coinfected samples had normal cytology. While *C. trachomatis* infection has been associated with increased risk of cervical cancer (Smith, 2004) few studies are available on the prevalence of *C. trachomatis* in abnormal cervical smears (Friedek *et al*, 2004; Fischer, 2002; Castle *et al*, 2003a), and the role of *C. trachomatis* infection in the development of cervical cancer is unclear. Some studies have cited *C. trachomatis* as a cofactor in the development of cervical lesions (Fischer, 2002) while others have not found any association between *C. trachomatis* infection and

neoplasia (Castle *et al*, 2003a; Friedek *et al*, 2004). More recent studies suggest a possible role of *C. trachomatis* infection in the maintenance of persistent HPV infections (Silins *et al*, 2005; Samoff *et al*, 2005). As *C. trachomatis* was detected in only 54 samples (5.4%) and concomitant *C. trachomatis* and HPV infections were detected in only 1% of our population, a study of greater magnitude would be necessary to make any accurate association between *C. trachomatis* infections and cytology.

4.4.3 *Smoking status as a risk factor for cervical neoplasia and HPV and C. trachomatis infection*

This is the first study conducted in Ireland examining smoking status in relation to cervical cytology, HPV and *C. trachomatis* infection. Smoking was significantly associated with abnormal cytology. Borderline cytology, CINI, CINII and CINIII lesions were more commonly found in smokers than in non-smokers. Only 81% of smokers had normal cytology compared to 91% of non-smokers. Other studies have demonstrated an association between current smoking and abnormal cytology, with some demonstrating a 2-fold increase in the risk of cervical cancer and a reduced survival rate in early stage carcinoma (Shiels *et al*, 2004; Wright *et al*, 2005). Greater smoking intensity and duration have also been associated with increased risk (McIntyre-Seltman *et al*, 2005). In a recent study, women with mildly abnormal cervical smears who were infected with oncogenic HPV and also smoked were three times more likely to be diagnosed with CINIII disease or higher than non-smokers (McIntyre-Seltman *et al*, 2005). In our study, smoking was significantly associated with HPV and *C. trachomatis* infections. Other studies have also demonstrated a positive association of HPV and *C. trachomatis* infection with current smoking (Boardman *et al*, 2005; Zbroch *et al*, 2004).

4.4.4 *Association of viral load with abnormal cytology*

The use of HPV viral load as a predictor of neoplasia remains controversial. One of the investigations conducted as part of this study was to determine if viral load was associated with abnormal cytology and to determine if viral load increased with grade of cervical abnormality. Overall, it was shown that abnormal cytological specimens had a higher viral load but no statistical difference between low-grade lesions and high-grade lesions was observed. High viral load has been linked with increased risk of development and the progression of cervical disease (Monnier-Benoit *et al*, 2005; Josefsson *et al*, 2000). Bigras and de Marval (2005) who assessed the viral load of 1,143 HPV positive samples also showed that higher viral load was associated with abnormal cytology. Gravitt *et al*, (2003a) in a study which compared real-time PCR with HCII for viral load determination showed that cumulative HPV viral load, indicated by HCII, could distinguish between cytologically normal women and women with abnormalities.

In our study women with borderline cytology (ASCUS/AGUS) had elevated HPV viral load when compared to women with normal cytology. It has been suggested that viral load may be a good indicator of women with borderline cytology who are at higher risk of development of CIN. Viral load data from the American ALTS study, which tested 2,198 women with ASCUS, based on cumulative viral load estimation by HCII analysis, found that women who were diagnosed with an histologically confirmed CIN had higher viral load than women with negative histopathology or colposcopy (Sherman *et al*, 2002). Santos *et al*, (2003) found that higher viral load in ASCUS/LSIL was associated with the diagnosis of CINII and III lesions on colposcopy. In our study, samples with CIN lesions had a higher viral load than samples with borderline cytology.

It has been suggested that HPV viral load can be used to determine grade of CIN and that viral load increases with disease severity (Healey *et al*, 2001; Sun *et al*, 2002; Dalstein *et al*, 2003) but other studies have refuted this theory (Clavel *et al*, 2001; Bory *et al*, 2002; Lorincz *et al*, 2002; Sherman *et al*, 2002). In this present study cumulative viral load was not linked to grade of CIN. Sherman *et al*, (2003) reported that the mean and median of HPV load associated with different grades of CIN were similar, ranged widely and showed considerable overlap when stratified according to number of HPV types detected. Even after adjusting for the number of HPV types detected, HPV load could not specifically identify patients with CINIII. In the same study, cases of CINIII in which CINI or II were not found in the surrounding mucosa were associated with lower viral load than cases in which CINIII lesion were surrounded by CINI. In CINIII cases, productive HPV infections of CINI lesions may account for the high viral load in particular in HPV infected cases of multiple types (Sherman *et al*, 2003). In cytology the clinical diagnosis represents the most severe pathology present in the smear. Thus high-grade lesions may also contain areas of lower grade neoplasia therefore heterogeneity may complicate the use of viral load as a predictive or prognostic indicator. Fluctuation in HPV viral load over short time intervals may also affect the accuracy of point prevalence measurements (Wheeler *et al*, 1996).

In our study, viral load decreased from CINI to CINII/III, however this decrease was not statistically significant. A study by Peitsaro *et al*, (2002) on the integration status of HPV suggested a decrease in viral load following the selection of integrated cell clones preceeding the development of CINIII. In the mouse polyomavirus-induced lymphoma model it was found that the quantity of viral early RNAs yielded by a single integrated copy was the same as that yielded by several thousand extrachromosomal copies of the viral genome (Mazur *et al*, 1995). The significance of high viral load may not be in the

production of huge quantities of transforming proteins but rather by creating a local environment where the chances of genomic integration are higher. It can be postulated that in high-grade lesions where integration rates are higher than in low-grade lesions (Ueda *et al*, 2003), particularly preceeding early stage carcinoma, production of high viral load is no longer a necessity. Further studies relating viral load, grade of lesion and viral mRNA expression are needed to determine the biological relationship between HPV load and grade of neoplasm. Some studies have suggested that the link between grade of CIN and viral load is type restricted and is particularly associated with HPV16 (Moberg *et al*, 2005; Swan *et al*, 1999; Lo *et al*, 2005).

4.4.5 *HPV genotype prevalence*

This is the first Irish study to generate population-based HPV genotype prevalence data. To date, two other studies have been performed in Ireland that involved HPV genotyping. One of these involved genotypic mapping of adenocarcinoma tumours (O'Leary *et al*, 1997) while the other correlated HPV status with cellular biomarkers predictive of squamous and glandular preinvasive lesions (Murphy *et al*, 2005). As reported by other studies, high-risk types predominated (Cuschieri *et al*, 2004). A similar proportion of infections were identified as high-risk by sequencing and HCII (74% versus 66%). High-risk HPV types were the most common types detected in all grades of cytology in this present study including normal smears. This has been reported by other studies (Munoz *et al*, 2004; Riethmuller *et al*, 1999).

In our study the most common high-risk types were HPV16 and 18. These genotypes were found in other European based studies. A recent German population-based study on 2,916 cytology samples, with a HPV prevalence of 32.5% identified 39 different HPV types by sequencing. HPV types occurring, in order of frequency were; 16

(26.2%), 31 (10.1%), 6 (5.7%), 18 (5.3%), 58 (4.5%), 61 (4.5%), 53 (4.4%), 42 (4.3%) and 51 (4%). Other types were detected at frequencies of 4% and all other types were detected only once (Speich *et al*, 2004). In a recent Danish study, the most commonly occurring high-risk types in order of prevalence were 16, 31, 33, 18, 58, 52 and the most commonly found low-risk types were 6, 53 and 11 (Johnson *et al*, 2003).

Interestingly, the third most common HPV type detected in this Irish cohort was HPV66, which was detected at a frequency of 11%. HPV66 has not previously been identified in the Irish population, however this may be as a consequence of using typing methods that do not identify a wide range of HPV types. Other studies in which HPV66 has been detected, have involved the application of the line-probe assay or direct sequencing of consensus HPV PCR products (Speich *et al*, 2004; Johnson *et al*, 2003). In these studies HPV66 was detected at moderate frequencies of 2% and 4% (Johnson *et al*, 2003; Speich *et al*, 2004). In a study by Melchers *et al*, (1999) conducted in the Netherlands, which used the LiPa assay and sequencing to identify HPV types in various grades of cytology, HPV66 was the second most common high-risk type identified. The study was based on the repeat cytology and colposcopy results of women who had previously had an abnormal smear. HPV66 was associated with abnormal cytology and was found in women with borderline cytology, low-grade lesions and high-grade lesions but was most common in the borderline cytology group. In our study 10/14 cases of HPV66 identified occurred in women under 30 years of age (Appendix G) and all but three cases of HPV66 were associated with abnormal cytology. Where HPV66 was identified in CINII/III lesions, these were in women in their early twenties. It is possible that this highly prevalent HPV66 is newly emerged among the young Irish population and may be contributing to the recent trend of reduced age of newly diagnosed women with high-grade lesions. It is proposed to verify

the HPV66 genotyping results by sequencing the MY09/11 PCR products in the opposite direction to that previously performed.

HPV16 and 18 are the most commonly found HPVs worldwide although they are only distantly related to one another and form two separate phylogenetic branches each including 6 closely related types (Prado *et al*, 2005). A third phylogenetic branch of high-risk HPV types has been identified unrelated to either HPV16 or 18. It includes types 30, 53, 56 and 66 (Prado *et al*, 2005). In our study types 66 and 53 accounted for 18% of overall HPV types and 19% of all high-risk types. HPV16 was the most common type in the normal, and CIN cytological categories while HPV66 was the most common type in borderline smears. Significantly this type is not tested for as part of the high-risk probe cocktail in the HCII assay but all samples typed as HPV66 were identified by the HCII assay indicating either cross-reactivity of the HCII probe cocktail with types outside of the assay range or multiple type infections. Based on these findings the utility of the HCII HPV test for the triage of Irish women with abnormal smears would be questionable. Further genotyping studies on a larger cohort of women with borderline smears are warranted in this respect.

In this study, 26% of multiplex HPV positive samples were not positive by HCII testing for 18 types. The reason these were not detected may be because the median viral load of these samples was lower than the overall median and HCII was performed on samples stored past the recommended three-week storage period. Another explanation may be the presence of HPV types outside the range of those detected by the HCII test. In a recent study by Johnson *et al*, (2003), 17% of HPV infections genotyped by sequencing would not have been identified by HCII. In the present study 20 samples which were multiplex PCR positive but HCII negative were genotyped and found to have high-risk HPV types 53, 54 (not tested for in the HCII assay) and the remainder

not detected by HCII were low-risk types. However, not all samples containing HPV53 were undetected by HCII. The ability of HCII probes to cross-react with other HPV types in particular HPV53, 54 and 66 has been documented previously (Castle *et al*, 2002; Poljak *et al*, 2002). A limitation of PCR-direct sequencing is that multiple HPV infections cannot be identified, however it is presumed that the dominating HPV type present is preferentially amplified. The linear array assay (LiPa; Roche Molecular Biochemicals) is a newly developed commercially available assay, which enables multiple HPV type detection. Studies to determine the suitability of the LiPa assay for clinical application in cervical cytology triage will be important in the future.

4.4.6 *Summary*

Summary

- Data was generated on the age-related prevalence of cervical cytological abnormalities.
- HPV detection by MY09/11 PCR was 100% sensitive in the detection of CINII and III lesions.
- *C. trachomatis* infection and concomitant HPV and *C. trachomatis* infection were not significantly associated with abnormal cytology.
- Current smoking was associated with abnormal cytology and positivity for HPV or *C. trachomatis*.
- Viral load was higher in abnormal and borderline cytology versus normal smears.
- HPV viral load was not associated with grade of CIN lesion.
- High-risk HPV genotypes predominated in all cytological categories including normal.
- HPV16 and 18 were the most common high-risk types followed by types 66, 53 and 33 (also high-risk).
- HPV6 was the most common low-risk type followed by 61 and 70.

CHAPTER 5

Discussion

HPV and *C. trachomatis* are the two most common sexually transmitted organisms to infect the female genital tract (Clifford *et al*, 2005; Beagley and Timms, 2000). *C. trachomatis* is a major cause of morbidity for women of reproductive age and is a contributor to the incidence of subacute conditions such as cervicitis and urethritis and more serious sequelae such as pelvic inflammatory disease, ectopic pregnancies and infertility (Paavonen *et al*, 1999). High-risk HPV infection is widely accepted as the progenitor of cellular abnormalities, which may progress to neoplasia and invasive cervical cancer in a small number of cases (Walboomers *et al*, 1999). Since the development of the Pap smear in the 1950's, there has been a dramatic fall off in the numbers of cervical cancer cases and mortalities in screened populations (Peto *et al*, 2004). However, this decrease in incidence in populations where screening is successful has plateaued, due to the technical limitations inherent in the cervical Pap smear (Peto *et al*, 2004). Recently, there has been a drive to improve cellular preparation methods for cytological screening through the development of liquid based cytology (Sherman *et al*, 1997). A revolutionizing feature of the new liquid based cytology preparation methods is that not only does the new technology provide enough cellular material for extra smear tests if needed but also that nucleic acids can be extracted from the residual cells. Ancillary molecular testing may be performed on these samples, which may provide further information on the sexual health of the individual (Cuschieri *et al*, 2005; Keegan *et al*, 2005b). The introduction of liquid based technology has created a platform, which may permit the development of a more integrated approach to women's sexual health issues.

One of the aims of this project was to investigate and develop inexpensive and robust nucleic acid based methods for the detection of HPV and *C. trachomatis* from liquid based PreservCyt cervical cytology samples and then to apply this technology to generate data on the prevalence and nature of these infections in the Irish female

population undergoing routine opportunistic cervical screening. As part of the project, a study was undertaken to investigate the feasibility of DNA extraction from PreservCyt using three DNA preparation procedures. A significant factor in the design of any large-scale screening study is cost, particularly if the study is being conducted in the context of a research setting. The efficiency of DNA extraction for the detection of *C. trachomatis* was investigated using two inexpensive in-house methods and one commercially available DNA extraction kit. Variability was encountered both in the quantity of *C. trachomatis* DNA liberated by the three extraction methods as determined by real-time quantitative PCR and also in the quality as measured by the success of PCR amplification of a large amplicon of the *momp* gene, but amplification was most successful using the commercial QIAamp kit (Keegan *et al*, 2005a).

The current trend in molecular diagnostics is towards kit-based standardized methods for both nucleic acid extraction and molecular testing. Often nucleic acid extraction procedures, such as that for Digene HCII are integrated as part of the whole testing procedure with each individual manufacturer providing their own transport medium and collection devices which must be used in conjunction with the testing kit to achieve valid results (Layfield and Qureshi, 2005). Apart from the cost of this, a major disadvantage is the inability to use the same sample for multiple diagnostic tests. A standardized kit based protocol for DNA extraction, which would be suitable for multiple types of nucleic acid based tests is desirable. Automated extraction is also under development, to facilitate large-scale testing for *C. trachomatis* and HPV from liquid-based cervical samples (Cuschieri *et al*, 2003).

During the second part of the project a multiplex PCR was developed by combining well characterised primers in a multiplex format for the simultaneous detection of HPV

and *C. trachomatis*. The multiplex was highly sensitive for the detection of HPV (89%) and *C. trachomatis* (100%). Sensitivity was comparable to that of other commercial tests for these organisms. The multiplex was adapted to a multiwell format, which afforded lower cost and higher throughput of samples than single testing making it ideal for population screening studies in resource limited settings. Few studies have explored the potential for screening for a number of sexually transmitted agents from PreservCyt samples (Hopwood *et al*, 2004; Koumans *et al*, 2003) by molecular based methods with fewer still investigating screening for both HPV and *C. trachomatis* (Samoff *et al*, 2005; Tabora *et al*, 2005). This is the first study to date to use multiplex PCR for the detection of both HPV and *C. trachomatis* from PreservCyt samples (Keegan *et al*, 2005b).

The establishment of baseline prevalence data is of prime importance before the introduction of population testing for any organism. Other Irish studies have reported the prevalence of these organisms in selected cohorts of limited numbers including males attending a university gym or orthopaedic clinic (Powell *et al*, 2004), high-risk populations (Mohammed *et al*, 1984) or in samples with prior pathological evidence of infection (O'Leary *et al*, 1997; Skyldberg *et al*, 1999). No Irish studies on HPV or *C. trachomatis* prevalence have been conducted of this magnitude. This is the first study to generate population prevalence data on HPV and *C. trachomatis* infections in the female Irish population.

In our study a 5% *C. trachomatis* prevalence rate was detected. This compares well to the European average and emphasises the need for investigations into the possibility of *C. trachomatis* screening in Ireland (Wilson *et al*, 2002). In 1999, in the U.K. a pilot screening project of 16,930 women recorded an overall prevalence of 9% (Pimenta *et al*, 2003). Following this, the National Chlamydia Screening Programme was

established in 2002. The programme has identified a prevalence of 10.1% in women less than 25 years of age outside of GUM setting (La Montagne *et al*, 2004). Our findings showed a prevalence of 10% in this same age group. Following from this study we conclude that the prevalence of infection in the less than 25 years age group, outside of GUM clinics is substantial and may warrant opportunistic screening.

Previous studies have shown that offering *C. trachomatis* screening to women under 25 years of age appears effective even in a setting with low to moderate prevalence rates (3%-6%), (Nelson and Helfand, 2001). Current guidelines from the CDC recommend that all sexually active women under 20 years of age are tested and women between 20 and 24 years if any of the following risk factors is present: inconsistent use of barrier contraceptives, or a new sexual partner or multiple sexual partners during the previous three months and women greater than 24 years of age if both risk factors are present (Peipert, 2003). In Sweden and parts of the USA where there are screening programmes for *C. trachomatis* the incidence of pelvic inflammatory disease, ectopic pregnancies and their sequelae has fallen dramatically (Kamwendo *et al*, 1998).

A review of 10 studies performed on the cost-effectiveness of screening for *C. trachomatis* found that screening is cost effective at a prevalence of 3.1% -10% (Honey *et al*, 2002). Based on our findings of a prevalence of 5.4% or 10% in women under 25 years of age, using these criteria, screening of the Irish population would be cost effective if age was used as a selection factor.

In our study a HPV prevalence of 20% was detected. Reports of HPV prevalence vary considerably worldwide. Prevalences of 27%, 13%, 26%, 28% and 62% have been reported in Australia, Gambia, Nigeria, China and Mexico respectively (Brestovac *et al*,

2005b; Wall *et al*, 2005; Thomas *et al*, 2004; Shen *et al*, 2003; Haws *et al*, 2005). Within Europe HPV had been reported in 14%, 33% and 20% of French, German and UK populations (Boulanger *et al*, 2004; Speich *et al*, 2004 and Cuschieri *et al*, 2004).

Great variation in HPV prevalence also exists in different population cohorts within countries. One study conducted in Vietnam reported a significant difference in HPV prevalence in the North (Hanoi; 2%) versus the South (Ho Chi Minh City; 10.9%) of the country (Pham *et al*, 2003). While in the USA a systematic review of studies published in the last decade on HPV prevalence in adolescent women revealed a range in prevalence of between 14% and 90% with the highest prevalence of infection in college students and those attending STD clinics identifying them as target populations for prevention interventions (Revzina and Diclemente, 2005). Our study was conducted in an urban setting and included areas of high population flux with large numbers of people living in rented accommodation as well as areas of more settled residential accommodation; therefore, it is a good representation of a modern urban Irish population.

In our study the correlation of HPV presence with cytological findings demonstrated the high predictive value of a negative HPV test result. All high-grade lesions were HPV positive. When cytology result was correlated with age it was found that the prevalence of CIN lesions was greatest in women less than 25 years of age (Table 4.1). Under the anticipated Irish Cervical Screening Programme it is proposed to offer cervical screening to women of 25 years of age and older. Our results suggest the potential inadequacies of the screening programme with respect to the recommended testing age. Apart from HPV, other risk factors have been identified that increase the risk of cervical cancer such as current and past smoking (Matos *et al*, 2005; McIntyre-Seltman *et al*, 2005). Another aim of our study was to establish the link between current smoking and

abnormal cytology. The incidence of smoking was elevated in all grades of cervical abnormalities confirming previous reports of this association (Harris *et al*, 2004; Boardman *et al*, 2002). Moreover, current smoking emerged as an associated behavioural risk factor for both HPV and *C. trachomatis* infection.

Acquisition of high-risk HPV is thought to be the single most important risk factor in the development of cervical cancer. In our study both genotyping by sequencing and determination of oncogenic risk by HCII classified the majority (74% and 66%) of HPV infections as high-risk. HPV16 and 18 followed by types 66, 33 and 53 respectively were the most commonly occurring high-risk types. Type 16 was the most commonly occurring type in all grades of cytology except the borderline category where type 66 predominated. Much research worldwide has focused on the pathogenesis of HPV16 and 18 with little attention paid to newly emerging and less prevalent oncogenic types such as types 66 and 53 (Prado *et al*, 2005). In our study the majority of HPV66 infections were associated with abnormal cytology and were more commonly seen in women in their twenties. Given the increasing trend of Irish people towards extended periods of worldwide travel during their twenties, it is possible that HPV66 may represent a newly introduced HPV type in Ireland with the capacity to reduce the mean time from acquisition of the virus to invasion. Based on these findings, any future HPV testing to be employed as part of a cervical screening algorithm should be capable of detecting HPV66, something that the currently available HCII cannot do. Studies on newly emerging HPV types are warranted particularly those involved in coinfections with known oncogenic types. Few commercially available systems have the ability to specifically type and also detect multiple infections. The LiPa Assay (Roche Molecular Biosystems) is one of the few systems to embrace this challenge. A growing number of studies are employing this method due to its ease of application and ability to identify persistent HPV infection (Cuschieri *et al*, 2004). Another recent technological advance

in the area has been the development of HPV DNA microarrays, which have the ability to detect and type HPV (Delrio-Lafreniere *et al*, 2004). The most common low-risk types reported in this study were types 6 and 61. As reported previously, the ability of HCII to detect HPV types outside that of the range of the probe cocktail was demonstrated (Poljak *et al*, 2002).

Another emerging area of HPV diagnostics is the determination of viral load. While numerous studies on HPV viral load have been conducted, the value of HPV viral load as a prognostic or diagnostic indicator has not yet been established. As seen in other studies, we demonstrated an association of HPV viral load with abnormal cytology (Monnier-Benoit *et al*, 2005; Josefsson *et al*, 2000). Other groups linked increasing viral load to increasing disease severity (Healey *et al*, 2001; Sun *et al*, 2002). Our study demonstrated a significant difference in viral load between borderline cytology cases and those with neoplastic lesions. However, an association between viral load and grade of lesion was not observed. The relationship of viral load to disease severity in samples with HPVs other than type 16 has not been well explored (Flores *et al*, 2005; Ho *et al*, 2005) and further studies exploring the association of viral load with oncogenic HPV types other than HPV16 are necessary before the full potential of viral load can be evaluated.

Current developments in the area of HPV research include the investigation of a HPV16/18 combined prophylactic vaccine by two leading pharmaceutical companies Merck and Glaxo-SmithKline. This combined vaccine is based on virus like particles of the L1 and L2 genes and is type-specific. While approximately 70% of cervical squamous carcinomas worldwide have been attributed to HPV16 or 18, the efficacy of the vaccine will vary as the prevalence of these types varies worldwide. The vaccine

will be targeted at adolescents but it will take many years for the effects to be realised. In addition to this, neoplasia arising from other oncogenic types will not be prevented, unless a multivalent vaccine is developed.

More immediately, developments in the area of cervical screening in Ireland include the possible rollout of a national cervical screening programme and the introduction of commercial HPV DNA testing by HCII. The Irish Health Service Executive is currently examining the need for a *C. trachomatis* screening programme. Together the results presented in this study may provide some valuable epidemiological data for clinicians involved in the decision-making processes on these and future issues pertaining to HPV and *C. trachomatis* infections in the Irish population.

REFERENCES

Adam E, Berkova Z, Daxnerova Z, Icenogle J, Reeves WC, Kaufman RH. Papillomavirus detection: demographic and behavioural characteristics influencing the identification of cervical disease. *Am J Obstet Gynecol* 2000; **182**:257-264.

Adams EJ, Charlett A, Edmunds WJ, Hughes G. *Chlamydia trachomatis* in the United Kingdom: a systematic review and analysis of prevalence studies. *Sex Transm Infect* 2004; **80**:354-362.

Anguenot JL, de Marval F, Vassilakos P, Auckenthaler R, Ibecheole V, Campana A. Combined screening for *Chlamydia trachomatis* and squamous intra-epithelial lesions using a single liquid-based cervical sample. *Hum Reprod* 2001; **16**:2206-2210.

Anttila T, Saikku P, Koskela P, Bloigu A, Dillner J, Ikaheimo I, Jellum E, Lehtinen M, Lenner P, Hakulinen T, Narvanen A, Pukkala E, Thoresen S, Youngman L, Paavonen J. Serotypes of *Chlamydia trachomatis* and risk for development of cervical squamous cell carcinoma. *JAMA* 2001; **285**:47-51.

Arends MJ, Donaldson YK, Duvall E, Wyllie AH, Bird CC. Human papillomavirus type 18 associates with more advanced cervical neoplasia than human papillomavirus type 16. *Hum Pathol* 1993; **24**:432-437.

Arora R, Kumar A, Prusty BK, Kailash U, Batra S, Das BC. Prevalence of high-risk human papillomavirus (HR-HPV) types 16 and 18 in healthy women with cytologically negative Pap smear. *Eur J Obstet Gynecol Reprod Biol* 2005; **121**:104-109.

Arroyo M, Bagchi S, Raychaudhuri P. Association of the human papillomavirus type 16 E7 protein with the S-phase-specific E2F-cyclin A complex. *Mol Cell Biol* 1993; **13**:6537-6546.

ASCUS-LSIL Triage Study (ALTS) Group. A randomized trial on the management of low-grade squamous intraepithelial lesion cytology interpretations. *Am J Obstet Gynecol* 2003a; **188**:1393-1400.

ASCUS-LSIL Triage Study (ALTS) Group. Results of a randomized trial on the management of cytology interpretations of atypical squamous cells of undetermined significance. *Am J Obstet Gynecol* 2003b; **188**:1383-1392.

Baay MF, Smits E, Tjalma WA, Lardon F, Weyler J, Van Royen P, Van Marck EA, Vermorken JB. Can cervical cancer screening be stopped at 50? The prevalence of HPV in elderly women. *Int J Cancer* 2004; **108**:258-261.

