Comparison of HPV detection technologies: Hybrid capture 2, PreTect™ HPV-Proofer and analysis of HPV DNA viral load in HPV16, HPV18 and HPV33 E6/E7 mRNA positive specimens

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ABSTRACT

Human papillomavirus (HPV) testing using molecular methods in liquid based cytology (LBC) specimens may be useful as an adjunct to cervical screening by cytology. We compared the positivity rate of the commercially available HPV DNA method hybrid capture 2 (hc2) and the commercially available E6/E7 mRNA method PreTect™ HPV-Proofer in cytological specimens ($n = 299$).

LBC specimens collected ($n = 299$) represented the following cervical cytological disease categories: Normal ($n = 60$), borderline nuclear abnormalities (BNA) ($n = 34$), CIN1 ($n = 121$), CIN2 ($n = 60$), CIN3 ($n = 24$). Overall, 69% (205/299) of the cases were positive by hc2 and 38% (112/299) of the cases were positive by PreTect™ HPV-Proofer. Concordance rates between the two tests were highest in the high-grade cytology cases (CIN2: 67% and CIN3: 83%) and the normal cytology cases (88%) and lowest in the BNA and CIN1 categories (56% and 52%). HPV DNA viral load analyses were carried out on HPV16 ($n = 55$), HPV18 ($n = 9$) and HPV33 ($n = 13$) samples that were positive by PreTect™ HPV-Proofer.

The sensitivity and specificity of PreTect™ HPV-Proofer and the hc2 DNA test for the detection of high-grade cytology (i.e. CIN2+) were 71.4% and 75.8% vs 100% and 43.7%, respectively.

The relatively low detection rate observed by PreTect™ HPV-Proofer in the whole range of cytological positive cases, combined with a relatively higher specificity and PPV, suggests that PreTect™ HPV-Proofer may be more useful than hc2 for triage and in predicting high-grade disease.

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1. Introduction

Cervical cancer is the second most common cause of mortality due to cancer in women worldwide. Cervical cytology screening for cervical intraepithelial neoplasia (CIN) has reduced the incidence of mortality worldwide and most dramatically in countries where cervical screening programmes have been implemented (van der Aa et al., 2008; Gunnell et al., 2007). However, cervical cytology has limited sensitivity and despite the introduction of liquid based cytology (LBC), the sensitivity of cytological testing for cervical disease relies on multiple screening events and referral to colposcopy, in particular in cases of low-grade or borderline cytology (Wright et al., 2006).

Infection with oncogenic human papillomavirus (HPV) is the main causative agent in the development of cervical cancer. HPV can be detected in 99.7% of invasive cervical carcinomas and high-risk HPV genotypes 16, 18, 31, 33 and 45 are the most commonly identified types in cervical tumours worldwide (Muñoz, 2000; Clifford et al., 2006). Therefore, it has been proposed that the performance of cervical screening programmes could be enhanced through the incorporation of molecular HPV testing (Cuschieri and Cubie, 2005). Most commercially available HPV tests are DNA based and involve the detection of a region of the L1 major capsid-forming gene. The hybrid capture test (hc2), Digene Ltd., UK, is a hybridisation based assay which has the ability to detect HPV DNA from 13 high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and

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68. hc2 is currently the only HPV test that has USA Food and Drugs Administration (FDA) approval for in vitro diagnostic use and has been approved for use as an adjunct to cervical screening in the US in women aged 30 years and over (Wright et al., 2004). However, as the overall prevalence of HPV DNA in cervical smear specimens is very high, particularly in young women (Coupé et al., 2008; Insinga et al., 2007; Keegan et al., 2007), and as most infections are transient, the potential use of pan-HPV DNA testing for early detection of cervical cancer is limited and would only marginally reduce the follow-up colposcopy and histology. This highlights the need to investigate additional prognostic markers such as HPV E6/E7 mRNA and HPV viral load for use in cervical screening programmes.

Unlike the hc2 test, which detects HPV DNA, the PreTect™ HPV-Proofer test, developed by NorChip AS (Klokkarstua, Norway), has the ability to detect HPV E6/E7 mRNA transcripts from the five most commonly found oncogenic HPV types: 16, 18, 31, 33 and 45. Expression of E6 and E7 mRNA from high-risk HPV types and the production of functional oncoproteins has been found to be necessary for conversion to malignancy, through the modulation of a number of host tumour suppressor and host regulatory products, such as p53 and retinoblastoma (pRB) (Münger and Howley, 2002). The PreTect™ NASBA assay has also been adapted to a diagnostic biochip format for the detection of HPV16 mRNA (Gulliksen et al., 2005). A further generation of this biochip is under development by the MicroActive Consortium (www.sintef.no/microactive). As the oncogenic potential of an HPV infection depends on the production of E6/E7 viral oncoproteins, the detection of HPV E6/E7 mRNA transcripts may be a more specific test in the determination of underlying pre-cancer disease and the risk potential of an HPV infection.

HPV viral load determination has been suggested as a biomarker for use in the area of cervical screening with adjunctive HPV testing, however its potential application has not yet been determined. Numerous studies have been performed to determine the significance of HPV viral load as a diagnostic or prognostic indicator. It has been postulated that the high viral load resulting from active viral replication may support viral persistence (Ylitalo et al., 2000). Viral load has also been used to determine the likelihood of lesional regression and viral clearance in abnormal cytology (van Duin et al., 2002). The significance of HPV viral load has been particularly associated with HPV16 viral load determination. Some studies have demonstrated that the grade of cervical disease is associated with HPV16 viral load (Lo et al., 2005) and HPV16 viral load has been shown to have the potential to predict the risk of cervical cancer before the development of CIN (Ylitalo et al., 2000; Josefsson et al., 2000). However, other studies have shown that in cervical carcinoma cases, patients with a higher viral load had a more favourable prognosis (Biedermann et al., 2004). This may be related to the process of viral integration into the human genome, which commonly is associated with viral episome loss (Pett et al., 2004). Thus, a correlation between viral load and prognosis may be found up to CIN 1 and 2; however, for the further progression towards CIN3 and cervi-

cal cancer, viral load does not seem to be significant as a prognostic indicator.

In this study, the use of hc2 and PreTect™ HPV-Proofer for the detection of HPV in cervical LBC specimens and in a subset of specimens with histological diagnosis were compared. HPV16, HPV33 or HPV18 DNA viral load in patients with a positive HPV E6/E7 mRNA result was also examined.

2. Materials and methods

2.1. Clinical specimens

Clinical specimens were obtained through the Department of Cytology at the Coombe Women's Hospital, Dublin, Ireland, during 2006. All samples were anonymised and ethical approval for the study was obtained from the Research Ethics Committee at the Coombe Women's Hospital. Cytological diagnosis was performed by specialised cytotechnologists and cytopathologists and diagnosis was consistent with BSCC (British Society for Clinical Cytology) guidelines for classification of abnormal smears (Evans et al., 1986). In total, 299 specimens were examined. The cytological diagnosis for these samples ranged from normal ($n = 60$) and BNA (borderline nuclear abnormalities) ($n = 34$) to CIN grades 1 ($n = 121$), 2 ($n = 60$) and 3 ($n = 24$). In Bethesda terminology, BNA corresponds to atypical squamous cells of undetermined significance (ASCUS), CIN1 corresponds to Low-Grade Squamous Intraepithelial Lesion (LSIL) while CIN2 and CIN3 correspond to High-Grade Squamous Intraepithelial Lesion (HSIL). Histological results following cervical biopsy were available for patients who underwent colposcopy following cytology ($n = 58$). Following preparation of a smear for cytological testing, total nucleic acids or DNA alone were extracted as described below.

2.2. HPV DNA testing by hybrid capture 2

DNA was extracted from an aliquot of 4 mL PreservCyt sample using the Sample Conversion Kit (Digene Ltd., UK) for HPV testing by hybrid capture (hc2, Digene Ltd., UK). The HPV DNA status of the specimens was assessed using the hc2 HPV kit for high-risk HPV detection of types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68, according to manufacturer's guidelines.