Badaracco G, Venuti A, Sedati A, Marcante ML. HPV16 and HPV18 in genital tumors: Significantly different levels of viral integration and correlation to tumor invasiveness. *J Med Virol* 2002; **67**:574-582.

Baker TS, Newcomb WW, Olson NH, Cowsert LM, Olson C, Brown JC. Structures of bovine and human papillomaviruses. Analysis by cryoelectron microscopy and three-dimensional image reconstruction. *Biophys J* 1991; **60:**1445-1456.

Baldauf JJ, Dreyfus M, Ritter J, Cuenin C, Tissier I, Meyer P. Cytology and colposcopy after loop electrosurgical excision: implications for follow-up. *Obstet Gynecol* 1998; **92:**124-130.

Baldwin SB, Djambazov B, Papenfuss M, Abrahamsen M, Denman C, Guernsey de Zapien J, Ortega L, Navarro Henze JL, Hunter J, Rojas M, Garcia F, Giuliano AR. Chlamydial infection in women along the US-Mexico border. *Int J STD AIDS* 2004; **15:**815-821.

Ballard RC, Fehler HG, Htuan Y, Radebe F, Jensen JS, Taylor-Robinson D. Coexistence of urethritis with genital ulcer disease in South Africa: influence on provision of syndromic management. *Sex Transm Infect* 2002; **78:**274-277.

Bassiri M, Mardh PA, Domeika M. Multiplex AMPLICOR PCR screening for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in women attenting non-sexually transmitted disease clinics. The European *Chlamydia* Epidemiology Group. *J Clin Microbiol* 1997; **35:**2556-2560.

Bavin PJ, Giles JA, Deery A, Crow J, Griffiths PD, Emery VC, Walker PG. Use of semi-quantitative PCR for human papillomavirus DNA type 16 to identify women with high grade cervical disease in a population presenting with a mildly dyskaryotic smear report. *Br J Cancer* 1993; **67:**602-605.

Beagley KW, Timms P. *Chlamydia trachomatis* infection: incidence, health costs and prospects for vaccine development. *J Reprod Immunol* 2000; **48:**47-68.

Beatty WL, Byrne GI, Morrison RP. Morphologic and antigenic characterization of interferon gamma-mediated persistent *Chlamydia trachomatis* infection *in vitro*. *Proc Natl Acad Sci U S A* 1993; **90:**3998-4002.

Beby-Defaux A, Bourgoin A, Ragot S, Battandier D, Lemasson JM, Renaud O, Bouguermouh S, Vienne Md Mde L, Agius G. Human papillomavirus infection of the cervix uteri in women attending a Health Examination Center of the French social security. *J Med Virol* 2004; **73:**262-268.

Bedell MA, Hudson JB, Golub TR, Turyk ME, Hosken M, Wilbanks GD, Laimins LA. Amplification of human papillomavirus genomes *in vitro* is dependent on epithelial differentiation. *J Virol* 1991; **65:**2254-2260.

Belinson J, Qiao YL, Pretorius R, Zhang WH, Elson P, Li L, Pan QJ, Fischer C, Lorincz A, Zahniser D. Shanxi Province Cervical Cancer Screening Study: a cross-sectional comparative trial of multiple techniques to detect cervical neoplasia. *Gynecol Oncol* 2001; **83:**439-444.

Berg HF, Maraha B, Bergmans AM, van der Zee A, Kluytmans JA, Peeters MF. Extraction of *Chlamydia pneumoniae* DNA from vascular tissue for use in PCR: an evaluation of four procedures. *Clin Microbiol Infect* 2003; **9:**135-139.

Bernal JN, Martinez MA, Dabancens A. Evaluation of proposed cytomorphologic criteria for the diagnosis of *Chlamydia trachomatis* in Papanicolaou smears. *Acta Cytol* 1989; **33:**309-313.

Bernard HU, Chan SY, Manos MM, Ong CK, Villa LL, Delius H, Peyton CL, Bauer HM, Wheeler CM. Identification and assessment of known and novel human papillomaviruses by polymerase chain reaction amplification, restriction fragment length polymorphisms, nucleotide sequence, and phylogenetic algorithms. *J Infect Dis* 1994; **170:**1077-1085.

Bianchi A, Moret F, Desrues JM, Champenois T, Dervaux Y, Desvouas O, Oursin A, Quinzat D, Dachez R, Bathelier C, Ronsin C. PreservCyt transport medium used for the ThinPrep Pap test is a suitable medium for detection of *Chlamydia trachomatis* by the COBAS Amplicor CT/NG test: results of a preliminary study and future implications. *J Clin Microbiol* 2002; **40:**1749-1754.

Biedermann K, Dandachi N, Trattner M, Vogl G, Doppelmayr H, More E, Staudach A, Dietze O, Hauser-Kronberger C. Comparison of real-time PCR signal-amplified *in situ* hybridization and conventional PCR for detection and quantification of human papillomavirus in archival cervical cancer tissue. *J Clin Microbiol* 2004; **42:**3758-3765.

Bigras G, de Marval F. The probability for a Pap test to be abnormal is directly proportional to HPV viral load: results from a Swiss study comparing HPV testing and liquid-based cytology to detect cervical cancer precursors in 13,842 women. *Br J Cancer* 2005; **93:**575-581.

Black CM, Marrazzo J, Johnson RE, Hook EW 3rd, Jones RB, Green TA, Schachter J, Stamm WE, Bolan G, St Louis ME, Martin DH. Head-to-head multicenter comparison of DNA probe and nucleic acid amplification tests for *Chlamydia trachomatis* infection in women performed with an improved reference standard. *J Clin Microbiol* 2002; **40:**3757-3763.

Black CM. Current methods of laboratory diagnosis *of Chlamydia trachomatis* infections. *Clin Microbiol Rev* 1997; **10:**160-184.

Blake DR, Lemay CA, Gaydos CA, Quinn TC. Performance of urine leukocyte esterase in asymptomatic male youth: another look with nucleic acid amplification testing as the gold standard for *Chlamydia* detection. *J Adolesc Heatlh* 2005; **36:**337-341.

Boardman LA, Adams AE, Peipert JF. Clinical predictors of cervical intraepithelial neoplasia 2 or greater in women with mildly abnormal Pap smears. *J Reprod Med* 2002; **47**:891-896.

Boardman LA, Stanko C, Weitzen S, Sung CJ. Atypical Squamous Cells of Undetermined Significance: Human Papillomavirus Testing in Adolescents. *Obstet Gynecol Surv* 2005; **60**:435-437.

Bontkes HJ, van Duin M, de Gruijl TD, Duggan-Keen MF, Walboomers JM, Stukart MJ, Verheijen RH, Helmerhorst TJ, Meijer CJ, Scheper RJ, Stevens FR, Dyer PA, Sinnott P, Stern PL. HPV 16 infection and progression of cervical intra-epithelial neoplasia: analysis of HLA polymorphism and HPV 16 E6 sequence variants. *Int J Cancer* 1998a; **78**:166-171.

Bontkes HJ, Walboomers JM, Meijer CJ, Helmerhorst TJ, Stern PL. Specific HLA class I down-regulation is an early event in cervical dysplasia associated with clinical progression. *Lancet* 1998b; **351**:187-188.

Bory JP, Cucherousset J, Lorenzato M, Gabriel R, Quereux C, Birembaut P, Clavel C. Recurrent human papillomavirus infection detected with the hybrid capture II assay selects women with normal cervical smears at risk for developing high grade cervical lesions: a longitudinal study of 3,091 women. *Int J Cancer* 2002; **102**:519-525.

Bosch FX, de Sanjose S. Chapter 1: Human papillomavirus and cervical cancer-burden and assessment of causality. *J Natl Cancer Inst Monogr* 2003; **31**:3-13.

Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J, Schiffman MH, Moreno V, Kurman R, Shah KV. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. *J Natl Cancer Inst* 1995; **87**:796-802.

Bosch FX, Munoz N, de Sanjose S, Franco EL, Lowy DR, Schiffman M, Franceschi S, Kjaer SK, Meijer CJ, Frazer IH, Cuzick J. Re: Cervical carcinoma and human papillomavirus: on the road to preventing a major human cancer. *J Natl Cancer Inst* 2001; **93**:1349-1350.

Boulanger JC, Sevestre H, Bauville E, Ghighi C, Harlicot JP, Gondry J. Epidemiology of HPV infection. *Gynecol Obstet Fertil* 2004; **32**:218-223.

Bozzetti M, Nonnenmacher B, Mielzinska I I, Villa L, Lorincz A, Breitenbach V V, Prolla J. Comparison between hybrid capture II and polymerase chain reaction results among women at low risk for cervical cancer. *Ann Epidemiol* 2000; **10**:466.

Brennan MM, Lambkin HA, Sheehan CD, Ryan DD, O'Connor TC, Kealy WF. Detection of high-risk subtypes of human papillomavirus in cervical swabs: routine use of the Digene Hybrid Capture assay and polymerase chain reaction analysis. *Br J Biomed Sci* 2001; **58**:24-29.

Brestovac B, Harnett GB, Smith DW, Frost F, Shellam GR. Multiplex nested PCR (MNP) assay for the detection of 15 high risk genotypes of human papillomavirus. *J Clin Virol* 2005a; **33:**116-122.

Brestovac B, Harnett GB, Smith DW, Shellam GR, Frost FA. Human papillomavirus genotypes and their association with cervical neoplasia in a cohort of Western Australian women. *J Med Virol* 2005b; **76:**106-110.

Bromberg-White JL, Meyers C. Comparison of the basal and glucocorticoid-inducible activities of the upstream regulatory regions of HPV18 and HPV31 in multiple epithelial cell lines. *Virology* 2003; **306:**197-202.

Brunelle BW, Nicholson TL, Stephens RS. Microarray-based genomic surveying of gene polymorphisms in *Chlamydia trachomatis*. *Genome Biol* 2004; **5:**R42.

Brunham RC, Paavonen J, Stevens CE, Kiviat N, Kuo CC, Critchlow CW, Holmes KK. Mucopurulent cervicitis--the ignored counterpart in women of urethritis in men. *N Engl J Med* 1984; **311:**1-6.

Burk RD, Ho GY, Beardsley L, Lempa M, Peters M, Bierman R. Sexual behavior and partner characteristics are the predominant risk factors for genital human papillomavirus infection in young women. *J Infect Dis* 1996a; **174:**679-689.

Burk RD, Kelly P, Feldman J, Bromberg J, Vermund SH, DeHovitz JA, Landesman SH. Declining prevalence of cervicovaginal human papillomavirus infection with age is independent of other risk factors. *Sex Transm Dis* 1996b; **23:**333-341.

Burke C, Hickey K. Inflammatory smears--is there a correlation between microbiology and cytology findings? *Ir Med J* 2004; **97:**295-296.

Burton MJ, Holland MJ, Makalo P, Aryee EA, Alexander ND, Sillah A, Faal H, West SK, Foster A, Johnson GJ, Mabey DC, Bailey RL. Re-emergence of *Chlamydia trachomatis* infection after mass antibiotic treatment of a trachoma-endemic Gambian community: a longitudinal study. *Lancet* 2005; **365:**1321-1328.

Calore EE, Pereira SM, Cavaliere MJ. Progression of cervical lesions in HIV-seropositive women: a cytological study. *Diagn Cytopathol* 2001; **24:**117-119.

Carlson JH, Hughes S, Hogan D, Cieplak G, Sturdevant DE, McClarty G, Caldwell HD, Belland RJ. Polymorphisms in the *Chlamydia trachomatis* cytotoxin locus associated with ocular and genital isolates. *Infect Immun* 2004; **72:**7063-7072.

Carozzi F, Lomardi FC, Zendron P, Confortini M, Sani C, Bisanzi S, Pontenani G, Ciatto S. Association of human papillomavirus with prostate cancer: analysis of a consecutive series of prostate biopsies. *Int J Biol Markers* 2004; **19**:257-261.

Castle PE, Escoffery C, Schachter J, Rattray C, Schiffman M, Moncada J, Sugai K, Brown C, Cranston B, Hanchard B, Palefsky JM, Burk RD, Hutchinson ML, Strickler HD. *Chlamydia trachomatis*, herpes simplex virus 2, and human T-cell lymphotrophic virus type 1 are not associated with grade of cervical neoplasia in Jamaican colposcopy patients. *Sex Transm Dis* 2003a; **30**:575-580.

Castle PE, Lorincz AT, Scott DR, Sherman ME, Glass AG, Rush BB, Wacholder S, Burk RD, Manos MM, Schussler JE, Macomber P, Schiffman M. Comparison between prototype hybrid capture 3 and hybrid capture 2 human papillomavirus DNA assays for detection of high-grade cervical intraepithelial neoplasia and cancer. *J Clin Microbiol* 2003b; **41**:4022-4030.

Castle PE, Schiffman M, Burk RD, Wacholder S, Hildesheim A, Herrero R, Bratti MC, Sherman ME, Lorincz A. Restricted cross-reactivity of hybrid capture 2 with nononcogenic human papillomavirus types. *Cancer Epidemiol Biomarkers Prev* 2002; **11**:1394-1399.

Castle PE, Schiffman M, Herrero R, Hildesheim A, Rodriguez AC, Bratti MC, Sherman ME, Wacholder S, Tarone R, Burk RD. A prospective study of age trends in cervical human papillomavirus acquisition and persistence in Guanacaste, Costa Rica. *J Infect Dis* 2005; **191**:1808-1816.

Castle PE, Solomon D, Hildesheim A, Herrero R, Concepcion Bratti M, Sherman ME, Cecilia Rodriguez A, Alfaro M, Hutchinson ML, Terence Dunn S, Kuypers J, Schiffman M. Stability of archived liquid-based cervical cytologic specimens. *Cancer* 2003c; **99**:89-96.

Castle PE, Wheeler CM, Solomon D, Schiffman M, Peyton CL; ALTS Group. Interlaboratory reliability of Hybrid Capture 2. *Am J Clin Pathol* 2004; **122**:238-245.

Castriciano S, Luinstra K, Jang D, Patel J, Mahony J, Kapala J, Chernesky M. Accuracy of results obtained by performing a second ligase chain reaction assay and PCR analysis on urine samples with positive or near-cutoff results in the LCx test for *Chlamydia trachomatis*. *J Clin Microbiol* 2002; **40**:2632-2634.

Cavuslu S, Mant C, Starkey WG, Bible JM, Biswas C, Kell B, Rice P, Best JM, Cason J. Analytic sensitivities of hybrid-capture, consensus and type-specific polymerase chain reactions for the detection of human papillomavirus type 16 DNA. *J Med Virol* 1996; **49**:319-324.

CDC. Centers for Disease Control and Prevention, USA. Sexually transmitted Diseases Surveillance 2000 Supplement, *Chlamydia* prevalence monitoring project Atlanta GA: US Department of Health and Human Services, Centers for Disease Control and Prevention, November 2001.

CDC. Centers for Disease Control and Prevention, USA. Reporting of chlamydial infection-Massachusetts, January – June 2003. *Morb Mortal Wkly Rep* 2005; **54**:558-560.

CDC. Centers for Disease Control and Prevention, USA. Recommendations for the prevention and management of *Chlamydia trachomatis* infections, 1993. *MMWR Recomm Rep* 1993; **42**(RR-12):1-39.

Chan EL, Brandt K, Olienus K, Antonishyn N, Horsman GB. Performance characteristics of the Becton Dickinson ProbeTec System for direct detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in male and female urine specimens in comparison with the Roche Cobas System. *Arch Pathol Lab Med* 2000; **124**:1649-1652.

Chan PK, Chan DP, To KF, Yu MY, Cheung JL, Cheng AF. Evaluation of extraction methods from paraffin wax embedded tissues for PCR amplification of human and viral DNA. *J Clin Pathol* 2001a; **54**:401-403.

Chan PK, Chan MY, Li WW, Chan DP, Cheung JL, Cheng AF. Association of human beta-herpesviruses with the development of cervical cancer: bystanders or cofactors. *J Clin Pathol* 2001b; **54**:48-53.

Chan PK, Cheung TH, Tam AO, Lo KW, Yim SF, Yu MM, To KF, Wong YF, Cheung JL, Chan DP, Hui M, Ip M. Biases in human papillomavirus genotype prevalence assessment associated with commonly used consensus primers. *Int J Cancer* 2005; **118**:243-245.

Chellappan S, Kraus VB, Kroger B, Munger K, Howley PM, Phelps WC, Nevins JR. Adenovirus E1A, simian virus-40 tumor antigen, and human papillomavirus E7 protein share the capacity to disrupt the interaction between transcription factor E2F and the retinoblastoma gene product. *Proc Natl Acad Sci USA* 1992; **89**:4549-4553.

Chernesky MA, Mahony JB, Castriciano S, Mores M, Stewart IO, Landis SJ, Seidelman W, Sargeant EJ, Leman C. Detection of *Chlamydia trachomatis* antigens by enzyme immunoassay and immunofluorescence in genital specimens from symptomatic and asymptomatic men and women. *J Infect Dis* 1986; **154**:141-148.

Chow VT, Loh E, Yeo WM, Tan SY, Chan R. Identification of multiple genital HPV types and sequence variants by consensus and nested type-specific PCR coupled with cycle sequencing. *Pathology* 2000; **32**:204-208.

Claas HC, Wagenvoort JH, Niesters HG, Tio TT, Van Rijsoort-Vos JH, Quint WG. Diagnostic value of the polymerase chain reaction for *Chlamydia* detection as determined in a follow-up study. *J Clin Microbiol* 1991; **29**:42-45.

Clavel C, Bory JP, Rihet S, Masure M, Duval-Binninger I, Putaud I, Lorenzato M, Quereux C, Birembaut P. Comparative analysis of human papillomavirus detection by hybrid capture assay and routine cytologic screening to detect high-grade cervical lesions. *Int J Cancer* 1998a; 75:525-528.

Clavel C, Cucherousset J, Lorenzato M, Caudroy S, Nou JM, Nazeyrollas P, Polette M, Bory JP, Gabriel R, Quereux C, Birembaut P. Negative human papillomavirus testing in normal smears selects a population at low risk for developing high-grade cervical lesions. *Br J Cancer* 2004; 90:1803-1808.

Clavel C, Masure M, Bory JP, Putaud I, Mangeonjean C, Lorenzato M, Nazeyrollas P, Gabriel R, Quereux C, Birembaut P. Human papillomavirus testing in primary screening for the detection of high-grade cervical lesions: a study of 7932 women. *Br J Cancer* 2001; 84:1616-1623.

Clavel C, Masure M, Putaud I, Thomas K, Bory JP, Gabriel R, Quereux C, Birembaut P. Hybrid capture II, a new sensitive test for human papillomavirus detection. Comparison with hybrid capture I and PCR results in cervical lesions. *J Clin Pathol* 1998b; 1:737-740.

Clifford GM, Gallus S, Herrero R, Munoz N, Snijders PJ, Vaccarella S, Anh PT, Ferreccio C, Hieu NT, Matos E, Molano M, Rajkumar R, Ronco G, de Sanjose S, Shin HR, Sukvirach S, Thomas JO, Tunsakul S, Meijer CJ, Franceschi S; IARC HPV Prevalence Surveys Study Group. Worldwide distribution of human papillomavirus types in cytologically normal women in the International Agency for Research on Cancer HPV prevalence surveys: a pooled analysis. *Lancet* 2005; 366:991-998.

Cohen CR, Brunham RC. Pathogenesis of *Chlamydia* induced pelvic inflammatory disease. *Sex Transm Infect* 1999; 75:21-24.

Comber H and Gavin A. National Cancer Regristry of Ireland. Trends in Cerivcal Cancer Incidence in Ireland. Proceedings of the Irish Cervical Cancer Screening Programme Conference, 18th June, 2005.

Cooper K, Herrington CS, Stickland JE, Evans MF, McGee JO. Episomal and integrated human papillomavirus in cervical neoplasia shown by non-isotopic *in situ* hybridisation. *J Clin Pathol* 1991; 44:990-996.

Cortinas P, Munoz MG, Loureiro CL, Pujol FH. Follicular fluid antibodies to *Chlamydia trachomatis* and human heat shock protein-60 kDa and infertility in women. *Arch Med Res* 2004; 35:121-125.

Coutlee F, Gravitt P, Kornegay J, Hankins C, Richardson H, Lapointe N, Voyer H, Franco E. Use of PGMY primers in L1 consensus PCR improves detection of human papillomavirus DNA in genital samples. *J Clin Microbiol* 2002; 40:902.

Cox JT. Epidemiology of cervical intraepithelial neoplasia: the role of human papillomavirus. *Baillieres Clin Obstet Gynaecol* 1995; 9:1-37.

Cruickshank ME, Chambers G, Murray G, McKenzie L, Donaldson C, Andrew J, Campbell MK, Kitchener HC. Age-restricted cervical screening: HPV testing at age 50 identifies a high risk group for cervical disease. *Int J Gynecol Cancer* 2002; **12**:735-740.

Crusius K, Rodriguez I, Alonso A. The human papillomavirus type 16 E5 protein modulates ERK1/2 and p38 MAP kinase activation by an EGFR-independent process in stressed human keratinocytes. *Virus Genes* 2000; **20**:65-69.

Cubie HA, Moore C, Waller M, Moss S; National Cervical Screening Committee LBC/HPV Pilot Steering Group. The development of a quality assurance programme for HPV testing within the UK NHS cervical screening LBC/HPV studies. *Clin Virol* 2005; **33**:287-292.

Cuschieri KS, Beattie G, Hassan S, Robertson K, Cubie H. Assessment of human papillomavirus mRNA detection over time in cervical specimens collected in liquid based cytology medium. *J Virol Methods* 2005; **124**:211-215.

Cuschieri KS, Cubie HA, Whitley MW, Seagar AL, Arends MJ, Moore C, Gilkisson G, McGoogan E. Multiple high risk HPV infections are common in cervical neoplasia and young women in a cervical screening population. *J Clin Pathol* 2004; **57**:68-72.

Cuschieri KS, Seagar AL, Moore C, Gilkison G, Kornegay J, Cubie HA. Development of an automated extraction procedure for detection of human papillomavirus DNA in liquid based cytology samples. *J Virol Methods* 2003; **107**:107-113.

Cuzick J, Beverley E, Ho L, Terry G, Sapper H, Mielzynska I, Lorincz A, Chan WK, Krausz T, Soutter P. HPV testing in primary screening of older women. *Br J Cancer* 1999a; **81**:554-558.

Cuzick J, Sasieni P, Davies P, Adams J, Normand C, Frater A, van Ballegooijen M, van den Akker-van Marle E. A systematic review of the role of human papilloma virus (HPV) testing within a cervical screening programme: summary and conclusions. *Br J Cancer* 2000; **83**:561-565.

Cuzick J, Sasieni P, Davies P, Adams J, Normand C, Frater A, van Ballegooijen M, van den Akker E. A systematic review of the role of human papillomavirus testing within a cervical screening programme. *Health Technol Assess* 1999b; **3**:1-196.

Cuzick J, Szarewski A, Cubie H, Hulman G, Kitchener H, Luesley D, McGoogan E, Menon U, Terry G, Edwards R, Brooks C, Desai M, Gie C, Ho L, Jacobs I, Pickles C, Sasieni P. Management of women who test positive for high-risk types of human papillomavirus: the HART study. *Lancet* 2003; **362**:1871-1876.

Cuzick J, Terry G, Ho L, Hollingworth T, Anderson M. HPV in cervical smears. *Lancet* 1992; **340**:112-113.

Cuzick J. HPV testing in cervical screening. *Sex Transm Infect* 1998; **74**:300-301.

Dalesio N, Marsiglia V, Quinn A, Quinn TC, Gaydos CA. Performance of the MagNA pure LC robot for extraction of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* DNA from urine and swab specimens. *J Clin Microbiol* 2004; **42**:3300-3302.

Dalstein V, Riethmuller D, Pretet JL, Le Bail Carval K, Sautiere JL, Carbillet JP, Kantelip B, Schaal JP, Mougin C. Persistence and load of high-risk HPV are predictors for development of high-grade cervical lesions: a longitudinal French cohort study. *Int J Cancer* 2003; **106**:396-403.

Daly LE and Bourke GJ. Bias and Measurement Error. *In* Interpretation and Uses of Medical Statistics. Eds Daly LE and Bourke GJ. Blackwell Science, Oxford, 5th edition 2000 12:412-414.

Danesh J, Collins R, Peto R. Chronic infections and coronary heart disease: is there a link? *Lancet* 1997; **350**:430-436.

Davies R, Hicks R, Crook T, Morris J, Vousden K. Human papillomavirus type 16 E7 associates with a histone H1 kinase and with p107 through sequences necessary for transformation. *J Virol* 1993; **67**:2521-2528.

De Francesco MA, Gargiulo F, Schreiber C, Ciravolo G, Salinaro F, Manca N. Detection and genotyping of human papillomavirus in cervical samples from Italian patients. *J Med Virol* 2005; **75**:588-592.

De Mets A. *Chlamydia trachomatis*. Retrieved from the World Wide Web on 7th November 2005; http://www.bact.wisc.edu/bact330/lecturechlamydia

de Roda Husman AM, Walboomers JM, van den Brule AJ, Meijer CJ, Snijders PJ. The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. *J Gen Virol* 1995; **76**:1057-1062.

de Villiers EM, Fauquet C, Broker TR, Bernard HU, zur Hausen H. Classification of papillomaviruses. *Virology* 2004; **324**:17-27.

Deacon JM, Evans CD, Yule R, Desai M, Binns W, Taylor C, Peto J. Sexual behaviour and smoking as determinants of cervical HPV infection and of CIN3 among those infected: a case-control study nested within the Manchester cohort. *Br J Cancer* 2000; **83**:1565-1572.

Delrio-Lafreniere SA, Browning MK, McGlennen RC. Low-density addressable array for the detection and typing of the human papillomavirus. *Diagn Microbiol Infect Dis* 2004; **48**:23-31

Demers GW. Espling E. Harry JB. Etscheid BG. Galloway DA. Abrogation of growth arrest signals by human papillomavirus type 16 E7 is mediated by sequences required for transformation. *J Virol* 1996; 70:6862-6869.

Depuydt CE. Vereecken AJ. Salembier GM. Vanbrabant AS. Boels LA. van Herck E. Arbyn M. Segers K. Bogers JJ. Thin-layer liquid-based cervical cytology and PCR for detecting and typing human papillomavirus DNA in Flemish women. *Br J Cancer* 2003; **88**:560-566.

Desaintes C. Demeret C. Control of papillomavirus DNA replication and transcription. *Semin Cancer Biol* 1996; 7:339-347.

Di Felice V. David S. Cappello F. Farina F. Zummo G. Is chlamydial heat shock protein 60 a risk factor for oncogenesis? *Cell Mol Life Sci* 2005; **62**:4-9.

Diagnostic Innovations: The Promise of Molecular Diagnostics. Retrieved from the World Wide Web on 21st July 2005:http://abbott.com/innovation/products.cfm

Dillner J. The serological response to papillomaviruses. *Semin Cancer Biol* 1999; **9**:423-430.

Doniger J. Muralidhar S. Rosenthal LJ. Human cytomegalovirus and human herpesvirus 6 genes that transform and transactivate. *Clin Microbiol Rev* 1999; **12**:367-382.

Doorbar J. The papillomavirus life cycle. *J Clin Virol* 2005; **32**:S7-15.

Duronio RJ. Brook A. Dyson N. O'Farrell PH. E2F-induced S phase requires cyclin E. *Genes Dev* 1996; **10**:2505-2513.

Durst M. Glitz D. Schneider A. zur Hausen H. Human papillomavirus type 16 (HPV 16) gene expression and DNA replication in cervical neoplasia: analysis by *in situ* hybridization. *Virology* 1992; **189**:132-140.

Dyson N. Howley PM. Munger K. Harlow E. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* 1989; **243**:934-937.

Eickhoff M. Laue T. Ruckes T. Cramer SO. Krupp G. Tiemann C. Ultra-rapid detection of *Chlamydia trachomatis* by real-time PCR in the LightCycler using SYBR green technology or 5'-nuclease probes. *Clin Lab* 2003; **49**:217-225.

Evander M. Frazer IH. Payne E. Qi YM. Hengst K. McMillan NA. Identification of the alpha6 integrin as a candidate receptor for papillomaviruses. *J Virol* 1997; **71**:2449-2456.

Everett K. *Chlamydia and Chlamydiales*: more than meets the eye. *Vet Microbiol* 2000; **75**:109-126.

Farencena A. Comanducci M. Donati M. Ratti G. Cevenini R. Characterization of a new isolate of *Chlamydia trachomatis* which lacks the common plasmid and has properties of biovar trachoma. *Infect Immun* 1997; 65:2965-2969.

Favre M. Orth G. Croissant O. Yaniv M. Human papillomavirus DNA: physical map. *Proc Natl Acad Sci USA* 1975; 72:4810-4814.

Fellerhoff B. Laumbacher B. Wank R. High risk of schizophrenia and other mental disorders associated with chlamydial infections: hypothesis to combine drug treatment and adoptive immunotherapy. *Med Hypotheses* 2005; 65:243-252.

Feoli-Fonseca JC. Oligny LL. Brochu P. Simard P. Falconi S. Yotov WV. Human papillomavirus (HPV) study of 691 pathological specimens from Quebec by PCR-direct sequencing approach. *J Med Virol* 2001; 63:284-292.

Fiel-Gan MD. Villamil CF. Mandavilli SR. Ludwig ME. Tsongalis GJ. Rapid detection of HSV from cytologic specimens collected into ThinPrep fixative. *Acta Cytol* 1999; 43:1034-1038.

Fischer N. *Chlamydia trachomatis* infection in cervical intraepithelial neoplasia and invasive carcinoma. *Eur J Gynaecol Oncol* 2002; 23:247-250.

Fitzpatrick C. McKenna P. Hone R. Teenage girls attending a Dublin sexually transmitted disease clinic: a socio-sexual and diagnostic profile. *Ir J Med Sci* 1992; 161:160-162.

Flannelly G. Jiang G. Anderson D. Melvin W. Mann E. Kitchener H. Serial quantitation of HPV-16 in the smears of women with mild and moderate dyskaryosis. *J Med Virol* 1995; 47:6-9.

Flores ER. Lambert PF. Evidence for a switch in the mode of human papillomavirus type 16 DNA replication during the viral life cycle. *J Virol* 1997; 71:7167-7179.

Flores R. Papenfuss M. Klimecki WT. Giuliano AR. Cross-sectional analysis of oncogenic HPV viral load and cervical intraepithelial neoplasia. *Int J Cancer* 2005 Sep 8; [Epub ahead of print].

Forslund O. Lindqvist P. Haadem K. Czegledy J. Hansson BG. HPV 16 DNA and mRNA in cervical brush samples quantified by PCR and microwell hybridization. *J Virol Methods* 1997; 69:209-222.

Franceschi S. The IARC commitment to cancer prevention: the example of papillomavirus and cervical cancer. *Recent Results Cancer Res* 2005; 166:277-297.

Friedek D. Ekiel A. Chelmicki Z. Romanik M. HPV, *Chlamydia trachomatis* and genital mycoplasmas infections in women with low-grade squamous intraepithelial lesions (LSIL). *Ginekol Pol* 2004; 75:457-463.

Funk JO, Waga S, Harry JB, Espling E, Stillman B, Galloway DA. Inhibition of CDK activity and PCNA-dependent DNA replication by p21 is blocked by interaction with the HPV-16 E7 oncoprotein. *Genes Dev* 1997; **11**:2090-100.

Gaydos CA, Theodore M, Dalesio N, Wood BJ, Quinn TC. Comparison of three nucleic acid amplification tests for detection of *Chlamydia trachomatis* in urine specimens. *J Clin Microbiol* 2004; **42**:3041-3045.

Geisler WM, Tang J, Wang C, Wilson CM, Kaslow RA. Epidemiological and genetic correlates of incident *Chlamydia trachomatis* infection in North American adolescents. *J Infect Dis* 2004; **190**:1723-1729.

Gen-Probe Receives FDA Clearance for STD Testing from Cytyc's ThinPrep® Pap Test Vial. Press Release August 10th 2005. Retrieved from the World Wide Web 1st November 2005: http://www.gen-probe.com/corp_overview/press_detail.asp?key=138

George JA, Panchatcharam TS, Paramasivam R, Balasubramanian S, Chakrapani V, Murugan G. Evaluation of diagnostic efficacy of PCR methods for *Chlamydia trachomatis* infection in genital and urine specimens of symptomatic men and women in India. *Jpn J Infect Dis* 2003; **56**:88-92.

Gerard HC, Freise J, Wang Z, Roberts G, Rudy D, Krauss-Opatz B, Kohler L, Zeidler H, Schumacher HR, Whittum-Hudson JA, Hudson AP. *Chlamydia trachomatis* genes whose products are related to energy metabolism are expressed differentially in active vs. persistent infection. *Microbes Infect* 2002; **4**:13-22.

Gerard HC, Krausse-Opatz B, Wang Z, Rudy D, Rao JP, Zeidler H, Schumacher HR, Whittum-Hudson JA, Kohler L, Hudson AP. Expression of *Chlamydia trachomatis* genes encoding products required for DNA synthesis and cell division during active versus persistent infection. *Mol Microbiol* 2001; **41**:731-741.

Giannoudis A, Herrington CS. Human papillomavirus variants and squamous neoplasia of the cervix. *J Pathol* 2001; **193**:295-302.

Girdner JL, Cullen AP, Salama TG, He L, Lorincz A, Quinn TC. Evaluation of the digene hybrid capture II CT-ID test for detection of *Chlamydia trachomatis* in endocervical specimens. *J Clin Microbiol* 1999; **37**:1579-1581.

Giroglou T, Florin L, Schafer F, Streeck RE, Sapp M. Human papillomavirus infection requires cell surface heparin sulfate. *J Virol* 2001; **75**:1565-1570.

Giuliano AR, Harris R, Sedjo RL, Baldwin S, Roe D, Papenfuss MR, Abrahamsen M, Inserra P, Olvera S, Hatch K. Incidence, prevalence, and clearance of type-specific human papillomavirus infections: The Young Women's Health Study. *J Infect Dis* 2002; 186:462-469.

Giuliano AR, Papenfuss MR, Denman CA, de Zapien JG, Abrahamsen M, Hunter JB. Human papillomavirus prevalence at the USA-Mexico border among women 40 years of age and older. *Int J STD AIDS* 2005; 16:247-251.

Golijow CD, Abba MC, Mouron SA, Laguens RM, Dulout FN, Smith JS. *Chlamydia trachomatis* and Human papillomavirus infections in cervical disease in Argentine women. *Gynecol Oncol* 2005; 96:181-186.

Gomes JP, Bruno WJ, Borrego MJ, Dean D. Recombination in the genome of *Chlamydia trachomatis* involving the polymorphic membrane protein C gene relative to ompA and evidence for horizontal gene transfer. *J Bacteriol* 2004; 186:4295-4306.

Gomes JP, Hsia RC, Mead S, Borrego MJ, Dean D. Immunoreactivity and differential developmental expression of known and putative *Chlamydia trachomatis* membrane proteins for biologically variant serovars representing distinct disease groups. *Microbes Infect* 2005; 7:410-420.

Gopalkrishna V, Francis A, Sharma JK, Das BC. A simple and rapid method of high quantity DNA isolation from cervical scrapes for detection of human papillomavirus infection. *J Virol Methods* 1992; 36:63-72.

Gravitt PE, Burk RD, Lorincz A, Herrero R, Hildesheim A, Sherman ME, Bratti MC, Rodriguez AC, Helzlsouer KJ, Schiffman M. A comparison between real-time polymerase chain reaction and hybrid capture 2 for human papillomavirus DNA quantitation. *Cancer Epidemiol Biomarkers Prev* 2003a; 12:477-484.

Gravitt PE, Peyton C, Wheeler C, Apple R, Higuchi R, Shah KV. Reproducibility of HPV 16 and HPV 18 viral load quantitation using TaqMan real-time PCR assays. *J Virol Methods* 2003b; 112:23-33.

Gravitt PE, Peyton CL, Alessi TQ, Wheeler CM, Coutlee F, Hildesheim A, Schiffman MH, Scott DR, Apple RJ. Improved amplification of genital human papillomaviruses. *J Clin Microbiol* 2000; 38:357-361.

Gravitt PE, Peyton CL, Apple RJ, Wheeler CM. Genotyping of 27 human papillomavirus types by using L1 consensus PCR products by a single-hybridization, reverse line blot detection method. *J Clin Microbiol* 1998; 36:3020-3027.

Gregory WW, Gardner M, Byrne GI, Moulder JW. Arrays of hemispheric surface projections on *Chlamydia psittaci* and *Chlamydia trachomatis* observed by scanning electron microscopy. *J Bacteriol* 1979; **138**:241-244.

Griffais R, Thibon M. Detection of *Chlamydia trachomatis* by the polymerase chain reaction. *Res Microbiol* 1989; **140**:139-141.

Gupta PK, Lee EF, Erozan YS, Frost JK, Geddes ST, Donovan PA. Cytologic investigations in *Chlamydia* infection. *Acta Cytol* 1979; **23**:315-320.

Habis AH, Vernon SD, Lee DR, Verma M, Unger ER. Molecular quality of exfoliated cervical cells: implications for molecular epidemiology and biomarker discovery. *Cancer Epidemiol Biomarkers Prev* 2004; **13**:492-496.

Hakama M, Lehtinen M, Knekt P, Aromaa A, Leinikki P, Miettinen A, Paavonen J, Peto R, Teppo L. Serum antibodies and subsequent cervical neoplasms: a prospective study with 12 years of follow-up. *Am J Epidemiol* 1993; **137**:166-170.

Hall S, Lorincz A, Shah F, Sherman ME, Abbas F, Paull G, Kurman RJ, Shah KV. Human papillomavirus DNA detection in cervical specimens by hybrid capture: correlation with cytologic and histologic diagnoses of squamous intraepithelial lesions of the cervix. *Gynecol Oncol* 1996; **62**:353-359.

Hammerschlag MR. The intracellular life of chlamydiae. *Semin Pediatr Infect Dis* 2002; **13**:239-248.

Harris DI. Implementation of *Chlamydia* screening in a general practice setting: a 6-month pilot study. *J Fam Plann Reprod Health Care* 2005; **31**:254-5.

Harris TG, Kulasingam SL, Kiviat NB, Mao C, Agoff SN, Feng Q, Koutsky LA. Cigarette smoking, oncogenic human papillomavirus, Ki-67 antigen, and cervical intraepithelial neoplasia. *Am J Epidemio* 2004; **159**:834-842.

Hart KW, Williams OM, Thelwell N, Fiander AN, Brown T, Borysiewicz LK, Gelder CM. Novel method for detection, typing, and quantification of human papillomaviruses in clinical samples. *J Clin Microbiol* 2001; **39**:3204-3212.

Hatch TP. Disulfide cross-linked envelope proteins: the functional equivalent of peptidoglycan in chlamydiae? *J Bacteriol* 1996; **178**:1-5.

Haws AL, Woeber S, Gomez M, Garza N, Gomez Y, Rady P, He Q, Zhang L, Grady JJ, McCormick JB, Fisher-Hoch SP, Tyring SK. Human papillomavirus infection and P53 codon 72 genotypes in a Hispanic population at high-risk for cervical cancer. *J Med Virol* 2005; **77**:265-272.

Healey SM, Aronson KJ, Mao Y, Schlecht NF, Mery LS, Ferenczy A, Franco EL. Oncogenic human papillomavirus infection and cervical lesions in aboriginal women of Nunavut, Canada. *Sex Transm Dis* 2001; **28**:694-700.

Helmerhorst TJ, Meijer CJ. Cervical cancer should be considered as a rare complication of oncogenic HPV infection rather than a STD. *Int J Gynecol Cancer* 2002; **12**:235-236.

Herbert A, Smith JA. Cervical intraepithelial neoplasia grade III (CIN III) and invasive cervical carcinoma: the yawning gap revisited and the treatment of risk. *Cytopathology* 1999; **10**:161-170.

Herrero R, Castle PE, Schiffman M, Bratti MC, Hildesheim A, Morales J, Alfaro M, Sherman ME, Wacholder S, Chen S, Rodriguez AC, Burk RD. Epidemiologic profile of type-specific human papillomavirus infection and cervical neoplasia in Guanacaste, Costa Rica. *J Infect Dis* 2005; **191**:1796-1807.

Herrero R, Hildesheim A, Bratti C, Sherman ME, Hutchinson M, Morales J, Balmaceda I, Greenberg MD, Alfaro M, Burk RD, Wacholder S, Plummer M, Schiffman M. Population-based study of human papillomavirus infection and cervical neoplasia in rural Costa Rica. *J Natl Cancer Inst* 2000; **92**:464-474.

Herrington CS. Does HPV testing have a role in primary cervical screening? *Cytopathology* 2001; **12**:71-74.

Herrington CS. Human papillomavirus and cervical neoplasia. I. Classification, virology, pathology and epidemiology. *J Clin Pathol* 1994; **47**:1066-1072.

Hertogs K, de Bethune MP, Miller V, Ivens T, Schel P, Van Cauwenberge A, Van Den Eynde C, Van Gerwen V, Azijn H, Van Houtte M, Peeters F, Staszewski S, Conant M, Bloor S, Kemp S, Larder B, Pauwels R. A rapid method for simultaneous detection of phenotypic resistance to inhibitors of protease and reverse transcriptase in recombinant human immunodeficiency virus type 1 isolates from patients treated with antiretroviral drugs. *Antimicrob Agents Chemother* 1998; **42**:269-276.

Hildesheim A, Schiffman MH, Gravitt PE, Glass AG, Greer CE, Zhang T, Scott DR, Rush BB, Lawler P, Sherman ME. Persistence of type-specific human papillomavirus infection among cytologically normal women. *J Infect Dis* 1994; **169**:235-240.

Ho CM, Yang SS, Chien TY, Huang SH, Jeng CJ, Chang SF. Detection and quantitation of human papillomavirus type 16, 18 and 52 DNA in the peripheral blood of cervical cancer patients. *Gynecol Oncol* 2005 Aug 10; [Epub ahead of print].

Ho GY, Bierman R, Beardsley L, Chang CJ, Burk RD. Natural history of cervicovaginal papillomavirus infection in young women. *N Engl J Med* 1998; **338**:423-428.

Ho L, Terry G, Londesborough P, Cuzick J, Lorenzato F, Singer A. Human papillomavirus DNA detection in the management of women with twice mildly abnormal cytological smears. *J Med Virol* 2003; **69**:118-121.

Hoffmeyer S, Burk O, von Richter O, Arnold HP, Brockmoller J, Johne A, Cascorbi I, Gerloff T, Roots I, Eichelbaum M, Brinkmann U. Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity *in vivo*. *Proc Natl Acad Sci* 2000; **97**:3473-3478.

Hogan RJ, Mathews SA, Mukhopadhyay S, Summersgill JT, Timms P. Chlamydial persistence: beyond the biphasic paradigm. *Infect Immun* 2004; **72**:1843-1855.

Holland MJ, Bailey RL, Conway DJ, Culley F, Miranpuri G, Byrne GI, Whittle HC, Mabey DC. T helper type-1 (Th1)/Th2 profiles of peripheral blood mononuclear cells (PBMC); responses to antigens of *Chlamydia trachomatis* in subjects with severe trachomatous scarring. *Clin Exp Immunol* 1996; **105**:429-435.

Honey E, Augood C, Templeton A, Russell I, Paavonen J, Mardh PA, Stary A, Stray-Pedersen B. Cost effectiveness of screening for *Chlamydia trachomatis*: a review of published studies. *Sex Transm Infect* 2002; **78**:406-412.

Hopwood J, Mallinson H, Hodgson E, Hull L. Liquid based cytology: examination of its potential in a *Chlamydia* screening programme. *Sex Transm Infect* 2004; **80**:371-373.

Hording U, Daugaard S, Bock JE. Detection of human papillomavirus (HPV) DNA in cervical swabs by the polymerase chain reaction: an evaluation of the sensitivity of the method in patients with HPV 16-harboring cervical lesions. *Int J Gynecol Pathol* 1994; **13**:139-142.

HPSC. Health Protection Surveillance Centre, Dublin, Ireland. 2004. Annual Summary Report 2003: Sexually transmitted infections in Ireland 2003 p 30-38. Retrieved 22nd February 2005 from the World Wide Web: http://www.ndsc.ie/AboutHPSC/AnnualReports/

HPSC. Health Protection Surveillance Centre, Dublin, Ireland. 2005. Sexually Transmitted Infections 2004 Annual Summary report p 1-18.

Huang LW, Chao SL, Chen PH, Chou HP. Multiple HPV genotypes in cervical carcinomas: improved DNA detection and typing in archival tissues. *J Clin Virol* 2004; **29**:271-276.

Hudelist G, Manavi M, Pischinger KI, Watkins-Riedel T, Singer CF, Kubista E, Czerwenka KF. Physical state and expression of HPV DNA in benign and dysplastic cervical tissue: different levels of viral integration are correlated with lesion grade. *Gynecol Oncol* 2004; **92**:873-880.

Husnjak K, Grce M, Magdic L, Pavelic K. Comparison of five different polymerase chain reaction methods for detection of human papillomavirus in cervical cell specimens. *J Virol Methods* 2000; **88**:125-134.

Hutchinson ML, Isenstein LM, Goodman A, Hurley AA, Douglass KL, Mui KK, Patten FW, Zahniser DJ. Homogenous sampling accounts for the increased diagnostic accuracy using the ThinPrep Processor. *Am J Clin Pathol* 1994; **101**:121-122.

Hwang TS, Jeong JK, Park M, Han HS, Choi HK, Park TS. Detection and typing of HPV genotypes in various cervical lesions by HPV oligonucleotide microarray. *Gynecol Oncol* 2003; **90**:51-56.

ICSP. Irish Cervical Screening Programme. Frequently asked questions: Cervical cancer: How common is cancer of the cervix? Retrieved from the World Wide Web on 1st November 2005. http://www.icsp.ie/faqs/defaultarticle.asp?FID=98

Ikenberg H, Sauerbrei W, Schottmuller U, Spitz C, Pfleiderer A. Human papillomavirus DNA in cervical carcinoma--correlation with clinical data and influence on prognosis. *Int J Cancer* 1994; **59**:322-326.

Itzhaki RF, Wozniak MA, Appelt DM, Balin BJ. Infiltration of the brain by pathogens causes Alzheimer's disease. *Neurobiol Aging* 2004; **25**:619-627.

Jacobs MV, Snijders PJ, van den Brule AJ, Helmerhorst TJ, Meijer CJ, Walboomers JM. A general primer GP5+/GP6(+)-mediated PCR-enzyme immunoassay method for rapid detection of 14 high-risk and 6 low-risk human papillomavirus genotypes in cervical scrapings. *J Clin Microbiol* 1997; **35**:791-795.

Jacobs MV, Zielinski D, Meijer CJ, Pol RP, Voorhorst FJ, de Schipper FA, Runsink AP, Snijders PJ, Walboomers JM. Papanicolaou-stained cervical smears: application to cervical smears from cancer patients starting with cytologically normal smears. *Br J Cancer* 2000; **82**:1421-1426.

Jenkins D, Sherlaw-Johnson C, Gallivan S. Can papilloma virus testing be used to improve cervical cancer screening? *Int J Cancer* 1996; **65**:768-773.

Johnson T, Bryder K, Corbet S, Fomsgaard A. Routine genotyping of human papillomavirus samples in Denmark. *APMIS* 2003; **111**:398-404.

Jones CS, Maple PA, Andrews NJ, Paul ID, Caul EO. Measurement of IgG antibodies to *Chlamydia trachomatis* by commercial enzyme immunoassays and immunofluorescence in sera from pregnant women and patients with infertility, pelvic inflammatory disease, ectopic pregnancy, and laboratory diagnosed *Chlamydia psittaci*/Chlamydia pneumoniae infection. *J Clin Pathol* 2003; **56**:225-229.

Jones DL, Alani RM, Munger K. The human papillomavirus E7 oncoprotein can uncouple cellular differentiation and proliferation in human keratinocytes by abrogating p21Cip1-mediated inhibition of cdk2. *Genes Dev* 1997; **11**:2101-2111.

Josefsson A, Livak K, Gyllensten U. Detection and quantitation of human papillomavirus by using the fluorescent 5' exonuclease assay. *J Clin Microbiol* 1999; **37**:490-496.

Josefsson AM, Magnusson PK, Ylitalo N, Sorensen P, Qwarforth-Tubbin P, Andersen PK, Melbye M, Adami HO, Gyllensten UB. Viral load of human papilloma virus 16 as a determinant for development of cervical carcinoma *in situ*: a nested case-control study. *Lancet* 2000; **355**:2189-2193.

Joyce AG, Thyagarajan SP, Rajendran P, Hari R, Balakrishnan P, Jeyaseelan L, Kurien T; STD Study Group. *Chlamydia trachomatis* genital infection in apparently healthy adult population of Tamil Nadu, India: a population-based study. *Int J STD AIDS* 2004; **15**:51-55.

Kalantari M, Karlsen F, Johansson B, Sigurjonsson T, Warleby B, Hagmar B. Human papillomavirus findings in relation to cervical intraepithelial neoplasia grade: a study on 476 Stockholm women, using PCR for detection and typing of HPV. *Hum Pathol* 1997; **28**:899-904.

Kamwendo F, Forslin L, Bodin L, Danielsson D. Programmes to reduce pelvic inflammatory disease--the Swedish experience. *Lancet* 1998; **351**:25-28.

Karinen L, Pouta A, Hartikainen AL, Bloigu A, Paldanius M, Leinonen M, Saikku P, Jarvelin MR. Antibodies to *Chlamydia trachomatis* heat shock proteins Hsp60 and Hsp10 and subfertility in general population at age 31. *Am J Reprod Immunol* 2004; **52**:291-297.

Karlsen F, Kalantari M, Jenkins A, Pettersen E, Kristensen G, Holm R, Johansson B, Hagmar B. Use of multiple PCR primer sets for optimal detection of human papillomavirus. *J Clin Microbiol* 1996; **34**:2095-2100.

Kashanchi F, Araujo J, Doniger J, Muralidhar S, Hoch R, Khleif S, Mendelson E, Thompson J, Azumi N, Brady JN, Luppi M, Torelli G, Rosenthal LJ. Human herpesvirus 6 (HHV-6) ORF-1 transactivating gene exhibits malignant transforming activity and its protein binds to p53. *Oncogene* 1997; **14**:359-367.

Keegan H, Boland C, Malkin A, Griffin M, Ryan F, Lambkin H. Comparison of DNA extraction from cervical cells collected in PreservCyt solution for the amplification of *Chlamydia trachomatis*. *Cytopathology* 2005a; **16**:82-87.

Keegan H, Malkin A, Griffin M, Ryan F, Lambkin H. Validation of a multiplex PCR assay for the simultaneous detection of human papillomavirus and *Chlamydia trachomatis* in cervical PreservCyt samples. *Clin Chem* 2005b; **51**:1301-1302.

Khan MJ, Castle PE, Lorincz AT, Wacholder S, Sherman M, Scott DR, Rush BB, Glass AG, Schiffman M. The elevated 10-year risk of cervical precancer and cancer in women with human papillomavirus (HPV) type 16 or 18 and the possible utility of type-specific HPV testing in clinical practice. *J Natl Cancer Inst* 2005; **97**:1072-1079.

Kinney WK, Manos MM, Hurley LB, Ransley JE. Where's the high-grade cervical neoplasia? The importance of minimally abnormal Papanicolaou diagnoses. *Obstet Gynecol* 1998; **91**:973-976.

Kjaer SK, Chackerian B, van den Brule AJ, Svare EI, Paull G, Walbomers JM, Schiller JT, Bock JE, Sherman ME, Lowy DR, Meijer CL. High-risk human papillomavirus is sexually transmitted: evidence from a follow-up study of virgins starting sexual activity (intercourse). *Cancer Epidemiol Biomarkers Prev* 2001; **10**:101-106.

Kjaer SK, Svare EI, Worm AM, Walboomers JM, Meijer CJ, van den Brule AJ. Human papillomavirus infection in Danish female sex workers. Decreasing prevalence with age despite continuously high sexual activity. *Sex Transm Dis* 2000; **27**:438-445.

Klaes R, Friedrich T, Spitkovsky D, Ridder R, Rudy W, Petry U, Dallenbach-Hellweg G, Schmidt D, von Knebel Doeberitz M. Overexpression of p16(INK4A) as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. *Int J Cancer* 2001; **92**:276-284.