2.3. HPV E6/E7 mRNA testing by PreTect™ HPV-Proofer

An aliquot of 5 mL PreservCyt was processed for total nucleic acid extraction using the Qiagen M48 BioRobot™ extraction method (Qiagen Ltd., UK). Cell lysis was performed prior to BioRobot™ extraction. Briefly, cells were centrifuged for 12 min @ $1\,130 \times g$, and washed in 1 mL 100% ethanol. Qiagen lysis buffer (Buffer RLT: 400 μL) was added to the cell pellet and the sample was vortexed for 1 min. Samples were transferred to the M48 BioRobot for nucleic acid extraction using the "Custom NorChip" programme in the Qiasoft™ software and the MagAttract™ RNA Cell Mini M48 kit (Qiagen Ltd., UK). Sample volume was set to 400 μL and elution volume was set to 50 μL. Extracted nucleic acids were stored at −80 °C. Samples were assessed for HPV E6/E7 mRNA from types 16, 18, 31, 33 and 45 and for an internal housekeeping control gene U1A, using the PreTect™ HPV-Proofer kit (NorChip AS, Norway), according to manufacturer's guidelines.

2.4. HPV16, HPV33 and HPV18 TaqMan® quantitative PCR

PCR primers and TaqMan® MGBNFQ (minor groove binders non-fluorescent quenchers) probes to the E6 region of HPV16, HPV33 and HPV18 genome were designed using Primer Express

Table 1
Sequences of HPV TaqMan® primers and probes.

Primer/Probe	Sequence (5′–3′)	Length (bp)	Dye
HPV16 E6F	gaacaacattagaacagcaatacaacaa	28	–
HPV16 E6R	tggcttttgacagttaatacacctaatt	28	–
HPV16 TP	ccgttgtgtgatttgt	16	6-FAM MGBNFQ
HPV33 E6F	tgatttgtgccaagcattgg	20	
HPV33 E6R	ttttgcattccacgcactg	19	
HPV33 TP	agacaactatacacaacattgaacta	26	6-FAM MGBNFQ
HPV18 E6F	gaggccagtgccattcgt	18	–
HPV18 E6R	tgtttctctgcgtcgttgga	20	–
HPV18 TP	caaccgagcacgacag	16	6-FAM MGBNFQ

Software Version 2.0 (Applied Biosystems, Foster City, CA, USA) (Table 1), and their specificities were confirmed using BlastN (www.ncbi.nlm.nih.gov/blast). All TaqMan® PCR probes were labeled with fluorescein molecule FAM at the 5′ end (Table 1).

The β-actin housekeeping gene was used as performance and integrity control in a TaqMan® PCR using the TaqMan® β-actin Control Reagents (Cat. No. AB 401846) from Applied Biosystems (Foster City, CA, USA). For HPV16 and HPV18, individual TaqMan® PCR reactions were performed in triplicate in a total volume of 25 μL using 1X Universal PCR Master Mix (Cat. No. AB4304437), 300 nM primers, 200 nM of TaqMan® probe and 1 μL of nucleic acid template, and for β-actin using 200 nM primers and 100 nM of TaqMan® probe. For HPV33, TaqMan® PCR was performed in triplicate in a total volume of 20 μL using 1X Universal PCR Master Mix, 300 nM primers, 200 nM of TaqMan® probe and 1 μL of nucleic acid template. TaqMan® PCRs were performed on an Applied Biosystems 7500 thermal cycler using the recommended Universal PCR Master Mix cycling conditions which were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 20 s, 60 °C for 1 min. Controls for TaqMan® PCRs included: no template control (water added as template), human control DNA, HPV16, HPV33 and HPV18 E6 plasmid clones. HPV copy number normalization was carried out using β-actin as a housekeeping gene. HPV quantitative TaqMan® PCR was performed by generating standard curves for HPV16, HPV33 and HPV18 E6 genes. This was achieved using serial dilutions of clones of E6 generated from HPV16, HPV33 and HPV18 over a linear dynamic range of 1×10^6 to 1×10^0 copies of HPV per reaction. Total nucleic acid extracted from cytology specimens as described above was amplified and quantified off standard curves.