Klaes R, Woerner SM, Ridder R, Wentzensen N, Duerst M, Schneider A, Lotz B, Melsheimer P, von Knebel Doeberitz M. Detection of high-risk cervical intraepithelial neoplasia and cervical cancer by amplification of transcripts derived from integrated papillomavirus oncogenes. *Cancer Res* 1999; **59**:6132-6136.

Kleter B, van Doorn LJ, Schrauwen L, Molijn A, Sastrowijoto S, ter Schegget J, Lindeman J, ter Harmsel B, Burger M, Quint W. Development and clinical evaluation of a highly sensitive PCR-reverse hybridization line probe assay for detection and identification of anogenital human papillomavirus. *J Clin Microbiol* 1999; **37**:2508-2517.

Kleter B, van Doorn LJ, ter Schegget J, Schrauwen L, van Krimpen K, Burger M, ter Harmsel B, Quint W. Novel short-fragment PCR assay for highly sensitive borad-spectrum detection of anogenital human papillomaviruses. *Am J Pathol* 1998; **153**:1731-1739.

Koenig MG, Kosha SL, Doty BL, Heath DG. Direct comparison of the BD ProbeTec ET system with in-house LightCycler PCR assays for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* from clinical specimens. *J Clin Microbiol* 2004; **42**:5751-5756.

Kohl KS, Markowitz LE, Koumans EH. Developments in the screening for *Chlamydia trachomatis*: a review. *Obstet Gynecol Clin North Am* 2003; **30**:637-658.

Kornegay JR, Roger M, Davies PO, Shepard AP, Guerrero NA, Lloveras B, Evans D, Coutlee F. International proficiency study of a consensus L1 PCR assay for the detection and typing of human papillomavirus DNA: evaluation of accuracy and intralaboratory and interlaboratory agreement. *J Clin Microbiol* 2003; **41**:1080-1086.

Koskela P, Anttila T, Bjorge T, Brunsvig A, Dillner J, Hakama M, Hakulinen T, Jellum E, Lehtinen M, Lenner P, Luostarinen T, Pukkala E, Saikku P, Thoresen S, Youngman L, Paavonen J. *Chlamydia trachomatis* infection as a risk factor for invasive cervical cancer. *Int J Cancer* 2000; **85**:35-39.

Koumans EH, Black CM, Markowitz LE, Unger E, Pierce A, Sawyer MK, Papp JR. Comparison of methods for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* using commercially available nucleic acid amplification tests and a liquid pap smear medium. *J Clin Microbiol* 2003; **41**:1507-1511.

Kraus I, Molden T, Erno LE, Skomedal H, Karlsen F, Hagmar B. Human papillomavirus oncogenic expression in the dysplastic portio: an investigation of biopsies from 190 cervical cones. *Br J Cancer* 2004; **90**:1407-1413.

Krayenbuehl PA, Wiesli P, Maly FE, Vetter W, Schulthess G. Progression of peripheral arterial occlusive disease is associated with *Chlamydia pneumoniae* seropositivity and can be inhibited by antibiotic treatment. *Atherosclerosis* 2005; **179**:103-110.

Kucera E, Sliutz G, Czerwenka K, Breitenecker G, Leodolter S, Reinthaller A. Is high-risk human papillomavirus infection associated with cervical intraepithelial neoplasia eliminated after conization by large-loop excision of the transformation zone? *Eur J Obstet Gynecol Reprod Biol* 2001; **100**:72-76.

Kulasingam SL, Hughes JP, Kiviat NB, Mao C, Weiss NS, Kuypers JM, Koutsky LA. Evaluation of human papillomavirus testing in primary screening for cervical abnormalities: comparison of sensitivity, specificity, and frequency of referral. *JAMA* 2002; **288**:1749-1757.

Kulmala SM, Syrjanen S, Shabalova I, Petrovichev N, Kozachenko V, Podistov J, Ivanchenko O, Zakharenko S, Nerovjna R, Kljukina L, Branovskaja M, Grunberga V, Juschenko A, Tosi P, Santopietro R, Syrjanen K. Human papillomavirus testing with the hybrid capture 2 assay and PCR as screening tools. *J Clin Microbiol* 2004; **42**:2470-2475.

Kuo CC, Shor A, Campbell LA, Fukushi H, Patton DL, Grayston JT. Demonstration of *Chlamydia pneumoniae* in atherosclerotic lesions of coronary arteries. *J Infect Dis* 1993; **167**:841-849.

Kuypers JM, Critchlow CW, Gravitt PE, Vernon DA, Sayer JB, Manos MM, Kiviat NB. Comparison of dot filter hybridization, Southern transfer hybridization, and polymerase chain reaction amplification for diagnosis of anal human papillomavirus infection. *J Clin Microbiol* 1993; **31**:1003-1006.

Labarca C, Paigen K. A simple, rapid, and sensitive DNA assay procedure. *Anal Biochem* 1980; **102**:344-352.

La Montagne DS, Patrick LE, Fine DN, Marrazzo JM, on behalf of the Region X Infertility Prevention Project. Re-evaluating selective screening criteria for Chlamydial infection among women in the U S Pacific Northwest. *Sex Transm Dis* 2004; **31**:283-289.

La Montagne DS, Fenton KA, Pimenta JM, Catchpole M, Rogers PA, Randall S, Hewitt WG, Mallinson H, Underhill GS, McLean L, Gleave T, Harindra V, Ghosh AK, Tobin JM. Using *Chlamydia* positivity to estimate prevalence: evidence from the *Chlamydia* Screening Pilot in England. *Int J STD AIDS* 2005; **16**:323-327.

Lamarcq L, Deeds J, Ginzinger D, Perry J, Padmanabha S, Smith-McCune K. Measurements of human papillomavirus transcripts by real time quantitative reverse transcription-polymerase chain reaction in samples collected for cervical cancer screening. *J Mol Diagn* 2002; **4**:97-102.

Lanham S, Herbert A, Basarab A, Watt P. Detection of cervical infections in colposcopy clinic patients. *J Clin Microbiol* 2001; **39**:2946-2950.

Lawson HW, Lee NC, Thames SF, Henson R, Miller DS. Cervical cancer screening among low-income women: results of a national screening program, 1991-1995. *Obstet Gynecol* 1998; **92**:745-752.

Layfield LJ, Qureshi MN. HPV DNA testing in the triage of atypical squamous cells of undetermined significance (ASCUS): cost comparison of two methods. *Diagn Cytopathol* 2005; **33**:138-143

Lee KJ, Lee JK, Saw HS. Can human papillomavirus DNA testing substitute for cytology in the detection of high-grade cervical lesions? *Arch Pathol Lab Med* 2004; **128**:298-302.

Lee SA, Kang D, Seo SS, Jeong JK, Yoo KY, Jeon YT, Kim JW, Park NH, Kang SB, Lee HP, Song YS. Multiple HPV infection in cervical cancer screened by HPVDNAChip. *Cancer Lett* 2003; **198**:187-192.

Lehmann M, Groh A, Rodel J, Nindl I, Straube E. Detection of *Chlamydia trachomatis* DNA in cervical samples with regard to infection by human papillomavirus. *J Infect* 1999; **38**:12-27.

Lentrichia BB, Hecht SS, Lapen D, Corkill MK. Potential for routine concurrent determination of *Chlamydia* and cervical abnormalities by single fluid-based sampling. *Prim Care Update Ob Gyns* 1998; 5:149-150.

Levidiotou S, Vrioni G, Papadogeorgaki H, Avdeliodi K, Kada H, Kaparos G, Kouskouni E, Fragouli E, Legakis NJ. *Chlamydia trachomatis* infections in Greece: first prevalence study using nucleic acid amplification tests. *Eur J Clin Microbiol Infect Dis* 2005; 24:207-213.

Lillo FB, Lodini S, Ferrari D, Stayton C, Taccagni G, Galli L, Lazzarin A, Uberti-Foppa C. Determination of human papillomavirus (HPV) load and type in high-grade cervical lesions surgically resected from HIV-infected women during follow-up of HPV infection. *Clin Infect Dis* 2005; **40**:451-457.

Lo KW, Wong YF, Chan MK, Li JC, Poon JS, Wang VW, Zhu SN, Zhang TM, He ZG, Wu QL, Li GD, Tam JS, Kahn T, Lam P, Cheung TH, Chung TK. Prevalence of human papillomavirus in cervical cancer: a multicenter study in China. *Int J Cancer* 2002; **100**:327-331.

Lo KW, Yeung SW, Cheung TH, Siu NS, Kahn T, Wong YF. Quantitative analysis of human papillomavirus type 16 in cervical neoplasm: a study in Chinese population. *J Clin Virol* 2005; **34**:76-80.

Loeffelholz MJ, Jirsa SJ, Teske RK, Woods JN. Effect of endocervical specimen adequacy on ligase chain reaction detection of *Chlamydia trachomatis*. *J Clin Microbiol* 2001; **39**:3838-3841.

Loeffelholz MJ, Lewinski CA, Silver SR, Purohit AP, Herman SA, Buonagurio DA, Dragon EA. Detection of *Chlamydia trachomatis* in endocervical specimens by polymerase chain reaction. *J Clin Microbiol* 1992; **30**:2847-2851.

Lorincz AT, Castle PE, Sherman ME, Scott DR, Glass AG, Wacholder S, Rush BB, Gravitt PE, Schussler JE, Schiffman M. Viral load of human papillomavirus and risk of CIN3 or cervical cancer. *Lancet* 2002; **360**:228-229.

Lorincz AT. Hybrid Capture method for detection of human papillomavirus DNA in clinical specimens: a tool for clinical management of equivocal Pap smears and for population screening. *J Obstet Gynaecol Res* 1996; **22**:629-636.

Lou YK, Qin H, Molodysky E, Morris BJ. Simple microwave and thermal cycler boiling methods for preparation of cervicovaginal lavage cell samples prior to PCR for human papillomavirus detection. *J Virol Methods* 1993; **44**:77-81.

Lukaszuk K, Liss J, Wozniak I, Emerich J, Wojcikowski C. Human papillomavirus type 16 status in cervical carcinoma cell DNA assayed by multiplex PCR. *J Clin Microbiol* 2003; **41**:608-612.

Mabey D, Peeling RW. Lymphogranuloma venereum. *Sex Transm Infect* 2002; **78**:90-92.

Madico G, Quinn TC, Boman J, Gaydos CA. Touchdown enzyme time release-PCR for detection and identification of *Chlamydia trachomatis*, *C. pneumoniae*, and *C. psittaci* using the 16S and 16S-23S spacer rRNA genes. *J Clin Microbiol* 2000; **38**:1085-1093.

Magnusson PK, Lichtenstein P, Gyllensten UB. Heritability of cervical tumours. *Int J Cancer* 2000; **88**:698-701.

Mahony JB, Luinstra KE, Sellors JW, Chernesky MA. Comparison of plasmid- and chromosome-based polymerase chain reaction assays for detecting *Chlamydia trachomatis* nucleic acids. *J Clin Microbiol* 1993; **31**:1753-1758.

Mahony JB, Song X, Chong S, Faught M, Salonga T, Kapala J. Evaluation of the NucliSens Basic Kit for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in genital tract specimens using nucleic acid sequence-based amplification of 16S rRNA. *J Clin Microbiol* 2001; **39**:1429-1435.

Mahony JB. Multiplex polymerase chain reaction for the diagnosis of sexually transmitted diseases. *Clin Lab Med* 1996; **16**:61-71.

Malle D, Pateinakis P, Chakka E, Destouni C. Experience with a thin-layer, liquid based cervical cytologic screening method. *Acta Cytol* 2003; **47**:129-134.

Manos MM, Ting Y, Wright DK, Lewis AJ, Broker TJ, Wolinsky SM. Use of PCR amplification for the detection of genital HPV. Cancer Cells 1989; **7**:209-214.

Matos A, Moutinho J, Pinto D, Medeiros R. The influence of smoking and other cofactors on the time to onset to cervical cancer in a southern European population. *Eur J Cancer Prev* 2005; **14**:485-491.

Matos E, Loria D, Amestoy GM, Herrera L, Prince MA, Moreno J, Krunfly C, van den Brule AJ, Meijer CJ, Munoz N, Herrero R; Proyecto Concordia Collaborative Group. Prevalence of human papillomavirus infection among women in Concordia, Argentina: a population-based study. *Sex Transm Dis* 2003; **30**:593-599.

Matsukura T, Koi S, Sugase M. Both episomal and integrated forms of human papillomavirus type 16 are involved in invasive cervical cancers. *Virology* 1989; **171**:63-72.

Matsukura T, Sugase M. Molecular cloning of a novel human papillomarvirus (type 58) from an invasive cervical carcinoma. *Virology* 1990; **177**:833-836.

Mazur S, Feunteun J, de La Roche Saint Andre C. Episomal amplification or chromosomal integration of the viral genome: alternative pathways in hamster polyomavirus-induced lymphomas. *J Virol* 1995; **69**:3059-3066.

McGoogan E. The cytopathology lecture: Morphology: Comparison of liquid based verses conventional smears. Institute of Biomedical Science Annual Congress 25th-27th September 2001, Birmingham. Retrieved from the World Wide Web.
http://www.ibms.org/index.cfm?method=science.cellular_pathology&subpage=cellular_pathology_morphology

McCormack WM, Alpert S, McComb DE, Nichols RL, Semine DZ, Zinner SH. Fifteen-month follow-up study of women infected with *Chlamydia trachomatis*. *N Engl J Med* 1979; **300**:123-125.

McIntyre-Seltman K, Castle PE, Guido R, Schiffman M, Wheeler CM; ALTS Group. Smoking is a risk factor for cervical intraepithelial neoplasia grade 3 among oncogenic human papillomavirus DNA-positive women with equivocal or mildly abnormal cytology. *Cancer Epidemiol Biomarkers Prev* 2005; **14**:1165-1170.

Melchers W, van den Brule A, Walboomers J, de Bruin M, Burger M, Herbrink P, Meijer C, Lindeman J, Quint W. Increased detection rate of human papillomavirus in cervical scrapes by the polymerase chain reaction as compared to modified FISH and southern-blot analysis. *J Med Virol* 1989; **27**:329-335.

Melchers WJ, Bakkers JM, Wang J, de Wilde PC, Boonstra H, Quint WG, Hanselaar AG. Short fragment polymerase chain reaction reverse hybridization line probe assay to detect and genotype a broad spectrum of human papillomavirus types. Clinical evaluation and follow-up. *Am J Pathol* 1999; **155**:1473-1478.

Metters JS. Department of Health, United Kingdom. 2001, September. *Chlamydia trachomatis*: Summary and conclusions of CMO's Expert Advisory Group on *Chlamydia trachomatis*. Retrieved 27th September 2004 from the World Wide Web.
http://www.dh.gov.uk/PolicyAndGuidance/HealthAndSocialCareTopics/Chlamydia

Mgone CS, Lupiwa T, Yeka W. High prevalence of *Neisseria gonorrhoeae* and multiple sexually transmitted diseases among rural women in the Eastern Highlands Province of Papua New Guinea, detected by polymerase chain reaction. *Sex Transm Dis* 2002; **29**:775-779.

Millman K, Black CM, Johnson RE, Stamm WE, Jones RB, Hook EW, Martin DH, Bolan G, Tavare S, Dean D. Population-based genetic and evolutionary analysis of *Chlamydia trachomatis* urogenital strain variation in the United States. *J Bacteriol* 2004; **186**:2457-2465.

Mittal A. Serovar distribution of *Chlamydia trachomatis* isolates collected from the cervix: use of the polymerase chain reaction and restriction endonuclease digestion. *Br J Biomed Sci* 1998; **55**:179-183.

Moberg M, Gustavsson I, Wilander E, Gyllensten U. High viral loads of human papillomavirus predict risk of invasive cervical carcinoma. *Br J Cancer* 2005; **92**:891-894.

Modarress KJ, Cullen AP, Jaffurs WJ Sr, Troutman GL, Mousavi N, Hubbard RA, Henderson S, Lorincz AT. Detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in swab specimens by the Hybrid Capture II and PACE 2 nucleic acid probe tests. *Sex Transm Dis* 1999; **26**:303-308.

Mohammed NR, McQuoid G, Hillary IB, Verling WH. Incidence of *Chlamydia trachomatis* infection in patients attending a Dublin venereal disease clinic. *Ir Med J* 1984; **77**:102-104.

Molano M, Meijer CJ, Morre SA, Pol R, van den Brule AJ. Combination of PCR targeting the VD2 of omp1 and reverse line blot analysis for typing of urogenital *Chlamydia trachomatis* serovars in cervical scrape specimens. *J Clin Microbiol* 2004; **42**:2935-2939.

Molano M, Meijer CJ, Weiderpass E, Arslan A, Posso H, Franceschi S, Ronderos M, Munoz N, van den Brule AJ. The natural course of *Chlamydia trachomatis* infection in asymptomatic Colombian women: a 5-year follow-up study. *J Infect Dis* 2005; **191**:907-916.

Monnier-Benoit S, Dalstein V, Riethmuller D, Lalaoui N, Mougin C, Pretet JL. Dynamics of HPV16 DNA load reflect the natural history of cervical HPV-associated lesions. *J Clin Virol 2005* Oct 5; [Epub ahead of print].

Monsonego J, Autillo-Touati A, Bergeron C, Dachez R, Liaras J, Saurel J, Zerat L, Chatelain P, Mottot C. Liquid-based cytology for primary cervical cancer screening: a multi-centre study. *Br J Cancer* 2001; **84**:360-366.

Monsonego J, Bohbot JM, Pollini G, Krawec C, Vincent C, Merignargues I, Haroun F, Sednaoui P, Monfort L, Dachez R, Syrjanen K. Performance of the Roche AMPLICOR human papillomavirus (HPV) test in prediction of cervical intraepithelial neoplasia (CIN) in women with abnormal PAP smear. *Gynecol Oncol* 2005; **99**:160-168.

Moreno V, Bosch FX, Munoz N, Meijer CJ, Shah KV, Walboomers JM, Herrero R, Franceschi S, International Agency for Research on Cancer. Multicentric Cervical Cancer Study Group. Effect of oral contraceptives on risk of cervical cancer in women with human papillomavirus infection: the IARC multicentric case-control study. *Lancet* 2002; **359**:1085-1092.

Morrison EA, Goldberg GL, Kadish AS, Burk RD. Polymerase chain reaction detection of human papillomavirus: quantitation may improve clinical utility. *J Clin Microbiol* 1992; **30**:2539-2543.

Moscicki AB. Cervical cytology screening in teens. *Curr Womens Health Rep* 2003; **3**:433-437.

Mouton JW, Peeters MF, van Rijssort-Vos JH, Verkooyen RP. Tubal factor pathology caused by *Chlamydia trachomatis*: the role of serology. *Int J STD AIDS* 2002; **13**:26-29.

Munger K, Phelps WC, Bubb V, Howley PM, Schlegel R. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *J Virol* 1989; **63**:4417-4421.

Munoz N, Bosch FX, Castellsague X, Diaz M, de Sanjose S, Hammouda D, Shah KV, Meijer CJ. Against which human papillomavirus types shall we vaccinate and screen? The international perspective. *Int J Cancer* 2004; **111**:278-285.

Munoz N, Bosch FX, de Sanjose S, Herrero R, Castellsague X, Shah KV, Snijders PJ, Meijer CJ. International Agency for Research on Cancer Multicenter Cervical Cancer Study Group. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med* 2003; **348**:518-527.

Munoz N, Bosch FX. The causal link between HPV and cervical cancer and its implications for prevention of cervical cancer. *Bull Pan Am Health Organ* 1996; **30**:362-377.

Munoz N. Human papillomavirus and cancer: the epidemiological evidence. *J Clin Virol* 2000; **19**:1-5.

Murphy N, Ring M, Heffron CC, King B, Killalea AG, Hughes C, Martin CM, McGuinness E, Sheils O, O'Leary JJ. p16INK4A, CDC6, and MCM5: predictive biomarkers in cervical preinvasive neoplasia and cervical cancer. *J Clin Pathol* 2005; **58**:525-534.

Myziuk L, Romanowski B, Brown M. Endocervical Gram stain smears and their usefulness in the diagnosis of *Chlamydia trachomatis*. *Sex Transm Infect* 2001; **77**:103-106.

Nagao S, Yoshinouchi M, Miyagi Y, Hongo A, Kodama J, Itoh S, Kudo T. Rapid and sensitive detection of physical status of human papillomavirus type 16 DNA by quantitative real-time PCR. *J Clin Microbiol* 2002; **40**:863-867.

NDSC. National Disease Surveillance Centre, Dublin, Ireland. 2002. Annual Summary Report 2001: Sexually transmitted infections in Ireland 2001 p 28-33. Retrieved from the World Wide Web: 20th July 2005.

Nelson HD, Helfand M. Screening for chlamydial infection. *Am J Prev Med* 2001; **20**:95-107.

Ness RB, Brunham RC, Shen C, Bass DC; PID Evaluation Clinical Health (PEACH) Study Investigators. Associations among human leukocyte antigen (HLA) class II DQ variants, bacterial sexually transmitted diseases, endometritis, and fertility among women with clinical pelvic inflammatory disease. *Sex Transm Dis* 2004; **31**:301-304.

Niederhauser C, Kaempf L. Improved sensitivity of the *Chlamydia trachomatis* Cobas Amplicor assay using an optimized procedure for preparation of specimens. *Eur J Clin Microbiol Infect Dis* 2003; **22**:118-121.

Nieuwenhuis RF, Ossewaarde JM, Gotz HM, Dees J, Thio HB, Thomeer MG, den Hollander JC, Neumann MH, van der Meijden WI. Resurgence of lymphogranuloma venereum in Western Europe: an outbreak of *Chlamydia trachomatis* serovar L2 proctitis in The Netherlands among men who have sex with men. *Clin Infect Dis* 2004; **39**:996-1003.

Nindl I, Greinke C, Zahm DM, Stockfleth E, Hoyer H, Schneider A. Human papillomavirus distribution in cervical tissues of different morphology as determined by hybrid capture assay and PCR. *Int J Gynecol Pathol* 1997; **16**:197-204.

Nindl I, Lorincz A, Mielzynska I, Petry U, Baur S, Kirchmayr R, Michels W, Schneider A. Human papillomavirus detection in cervical intraepithelial neoplasia by the second-generation hybrid capture microplate test, comparing two different cervical specimen collection methods. *Clin Diagn Virol* 1998; **10**:49-56.

Nobbenhuis MA, Helemerhorst TJ, van den Brule AJ, Rozendaal L, Voorhorst FJ, Bezemer PD, Verheijen RH, Meijer CJ. Cytological regression and clearance of high-risk human papillomavirus in women with an abnormal cervical smear. *Lancet* 2001; **358**:1782-1783.

Nobbenhuis MA, Walboomers JM, Helmerhorst TJ, Rozendaal L, Remmink AJ, Risse EK, van der Linden HC, Voorhorst FJ, Kenemans P, Meijer CJ. Relation of human papillomavirus status to cervical lesions and consequences for cervical-cancer screening: a prospective study. *Lancet* 1999; **354**:20-25

Nonogaki S, Wakamatsu A, Longatto Filho A, Pereira SM, Utagawa ML, Ferreira Alves VA, Di Loreto C, Sakamoto Maeda MY, Lima TP, Roteli-Martins C, Syrjanen K. Hybrid capture II and polymerase chain reaction for identifying HPV infections in samples collected in a new collection medium: a comparison. *Acta Cytol* 2004; **48**:514-520.

O'Leary JJ, Landers RJ, Crowley M, Healy I, Kealy WF, Hogan J, Cullinane C, Kelehan P, Doyle CT. Genotypic mapping of HPV and assessment of EBV prevalence in endocervical lesions. *J Clin Pathol* 1997; **50**:904-910.

Osterlund A, Persson T, Persson I, Lysen M, Herrmann B. Improved contact tracing of *Chlamydia trachomatis* in a Swedish county--is genotyping worthwhile? *Int J STD AIDS* 2005; **16**:9-13.

Ozbun MA, Meyers C. Temporal usage of multiple promoters during the life cycle of human papillomavirus type 31b. *J Virol* 1998; **72**:2715-2722.

Paavonen J, Eggert-Kruse W. *Chlamydia trachomatis*: impact on human reproduction. *Hum Reprod Update* 1999; **5**:433-447.

Paavonen J, Karunakaran KP, Noguchi Y, Anttila T, Bloigu A, Dillner J, Hallmans G, Hakulinen T, Jellum E, Koskela P, Lehtinen M, Thoresen S, Lam H, Shen C, Brunham RC. Serum antibody response to the heat shock protein 60 of *Chlamydia trachomatis* in women with developing cervical cancer. *Am J Obstet Gynecol* 2003; **189**:1287-1292.

Paavonen J. Pelvic inflammatory disease. *Duodecim* 1985; **101**:1486-1495.

Paler RJ Jr, Simpson DR, Kaye AM, Gunn S, Felix JC. The relationship of inflammation in the papanicolaou smear to *Chlamydia trachomatis* infection in a high-risk population. *Contraception* 2000; **61**:231-234.

Palmer L, Falkow S. A common plasmid of *Chlamydia trachomatis*. *Plasmid* 1986; **16**:52-62.

Pannekoek Y, Westenberg SM, de Vries J, Repping S, Spanjaard L, Eijk PP, van der Ende A, Dankert J. PCR assessment of *Chlamydia trachomatis* infection of semen specimens processed for artificial insemination. *J Clin Microbiol* 2000; **38**:3763-3767.

Pasternack R, Vuorinen P, Miettinen A. Effect of urine specimen dilution on the performance of two commercial systems in the detection of *Chlamydia trachomatis* infection in men. *Eur J Clin Microbiol Infect Dis* 1998; **17**:676-678.

Patton DL, Sweeney YC, Bohannon NJ, Clark AM, Hughes JP, Cappuccio A, Campbell LA, Stamm WE. Effects of doxycycline and antiinflammatory agents on experimentally induced chlamydial upper genital tract infection in female macaques. *J Infect Dis* 1997; **175**:648-654.

Peipert JF. Clinical practice. Genital chlamydial infections. *N Engl J Med* 2003; **349**:2424-2430.

Peitsaro P, Johansson B, Syrjanen S. Integrated human papillomavirus type 16 is frequently found in cervical cancer precursors as demonstrated by a novel quantitative real-time PCR technique. *J Clin Microbiol* 2002; **40**:886-891.

Peto J, Gilham C, Fletcher O, Matthews FE. The cervical cancer epidemic that screening has prevented in the UK. Lancet 2004; **364**:249-56.