2.5. Data analysis

HPV detection rates, concordance rates, and sensitivity, specificity and positive predictive values for detecting cytological abnormalities, CIN2+ were calculated for both HPV tests hc2 and PreTect™ HPV-Proofer. As histological data was not available for all cases and the primary objective was to compare HPV tests for the detection of cytological abnormalities we used cytology as the gold-standard test for the majority of these calculations except where otherwise stated. Sensitivity and specificity were calculated using the following formula whereby:

	Test +	Test −	Sum
Gold standard +	*a*	*c*	*g*
Gold standard −	*b*	*d*	*h*
Sum	*e*	*f*	*n*

Population size: *n*, sensitivity: *a/g*, PPV: *a/e*, specificity: *d/h*.

3. Results

3.1. Comparison of HPV detection by hybrid capture 2 (hc2) and PreTect™ HPV-Proofer

Cytological diagnoses of specimens recruited to the study are outlined in Table 2. Cytological diagnosis was performed by specialised cytotechnologists and cytopathologists and diagnosis was

Table 2
Cytological diagnosis of cervical specimens (*n* = 299).

Cytology	Normal	BNA[a]	CIN[b] 1	CIN2	CIN3
No. of samples (*n* = 299)	60 (20%)	34 (11%)	121 (41%)	60 (20%)	24 (8%)

[a] Borderline nuclear abnormality.
[b] Cervical intraepithelial neoplasia.

Table 3
Comparison of HPV detection technologies for the detection of HPV in cytology specimens (*n* = 299).

| Cytology	HC2		PreTect™ HPV-Proofer		Concordance
	+	−	+	−	
Normal (*n* = 60)	5/60	55/60	2/60	58/60	88%
BNA[a] (*n* = 34)	16/34	18/34	5/34	29/34	19%
CIN1[b] (*n* = 121)	100/121	21/121	45/121	76/121	40%
CIN2 (*n* = 60)	60/60	–	40/60	20/60	67%
CIN3 (*n* = 24)	24/24	–	20/24	4/24	83%

[a] Borderline nuclear abnormality.
[b] Cervical intraepithelial neoplasia.

consistent with BSCC (British Society for Clinical Cytology) guidelines for classification of abnormal smears (Evans et al., 1986) which uses the following categories to grade cytological specimens Normal, CIN grades 1–3 and Borderline Nuclear Abnormalities (BNA). The HPV status of the clinical specimens was determined by hc2 and by PreTect™ HPV-Proofer.

The overall prevalence of HPV in the study sample population (*n* = 299) was 68.6% by hc2 and 37.5% by PreTect™ HPV-Proofer. HPV DNA was detected in a higher percentage of specimens from each cytological category than HPV mRNA (Table 3). HPV DNA was detected in 8.3% of normal, 47.1% of BNA, 83% of CIN1, and 100% of CIN2 and CIN3 specimens, while HPV mRNA was detected in 3.3% of normal, 14.7% of BNA, 37% of CIN1, 67% of CIN2 and 83% of CIN3 specimens. There was good concordance between the methods for the detection of HPV in cases with high-grade cytology CIN3 (83%) and normal cytology (88%) (Table 3; Fig. 1). Concordance rates in the CIN2 category were 67% while the BNA/CIN1 category was lower at 19% and 40%, respectively.

3.2. Clinical sensitivity and specificity of hc2 and PreTect™ HPV-Proofer

In this cohort of specimens, which represents a broad spectrum of cervical pre-cancer disease categories, the positive predictive value and specificity of the hc2 DNA test was 97.6% and 91.7%, respectively, compared with 98.2% and 96.7% of the PreTect™ HPV-Proofer E6/E7 mRNA test for the detection of overall abnormal cytology. The sensitivity of hc2 and PreTect™ HPV-Proofer were 83.7% and 46%, respectively. If we specifically focus on high-grade disease (cytological CIN2+), the sensitivity of PreTect™
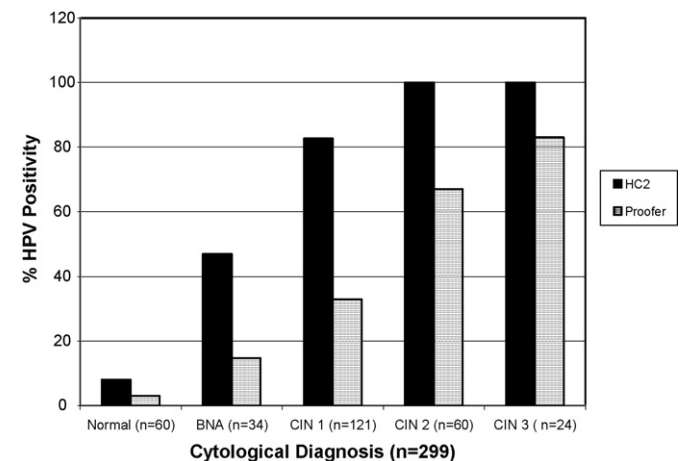
Fig. 1. Comparison of HPV detection technologies: hybrid capture (hc2) for the detection of HPV DNA and PreTect™ HPV-Proofer for the detection of HPV E6/E7 mRNA in cervical cytology specimens.

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Table 4
HPV genotype prevalence by PreTect™ HPV-Proofer ($n = 112$).

Cytology	PreTect™ HPV-Proofer genotype (no. of positive samples)										Total
	16	18	31	33	45	16&33	18&33	16&18	31&33	33&45	
Normal $n = 60$	2										2
BNA $n = 34$	2		1	1		1					5
CIN1 $n = 121$	24	3	1	7	6	2	1	1	1		46
CIN2 $n = 60$	26	5	3	4	1					1	40
CIN3 $n = 24$	13	2		2		3					20

HPV-Proofer increases to 71.4% with a specificity of 75.8%, while the sensitivity and specificity of the hc2 DNA test are 100% and 43.7% respectively. These figures were calculated using cytology as the gold-standard method. The primary objective of this study was to compare HPV detection technologies in cytology specimens and to correlate this with cytological diagnoses. The majority of specimens examined in this study were normal and low-grade disease ($n = 215$). Therefore, in the majority of cases, tissue biopsies following colposcopy were not available. However, histological data on a small subset of the study group ($n = 58$) were available for analysis and comparison.

Using the follow-up histology result (CIN2+) as the gold standard for these specimens, clinical sensitivity, specificity, and positive predictive value (PPV) could be calculated. Given that histology data was only available for women with a cytologically high-grade lesion the authors recognise that this type of calculation will have a strong verification bias towards cytology. With this in mind, the sensitivity and PPV of hc2 were 100%, and 87.9%, respectively, and of PreTect™ HPV-Proofer, 74.5%, and 92.7% respectively.

3.3. HPV genotype distribution in cervical cytology specimens

In total, 112 of the 299 cases (37%) tested were positive for HPV E6/E7 mRNA using the PreTect™ HPV-Proofer assay. HPV16 was the most prevalent single type infection, representing 60% of the positive cases, followed by HPV33 (12.5%), HPV18 (9%), HPV45 (6.3%) and HPV31 (4.5%) (Table 4). In addition, co-infection with more than one HPV type was observed in 10/112 (9%) of the positive cases. This co-infection was predominantly with HPV16 and 33 (6 of 10 co-infected cases). These multiple infections with two or more types of HPV were found across each of the cytology disease categories; BNA (1/34), CIN1 (5/121), CIN2 (1/60) and CIN3 (3/24) (Table 4).
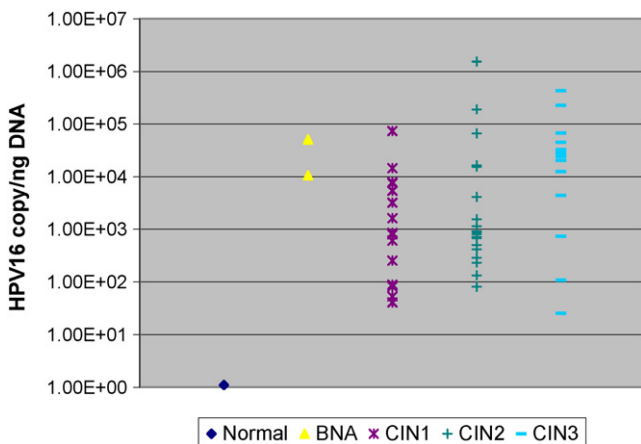