Peyton CL, Schiffman M, Lorincz AT, Hunt WC, Mielzynska I, Bratti C, Eaton S, Hildesheim A, Morera LA, Rodriguez AC, Herrero R, Sherman ME, Wheeler CM. Comparison of PCR- and hybrid capture-based human papillomavirus detection systems using multiple cervical specimen collection strategies. *J Clin Microbiol* 1998; **36**:3248-3254.

Pham TH, Nguyen TH, Herrero R, Vaccarella S, Smith JS, Nguyen Thuy TT, Nguyen HN, Nguyen BD, Ashley R, Snijders PJ, Meijer CJ, Munoz N, Parkin DM, Franceschi S. Human papillomavirus infection among women in South and North Vietnam. *Int J Cancer* 2003; **104**:213-220.

Pickett MA, Everson JS, Pead PJ, Clarke IN. The plasmids of *Chlamydia trachomatis* and *Chlamydophila pneumoniae* (N16): accurate determination of copy number and the paradoxical effect of plasmid-curing agents. *Microbiology* 2005; **151**:893-903.

Pimenta JM, Catchpole M, Rogers PA, Hopwood J, Randall S, Mallinson H, Perkins E, Jackson N, Carlisle C, Hewitt G, Underhill G, Gleave T, McLean L, Ghosh A, Tobin J, Harindra V. Opportunistic screening for genital chlamydial infection. II: prevalence among healthcare attenders, outcome, and evaluation of positive cases. *Sex Transm Infect* 2003; **79**:22-27.

Pirami L, Giache V, Becciolini A. Analysis of HPV16, 18, 31, and 35 DNA in pre-invasive and invasive lesions of the uterine cervix. *J Clin Pathol* 1997; **50**:600-604.

Poljak M, Marin IJ, Seme K,Vince A. Hybrid Capture II HPV Test detects at least 15 human papillomavirus genotypes not included in its current high-risk probe cocktail. *J Clin Virol* 2002; **25**:89-97.

Popescu NC, DiPaolo JA. Preferential sites for viral integration on mammalian genome. *Cancer Genet Cytogenet* 1989; **42**:157-171.

Powell J, O'Connor C, O'hIarlaithe M, De Freitas J. *Chlamydia trachomatis* prevalence in men in the mid-west of Ireland. *Sex Transm Infect* 2004; **80**:349-353.

Prado JC, Calleja-Macias IE, Bernard HU, Kalantari M, Macay SA, Allan B, Williamson AL, Chung LP, Collins RJ, Zuna RE, Dunn ST, Ortiz-Lopez R, Barrera-Saldana HA, Cubie HA, Cuschieri K, von Knebel-Doeberitz M, Sanchez GI, Bosch FX, Villa LL. Worldwide genomic diversity of the human papillomaviruses-53, 56, and 66, a group of high-risk HPVs unrelated to HPV-16 and HPV-18. *Virology* 2005; **340**:95-104.

Pretet JL, Dalstein V, Monnier-Benoit S, Delpeut S, Mougin C. High risk HPV load estimated by Hybrid Capture II correlates with HPV16 load measured by real-time PCR in cervical smears of HPV16-infected women. *J Clin Virol* 2004; **31**:140-147.

Prokopczyk B, Cox JE, Hoffman D, Waggoner SE. Identification of tobacco-specific carcinogen in the cervical mucus of smokers and nonsmokers. *J Natl Cancer Inst* 1997; **89**:868-873.

Punnonen R, Terho P, Nikkanen V, Meurman O. Chlamydial serology in infertile women by immunofluorescence. *Fertil Steril* 1979; **31**:656-659.

Quinn TC, Gupta PK, Burkman RT, Kappus EW, Barbacci M, Spence MR Detection of *Chlamydia trachomatis* cervical infection: a comparison of Papanicolaou and immunofluorescent staining with cell culture. *Am J Obstet Gynecol* 1987; **157**:394-399.

Rabelo-Santos SH, Levi JE, Derchain SF, Sarian LO, Zeferino LC, Messias S, Moraes DL, Campos EA, Syrjanen KJ. DNA recovery from Hybrid Capture II samples stored in specimen transport medium with denaturing reagent, for the detection of human papillomavirus by PCR. *J Virol Methods* 2005; **126**:197-201.

Rassu M, Bertoloni G, Mengoli C, Peron A, Benedetti P, Palu' G. HPV genotype prevalence in cervical specimens with abnormal cytology: a report from north-east Italy. *Scand J Infect Dis* 2005; **37**:476-481.

Raulston JE. Chlamydial envelope components and pathogen-host cell interactions. *Mol Microbiol* 1995; **15**:607-616.

Remmerbach TW, Brinckmann UG, Hemprich A, Chekol M, Kuhndel K, Liebert UG. PCR detection of human papillomavirus of the mucosa: comparison between MY09/11 and GP5+/6+ primer sets. *J Clin Virol* 2004; **30**:302-308.

Revzina NV, Diclemente RJ. Prevalence and incidence of human papillomavirus infection in women in the USA: a systematic review. *Int J STD AIDS* 2005; **16**:528-537.

Richardson H, Kelsall G, Tellier P, Voyer H, Abrahamowicz M, Ferenczy A, Coutlee F, Franco EL. The natural history of type-specific human papillomavirus infections in female university students. *Cancer Epidemiol Biomarkers Prev* 2003; **12**:485-490.

Riethmuller D, Gay C, Bertrand X, Bettinger D, Schaal JP, Carbillet JP, Lassabe C, Arveux P, Seilles E, Mougin C. Genital human papillomavirus infection among women recruited for routine cervical cancer screening or for colposcopy determined by Hybrid Capture II and polymerase chain reaction. *Diagn Mol Pathol* 1999; **8**:157-164.

Rintala MA, Grenman SE, Puranen MH, Isolauri E, Ekblad U, Kero PO, Syrjanen SM. Transmission of high-risk human papillomavirus (HPV) between parents and infant: a prospective study of HPV in families in Finland. *J Clin Microbiol* 2005; **43**:376-381.

Roberts JM, Gurley AM, Thurloe JK, Bowditch R, Laverty CR. Evaluation of the ThinPrep Pap test as an adjunct to the conventional Pap smear. *Med J Aust* 1997; **167**:466-469.

Roosendaal R, Walboomers JM, Veltman OR, Melgers I, Burger C, Bleker OP, MacClaren DM, Meijer CJ, van den Brule AJ. Comparison of different primer sets for detection of Chlamydia trachomatis by the polymerase chain reaction. *J Med Microbiol* 1993; **38**:426-433.

Rozendaal L, Westerga J, van der Linden JC, Walboomers JM, Voorhorst FJ, Risse EK, Boon ME, Meijer CJ. PCR based high risk HPV testing is superior to neural network based screening for predicting incident CIN III in women with normal cytology and borderline changes. *J Clin Pathol* 2000; **53**:606-611.

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Biotechnology* 1992; **24**:476-480.

Sailors J, Gander R, Saboorian MH, Berkley P, Foster B, Ashfaq R. Stability of PreservCyt for Hybrid Capture (HC II) HPV test. *Diagn Cytopathol* 2005; **32**:260-263.

Saito T, Ohnishi J, Mori Y, Iinuma Y, Ichiyama S, Kohi F. Infection by *Chlamydophilia avium* in an elderly couple working in a pet shop. *J Clin Microbiol* 2005; **43**:3011-3013.

Samoff E, Koumans EH, Markowitz LE, Sternberg M, Sawyer MK, Swan D, Papp JR, Black CM, Unger ER. Association of *Chlamydia trachomatis* with persistence of high-risk types of human papillomavirus in a cohort of female adolescents. *Am J Epidemiol* 2005; **162**:668-675.

Santos AL, Derchain SF, Martins MR, Sarian LO, Martinez EZ, Syrjanen KJ. Human papillomavirus viral load in predicting high-grade CIN in women with cervical smears showing only atypical squamous cells or low-grade squamous intraepithelial lesion. *Sao Paulo Med J* 2003; **121**:238-243.

Sapp M, Volpers C, Muller M, Streeck RE. Organization of the major and minor capsid proteins in human papillomavirus type 33 virus-like particles. *J Gen Virol* 1995; **76**:2407-2412.

Sato S, Maruta J, Konno R,Yajima A. *In situ* detection of HPV in a cervical smear with *in situ* hybridization. *Acta Cytol* 1998; **42**:1483-1485.

Schachter J, Dawson CR (Eds). Human Chlamydial Infections. PSG Publishing Littleton, Massachusetts. 1978; 56-59,157-158, 186.

Schachter J, Hill EC, King EB, Coleman VR, Jones P, Meyer KF. Chlamydial infection in women with cervical dysplasia. *Am J Obstet Gynecol* 1975; **123**:753-757.

Schachter J, Hook EW 3rd, McCormack WM, Quinn TC, Chernesky M, Chong S, Girdner JI, Dixon PB, DeMeo L, Williams E, Cullen A, Lorincz A. Ability of the digene hybrid capture II test to identify *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in cervical specimens. *J Clin Microbiol* 1999; **37**:3668-3671.

Schachter J, Meyer KF. Lymphogranuloma venereum. II. Characterization of some recently isolated strains. *J Bacteriol* 1969; **99**:636-638.

Schiffman MH. Recent progress in defining the epidemiology of human papillomavirus infections and cervical neoplasia. *J Natl Cancer Inst* 1992; **84**:394-398.

Schlott T, Eiffert H, Bohne W, Landgrebe J, Brunner E, Spielbauer B, Knight B. *Chlamydia trachomatis* modulates expression of tumor suppressor gene caveolin-1 and oncogene C-myc in the transformation zone of non-neoplastic cervical tissue. *Gynecol Oncol* 2005; **98**:409-419.

Schneider A, Hoyer H, Lotz B, Leistritza S, Kuhne-Heid R, Nindl I, Muller B, Haerting J, Durst M. Screening for high-grade cervical intra-epithelial neoplasia and cancer by testing for high-risk HPV, routine cytology or colposcopy. *Int J Cancer* 2000; **89**:529-534.

Sepp R, Szabo I, Uda H, Sakamoto H. Rapid techniques for DNA extraction from routinely processed archival tissue for use in PCR. *J Clin Pathol* 1994; **47**:318-323.

Serth J, Panitz F, Paeslack U, Kuczyk MA, Jonas U. Increased levels of human papillomavirus type 16 DNA in a subset of prostate cancers. *Cancer Res* 1999; **59**:823-825.

Shattock RM, Patrizio C, Simmonds P, Sutherland S. Detection of *Chlamydia trachomatis* in genital swabs: comparison of commercial and in house amplification methods with culture. Sex Transm Infect 1998; **74**:289-293.

Shen YH, Chen F, Huang MN, Liu B, Wang XX, Zhao FH, Li SM, Li N, Wu LY, Rong SD, Zhang WH, Ren SD, Huang RD, Qiao YL. Population-based study of human papillomavirus infection in high-risk area for cervical cancer in Shanxi Province, China. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao* 2003; **25**:381-385.

Sherman ME, Schiffman M, Cox JT; Atypical Squamous Cells of Undetermined Significance/Low-Grade Squamous Intraepithelial Lesion Triage Study Group. Effects of age and human papilloma viral load on colposcopy triage: data from the randomized Atypical Squamous Cells of Undetermined Significance/Low-Grade Squamous Intraepithelial Lesion Triage Study (ALTS). *J Natl Cancer Inst* 2002; **94**:102-107.

Sherman ME, Schiffman MH, Lorincz AT, Herrero R, Hutchinson ML, Bratti C, Zahniser D, Morales J, Hildesheim A, Helgesen K, Kelly D, Alfaro M, Mena F, Balmaceda I, Mango L, Greenberg M. Cervical specimens collected in liquid buffer are suitable for both cytologic screening and ancillary human papillomavirus testing. *Cancer* 1997; **81**:89-97.

Sherman ME, Wang SS, Wheeler CM, Rich L, Gravitt PE, Tarone R, Schiffman M. Determinants of human papillomavirus load among women with histological cervical intraepithelial neoplasia 3: dominant impact of surrounding low-grade lesions. *Cancer Epidemiol Biomarkers Prev* 2003; **12**:1038-1044.

Shields TS, Brinton LA, Burk RD, Wang SS, Weinstein SJ, Ziegler RG, Studentsov YY, McAdams M, Schiffman M. A case-control study of risk factors for invasive cervical cancer among U.S. women exposed to oncogenic types of human papillomavirus. *Cancer Epidemiol Biomarkers Prev* 2004; **13**:1574-1582.

Shrier LA, Dean D, Klein E, Harter K, Rice PA. Limitations of screening tests for the detection of *Chlamydia trachomatis* in asymptomatic adolescent and young adult women. *Am J Obstet Gynecol* 2004; **190**:654-662.

Silins I, Ryd W, Strand A, Wadell G, Tornberg S, Hansson BG, Wang X, Arnheim L, Dahl V, Bremell D, Persson K, Dillner J, Rylander E. *Chlamydia trachomatis* infection and persistence of human papillomavirus. *Int J Cancer* 2005; **116**:110-115.

Singh V, Rastogi S, Garg S, Kapur S, Kumar A, Salhan S, Mittal A. Polymerase chain reaction for detection of endocervical *Chlamydia trachomatis* infection in women attending a gynecology outpatient department in India. *Acta Cytol* 2002; **46**:540-544.

Singh V, Salhan S, Das BC, Mittal A. Predominance of *Chlamydia trachomatis* serovars associated with urogenital infections in females in New Delhi, India. *J Clin Microbiol* 2003; **41**:2700-2702.

Skyldberg BM, Murray E, Lambkin H, Kelehan P, Auer GU. Adenocarcinoma of the uterine cervix in Ireland and Sweden: human papillomavirus infection and biologic alterations. *Mod Pathol* 1999; **12**:675-682.

Smith JS, Bosetti C, Munoz N, Herrero R, Bosch FX, Eluf-Neto J, Meijer CJ, Van Den Brule AJ, Franceschi S, Peeling RW; IARC multicentric case control study. *Chlamydia trachomatis* and invasive cervical cancer: a pooled analysis of the IARC multicentre case-control study. *Int J Cancer* 2004; **111**:431-439.

Smith JW, Rogers RE, Katz BP, Brickler JF, Lineback PL, Van der Pol B, Jones RB. Diagnosis of chlamydial infection in women attending antenatal and gynecologic clinics. *J Clin Microbiol* 1987; **25**:868-872.

Solinas-Toldo S, Durst M, Lichter P. Specific chromosomal imbalances in human papillomavirus-transfected cells during progression toward immortality. *Proc Natl Acad Sci USA* 1997; **94**:3854-3859.

Soloman D, Schiffman M, Tarone R; ALTS Study group. Comparison of three management strategies for patients with atypical squamous cells of undetermined significance: baseline results from a randomized trial. *J Natl Cancer Inst* 2001; **93**:293-299.

Solomon AW, Holland MJ, Burton MJ, West SK, Alexander ND, Aguirre A, Massae PA, Mkocha H, Munoz B, Johnson GJ, Peeling RW, Bailey RL, Foster A, Mabey DC. Strategies for control of trachoma: observational study with quantitative PCR. *Lancet* 2003; **362**:198-204.

Sotlar K, Diemer D, Dethleffs A, Hack Y, Stubner A, Vollmer N, Menton S, Menton M, Dietz K, Wallwiener D, Kandolf R, Bultmann B. Detection and typing of human papillomavirus by e6 nested multiplex PCR. *J Clin Microbiol* 2004; **42**:3176-3184.

Southern SA, Herrington CS. Disruption of cell cycle control by human papillomaviruses with special reference to cervical carcinoma. *Int J Gynecol Cancer* 2000; **10**:263-274.

Southern SA, Herrington CS. Molecular events in uterine cervical cancer. *Sex Transm Infect* 1998; **74**:101-109.

Spaargaren J, Fennema HS, Morre SA, de Vries HJ, Coutinho RA. New lymphogranuloma venereum *Chlamydia trachomatis* variant, Amsterdam. *Emerg Infect Dis* 2005; **11**:1090-1092.

Speich N, Schmitt C, Bollmann R, Bollmann M. Human papillomavirus (HPV) study of 2916 cytological samples by PCR and DNA sequencing: genotype spectrum of patients from the west German area. *J Med Microbiol* 2004; **53**:125-128.

Spitzer M. Cervical screening adjuncts: recent advances. *Am J Obstet Gynecol* 1998; **179**:544-556.

Stamm WE, Wagner KF, Amsel R, Alexander ER, Turck M, Counts GW, Holmes KK. Causes of the acute urethral syndrome in women. *N Engl J Med* 1980; **303**:409-415.

Stephens RS. Chlamydial evolution:a billion years and counting. *In* Chlamydial Infections. Proceedings of the Tenth International Symposium on Human Chlamydial Infections: 16-21 June 2002. Ed: Schachter J, Chrstiansen G, Clarke IN, Hammerschlag MR, Kaltenbock B, Kuo C_C, Rank GL, Ridgeway GL, Saikku P, Stamm WE, International *Chlamydia* Symposium, Antalya, Turkey; 2002:3-16.

Stoler MH, Rhodes CR,Whitbeck A, Wolinsky SM, Chow LT, Broker TR. Human papillomavirus type 16 and 18 gene expression in cervical neoplasias. *Hum Pathol* 1992; **23**:117-128.

Straight SW, Herman B, McCance DJ. The E5 oncoprotein of human papillomavirus type 16 inhibits the acidification of endosomes in human keratinocytes. *J Virol* 1995; **69**:3185-3192.

Strauss S, Jordens JZ, Desselberger U,Gray JJ. Single-tube real-time nested polymerase chain reaction for detecting human papillomavirus DNA. *Diagn Mol Pathol* 2000; **9**:151-157.

Sun CA, Liu JF, Wu DM, Nieh S, Yu CP, Chu TY. Viral load of high-risk human papillomavirus in cervical squamous intraepithelial lesions. *Int J Gynaecol Obstet* 2002; **76**:41-47.

Sun XW, Ferenczy A, Johnson D, Koulos JP, Lungu O, Richart RM, Wright TC Jr. Evaluation of the Hybrid Capture human papillomavirus deoxyribonucleic acid detection test. *Am J Obstet Gynecol* 1995; **173**:1432-1437.

Swain GR, McDonald RA, Pfister JR, Gradus MS, Sedmak GV, Singh A Decision Analysis: Point-of-care *Chlamydia* Testing vs. Laboratory-based Methods. *Clin Med Res* 2004; **46**:540-544.

Swan DC, Tucker RA, Tortolero-Luna G, Mitchell MF, Wideroff L, Unger ER, Nisenbaum RA, Reeves WC, Icenogle JP. Human papillomavirus (HPV) DNA copy number is dependent on grade of cervical disease and HPV type. *J Clin Microbiol* 1999; **37**:1030-1034.

Sweet D, Lorente M, Valenzuela A, Lorente JA, Alvarez JC. Increasing DNA extraction yield from saliva stains with a modified Chelex method. *Forensic Sci Int* 1996; **83**:167-177.

Syrjanen S. Human papillomavirus (HPV) in head and neck cancer. *J Clin Virol* 2005; **32**:59-66.

Szoke K, Sapy T, Krasznai Z, Hernadi Z, Szladek G, Veress G, Dillner J, Gergely L, Konya J. Moderate variation of the oncogenic potential among high-risk human papillomavirus types in gynecologic patients with cervical abnormalities. *J Med Virol* 2003; **71**:585-592.

Szuhai K, Sandhaus E, Kolkman-Uljee SM, Lemaitre M, Truffert JC, Dirks RW, Tanke HJ, Fleuren GJ, Schuuring E, Raap AK. A novel strategy for human papillomavirus detection and genotyping with SybrGreen and molecular beacon polymerase chain reaction. *Am J Pathol* 2001; **159**:1651-1660.

Tabora N, Zelaya A, Bakkers J, Melchers WJ, Ferrera A. *Chlamydia trachomatis* and genital human papillomavirus infections in female university students in Honduras. *Am J Trop Med Hyg* 2005; **73**:50-53.

Tabrizi SN, Stevens M, Chen S, Rudland E, Kornegay JR, Garland SM. Evaluation of a modified reverse line blot assay for detection and typing of human papillomavirus. *Am J Clin Pathol* 2005; **123**:896-899.

Tarkowski TA, Rajeevan MS, Lee DR, Unger ER. Improved detection of viral RNA isolated from liquid-based cytology samples. *Mol Diagn* 2001; **6**:125-130.

Tayal SC, Robertson A, Doxford L. An audit of *Chlamydia trachomatis* screening in colposcopy--Hartlepool experience. *Int J STD AIDS* 2005; **16**:500-501.

Thomas JO, Herrero R, Omigbodun AA, Ojemakinde K, Ajayi IO, Fawole A, Oladepo O, Smith JS, Arslan A, Munoz N, Snijders PJ, Meijer CJ, Franceschi S. Prevalence of papillomavirus infection in women in Ibadan, Nigeria: a population-based study. *Br J Cancer* 2004; **90**:638-645.

Thomas M, Banks L. Human papillomavirus (HPV) E6 interactions with Bak are conserved amongst E6 proteins from high and low risk HPV types. *J Gen Virol* 1999; **80**:1513-1517.

Thomas M, Pim D, Banks L. The role of the E6-p53 interaction in the molecular pathogenesis of HPV. *Oncogene* 1999; **18**:7690-7700.

Tibbs RF, Wong JY, Logrono R. Enhancing recovery of endocervical component on gynecologic cytology specimens processed by thin-layer technology. *Acta Cytol* 2003; **47**:172-176.

Tieben LM, ter Schegget J, Minnaar RP, Bouwes Bavinck JN, Berkhout RJ, Vermeer BJ, Jebbink MF, Smits HL. Detection of cutaneous and genital HPV types in clinical samples by PCR using consensus primers. *J Virol Methods* 1993; **42**:265-279.

Tondella ML, Talkington DF, Holloway BP, Dowell SF, Cowley K, Soriano-Gabarro M, Elkind MS, Fields BS. Development and evaluation of real-time PCR-based fluorescence assays for detection of *Chlamydia pneumoniae*. *J Clin Microbiol* 2002; **40**:575-583.

Torrisi A, Del Mistro A, Onnis GL, Merlin F, Bertorelle R, Minucci D. Colposcopy, cytology and HPV-DNA testing in HIV-positive and HIV-negative women. *Eur J Gynaecol Oncol* 2000; **21**:168-172.

Touze A, de Sanjose S, Coursaget P, Almirall MR, Palacio V, Meijer CJ, Kornegay J, Bosch FX. Prevalence of anti-human papillomavirus type 16, 18, 31, and 58 virus-like particles in women in the general population and in prostitutes. *J Clin Microbiol* 2001; **39**:4344-4348.

Treanor D, Magee D, Turner L, Carroll B, Gibbons D. Incidence of cervical dyskaryosis in Ireland: report of a five-year audit. *Ir Med J* 2002; **95**:206-208.

Tucker RA, Johnson PR, Reeves WC, Icenogle JP. Using the polymerase chain reaction to genotype human papillomavirus DNAs in samples containing multiple HPVs may produce inaccurate results. *J Virol Methods* 1993; **43**:321-333.

Tucker RA, Unger ER, Holloway BP, Swan DC. Real-time PCR-based fluorescent assay for quantitation of human papillomavirus types 6, 11, 16, and 18. *Mol Diagn* 2001; **6**:39-47.

Ueda Y, Enomoto T, Miyatake T, Ozaki K, Yoshizaki T, Kanao H, Ueno Y, Nakashima R, Shroyer KR, Murata Y. Monoclonal expansion with integration of high-risk type human papillomaviruses is an initial step for cervical carcinogenesis: association of clonal status and human papillomavirus infection with clinical outcome in cervical intraepithelial neoplasia. *Lab Invest* 2003; **83**:1517-1527.

Vainio H, Wilbourn JD, Sasco AJ, Partensky C, Gaudin N, Heseltine E, Eragne I. Identification of human carcinogenic risks in IARC monographs. *Bull Cancer* 1995; **82**:339-348.

van den Akker-van Marie ME, van Ballegooijen M, Rozendaal L, Meijer CJ, Habbema JD. Extended duration of the detectable stage by adding HPV test in cervical cancer screening. *Br J Cancer* 2003; **89**:1830-1833.

van den Brule AJ, Pol R, Fransen-Daalmeijer N, Schouls LM, Meijer CJ, Snijders PJ. GP5+/6+ PCR followed by reverse line blot analysis enables rapid and high-throughput identification of human papillomavirus genotypes. *J Clin Microbiol* 2002; **40**:779-787.

van den Hoek JA, Mulder-Folkerts DK, Coutinho RA, Dukers NH, Buimer M, van Doornum GJ. Opportunistic screening for genital infections with *Chlamydia trachomatis* among the sexually active population of Amsterdam. II Over 90% participation and almost 5% prevalence. *Ned Tijdschr Geneeskd* 1999; **143**:668-672.

Van Der Pol B, Quinn TC, Gaydos CA, Crotchfelt K, Schachter J, Moncada J, Jungkind D, Martin DH, Turner B, Peyton C, Jones RB. Multicenter evaluation of the AMPLICOR and automated COBAS AMPLICOR CT/NG tests for detection of *Chlamydia trachomatis*. *J Clin Microbiol* 2000; **38**:1105-1112.

Van Der Pol B, Williams JA, Smith NJ, Batteiger BE, Cullen AP, Erdman H, Edens T, Davis K, Salim-Hammad H, Chou VW, Scearce L, Blutman J, Payne WJ. Evaluation of the Digene Hybrid Capture II Assay with the Rapid Capture System for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. *J Clin Microbiol* 2002; **40**:3558-3564.

van Duin M, Snijders PJ, Schrijnemakers HF, Voorhorst FJ, Rozendaal L, Nobbenhuis MA, van den Brule AJ, Verheijen RH, Helmerhorst TJ, Meijer CJ. Human papillomavirus 16 load in normal and abnormal cervical scrapes: an indicator of CIN II/III and viral clearance. *Int J Cancer* 2002; **98**:590-595.

Vandahl BB, Birkelund S, Christiansen G. Genome and proteome analysis of *Chlamydia*. *Proteomics* 2004; **4**:2831-2842.

Verhoeven V, Avonts D, Meheus A, Ieven M, Goossens H, Van Royen P. Performance of the recommendations of a British advisory group for screening for *Chlamydia* in a sample of women in general practice. *J Med Screen* 2003; **10**:14-15.

Wagatsuma M, Hashimoto K, Matsukura T. Analysis of integrated human papillomavirus type 16 DNA in cervical cancers: amplification of viral sequences together with cellular flanking sequences. *J Virol* 1990; **64**:813-821.

Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Peto J, Meijer CJ, Munoz N. Human Papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol* 1999; **189**:12-19.

Wall SR, Scherf CF, Morison L, Hart KW, West B, Ekpo G, Fiander AN, Man S, Gelder CM, Walraven G, Borysiewicz LK. Cervical human papillomavirus infection and squamous intraepithelial lesions in rural Gambia, West Africa: viral sequence analysis and epidemiology. *Br J Cancer* 2005; **93**:1068-1076.

Wallin KL, Wiklund F, Luostarinen T, Angstrom T, Anttila T, Bergman F, Hallmans G, Ikaheimo I, Koskela P, Lehtinen M, Stendahl U, Paavonen J, Dillner J. A population-based prospective study of *Chlamydia trachomatis* infection and cervical carcinoma. *Int J Cancer* 2002; **101**:371-374.

Wang Q, Griffin H, Southern S, Jackson D, Martin A, McIntosh P, Davy C, Masterson PJ, Walker PA, Laskey P, Omary MB, Doorbar J. Functional analysis of the human papillomavirus type 16 E1=E4 protein provides a mechanism for *in vivo* and *in vitro* keratin filament reorganization. *J Virol* 2004; **78**:821-833.

Wang SP, Grayston JT. Population prevalence of antibody to *Chlamydia pneumoniae*, strain TWAR. *In* Chlamydial Infections. Proceedings of the Seventh International Symposium on Human Chlamydial infections. Ed W.R. Bowie, H.D. Caldwell, R.P. Jones, P-A Mardh, G. L. Ridgeway, J. Schachter, W.E. Stamm, and M.E. Ward. Cambridge University Press. Cambridge, United Kingdom 1990: p 402-405.

Wang SP, Grayston JT. Immunologic relationship between genital TRIC, lymphogranuloma venereum, and related organisms in a new microtiter indirect immunofluorescence test. *Am J Ophthalmol* 1970; **70**:367-374.

Wang SP, Grayston JT. Classification of trachoma virus strains by protection of mice from toxic death. *J Immunol* 1963; **90**:849-856.

Wang-Johanning F, Lu DW, Wang Y, Johnson MR, Johanning GL. Quantitation of human papillomavirus 16 E6 and E7 DNA and RNA in residual material from ThinPrep Papanicolaou tests using real-time polymerase chain reaction analysis. *Cancer* 2002; **94**:2199-2210.

Wentzensen N, Vinokurova S, von Knebel Doeberitz M. Systematic review of genomic integration sites of human papillomavirus genomes in epithelial dysplasia and invasive cancer of the female lower genital tract. *Cancer Res* 2004; **64**:3878-3884.

Westrom L, Bengtsson LP, Mardh PA. Incidence, trends, and risks of ectopic pregnancy in a population of women. *Br Med J* 1998; **282**:15-18.

Westrom LV. Sexually transmitted diseases and infertility. *Sex Trans Dis* 1994; **21**:32-37.

Wheeler CM, Greer CE, Becker TM, Hunt WC, Anderson SM, Manos MM. Short-term fluctuations in the detection of cervical human papillomavirus DNA. *Obstet Gynecol* 1996; **88**:261-268.

Whitcher JP, Srinivasan M, Upadhyay MP. Corneal blindness: a global perspective. *Bull World Health Organ* 2001; **79**:214-221.

WHO. World Health Organisation. Three-hundred, thirty-three million new STD curable cases in 1995. *AIDS Wkly* 1995; Sept 18:15-16.

Wick MJ. Diagnosis of human papillomavirus gynecologic infections. *Clin Lab Med* 2000; **20**:271-287.

Wilson DP, Mathews S, Wan C, Pettitt AN, McElwain DL. Use of a quantitative gene expression assay based on micro-array techniques and a mathematical model for the investigation of chlamydial generation time. *Bull Math Biol* 2004; **66**:523-537.

Wilson JS, Honey E, Templeton A, Paavonen J, Mardh PA, Stray-Pedersen B; EU Biomed Concerted Action Group. A systematic review of the prevalence of *Chlamydia trachomatis* among European women. *Hum Reprod Update* 2002; **8**:385-394.

Wittwer CT, Fillmore GC, Garling DJ. Minimizing the time required for DNA amplification by efficient heat transfer to small samples. *Anal Biochem* 1990; **186**:328-331.

Wood H, Reischl U, Peeling RW. Rapid detection and quantification of *Chlamydia trachomatis* in clinical specimens by LightCycler PCR. *In* Rapid Cycle Real-Time PCR-Methods and Applications. Eds Reischl U, Wittwer C, Cockerill F. Springer, New York. 2002;115-132.

Woodman CB, Collins S, Winter H, Bailey A, Ellis J, Prior P, Yates M, Rollason TP, Young LS. Natural history of cervical human papillomavirus infection in young women: a longitudinal cohort study. *Lancet* 2001; **357**:1831-1836.

Woodworth CD, McMullin E, Iglesias M, Plowman GD. Interleukin 1 alpha and tumor necrosis factor alpha stimulate autocrine amphiregulin expression and proliferation of human papillomavirus-immortalized and carcinoma-derived cervical epithelial cells. *Proc Natl Acad Sci U S A* 1995; **92**:2840-2844.

Wright JD, Li J, Gerhard DS, Zhang Z, Huettner PC, Powell MA, Gibb RK, Herzog TJ, Mutch DG, Trinkaus KM, Rader JS. Human papillomavirus type and tobacco use as predictors of survival in early stage cervical carcinoma. *Gynecol Oncol* 2005; **98**:84-91.

Wright TC Jr, Schiffman M, Solomon D, Cox JT, Garcia F, Goldie S, Hatch K, Noller KL, Roach N, Runowicz C, Saslow D. Interim guidance for the use of human papillomavirus DNA testing as an adjunct to cervical cytology for screening. *Obstet Gynecol* 2004; **103**:304-309.

Yarkin F, Chauvin S, Konomi N, Wang W, Mo R, Bauchman G, Diaz A, Burstein D, Szporn A, Hauptman E, Zhang DY. Detection of HPV DNA in cervical specimens collected in cytologic solution by litigation-dependent PCR. *Acta Cytol* 2003; **47**:450-456.

Yen TY, Pal S, de la Maza LM. Characterization of the disulfide bonds and free cysteine residues of the *Chlamydia trachomatis* mouse pneumonitis major outer membrane protein. *Biochemistry* 2005; **44**:6250-6256.

Ying S, Seiffert BM, Hacker G, Fischer SF. Broad degradation of proapoptotic proteins with the conserved Bcl-2 homology domain 3 during infection with *Chlamydia trachomatis*. *Infect Immun* 2005; **73:**1399-1403.

Ylitalo N, Sorensen P, Josefsson AM, Magnusson PK, Andersen PK, Ponten J, Adami HO, Gyllensten UB, Melbye M. Consistent high viral load of human papillomavirus 16 and risk of cervical carcinoma *in situ*: a nested case-control study. *Lancet* 2000; **355:**2194-2198.

Yoshikawa H, Kawana T, Kitagawa K, Mizuno M, Yoshikura H, Iwamoto A. Detection and typing of multiple genital human papillomaviruses by DNA amplification with consensus primers. *Jpn J Cancer Res* 1991; **82:**524-531.

Young DC, Craft S, Day MC, Davis B, Hartwell E, Tong S. Comparison of Abbott LCx *Chlamydia trachomatis* assay with Gen-Probe PACE2 and culture. *Infect Dis Obset Gynecol* 2000; **8:**112-115.

Zbroch T, Knapp P, Blonska E, Kobylec M, Knapp P. Life style, *Chlamydia trachomatis* infection, bacterial vaginosis and their impact on the frequency of cervical lesions. *Ginekol Pol* 2004; **75:**538-544.

Zerbini M, Venturoli S, Cricca M, Gallinella G, De Simone P, Costa S, Santini D, Musiani M. Distribution and viral load of type specific HPVs in different cervical lesions as detected y PCR-ELISA. *J Clin Pathol* 2001; **54:**377-380.

Zerfass-Thome K, Zwerschke W, Mannhardt B, Tindle R, Botz JW, Jansen-Durr P. Inactivation of the cdk inhibitor p27KIP1 by the human papillomavirus type 16 E7 oncoprotein. *Oncogene* 1996; **13:**2323-2330.

Zhang W, Cohenford M, Lentrichia B, Isenberg HD, Simson E, Li H, Yi J, Zhang DY. Detection of *Chlamydia trachomatis* by isothermal ramification amplification method: a feasibility study. *J Clin Microbiol* 2002; **40:**128-132.

Zielinski GD, Rozendaal L, Voorhorst FJ, Berkhof J, Snijders PJ, Risse EJ, Runsink AP, de Schipper FA, Meijer CJ. HPV testing can reduce the number of follow-up visits in women treated for cervical intraepithelial neoplasia grade 3. *Gynecol Oncol* 2004; **91:**67-73.

zur Hausen H. Human genital cancer: synergism between two virus infections or synergism between a virus infection and initiating events? *Lancet* 1982; **2:**1370-1372.

zur Hausen H. Papillomaviruses in human cancers. *Proc Assoc Am Physicians* 1999; **111:**581-587.

zur Hausen H. Viruses in human cancers. *Science* 1991; **254:**1167.

APPENDICES

Appendix A: QIAamp DNA extraction protocol

1. Pipet 20 µL QIAGEN Protease into the bottom of a 1.5 mL microcentrifuge tube.

2. Add 200 µL sample to the microcentrifuge tube.

3. Add 200 µL Buffer AL to the sample. Mix by pulse-vortexing for 15 s.

4. Incubate at 56°C for 10 min.

5. Briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from the inside of the lid.

6. Add 200 µL ethanol (96-100%) to the sample, and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from the inside of the lid.

7. Carefully apply the mixture from step 6 to the QIAamp Spin Column (in a 2 mL collection tube) without wetting the rim, close the cap, and centrifuge at 6,000 x g (8,000 rpm) for 1 min. Place the QIAamp Spin Column in a clean 2 mL collection tube and discard the tube containing the filtrate.

8. Carefully open the QIAamp Spin Column and add 500 µL Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6,000 x g (8,000 rpm) for 1 min. Place the QIAamp Spin Column in a clean 2 mL collection tube and discard the collection tube containing the filtrate.

9. Carefully open the QIAamp Spin Column and add 500 µL buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.

10. Place the QIAamp Spin column in a clean 1.5 mL microcentrifuge tube and discard the collection tube containing the filtrate. Carefully open the QIAamp Spin Column and

add 200 μL buffer AE. Incubate at room temperature for 1 min and centrifuge at 6,000 x g (8,000 rpm for 1 min).

Appendix B: Working protocols for PCR

CTP PCR:

Stock	Conc. Required	Quantity in 20 µL PCR reaction
PCR Buffer 10x	1x	2 µL
DNTPs 2 mM	200 µM	2 µL
$MgCl_2$	1.5 mM	0.6 µL
CTP1/2 100 pmol/µL	25 pmol/µL	2 µL
***Taq* polymerase 5U/µL**	1 U	0.2 µL
H_2O		8.1 µL
Sample DNA		5 µL

PCR cycle: 95°C for 5 min, 40x(95°C for 1 min, 55°C for 1 min, 72°C for 2 min), 72°C for 5 min

Real-time Hsp60 (650 bp) PCR:

Stock	Conc. Required	Quantity in 10 µL PCR reaction
Faststart DNA-Mastermix Enzyme		1 µL
$MgCl_2$ 25 mM	3 mM	0.8 µL
Hsp60 5 µM	0.5 µM	2 µL
H_2O		5.2 µL
Sample DNA		1 µL

PCR cycle: 95°C for 10, 40x (95°C for 5 s, 68°C for 10 s, decreasing to 65°C at a rate of 1°C/cycle, 72°C for 30 s).

MOMP PCR:

Stock	Conc. Required	Quantity in 20 μL PCR reaction
PCR Buffer 10x	1x	2 μL
DNTPs 2 mM	200 μM	2 μL
$MgCl_2$ 50 mM	1.5 mM	0.6 μL
MOMP 100 pmol/μL	25 pmol/μL	2 μL
***Taq* polymerase 5U/μL**	1 U	0.2 μL
H_2O		8.1 μL
Sample DNA		5 μL

PCR cycle: 95°C for 5 min, 40x (95°C for 1 min, 55°C for 1 min, 72°C for 2 min), 72°C for 5 min.

Multiplex PCR:

Stock	Conc. Required	Quantity in 20 μL PCR reaction
PCR Buffer 10x	1x	2 μL
DNTPs 2 mM	200 μM	2 μL
$MgCl_2$ 50 mM	2 mM	0.8 μL
PCO3/4 100 pmol/μL	5 pmol/μL	2 μL
CTP1/2 100 pmol/μL	5 pmol/μL	2 μL
MY09/11 100 pmol/μL	10 pmol/μL	2 μL
***Taq* polymerase 5U/μL**	1 U	0.2 μL
H_2O		4 μL
Sample DNA		5 μL

PCR cycle: 95°C for 10 min, 40x (95 °C for 30 s, 57°C for 1 min, 72°C for 1 min), 72°C for 10 min.

Real-time MY09/11 PCR:

Stock	Conc. Required	Quantity in 20 μL PCR reaction
PCR Buffer 10x	1x	2 μL
DNTPs 2 mM	200 μM	2 μL
$MgCl_2$ 50 mM	2 mM	0.8 μL
MY09/11 100 pmol/μL	10 pmol/μL	2 μL
***Taq* polymerase 5U/μL**	1 U	0.2 μL
BSA (10 mg/mL)		1 μL
SYBR Green (1:3000)	1:30000	2 μL
H_2O		5 μL
Sample DNA		5 μL

PCR cycle: 95°C for 5 min, 45x (95°C for 5 s, 60°C for 1 min, decreasing to 55°C at a rate of 1°C/cycle, 72°C for 1 min.

PCO3/4 PCR:

Stock	Conc. Required	Quantity in 20 μL PCR reaction
PCR Buffer 10x	1x	2 μL
DNTPs 2 mM	200 μM	2 μL
$MgCl_2$ 50 mM	2 mM	0.8 μL
PCO3/4 100 pmol/μL	50 pmol/μL	2 μL
***Taq* polymerase 5U/μL**	1 U	0.2 μL
H_2O		8 μL
Sample DNA		5 μL

PCR cycle: 95°C for 5 min, 40x (95°C for 1 min, 55°C for 1 min, 72°C for 1 min), 72°C for 5 min.

Hsp60R2 (309 bp) PCR:

Stock	Conc. Required	Quantity in 20 µL PCR reaction
PCR Buffer 10x	1x	2 µL
DNTPs 2 mM	200 µM	2 µL
$MgCl_2$ 50 mM	2 mM	0.8 µL
Hsp60 100 pmol/µL	10 pmol/µL	2 µL
***Taq* polymerase 5U/µL**	1 U	0.2 µL
H_2O		8 µL
Sample DNA		5 µL

PCR cycle: 95°C for 5 min, 40x (95°C for 1 min, 55°C for 1 min, 72°C for 1 min), 72°C for 5 min.

MY09/11 Sequencing PCR:

Stock	Conc. Required	Quantity in 50 µL PCR reaction
PCR Buffer 10x	1x	5 µL
DNTPs 2 mM	200 µM	5 µL
$MgCl_2$ 50 mM	2 mM	2 µL
My09/11 100 pmol/µL	10 pmol/µL	5 µL
***Taq* polymerase 5U/µL**	1 U	0.5 µL
H_2O		20 µL
Sample DNA		12.5 µL

PCR cycle: 95°C for 5 min, 35x (95°C for 1 min, 57°C for 1 min, 72°C for 1 min), 72°C for 5 min.

GP5+/6+ PCR:

Stock	**Conc. Required**	**Quantity in 20 μL PCR reaction**
PCR Buffer 10x	1x	2 μL
DNTPs 2 mM	200 μM	2 μL
$MgCl_2$ 50 mM	2 mM	0.8 μL
GP5+/6+ 100 pmol/μL	50 pmol/μL	2 μL
***Taq* polymerase 5U/μL**	1 U	0.2 μL
H_2O		8 μL
Sample DNA		5 μL

PCR cycle: 95°C for 5 min, 40x(95°C for 1 min, 40°C for 1 min, 72°C for 1 min), 72°C for 5 min.

Appendix C: Sensitivity and specificity formula

Determination of sensitivity and specificity of multiplex PCR using Bayesian Formula (Daly and Bourke, 2000).

True Positives = (TP)

True Negatives = (TN)

False Positives = (FP)

False Negatives = (FN)

Sensitivity formula: Sensitivity = TP/TP+FN

Specificity formula: Specificity = TN/FP+TN

Appendix D: Hybrid capture protocols

A. Digene Sample Conversion Kit protocol

1. Shake each PreservCyt Solution vial until cells appear to be homogenously dispersed.
2. Uncap and immediately pipette 4 mL of PreservCyt Solution specimen into a 10 mL conical Sarstedt tube as cells settle very quickly. Label the tube with the specimen identification.
3. Add 0.4 mL Sample Conversion Buffer.
4. Mix each tube thoroughly by vortexing.
5. Centrifuge tubes in a swinging bucket centrifuge at 2,900 +/- 150 g for 15 +/- 2 min.
6. During centrifugation, prepare the Denaturation reagent and the mixture of Specimen Transport Medium + Denaturation Reagent. To prepare Denaturation Reagent, add 3 drops of Indicator Dye to the bottle of Denaturation Reagent and mix well. After addition of Indicator Dye, Denaturation Reagent should be a uniform, dark purple colour. Once prepared, the Denaturation Reagent is stable for 3 months when stored at 2-8°C. Label it with the new expiration date. If the colour fades, add 3 additional drops of Indicator Dye and shake by hand until contents are a uniform colour. To prepare the mixture of Specimen Transport Medium + Denaturation Reagent. Mix 1 volume of Specimen Transport Medium with a ½ volume of Denaturation Reagent (containing Indicator Dye). For a PreservCyt Solution starting volume of 4 mL, 150 μL of Specimen Transport Medium + Denaturation Reagent is required per sample (100 μL Specimen Transport Medium + 50 μL Denaturation Reagent). However, prepare 180 μL final volume for each sample to be processed to allow for loss during transfer

(120 μL Specimen Transport Medium + 60 μL Denaturation Reagent). **Solution must be prepared fresh each day.**

7. Remove tubes from the centrifuge one tube at a time, and place them into a rack. Look for a pink/orange pellet.
8. Carefully decant supernatant, a gently blot (~ 6 times) each tube on absorbent lint-free paper towels until liquid no longer drips from tube. **Do not** let cell pellets slide down the tube during blotting. Return tubes to the rack.
9. Add 180 μL Specimen Transport Medium + Denaturation Reagent to each pellet. Resuspend each pellet by vortexing each tube individually for at least 30 s at the highest speed setting. If a pellet is difficult to resuspend, vortex for an additional 10-30 s or until the pellet floats loose from the bottom of the tube. If a pellet remains undissolved after additional vortexing (2 min maximum), note the sample identification and proceed to the next step.
10. Place tubes in 65 +/- 2°C water bath for 15 +/- 2 min.
11. Remove the rack with specimens from the water bath and vortex samples individually for about 15 – 30 s. Make sure that all pellets are completely resuspended at this point. Samples that still have visible pellets are not acceptable for testing.
12. Return the rack to the 65 +/- 2°C water bath and continue denaturation for another 30 +/- 3 min.
13. Proceed to the *Hyridization Step* as described in HCII HPV DNA Tests' package inserts.

B. Preparation of QIAamp DNA samples for HCII

1. DNA was extracted from PreservCyt samples as per section 2.2.2 using the QIAamp DNA extraction protocol.
2. 60 μL of DNA was added to 60 μL of Digene specimen transport medium and 60 μL of Digene denaturation reagent.
3. HCII was performed as per HCII HPV DNA test protocol.

C. HCII HPV DNA test protocol

Summary of hc2 HPV DNA Test

HYBRIDIZATION

Microplater Heater Method

Combined -Probe Cocktail Method

Mix denatured specimen well and pipette 75 µL into microplate wells.

Incubate for 10+/- 2 minutes at 20-25°C

Pipette 25 µL Combined - Probe Cocktail into hybridization microplate wells.

OR

Dual Probe Method

Mix denatured specimen well and pipette 75 µL into "LR" microplate wells and 75 µL into "HR" microplate wells.

Incubate for 10 +/- 2 minutes at 20-25°C

Pipette 25 µL Low-Risk HPV Probe Mix into "LR" microplate wells.
Pipette 25 µL High-Risk HPV Probe Mix into "HR" microplate wells.

Cover microplate with a plate lid and shake on Rotary Shaker I at 1,100 +/- 100 rpm for 3 +/- 2 minutes. Check that all tubes show yellow colour.

Incubate at 65 +/- 2°C for 60 +/- 5 minutes. Prepare capture microplate.

HYBRID CAPTURE

Transfer contents from each Hybridization Plate Well or microtube to corresponding well in capture microplate using an 8-channel pipettor. Cover with a plate lid or plate sealer. Shake at 1,100 +/- 100 rpm at 20-25°C for 60 +/- 5 minutes. Prepare Wash Buffer.

	↓ Decant and blot capture microplate ↓
HYBRID DETECTION	Pipette 75 µL Detection Reagent 1 into each well of capture microplate. Cover capture microplate with Plate Lid, Parafilm or equivalent. Incubate at 20-25°C for 30-45 minutes. Wash plate using desired method. ↓
WASHING	**Automated Plate Washer Method** Place plate on washer and press "Start/Stop" to begin. ↓
SIGNAL AMPLIFICATION	Pipette 75 µL Detection Reagent 2 into each well of capture microplate. Incubate at 20-25°C for 15-30 minutes. ↓
READING	Read capture microplate on DML 2000 instrument. ↓ Validate assay and interpret specimen result.

Appendix E: Study consent form and ethical approval

A. Consent form:

ST. JAMES'S HOSPITAL

James's Street, Dublin 8

Telephone (+353 1) 410 3000

www.stjames.ie

St James's Hospital and Dublin Institute of Technology Cervical Smear Research Study 2003-2004

Your cervical smear test is examined at St James's hospital, cytology laboratory. Not all the sample is used for routine screening and the remainder is discarded following completion of the test. We would like to use this residual material for research.

The purpose of this research study is to establish new tests for detection of infections in Cervical smear samples. This study aims to establish new molecular methods for detection, genotyping and quantitation of Human papillomavirus and Chlamydia trachomatis infections in the female population.

This is a research study using only the leftover smear sample material and will have no effect on your cervical smear results.

<u>Consent Form</u>

Your cervical smear test is examined at St James's hospital, cytology laboratory. Not all the sample is used for routine screening and the remainder is discarded following completion of the test.

As part of a research study, we seek your consent to use this residual sample that is normally discarded.

The study will be anonymised, which means that the researchers will not have any name or other identifiers on the sample.

I allow my residual cervical sample to be used for research.

Signed ______________________ **Date** ________

Smoker ☐ **Non-smoker** ☐

University Teaching Hospital of Trinity College Dublin

B. Letter of ethical approval:

THIS NOTEPAPER MUST NOT BE USED FOR PRESCRIPTIONS OR INVOICING PURPOSES

THE ADELAIDE & MEATH HOSPITAL, DUBLIN
INCORPORATING
THE NATIONAL CHILDREN'S HOSPITAL

TALLAGHT, DUBLIN 24, IRELAND
TELEPHONE +353 1 4142000

Dr. Helen Lambkin,
School of Biological Sciences,
Dublin Institute of Technology,
Kevin Street,
Dublin 8.

Dr. Mairead Griffin,
Cytopathology Dept.,
St. James's Hospital,
James's Street,
Dublin 8.

15th October 2003

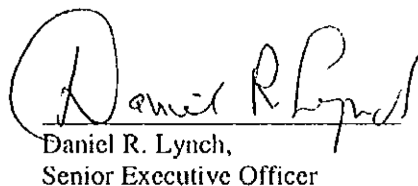
Re. Detection, quantification and typing of micro-organisms in liquid cervical PreservCyt samples to the normal female population.

Please quote this reference in any follow-up to this letter: 2003 / 33 /4

Dear Dr. Lambkin,

Thank you for your letter dated 3/09/2003 in which you respond to the conditions attached to the initial Ethical Approval of the above study. This response has been approved by the Chairman on behalf of the Joint Research ethics Committee. Ethical Approval for this study is, therefore, complete.

Yours sincerely



Daniel R. Lynch,
Senior Executive Officer

Appendix F: Sigma GenElute PCR Clean-up Kit protocol

1. Insert a GenElute Miniprep binding column into a provided collection tube, if not already assembled. Add 0.5 mL of the column preparation solution to each miniprep column and centrifuge at 12,000 x g for 30 s to 1 min. Discard the flow-through liquid.
2. Add 5 volumes of binding solution to 1 volume of PCR reaction and mix. For example, add 500 μL of binding solution to 100 μL of the PCR reaction. Transfer the solution into the binding column. Centrifuge the column at maximum speed 12,000-16,000 x g for 1 min. Discard the flow-through liquid, but retain the collection tube.
3. Replace the binding column into the collection tube. Apply 0.5 mL of diluted Wash solution to the column and centrifuge at maximum speed for 1 min. Discard the flow-through liquid, but retain the collection tube.
4. Replace the column into the collection tube. Centrifuge the column at maximum speed for 2 min without any additional wash solution, to remove excess ethanol. Discard any residual flow-through as well as the collection tube.
5. Transfer the column to a fresh 2 mL collection tube. Apply 50 μL of Elution Solution or water to the center of each column. Incubate at room temperature for 1 min.
6. To elute the DNA, centrifuge the column at maximum speed for 1 min. The PCR amplification product is now present in the eluate and is ready for immediate use or storage at –20C.