Fig. 2. Distribution of HPV16 viral load in cervical cytology specimens.

3.4. HPV16, HPV33 and HPV18 viral load quantitation by TaqMan® PCR

HPV16 DNA viral load was determined in 55 cytology specimens positive for HPV16 E6/E7 mRNA by PreTect™ HPV-Proofer. The range of HPV16 copy numbers across all of the specimens varied from 0 to 1.5×10^6 copies/ng. We did not detect a statistically significant difference in HPV16 viral load across the cytology categories ($P = 0.03$) (Fig. 2). However, it should be noted that the number of HPV16 positive cases in each group was low. The viral load of HPV33 was determined in 13 specimens positive for HPV33 E6/E7 mRNA by PreTect™ HPV-Proofer. The range of HPV33 copy numbers across all of the specimens varied from 6.49×10^2 to 1.6×10^5 copies/ng (Table 5) and there was no correlation between HPV33 viral load and cytology. The viral load of HPV18 was determined in nine specimens positive for HPV18 E6/E7 mRNA by PreTect™ HPV-Proofer. The range of HPV18 copy numbers across all of the specimens varied from 1×10^2 to 1.8×10^4 copies/ng (Table 5) and no correlation was seen between HPV18 viral load and cytology.

4. Discussion

Adjunctive molecular testing of cytology specimens for HPV has mainly involved the use of DNA based assays, in particular the hc2 test. However, as HPV DNA is highly prevalent and the life-time risk for HPV is estimated to be up to 80%, more specific biological markers for early detection of cervical cancer are needed. It is now widely known that the high-risk HPV oncoproteins E6 and E7 are necessary for conversion to and maintenance of malignancy. With this in mind, the PreTect™ HPV-Proofer assay has been designed to detect type-specific E6/E7 mRNA transcripts of the five most commonly found high-risk types in cervical carcinomas (Molden et al., 2007). Our study compares the detection of high-risk HPV by hc2 and PreTect™ HPV-Proofer in liquid based cervical cytology specimens ranging in their cytological diagnoses from normal to CIN3. Follow-up histology results were available for 58 samples with cytological CIN2 or CIN3. HPV DNA viral load analyses were performed on a subset of HPV16, HPV33 and HPV18 E6/E7 mRNA positive specimens with various cervical cytological diagnoses.

The overall prevalence of HPV DNA (68.6%) as detected by hc2 was greater than that of E6/E7 mRNA (37.5%) as detected by PreTect™ HPV-Proofer, and this was the case for all cytological categories including CIN2 and CIN3. This is to be expected, as not all HPV DNA infected cells will have high-level expression of E6/E7 mRNA. In addition the limitations of the PreTect™ HPV-Proofer test with respect to the smaller number of HPV genotypes detected (5 high-risk types) compared with hc2 which detects 13 high-risk types, may contribute to the higher detection rate observed for hc2. Similar data presented by Andersson et al. (2006) showed that 69% of women with any grade of cytological abnormality, detected in a population-based primary screening setting, were HPV DNA positive using the Quantovir HPV detection system (Quantovir AB, Sweden) while 40% were HPV E6/E7 mRNA positive by PreTect™ HPV-Proofer (Andersson et al., 2006). More importantly, in the

present study, HPV prevalence in women with BNA was 47% and 15% for HPV DNA and E6/E7 mRNA, respectively and in women with CIN1, 83% and 37%, respectively. This preliminary data would suggest that secondary screening using PreTect[TM] HPV-Proofer may have a higher triage effect and may be more cost effective, reducing the number of positive cases that are referred to colposcopy. However, a larger trial with appropriate patient follow-up is required to investigate this. Also, a high rate of concordance between HPV DNA and mRNA detection was observed in specimens with normal cytology and CIN3 cytological categories. In the normal cytological category, the 88% concordance rate predominantly reflected HPV negative results and the rate of detection of HPV DNA (8.3%) was higher than that of E6/E7 mRNA (3.3%).

For samples in the BNA and CIN1 categories, a much lower rate of concordance was observed between the HPV DNA test and the mRNA test (18% and 40%, respectively) (Table 2, Fig. 2). This is to be expected given that less than 20% of BNA and CIN1 cases progress to pre-cancer and that the two technologies detect different targets (DNA vs E6/E7 mRNA), with hc2 detecting a larger number of HPV genotypes (13 high-risk HPV types) than the PreTect[TM] HPV-Proofer assay (5 high-risk HPV types). There is evidence to suggest that detection of a HPV mRNA result as opposed to a DNA result within these categories may be indicative of an infection of higher biological significance and a positive HPV E6/E7 mRNA result may be of greater prognostic value (Molden et al., 2005). This study, which looked specifically at the BNA/CIN1 categories using PreTect[TM] HPV-Proofer, found that a positive HPV mRNA result in women with low-grade abnormalities, increased the relative risk of a histological CIN2+ diagnosis by 69.8-fold (Molden et al., 2005). Another study which used both DNA and mRNA detection techniques on normal cytology specimens has demonstrated that HPV mRNA detection is less sensitive but more specific than HPV DNA for the detection of disease during a 2 years follow-up (Cuschieri et al., 2004). In the same study, women who were positive for both HPV DNA and mRNA at baseline were more likely to have a persistent HPV infection. In the American ASCUS LSIL study (ALTS), it was concluded that the detection of HPV DNA by hc2 in women with LSIL was of limited value in their clinical management (Schiffman and Solomon, 2003). Combining the data from these published studies would suggest that detection of mRNA as opposed to HPV DNA in women with BNA/CIN1 cytology might reduce the number of women who are referred to follow-up without compromising the identification of women most likely to progress to CIN2+ or invasive cancer. In our study, a higher rate of concordance between the hc2 and the PreTect[TM] HPV-Proofer test was observed in the CIN3 (83%) categories (Fig. 1, Table 3), however this study lacks patient follow-up and has limited information in relation to histological outcomes. Based on these preliminary findings a larger trial examining the impact of HPV DNA and mRNA testing across all disease categories with appropriate patient follow is urgently required.

The overall aim of this study was to examine the utility of HPV DNA and mRNA detection technologies for detecting HPV in cytological specimens. For this reason, calculations in relation to specificity, sensitivity and PPV are based on cytology as gold-standard method for detecting cytological abnormalities. Overall, the sensitivity of the DNA based test for the detection of abnormal cytology was 83.7% while the sensitivity of the PreTect[TM] HPV-Proofer assay was much lower at 46% indicating that fewer false negatives were detected by the hc2 assay. Indeed the high negative predictive value of the hc2 assay has made it very suitable for use in large scale clinical studies such as the ALTS trial (Schiffman et al., 2003). The positive predictive value and specificity of PreTect HPV-Proofer (98.2% and 96.7%) were greater than those of hc2 (97.6% and 91.7%) for the detection of abnormal cytology. Focussing specifically on the detection of high-grade disease, i.e. CIN2+ in

cytology, the sensitivity of the mRNA test (71.4%) was lower than that of the DNA test (100%), however the specificity of the mRNA test (75.8%) was much greater than that of the DNA test (43.7%). A recent study by Varnai et al. (2008) reported similar rates of sensitivity (87%) and specificity (56%) of the PreTect[TM] HPV-Proofer test for the detection of prevalent cytological disease (HSIL). In CIN2+ cases with histological follow-up ($n = 58$), the sensitivity of the DNA test (100%) was greater than that of the mRNA test (74.5%) for the detection of CIN2+, however the PPV of the mRNA test was higher than that of the DNA test (92.7% vs 87.9%). The higher PPV of a PreTect[TM] HPV-Proofer positive result may be explained biologically, as the production of neoplastic lesions requires a stable expression from the E6 and E7 genes and a subsequent high and stable production of the transforming viral oncoproteins. HPV DNA detection at a single time point by hc2 indicates however viral presence only and would not give any information about the presence of a transforming infection.