Appendix G: Summary of HPV positive samples

Sample No.	HPV multiplex	C. trach multiplex	HCII High-risk	HCII Low-risk	HPV Viral load copy/ug DNA	Genotype	Cytology	Age
3	+	-	ND	ND	1.3E+05	ND	normal	26
5	+	-	-	-	ND	ND	normal	39
6	+	-	-	+	1.4E+03	66	borderline	26
10	+	-	ND	ND	4.5E+08	16	CIN I	18
18	+	-	+	ND	6.7E+05	ND	normal	24
23	+	-	-	+	2.4E+05	6	normal	25
26	+	-	ND	ND	6.9E+05	33	normal	21
27	+	-	ND	ND	8.6E+04	ND	CIN I	25
36	+	-	ND	ND	9.4E+06	ND	normal	22
43	+	-	ND	ND	8.7E+05	ND	normal	23
48	+	-	-	-	1.8E+06	61	normal	33
56	+	-	+	ND	1.5E+08	53	borderline	39
57	+	-	-	-	1.3E+05	ND	normal	33
59	+	-	+	ND	5.3E+06	ND	CIN I	33
62	+	-	ND	ND	7.0E+05	59	borderline	33
65	+	-	-	-	7.2E+06	ND	normal	23
68	+	-	+	ND	3.2E+03	ND	borderline	36
71	+	-	ND	ND	9.1E+04	ND	CIN III	39
73	+	-	ND	ND	3.3E+05	ND	borderline	35
79	+	-	ND	ND	1.4E+03	54	normal	32
80	+	-	+	ND	8.4E+06	70	normal	48
85	+	-	+	ND	3.7E+06	66	normal	27
86	+	-	ND	ND	1.1E+03	61	normal	27
88	+	-	ND	ND	1.7E+10	88	CIN I	33
94	+	-	ND	ND	4.8E+07	31	CIN I	24
104	+	-	ND	ND	4.4E+04	53	normal	21
105	+	-	ND	ND	2.5E+07	33	CIN I	32
106	+	-	ND	ND	1.8E+04	ND	normal	23
109	+	-	ND	ND	1.3E+07	61	normal	29
111	+	-	+	ND	2.2E+03	33	CIN II	24
114	+	-	ND	ND	8.8E+02	ND	normal	53
115	+	-	-	+	5.1E+03	70	normal	27
118	+	-	+	ND	3.4E+05	53	normal	34
119	+	-	ND	ND	7.5E+04	ND	borderline	27
123	+	-	+	ND	2.5E+09	16	CIN I	21
124	+	-	ND	ND	6.6E+06	66	CIN I	28
127	+	-	ND	ND	8.5E+07	6	normal	19
131	+	-	ND	ND	1.6E+05	ND	normal	29
138	+	+	ND	ND	6.7E+04	6	normal	18

Sample No.	HPV multiplex	C. trach multiplex	HCII High-risk	HCII Low-risk	HPV Viral load copy/ug DNA	Genotype	Cytology	Age
141	+	-	-	-	7.7E+03	ND	normal	59
142	+	-	-	+	6.7E+08	6	borderline	20
143	+	-	-	-	1.3E+07	81	normal	31
144	+	-	+	-	8.6E+08	16	CIN I	27
147	+	-	+	-	7.0E+07	33	normal	50
150	+	+	-	+	9.4E+06	6	normal	20
151	+	-	ND	ND	9.9E+03	70	normal	16
159	+	-	ND	-	1.2E+02	ND	normal	41
166	+	-	+	ND	2.0E+06	ND	normal	27
176	+	-	+	ND	5.8E+07	ND	CIN III	48
177	+	-	ND	ND	2.5E+05	ND	borderline	36
186	+	-	-	-	4.2E+04	62	normal	26
193	+	-	-	-	4.5E+04	ND	normal	46
196	+	-	ND	ND	7.2E+07	70	borderline	29
200	+	-	ND	ND	9.2E+04	ND	normal	40
201	+	-	-	-	2.9E+03	ND	normal	28
205	+	-	+	ND	7.9E+07	ND	CIN I	27
208	+	-	-	+	4.6E+05	ND	normal	30
221	+	-	+	ND	1.8E+08	66	normal	24
223	+	-	+	ND	2.2E+07	ND	CIN I	24
226	+	-	ND	ND	1.7E+05	73	CIN I	24
228	+	-	+	ND	1.2E+04	33	borderline	23
229	+	-	ND	ND	2.4E+05	ND	borderline	42
231	+	+	+	ND	1.9E+05	ND	normal	25
232	+	-	+	ND	7.3E+05	33	normal	32
248	+	-	ND	ND	6.5E+05	18	CIN I	25
251	+	-	+	-	1.7E+05	53	normal	22
258	+	-	ND	ND	2.7E+06	52	CIN I	26
276	+	-	+	ND	5.9E+08	33	CIN I	21
290	+	-	+	ND	3.4E+07	ND	CIN III	30
303	+	-	+	ND	9.7E+05	16	normal	26
306	+	-	+	ND	1.7E+07	66	CIN I	24
311	+	-	+	ND	6.6E+05	16	normal	20
312	+	-	+	ND	2.7E+06	ND	normal	24
325	+	-	+	ND	2.8E+06	ND	CIN I	27
326	+	-	-	-	8.1E+06	16	normal	33
338	+	-	-	-	1.3E+06	ND	normal	31
339	+	-	ND	ND	2.2E+05	ND	borderline	22
345	+	-	+	ND	2.2E+06	ND	CIN III	31
346	+	-	-	-	1.8E+03	ND	normal	27
347	+	-	+	ND	4.6E+04	ND	normal	47

Sample No.	HPV multiplex	C. trach multiplex	HCII High-risk	HCII Low-risk	HPV Viral load copy/ug DNA	Genotype	Cytology	Age
358	+	-	+	ND	2.4E+04	58	normal	30
363	+	-	-	-	8.7E+06	ND	normal	31
395	+	-	+	ND	5.1E+05	81	borderline	40
398	+	-	+	ND	2.6E+07	66	borderline	25
399	+	-	+	ND	1.3E+04	68	normal	37
404	+	-	-	+	ND	61	normal	31
407	+	-	+	ND	4.9E+05	61	CIN III	40
412	+	-	+	ND	2.3E+06	31	normal	26
415	+	-	ND	ND	ND	61	normal	35
422	+	+	-	-	2.3E+03	ND	normal	32
424	+	-	+	ND	8.3E+05	16	borderline	26
427	+	-	ND	ND	1.4E+06	58	CIN II	21
429	+	-	-	+	7.8E+08	6	CIN I	19
432	+	-	ND	ND	2.8E+05	31	borderline	30
441	+	-	+	ND	1.9E+07	16	CIN I	39
444	+	-	-	-	1.8E+08	83	normal	42
447	+	-	+	ND	9.5E+04	16	CIN I	26
453	+	-	+	ND	7.2E+07	53	CIN I	21
454	+	-	+	ND	3.0E+06	ND	normal	23
457	+	-	-	-	1.3E+03	ND	normal	29
458	+	-	ND	-	9.5E+07	18	CIN I	38
459	+	-	-	-	9.4E+04	18	normal	40
461	+	-	-	-	3.8E+07	18	normal	32
462	+	-	-	-	7.2E+05	18	normal	31
463	+	-	-	+	3.5E+05	18	CIN I	30
464	+	-	-	-	2.3E+05	18	normal	47
466	+	+	-	-	1.4E+04	18	normal	63
467	+	+	-	-	1.3E+05	ND	normal	72
468	+	-	+	ND	4.8E+03	ND	normal	26
473	+	-	+	ND	4.2E+04	16	normal	23
474	+	+	-	-	9.8E+03	18	normal	21
481	+	+	-	-	1.5E+05	18	normal	27
482	+	+	+	ND	1.1E+05	18	normal	19
484	+	-	ND	ND	8.2E+06	16	CIN I	27
487	+	-	+	ND	1.7E+07	66	CIN I	38
489	+	-	+	ND	3.7E+07	ND	CIN I	29
506	+	-	ND	ND	1.9E+06	68	CIN I	27
507	+	+	ND	ND	7.2E+06	ND	borderline	20
509	+	-	ND	ND	2.1E+05	ND	CIN II	30
511	+	-	+	ND	2.1E+06	ND	normal	47
513	+	-	+	-	9.9E+05	68	CIN I	31

Sample No.	HPV multiplex	C. trach multiplex	HCII High-risk	HCII Low-risk	HPV Viral load copy/ug DNA	Genotype	Cytology	Age
515	+	-	ND	ND	1.3E+06	ND	borderline	35
516	+	-	+	-	6.7E+06	ND	CIN II	24
522	+	-	+	+	4.6E+05	66	CIN II	21
524	+	-	+	-	1.1E+06	70	normal	22
525	+	-	+	-	1.3E+06	61	CIN I	25
526	+	-	+	-	1.2E+05	61	normal	40
530	+	-	+	+	1.6E+07	ND	CIN I	19
531	+	-	+	-	1.9E+05	18	normal	34
537	+	+	+	-	9.7E+05	83	normal	24
539	+	-	+	ND	2.6E+05	31	normal	32
541	+	-	-	+	1.7E+05	6	normal	39
553	+	-	+	-	1.8E+07	16	CIN I	35
554	+	-	+	-	1.1E+06	31	normal	26
568	+	-	+	-	4.9E+05	16	normal	24
571	+	-	+	-	8.6E+06	ND	CIN II	44
584	+	-	+	ND	1.6E+07	16	CIN I	31
585	+	-	+	-	6.2E+05	62	normal	25
587	+	-	+	-	4.4E+06	53	CIN I	23
595	+	-	+	ND	7.6E+07	33	CIN I	28
608	+	-	+	ND	1.7E+06	16	borderline	22
613	+	-	+	-	1.3E+05	ND	normal	35
614	+	-	ND	ND	5.3E+08	16	CIN III	17
652	+	-	+	+	3.5E+08	16	CIN I	24
658	+	-	+	-	3.2E+07	66	borderline	35
665	+	-	ND	ND	4.0E+04	ND	CIN I	27
671	+	-	ND	ND	2.8E+07	59	CIN I	26
680	+	-	+	-	3.4E+07	58	CIN I	48
682	+	-	+	-	1.8E+05	18	normal	42
684	+	-	+	ND	8.1E+06	18	borderline	19
686	+	-	+	ND	1.9E+07	16	normal	22
697	+	-	-	+	5.4E+07	11	normal	30
699	+	-	ND	ND	5.3E+07	18	CIN II	25
706	+	-	+	ND	2.2E+07	ND	borderline	20
715	+	-	+	ND	5.6E+07	16	normal	33
717	+	-	+	ND	8.6E+05	58	normal	21
735	+	-	ND	ND	1.4E+08	ND	CIN III	21
736	+	-	+	ND	8.9E+05	16	normal	26
740	+	-	+	ND	7.1E+07	31	borderline	29
763	+	-	ND	ND	3.2E+06	16	CIN III	29
767	+	-	+	ND	3.7E+07	ND	CIN I	50
774	+	-	ND	ND	2.8E+06	ND	borderline	48

Sample No.	HPV multiplex	C. trach multiplex	HCII High-risk	HCII Low-risk	HPV Viral load copy/ug DNA	Genotype	Cytology	Age
783	+	-	+	ND	1.1E+09	53	borderline	33
784	+	-	-	-	6.2E+03	16	normal	33
788	+	-	-	-	2.5E+02	ND	normal	21
792	+	-	+	ND	4.5E+10	66	borderline	44
793	+	-	+	ND	1.7E+08	16	CIN II	23
797	+	-	+	ND	8.2E+05	ND	normal	27
801	+	-	-	-	5.4E+05	ND	normal	30
806	+	-	+	ND	5.5E+07	16	CIN III	25
818	+	-	+	ND	1.3E+07	18	borderline	26
820	+	-	+	ND	8.8E+04	ND	normal	32
823	+	-	-	-	1.9E+06	54	normal	41
825	+	-	-	-	4.8E+07	81	normal	39
828	+	-	-	+	1.2E+08	6	normal	19
836	+	-	-	-	2.2E+08	84	borderline	20
841	+	-	+	ND	9.0E+06	83	CIN II	40
842	+	-	ND	ND	2.7E+05	ND	borderline	43
848	+	-	+	ND	4.1E+08	6	CIN I	18
852	+	-	+	ND	1.7E+06	62	borderline	25
855	+	-	+	ND	2.3E+06	66	normal	25
870	+	-	+	ND	5.2E+07	16	CIN II	34
877	+	-	-	-	3.9E+05	70	normal	55
891	+	-	ND	ND	2.2E+05	ND	borderline	28
909	+	-	ND	ND	6.3E+06	ND	normal	34
915	+	-	ND	ND	2.5E+05	ND	CIN I	37
926	+	-	+	ND	1.3E+07	66	borderline	39
932	+	-	+	ND	8.1E+05	16	normal	29
935	+	-	ND	ND	9.9E+06	66	CIN II	23
942	+	-	+	ND	3.6E+07	66	borderline	20
943	+	-	-	-	4.5E+06	83	normal	31
948	+	-	+	ND	3.3E+06	58	normal	36
966	+	-	-	-	1.1E+06	53	normal	32
971	+	-	-	-	1.3E+05	53	normal	46
981	+	-	+	ND	5.3E+06	58	normal	39
987	+	-	+	ND	5.7E+06	33	borderline	22
992	+	-	+	ND	1.4E+08	33	borderline	31

HCII – Digene hybrid capture HPV test
ND = not determined
CIN – cervical intraepithelial neoplasia
\+ – positive test result
\- – negative test result

PUBLICATIONS AND PRESENTATIONS

Poster Presentations

- **Development of a Multiplex PCR Protocol for Rapid Screening of ThinPrep Cervical Samples for HPV and *Chlamydia trachomatis*.** British Society of Clinical Cytology 42nd Annual Meeting, September 2003, Queen's University Belfast and Annual Academy of Medical Laboratory Sciences Meeting, Galway, December 2003.

- **Combined Detection of Human papillomavirus and *C. trachomatis* from a single PreservCyt sample using PCR**. Annual TecNet Conference, Galway and Mayo Institute of Technology, December 2003.

Oral Presentations

- **Development of a Multiplex PCR Protocol for Rapid Screening of ThinPrep Cervical Samples for HPV and *Chlamydia trachomatis*.** British Society of Clinical Cytology 42nd Annual Meeting, September 2003, Queen's University Belfast, Northern Ireland.

- **Combined Screening for HPV and *Chlamydia trachomatis* from PreservCyt samples in a normal Irish female population using multiplex PCR.** European Research Organisation on Genital Infection and Neoplasia (EUROGIN) International Expert meeting "HPV Infection and Cervical Cancer Prevention" October 2004, Nice, France.

Publications

- **Keegan H,** Lambkin H, Malkin A, Griffin M, and Ryan F. Development of a multiplex PCR protocol for rapid screening of ThinPrep cervical samples for HPV and *Chlamydia trachomatis*. *Cytopathology* 2003: **14**;(Suppl.1).

- **Keegan H,** Boland C, Malkin A, Griffin M, Ryan F, Lambkin H. Comparison of DNA extraction from cervical cells collected in PreservCyt solution for the amplification of *Chlamydia trachomatis*. *Cytopathology* 2005: **16**;82-87.

- **Keegan H,** Malkin A, Griffin M, Ryan F, Lambkin H. Validation of a Multiplex PCR Assay for the Simultaneous Detection of Human Papillomavirus and *Chlamydia trachomatis* in Cervical PreservCyt Samples. *Clin Chem* 2005: **51**;1301-1302.

Comparison of DNA extraction from cervical cells collected in PreservCyt solution for the amplification of *Chlamydia trachomatis*

H. Keegan*, C. Boland*, A. Malkin†, M. Griffin†, F. Ryan* and H. Lambkin*

*Molecular Biomedical Research, School of Biological Sciences, Dublin Institute of Technology and †Clinical Cytology Laboratory, Department of Pathology, St James' Hospital, Dublin, Ireland

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H. Keegan, C. Boland, A. Malkin, M. Griffin, F. Ryan and H. Lambkin

Comparison of DNA extraction from cervical cells collected in PreservCyt solution for the amplification of *Chlamydia trachomatis*

Objective: The aim of this study was to compare and evaluate three methods of DNA extraction for the amplification of *Chlamydia trachomatis* in uterine cervical samples collected in PreservCyt solution. ThinPrep is the trade name for the slide preparation.

Methods: Thirty-eight samples collected in LCx buffer medium, which were identified as *C. trachomatis* infected by ligase chain reaction (LCR), were selected for this study. DNA from the PreservCyt samples was extracted by three methods: (i) QIAamp kit, (ii) boiling in Tris-EDTA buffer with Chelex purification, and (iii) Proteinase K digestion with Chelex purification. Sample DNA was tested for the presence of *C. trachomatis* by PCR using cryptic plasmid research (CTP) primers and major outer membrane protein research *momp* gene (MOMP) primers. Real-time (LightCycler) PCR for relative *C. trachomatis* quantification following DNA extraction was performed using primers (Hsp 60) for the 60 kDa heat-shock protein *hsp60* gene.

Results: Amplification using CTP primers was the most successful with each of the extraction protocols. Boiling in buffer was the least successful extraction method. QIAamp was the best extraction method, yielding the most positives with both the CTP and MOMP primers. Proteinase K-Chelex extraction gave similar sensitivity to QIAamp extraction with CTP primers but lower for MOMP primers.

Conclusions: The DNA extraction method must be carefully selected to ensure that larger PCR amplicons can be successfully produced by PCR and to ensure high sensitivity of detection of *C. trachomatis*. In this study it was found that the QIAamp extraction method followed by PCR with the CTP primers was the most successful for amplification of *C. trachomatis* DNA.

Keywords: DNA extraction, cervical cells, PreservCyt, *Chlamydia trachomatis*, PCR, LightCycler

Introduction

Gynaecological cytology is rapidly changing due to the development of new technologies for smear preparation, automated evaluation and molecular analysis of cervical smear samples.[1,2]

Liquid-based cervical smear preparation is overtaking conventional smear preparation as the method of choice for cytological evaluation due to the improvement in rates of detection of preneoplastic lesions, ease of slide interpretation and decrease in number of unsatisfactory (repeat) smears.[3,4] These liquid-based cervical samples are rich in cellular material, with thousands of residual cells remaining in the solution after the preparation of a monolayer smear. This cellular material is ideal for molecular analysis of both host cell genome and for detection and quantification of micro-organisms,[5–7] thus offering the possibility of additional molecular tests for patients who have abnormal epithelial cells or features of inflammation on routine cytology.

Correspondence:
Dr Helen Lambkin, School of Biological Sciences, Dublin Institute of Technology, Kevin St, Dublin 8, Ireland.
Tel.: +353 1 402 4911; Fax: +353 1 402 4995;
E-mail: helen.lambkin@dit.ie

Detection of human papillomavirus in cervical samples has been exhaustively studied in both conventional and liquid-based cytology samples,[8,9] however, detection and quantitation of *Chlamydia trachomatis* has been less thoroughly explored.[10,11] *Chlamydia trachomatis* is an obligate intracellular bacterium which may be present in between 2% and 17% of cervical smear samples from healthy asymptomatic females depending on the population studied.[12] Infection with *C. trachomatis* may result in pelvic inflammatory disease, infertility and is a major cause of ectopic pregnancy. Numerous studies have suggested that screening of young women for *C. trachomatis* would be cost effective due to the ultimate burden on the health service of the complications of this infection.[13–15]

The main detection methods for *C. trachomatis* include culture, enzyme immunoassay and nucleic acid-based techniques (NAAT).[16] Recent studies indicate that the NAAT methods, which are mostly based on PCR amplification of the *C. trachomatis* cryptic plasmid sequences, offer the highest sensitivity for detection of the organism.[17,18] A hybrid capture (HC) system has also been developed for *Chlamydia* detection, similar to the HCII system for HPV detection (Digene Corporation).[19]

Methods for DNA extraction from cervical cells are varied, mostly relying on some form of Proteinase K extraction, with or without a further purification step.[20,21] Other protocols have been applied to cervical cell samples, including simple boiling in buffer, microwaving and automated DNA extraction techniques.[22,23] In this study three methods of DNA extraction from uterine cervical cell samples collected into PreservCyt solution were evaluated, by the sensitivity of detection and relative quantitation of *C. trachomatis* by molecular (PCR) amplification methods compared to a gold standard of routine cervical samples tested by LCR.

Methods

Study population and clinical specimens

Cervical samples were taken from women attending a genitourinary medicine clinic at St James' Hospital, Dublin, Ireland, and placed in LCx transport medium (LCx; Abbott Laboratories, Chicago, IL, USA). These were tested for *C. trachomatis* using the Ligase Chain Reaction assay (LCx; Abbott Laboratories). A second cervical sample was taken on the same visit and placed in a vial of PreservCyt medium and transported to the cytology laboratory of the hospital where a cervical smear was made. Specimens were then kept at room temperature and the DNA was extracted within 6 weeks. The sample population in this study consisted of 38 women who tested positive for *C. trachomatis* by the LCx.

DNA extraction

Fifteen millilitres of PreservCyt specimen was vortexed briefly and divided into three 5-ml aliquots. These were then centrifuged at 13000 *g* and the pellet was washed twice with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and resuspended in a final volume of 200 µl TE buffer. In extraction method A (TE-Chelex), the cell suspension was boiled for 10 minutes with 0.1% Chelex solution (Sigma-Aldrich, St. Louis, MO, USA). In method B (Proteinase K-Chelex), the cells were resuspended in 200 µl of cell lysis buffer (10 mM Tris-HCl, 400 mM NaCl, 2 mM Na_2EDTA, pH 8.2), with 20 µl of Proteinase K (20 mg/ml) and 0.5% sodium dodecyl sulphate and incubated at 65 °C for 2.5 hours. This was then boiled for 20 minutes with 0.1% Chelex-100 solution. In method C (QIAamp), the QIAamp DNA Mini Kit (Qiagen Ltd, Crawley, UK) was used according to the manufacturer's instructions. DNA was extracted from a single sample using the three extraction methods within the same 48-hour period. Following DNA extraction samples were stored at −20 °C, until required for PCR.

PCR amplification of C. trachomatis plasmid and omp gene

The following primer sets were used for detection of *C. trachomatis*: a plasmid primer set (CTP)[24] (201 bp), and a primer set (MOMP)[25] for the major outer membrane protein gene (540 bp), (Table 1, Figure 1). PCR was performed in 20 µl reaction volume, containing 2 µl PCR buffer (Invitrogen Ltd, Renfrew, UK), 1.5 mM $MgCl_2$ 200 µM of each dNTP, 25 pmol of each primer set, 1 U of *Taq* DNA polymerase (Invitrogen Ltd) and 5 µl of DNA sample. The PCR reaction mixture was performed in a Hybaid Omni-E thermal cycler (Hybaid Ltd, Ashford, UK), with an initial denaturation of 95 °C for 5 minutes followed by 40 cycles of 95 °C for 1 minute, 55 °C for 1 minute and 72 °C for 2 minutes. After 40 cycles, a further elongation step was carried out at 72 °C for 5 minutes. The products were run on a 1% agarose gel containing 0.5 µg/µl ethidium bromide.

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Table 1. DNA oligonucleotides used for polymerase chain reaction

Gene target	Primer name	Primer sequence	Fragment amplified (bp)
Plasmid	CTP 1	5′-TAGTAACTGCCACTTCATCA-3′[24]	201
	CTP 2	5′-TTCCCCTTGTAATTCGTTGC-3′	
momp	MOMP A	5′-TATACAAAAATGGCTCTCTGCTT-3′[25]	540
	MOMP B	5′-CCCATTTGGAATTCTTTATTCACATC-3′	
hsp60	Hsp 60 F	5′-GATGGTGTTACCGTTGCGA-3′[26]	650
	Hsp 60 R	5′- CCTCCACGAATTCTGTTCAC-3′	

Real-time PCR detection and quantitation of C. trachomatis DNA

Real-time PCR for detection and quantitation of *C. trachomatis* was performed on all DNA samples using a primer set (Hsp 60)[26] specific for the heat-shock protein 60 gene (Table 1). An aliquot of 1 μl of each sample was added to 9 μl of a reaction mixture containing 3 mM $MgCl_2$, 0.5 μM primers and 1 μl of LC DNA FastSTART Master SYBR Green I enzyme mix (Roche Biochemicals, Mannheim, Germany). Samples were amplified on a LightCycler (Roche) under the following cycle conditions: an initial 10 minutes at 95 °C for activation of the FastSTART *Taq* DNA polymerase, followed by 40 cycles of 5 seconds of denaturation at 95 °C, 10 seconds of annealing at 68 °C, decreasing to 65 °C at a rate of 1 °C/cycle and 30 seconds of extension at 72 °C. Data were obtained after the extension period in the single acquisition mode. The Hsp 60 PCR product was cloned into a pBSII vector and serially diluted cloned copies were used to create a standard curve (10^5 to 10^2 copies) for quantitation of chlamydial copy numbers. These standards were run with each LightCycler run and a calculation of *C. trachomatis* copy numbers was taken by the machine at the crossing point of each sample during the exponential phase of amplification. A melt curve step was included to confirm the amplification. Samples which gave copy numbers outside the range of the standards or whose melt-temperature (T_m) was outside those of the standards had their real-time PCR product analysed by agarose gel electrophoresis. A sample was positive if amplification of the 650 bp product occurred during the amplification programme of 40 cycles.

Results

DNA isolated from the 38 LCx-positive samples by the three extraction protocols was analysed for *C. trachomatis* positivity by PCR and real-time PCR and quantified using real-time PCR. A positive result for either the CTP or the MOMP primer set was determined by the presence of 201 bp product for the CTP primer set and a 540-bp product for the MOMP primer set on agarose gel electrophoresis. The Hsp 60 real-time PCR assay was positive if quantified *C. trachomatis* copy numbers were inside the range of the standards or if running of the product on an agarose gel gave the required 650 bp product size. 5 TE-Chelex, 5 Proteinase K-Chelex, and 2 QIAamp samples had copy numbers below the range of the standards (<100) but were positive on gel electrophoresis of the PCR product.

Detection of C. trachomatis by plasmid, major outer membrane protein and heat-shock protein 60 real-time PCR

Ninety-five percent (36/38) of samples extracted using either the TE-Chelex method or the Proteinase K-Chelex method were positive for *C. trachomatis* by either of the three PCRs. The QIAamp samples gave a slightly higher overall positivity rate of 97% (37/38) with a sensitivity of 95% for detection using plasmid PCR, 90% by MOMP PCR and 95% by Hsp 60 real-time PCR (Table 2). Of the QIAamp samples 90% (34/38) amplified for each of the three PCRs as opposed to 71% (27/38) of the Proteinase K-Chelex samples and only 50% (19/38) of the TE-Chelex samples (Table 2). The plasmid primers were the most successful for the amplification of *C. trachomatis* DNA extracted by each of the three methods, followed by the Hsp 60 primer set and then the MOMP primer set (Table 2).

Real-time LightCycler PCR analyses of C. trachomatis copy numbers

Real-time quantitation of *C. trachomatis* copy numbers in samples was carried out to determine if reduced sensitivity of detection by PCR was associated with low copy numbers of the organism and to determine the relative yield of *C. trachomatis* copy numbers for each extraction method.