In our study population, HPV16 was the most predominant single type infection (59%), followed by HPV33 (12.5%), HPV18 (9%), HPV45 (6.2%) and HPV31 (4.5%. HPV16 was the most prevalent HPV type in all cytological categories, whereas the prevalence of the other HPV types detected by PreTect[TM] HPV-Proofer varied. The rate of co-infections was 8% (9/112), with HPV16 and 33 being the most commonly occurring co-infection. Other studies that have used the PreTect[TM] HPV-Proofer assay have detected multiple type infections in 2.8% of cases of various grade cytology (Andersson et al., 2006) and in 5.6% of women with normal cytology but a positive DNA result for either HPV16, 18, 31, 33 or 45 (Cuschieri et al., 2004). By comparison, it is estimated that 20-30% of women infected with HPV DNA will have multiple type infections regardless of cytology (Moscicki et al., 2006). In a recent Irish study, which genotyped HPV positive samples from general practitioners in the Dublin area by sequencing of the L1 gene, the most commonly detected HPV types found that are included in the PreTect[TM] HPV-Proofer assay were HPV16 (20%) followed by HPV18 (12%) and HPV33 (8%) (Keegan H et al., 2007), which corresponds with the findings of our study. The higher prevalence of HPV in our study population reflects the greater number of high-grade cytology specimens examined.

In addition to HPV type, there is evidence to suggest that HPV viral load can be used to determine the grade of CIN, increasing with disease severity (Healey et al., 2001; Sun et al., 2002; Dalstein et al., 2003). The ALTS study, conducted on 2198 women with ASCUS, showed that high viral load can be used to identify women with ASCUS who had an underlying CIN which was confirmed on histological follow-up (Sherman et al., 2003). In our study, no correlation between HPV viral load and grade of abnormal cytology was observed for HPV16, HPV18 or HPV33 infected patients; however, it should be noted that viral load was only assessed in cases expressing E6/E7 mRNA. Moreover, there is further evidence to suggest that there is no correlation between HPV viral load and expression of HPV16 or HPV18/45 E6/E7 mRNA in cases with abnormal cytology (Andersson et al., 2006). This may be explained by viral integration into the host genome and the observed loss of viral replication upon integration (Doorbar, 2006). It is therefore plausible that in patients with high-grade disease, the HPV DNA viral load is relatively low compared to mRNA expression. In addition, increased stable expression of E6/E7 is commonly a result of deregulated transcription of integrated HPV DNA copies, in turn being independent of the number of integrated copies.

One concern in evaluating the use of HPV viral load as a biomarker for cervical screening is the method of cervical scraping that is used. In our study, the range of viral load was much less for HPV18 and HPV33 positive specimens than for HPV16. It has been shown previously, that HPV18 resides in the lower layers of the epidermis and is to a higher degree associated with glandu-

lar lesions and adenocarcinomas than squamous cell carcinomas (Castellsagué et al., 2006). Thus, sampling methods may explain the lower overall HPV18 viral load. Alternatively, the natural history of HPV infections may be type specific and is yet not well understood.

In conclusion, no correlation between HPV viral load and grade of abnormal cytology was observed for HPV16, HPV18 or HPV33 E6/E7 mRNA positive patients. Further studies are needed in order to reveal whether testing for viral load may have a potential value for use in cervical cancer screening. Moreover, our results suggest that the detection of E6/E7 mRNA by PreTect HPV-Proofer may indicate underlying high-grade cervical abnormalities and pre-cancer, supported by the higher specificity and PPV compared with the hc2 DNA test for the detection of abnormal cytology, in particular for the detection of CIN2+.

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