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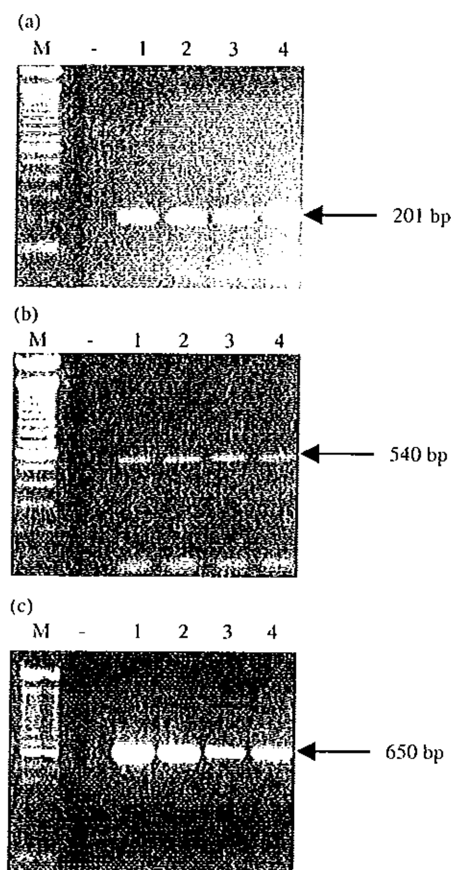

Figure 1. (a) Examples of PCR amplification of a region of *Chlamydia trachomatis* plasmid (201 bp) using the CTP primer set. (b) Examples of PCR amplification of *C. trachomatis* major outer membrane protein gene (540 bp) using the MOMP primer set. (c) Examples of PCR amplification of the *C. trachomatis* heat-shock protein 60 gene (650 bp) using the Hsp 60 primer set. M = 100 bp DNA marker; -: negative control; 1: positive control; 2–4: different samples.

The QIAamp and the Proteinase K-Chelex samples most commonly gave copy numbers of 10^4/μl of extracted DNA as opposed to the TE-Chelex samples which gave a lower yield of 10^2/μl of extracted DNA (Table 2). The distribution of copy numbers was evenly spread for the Proteinase K-Chelex samples, when compared with the TE-Chelex and QIAamp samples, which had copy numbers concentrated at the lower and upper end of the range respectively (Table 2).

Negative results by the plasmid and the MOMP PCRs were frequently associated with low copy numbers. Of the four TE-Chelex extracted samples negative by plasmid PCR, three were below the detection level of 100 copies of *C. trachomatis*/μl of extracted DNA and of the 15 MOMP-negative samples three samples had <1000 copies and 12 had <100 copies of *C. trachomatis* by quantitative PCR. Of the four QIAamp samples which did not amplify for all three genes, one sample was positive by Hsp 60 real-time PCR only, with a copy number of 10^2. The other two amplified for the plasmid but not the *momp* gene; the real-time PCR result was negative for one and copy numbers <100/μl DNA for the other).

Table 2. Comparison of positive PCR results for each extraction method using the CTP, MOMP and Hsp 60 primers, with quantification of *Chlamydia trachomatis* copy numbers by Hsp 60 real-time PCR

	No. of positive tests*		
Primer set	TE-Chelex (%)†	PK-Chelex (%)‡	QiAmp (%)§
CTP	34 (90)	35 (92)	36 (95)
MOMP	23 (61)	28 (74)	34 (90)
Hsp 60	28 (74)	32 (84)	36 (95)
Chlamydial copy/μl	No of positive tests in each copy number level		
<100	5	5	2
10^2	14	6	8
10^3	6	6	11
10^4	2	8	13
10^5	1	6	2
10^6	0	1	0

*All samples in the study were *C. trachomatis* positive by the LCx assay.
†Boiling in Tris-EDTA buffer followed by Chelex purification.
‡Proteinase K digestion followed by Chelex purification.
§QIAamp DNA extraction kit.

Discussion

The advent of liquid-based cervical cytology may enable gynaecologists to screen for both cervical preneoplastic lesions and sexually transmitted infectious agents from the same sample. This is based on the ability of liquid-fixed cells to yield nucleic acids suitable for molecular-based assays.[27,28] There are a growing number of publications using PreservCyt specimens for molecular detection of organisms including HPV, herpes simplex viruses, *Trichomonas vaginalis* and *C. trachomatis*.[29,30] As DNA extracted from PreservCyt specimens may be used to screen for multiple organisms there is a need for the development of simple rapid inexpensive DNA extraction methods,

which can readily be applied in the diagnostic setting. The main aim of this study therefore was to compare the efficiency of two inexpensive in-house DNA extraction procedures (Tris-EDTA-Chelex and Proteinase K-Chelex) and one kit-based method (QIAamp) for the detection of *C. trachomatis* by PCR.

The results of this study indicate that the commercial QIAamp extraction kit was the most successful extraction method for amplification of the three different target genes, but that the Proteinase K-Chelex method had a similar success rate for *C. trachomatis* amplification when the plasmid primers were used. However, amplification of larger PCR products was less successful from Proteinase K-Chelex than with the commercial extraction kit which would have implications for application of other molecular methods, particularly restriction enzyme digestion for restriction fragment length polymorphism analysis.

In this study there was 90%, 92% and 95% sensitivity of *C. trachomatis* detection by plasmid PCR amplification by TE-Chelex, Proteinase K-Chelex and QIAamp respectively. Koumans *et al.* also analysed PreservCyt samples for *C. trachomatis* detection using a commercial extraction and detection system and reported 97% sensitivity for *C. trachomatis* detection.[11] On comparison of commercial DNA extraction kits QIAamp has been reported as the most successful.[31] In this study amplification for the plasmid gene was the most successful method for detection of *C. trachomatis* and showed the greatest concordance for the three extraction methods. Other studies have shown that plasmid primers are more sensitive than MOMP primers for the detection of *C. trachomatis* due to the presence of multiple plasmids per organism.[32,33]

Some studies have reported lack of reproducibility of *C. trachomatis* detection, even with commercial systems, and recommend duplicate samples or the application of two NAAT detection methods.[34] In this study all samples were amplified for three different genes to increase the specificity of detection. This study recommends the combined use of the plasmid and heat-shock protein 60 gene primers for PCR detection of *C. trachomatis*.

Real-time PCR is a fast and effective way for the detection and quantitation[35] of bacterial load in clinical samples and for validation of DNA extraction methods. Real-time PCR quantitation was used in this study to show that a drop off in sensitivity of detection, particularly for the larger PCR products, was associated with low copy numbers of *C. trachomatis*.

This study shows that a single PreservCyt cervical specimen can be used as a source of high quality DNA for testing for sexually transmitted infections. Optimization of the method of DNA extraction from PreservCyt is essential to avoid false negatives and ensure adequate sensitivity of detection. Careful selection of genes to be amplified and the PCR product size for the detection of *C. trachomatis* is recommended. Real-time PCR quantitation is a valuable method for validation of the sensitivity of PCR detection methods.

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References

1. Costa S, Sideri M, Syrjanen K *et al.* Combined Pap smear, cervicography and HPV DNA testing in the detection of cervical intraepithelial neoplasia and cancer. *Acta Cytol* 2000;44:310–8.
2. Alasio LM, Alphandery C, Grassi P *et al.* Performance of the AutoPap Primary Screening System in the detection of high-risk cases in cervicovaginal smears. *Acta Cytol* 2001;45:704–8.
3. Monsonego J, Autillo-Touati A, Bergeron C *et al.* Liquid-based cytology for primary cervical cancer screening: a multi-centre study. *Br J Cancer* 2001;**84**:360–6.
4. Wang N, Emancipator SN, Rose P, Rodriguez M, Abdul-Karim FW. Histologic follow-up of atypical endocervical cells. Liquid-based, thin-layer preparation vs. conventional Pap smear. *Acta Cytol* 2002;**46**:453–7.
5. Murphy N, Ring M, Killalea AG *et al.* p16INK4A as a marker for cervical dyskaryosis: CIN and cGIN in cervical biopsies and ThinPrep smears. *J Clin Pathol* 2003;**56**: 56–63.
6. Cubie HA, Seagar AL, McGoogan E *et al.* Rapid real time PCR to distinguish between high risk human papillomavirus types 16 and 18. *Mol Pathol* 2001;54:24–9.
7. Tucker RA, Unger ER, Holloway BP *et al.* Real-time PCR-based fluorescent assay for quantitation of human papillomavirus types 6, 11, 16, and 18. *Mol Diagn* 2001;6: 39–47.
8. Schiffman M, Castle PE. Human papillomavirus: epidemiology and public health. *Arch Pathol Lab Med* 2003;**127**: 930–4.
9. van Duin M, Snijders PJ, Schrijnemakers HF *et al.* Human papillomavirus 16 load in normal and abnormal cervical

scrapes: an indicator of CIN II/III and viral clearance. *Int J Cancer* 2002;**98**:590–5.

10. Bianchi A, Moret F, Desrues M *et al.* PreservCyt transport medium used for the ThinPrep Pap test is a suitable medium for detection of *Chlamydia trachomatis* by the COBAS Amplicor CT/NG test: results of a preliminary study and future implications. *J Clin Microbiol* 2002;**40**:1749–54.
11. Koumans EH, Black CM, Markowitz LE *et al.* Comparison of methods for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* using commercially available nucleic acid amplification tests and a liquid pap smear medium. *J Clin Microbiol* 2003;**41**:1507–11.
12. Wilson JS, Honey E, Templeton A *et al.* A systematic review of the prevalence of *Chlamydia trachomatis* among European women. *Hum Reprod Update* 2002;**8**:385–94.
13. Howell MR, Quinn TC, Gaydos CA. Screening for *Chlamydia trachomatis* in asymptomatic women attending family planning clinics. A cost-effectiveness analysis of three strategies. *Ann Intern Med* 1998;**128**:277–84.
14. Howell MR, Quinn TC, Braithwaite W, Gaydos CA. Screening women for *Chlamydia trachomatis* in family planning clinics: the cost-effectiveness of DNA amplification assays. *Sex Transm Dis* 1998;**25**:108–17.
15. Howell MR, Gaydos JC, McKee KT Jr, Quinn TC, Gaydos CA. Control of *Chlamydia trachomatis* infections in female army recruits: cost-effective screening and treatment in training cohorts to prevent pelvic inflammatory disease. *Sex Transm Dis* 1999;**26**:519–26.
16. Black CM. Current methods of laboratory diagnosis of *Chlamydia trachomatis* infections. *Clin Microbiol Rev* 1997;**10**:160–84.
17. Pasternak R, Vuorinen P, Pitkajarvi T *et al.* Comparison of manual amplicor PCR, Cobas Amplicor PCR, and LCx assays for detection of *Chlamydia trachomatis* infection in women by using urine specimens. *J Clin Microbiol* 1997;**35**:402–5.
18. Land S, Tabrizi S, Gust A *et al.* External quality assessment program for *Chlamydia trachomatis* diagnostic testing by nucleic acid amplification assays. *J Clin Microbiol* 2002;**40**:2893–6.
19. Girdner JL, Cullen AP, Salama TG *et al.* Evaluation of the digene hybrid capture II CT-ID test for detection of *Chlamydia trachomatis* in endocervical specimens. *J Clin Microbiol* 1999;**37**:1579–81.
20. Gopalkrishna V, Francis A, Sharma JK, Das BC. A simple and rapid method of high quantity DNA isolation from cervical scrapes for detection of human papillomavirus infection. *J Virol Methods* 1992;**36**: 63–72.
21. Jacobs MV, Zielinski D, Meijer CJ *et al.* A simplified and reliable HPV testing of archival Papanicolaou-stained cervical smears: application to cervical smears from cancer patients starting with cytologically normal smears. *Br J Cancer* 2000;**82**:1421–6.
22. Lou YK, Qin H, Molodysky E, Morris BJ. Simple microwave and thermal cycler boiling methods for preparation of cervicovaginal lavage cell samples prior to PCR for human papillomavirus detection. *J Virol Methods* 1993;**44**:77–81.
23. Cuschieri KS, Seagar AL, Moore C *et al.* Development of an automated extraction procedure for detection of human papillomavirus DNA in liquid-based cytology samples. *J Virol Methods* 2003;**107**:107–13.
24. Griffais R, Thibon M. Detection of *Chlamydia trachomatis* by the polymerase chain reaction. *Res Microbiol* 1989;**140**:139–41.
25. Mittal A. Serovar distribution of *Chlamydia trachomatis* isolates collected from the cervix: use of the polymerase chain reaction and restriction endonuclease digestion. *Br J Biomed Sci* 1998;**55**:179–83.
26. Wood H, Reischl U, Peeling RW. Rapid detection and quantification of *Chlamydia trachomatis* in clinical specimens by LightCycler PCR. In: *Rapid Cycle Real-Time PCR – Methods and Applications*. Reischl U, Witter C, Cockerill F (eds). New York: Springer; 2002: 115 pp.
27. Habis AH, Vernon SD, Lee DR, Verma M, Unger ER. Molecular quality of exfoliated cervical cells: implications for molecular epidemiology and biomarker discovery. *Cancer Epidemiol Biomarkers Prev* 2004;**13**:492–6.
28. Tarkowski TA, Rajeevan MS, Lee DR, Unger ER. Improved detection of viral RNA isolated from liquid-based cytology samples. *Mol Diagn* 2001;**6**:125–30.
29. Lanham S, Herbert A, Basarab A, Watt P. Detection of cervical infections in colposcopy clinic patients. *J Clin Microbiol* 2001;**39**:2946–50.
30. Fiel-Gan MD, Villamil CF, Mandavilli SR *et al.* Rapid detection of HSV from cytologic specimens collected into ThinPrep fixative. *Acta Cytol* 1999;**43**:1034–8.
31. Berg HF, Maraha B, Bergmans AM *et al.* Extraction of *Chlamydia pneumoniae* DNA from vascular tissue for use in PCR: an evaluation of four procedures. *Clin Microbiol Infect* 2003;**9**:135–9.
32. Mahony JB, Luinstra KE, Sellors JW, Chernesky MA. Comparison of plasmid- and chromosome-based polymerase chain reaction assays for detecting *Chlamydia trachomatis* nucleic acids. *J Clin Microbiol* 1993;**31**:1753–8.
33. Roosendaal R, Walboomers JM, Veltman OR *et al.* Comparison of different primer sets for detection of *Chlamydia trachomatis* by the polymerase chain reaction. *J Med Microbiol* 1993;**38**:426–33.
34. Castriciano S, Luinstra K, Jang D *et al.* Accuracy of results obtained by performing a second ligase chain reaction assay and PCR analysis on urine samples with positive or near-cutoff results in the LCx test for *Chlamydia trachomatis*. *J Clin Microbiol* 2002;**40**:2632–4.
35. Tondella ML, Talkington DF, Holloway BP *et al.* Development and evaluation of real-time PCR-based fluorescence assays for detection of *Chlamydia pneumoniae*. *J Clin Microbiol* 2002;**40**:575–83.

assisted laser desorption/ionization (MALDI)-TOF MS, with its higher resolution, is the capability of SELDI to enrich low-abundance proteins from complex matrices such as plasma, through, for example, the coupling of specific antibodies to the chip surfaces *(4)*. Such an approach is an important methodologic aspect in "second phase" proteomics, which are characterized by repetitive investigation of the same protein to validate the protein phenotype in large population-based studies. This provides a basis for diagnostic progress in personalized medicine *(5)*. For TTR, such an approach is relevant not only in the diagnosis of TTR-related amyloidosis but also in other diseases.

The true problem with on-chip immunopurification is not the resolution of the MS, which can be solved by use of specific available interfaces, but the efficient coupling of the antibody to the chip surface. When an on-chip immunoassay format is being used, it is important that the protein chip retains the antibody in an active state at high density. Results are greatly affected by functionality characteristics, such as the stability, affinity, and specificity of the antibody. On the basis of studies relating to microarrays, only 5%–20% of commercially available antibodies are suitable for one or the other microarray format *(6)*.

Florian J. Schweigert*
Jens Raila

Department of Physiology and Pathophysiology
University of Potsdam
Potsdam-Rehbrucke, Germany

*Address correspondence to this author at: Institute of Nutritional Science, University of Potsdam, Scheunert-Allee 114-116, Potsdam-Rehbrucke, D-14558 Germany. E-mail fjschwei@rz.uni-potsdam.de.

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References

1. Theberge R, Connors LH, Skinner M, Costello CE. Detection of transthyretin variants using immunoprecipitation and matrix-assisted laser desorption/ionization bioreactive probes: a clinical application of mass spectrometry. J Am Soc Mass Spectrom 2000;11:172–5.
2. Wang Z, Yip C, Ying Y, Wang J, Meng XY, Lomas L, et al. Mass spectrometric analysis of protein markers for ovarian cancer. Clin Chem 2004;50:1939–42.
3. Schweigert FJ, Wirth K, Raila J. Characterization of the microheterogeneity of transthyretin in plasma and urine using SELDI-TOF-MS immunoassay. Proteome Sci 2004;2:5.
4. Schweigert FJ. Characterization of protein microheterogeneity using mass spectrometry based immunoassays. Brief Funct Genomic Proteomic 2005;4:7–15.
5. Hanash S. Disease proteomics. Nature 2003;422:226–32.
6. MacBeath G. Protein microarrays and proteomics. Nat Genet 2002;32:526–32.

Validation of a Multiplex PCR Assay for the Simultaneous Detection of Human Papillomavirus and *Chlamydia trachomatis* in Cervical PreservCyt Samples

To the Editor:

Chlamydia trachomatis is the most common sexually transmitted bacterium worldwide *(1)* and a leading cause of infertility in women *(2)*. Human papillomaviruses (HPVs) are the most important single agent causing carcinoma of the uterine cervix *(3)*. Combined molecular screening for *C. trachomatis* and HPV could be justified given their propensity to cause asymptomatic infections, particularly in high-risk groups. Features of HPV infection of cells of the uterine cervix are traditionally reported by the Pap smear method *(4)*. The introduction of liquid-based cytology, such as the ThinPrep® Pap Test™, has had the effect of improving the sensitivity of conventional cytologic screening with the potential for HPV testing of residual cellular material in borderline or difficult cases *(5–7)*. The US Food and Drug Administration (FDA) has recently cleared a hybrid capture-based system (HCII; Digene) for screening women over 30 years of age as an adjunct to Pap testing *(8)*. Researchers have developed consensus primers for the detection of HPV DNA by PCR *(9, 10)*. We developed and evaluated a multiplex PCR for the simultaneous detection of HPV and *C. trachomatis* from PreservCyt™ (ThinPrep) solution.

The multiplex PCR was performed on 100 cervical PreservCyt fluid specimens collected from women attending their general practitioners for routine cervical screening. We used the MY09/11 primers *(9)* for HPV and plasmid primers for *C. trachomatis (11)*, which generated fragments of 450 and 201 bp, respectively. Primers for the human *β-globin* gene were included in the multiplex as an internal DNA amplification control, generating a 110-bp product *(12)* (see Fig. 1 in the Data Supplement that accompanies the online version of this Letter at http://www.clinchem.org/content/vol51/issue7/). The PCR mixture contained 5 pmol of each of the forward and reverse primers of the MY09/11 and CTP1/2 primer sets and 10 pmol of each of the forward and reverse primers of the PCO3/4 primer set, 200 μM deoxynucleoside triphosphates, 10× PCR buffer [containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl], 2 mM $MgCl_2$, and 1 U of Platinum *Taq* DNA polymerase (Invitrogen Ltd.) in a final volume of 20 μL. The PCR was initiated by a 10-min denaturation and enzyme activation step at 95 °C and was completed by a 10-min extension step at 72 °C. The

Table 1. Comparison of multiplex and single PCR for the detection of HPV and *C. trachomatis* in PreservCyt cervical samples.

	Multiplex positive, n		Single PCR positive, n			
Sample cohort	MY09/11[a]	CTP1/2[b]	MY09/11	GP5+/6+[a]	CTP1/2	Hsp60[b]
GP[c] clinic (n = 100)	21	2	24	14	2	2
GUM clinic (n = 34)	10	34	10	10	34	34

[a] Primers for the detection of HPV.
[b] Primers for the detection of *C. trachomatis*.
[c] Women who attended general practitioner (GP) clinics for routine cervical screening.

temperature cycles were as follows: 40 cycles of 30 s at 95 °C, 1 min at 57 °C, and 1 min at 72 °C.

The detection limit was estimated on serial dilutions, from 10^8 to 10^1 copies/μL, of cloned HPV MY09/11 and the *C. trachomatis* CTP PCR product in the pCR® 2.1-TOPO® (Invitrogen Ltd). To evaluate the sensitivity and specificity of the multiplex PCR for the detection of each organism, we performed single PCRs on all samples: one PCR that detects HPV by use of the GP5+/6+ primers *(10)*, generating a 150-bp product; and one that detects *C. trachomatis* by use of primers specific to the *hsp60* gene of *C. trachomatis* (sense, 5'-GAT GGT GTT ACC GTT GCG A-3'; antisense, 5'-TAA TAA TCG TCT TTA ACA ACG T-3'), generating a truncated version (309 bp) of a previously described product *(13)*. PCRs were also performed with the MY09/11 and CTP1/2 primer sets singly.

In the screened population, 21% (21 of 100) of samples were positive for HPV by the multiplex PCR (Table 1). The MY09/11 primers identified 3 other samples as HPV positive that were not detected by the multiplex assay (Table 1). Either the MY09/11 or the GP5+/6+ primers confirmed all 21 of the samples positive by the multiplex assay. Two samples were positive for *C. trachomatis* in the multiplex assay (Table 1). These were confirmed positive by the CTP1/2 and Hsp60 primers in single PCRs, and no additional positive samples were detected. A positive sample was defined as positive by either the multiplex or any of the single PCRs for that organism. The sensitivity and specificity of the multiplex with respect to single PCR for the detection of HPV in the opportunistically screened samples were 89% (95% confidence interval, 70%–97%) and 100% (94%–100%; Table 1). The multiplex assay was 100% specific and sensitive for the detection of *C. trachomatis* with respect to single PCR in the screened population (Table 1).

To estimate the diagnostic sensitivity of the multiplex assay for the detection of *C. trachomatis*, we performed multiplex PCR on 34 PreservCyt fluid specimens from women attending a genitourinary (GUM) outpatient clinic who had tested positive for *C. trachomatis* by the LCx (Abbott Laboratories). The sensitivity of the multiplex assay for the detection of *C. trachomatis* in the GUM clinic population was 100% with respect to the commercial LCx assay (Table 1). Ten of the 34 samples were positive for HPV by the multiplex assay. These results were confirmed by single PCRs (Table 1). The multiplex assay could detect as few as 100 copies of the *C. trachomatis* plasmid and 100 copies of the HPV genome per microliter of extracted DNA.

This is the first study looking at the detection of both HPV and *C. trachomatis* from a single ThinPrep sample by use of multiplex PCR. This simple multiplex is rapid and could be used to screen cervical ThinPrep samples for both HPV and *C. trachomatis*, particularly in a high-risk population.

References

1. World Health Organization. State of the art of new vaccines research and development: Initiative for Vaccine Research. Geneva: WHO, April 2003:21. http://www.who.int/vaccine_research/documents/en/stateofart_excler.pdf (accessed October 10, 2004).
2. Mardh PA. Tubal factor infertility, with special regard to chlamydial salpingitis. Curr Opin Infect Dis 2004;17:49–52.
3. Munoz N. Human papillomavirus and cancer: the epidemiological evidence. J Clin Virol 2000; 19:1–5.
4. Renshaw AA, Young AN, Birdsong G, Styer P, Davey D, Mody D, et al. Comparison of performance of conventional and ThinPrep gynecologic preparations in the College of American Pathologists Gynecologic Cytology Program. Arch Pathol Lab Med 2004;128:17–22.
5. Hoelund B. Implementation of liquid-based cytology in the screening programme against cervical cancer in the County of Funen, Denmark, and status for the first year. Cytopathology 2003;14:269–74.
6. Cheung A, Szeto NE, Leung B, Khoo U, Ng A. Liquid-based cytology and conventional cervical smears: a comparison study in an Asian screening population. Cancer 2003;99:331–5.
7. Schiffman M, Solomon D. Findings to date from the ASCUS-LSIL Triage Study (ALTS). Arch Pathol Lab Med 2003;127:946–9.
8. FDA News. FDA approves expanded use of HPV test. Washington: FDA, March 31, 2003. http://www.fda.gov/bbs/topics/news/2003/new00890.html (accessed October 10, 2004).
9. Manos MM, Ting Y, Wright D, Lewis A, Broker T, Wolinsky S. Use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. Cancer Cells 1989;7:209–14.
10. de Roda Husman AM, Walboomers JM, van den Brule AJ, Meijer CJ, Snijders PJ. The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. J Gen Virol 1995;76:1057–62.
11. Griffais R, Thibon M. Detection of *Chlamydia trachomatis* by the polymerase chain reaction. Res Microbiol 1989;140:139–40.
12. Saiki RK, Scharf S, Faloona F, Mullis K, Horn G, Erlich H, et al. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia. Biotechnology 1992;24:476–80.
13. Wood H, Reischl U, Peeling R. Rapid detection and quantification of *Chlamydia trachomatis* in clinical specimens by LightCycler PCR. In: Reischl U, Wittwer C, Cockerill F, eds. Rapid cycle real-time PCR—methods and applications. New York: Springer, 2002:115–32.

Helen Keegan[1]
Alison Malkin[2]
Mairead Griffin[2]
Fergus Ryan[1]
Helen Lambkin[1*]

[1] *Biomedical Research Group*
School of Biological Sciences
Dublin Institute of Technology
Dublin, Ireland

[2] *Department of Clinical Cytology*
Central Pathology Laboratory
St. James' Hospital
Dublin, Ireland

*Address correspondence to this author at: School of Biological Sciences, Dublin Institute of Technology, Kevin St., Dublin 8, Ireland. Fax 353-1-402-4995; e-mail helen.lambkin@dit.ie.

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Detection of Heavy Chain Disease by Capillary Zone Electrophoresis

To the Editor:

Luraschi et al. *(1)* recently published the first report describing the use of capillary zone electrophoresis (CZE) coupled with immunosubtraction to detect and characterize low concentrations of free γ heavy chains in serum. By contrast, we describe a case in which CZE failed to detect and characterize μ heavy chain disease.

A 90-year-old woman who complained of weight loss presented with progressive enlargement of the left parotid gland, splenomegaly, and palpable inguinal lymph node