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Human papillomavirus detection and genotyping, by HC2, Full-Spectrum HPV and Molecular Beacon Real-Time HPV assay in an Irish colposcopy clinic.

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Abstract

Cervical screening programmes are moving towards HPV testing as part of the screening process and as a triage for colposcopy. Three HPV detection methods were evaluated using cervical cytology specimens from colposcopy patients. PreservCyt™ liquid based cytology specimens from 241 women attending colposcopy clinics with greater than 2 persistently abnormal smears were recruited through the Coombe Women and Infants University Hospital, Dublin. HPV DNA was detected by Hybrid Capture (HC2) for 13 high-risk HPV types, Full-Spectrum HPV (FS-HPV) for 49 high and low-risk types and Molecular Beacon Real-Time HPV assay (MBRT-HPV) for 16 high and low-risk types. HPV genotyping was performed using Linear Array HPV Assay (LA-HPV). HPV was detected in 83.3% (195/234), 91.9% (217/236) and 80.1% (169/211) of cytology specimens by HC2, FS-HPV and MBRT-HPV, HPV DNA detection assays. The sensitivity of the assays for the detection of high-risk HPV in cytology specimens that had a Cervical Intraepithelial Neoplasia Grade 2+ result by histology were, 98%, 97% and 94% for HC2, FS-HPV and MBRT-HPV assays with positive predictive values of 94.1%, 94.1% and 97.3%. The most common HPV genotypes were HPV 16, 31, 33, 58, 42, 61 and 53, and the most common high-risk HPV genotypes were HPV 16, 31, 33, 58, 18, 45, 59, 51, 56 and 39, with detection of multiple infections in 57.7% of all cases. FS-HPV and MBRT-HPV are highly sensitive and have a similarly high PPV as the HC2 assay for detection of HPV in patients with Cervical Intraepithelial Neoplasia Grade 2+ disease. HPV genotyping of women with persistent abnormalities is warranted prior to the introduction of HPV DNA testing in a colposcopy setting.

1. Introduction

Cervical screening programmes worldwide are moving towards HPV DNA testing as part of the screening process as either a co-test alongside cytology (Naucler et al., 2009; Castle et al., 2010), in primary screening or in triage of women with Low-Grade Squamous Intraepithelial Lesions or Atypical Squamous Cells of Undetermined Significance (Castle et al., 2010; Ronco et al., 2007; ALTS Group, 2003), in particular for women over the age of 30-35 years and as a test of cure for post treatment disease surveillance (Kreimer et al., 2006; Kreimer et al., 2007). As a consequence of the increased utility of HPV DNA testing, many new HPV DNA tests are becoming rapidly available, and existing HPV tests are evolving constantly to meet the needs of the end-user. At least 125 distinct HPV tests have been identified, with 84 variants of the same and for more than 75% of the HPV tests currently on the market, there is no single reference publication in the peer-reviewed literature (Poljak et al., 2012). The most commonly used commercially available HPV DNA tests include Hybrid Capture 2 for high-risk HPV (HC2), (Qiagen, Hilden, Germany), (Giorgi-Rossi et al., 2011), HC2 for detection of HPV 16, HPV 18 and HPV 45 (Qiagen, Hilden, Germany), (Thai et al., 2009), the Cervista™ HR HPV test (Hologic, Bedford, MA, USA), (Youens et al., 2011), the Cervista™ HPV 16, HPV 18 test (Hologic, Bedford, MA, USA), (Einstein et al., 2011), the Cobas 4800 HPV test (Roche Diagnostics, Mannheim, Germany), (Stoler et al., 2011), Papillocheck (Greiner Bio-One, Frickenhausen, Germany), (Dalstein et al., 2009), the Full-Spectrum HPV Amplification and Detection System (GenoID, Budapest, Hungary), (Jeney et al., 2007), GenoID Real-Time HPV Assay (GenoID, Budapest, Hungary), (Takacs et al., 2008) and the Linear Array HPV Genotyping Test (Roche Diagnostics, Mannheim, Germany), (Dobec et al., 2011), to name but a few. The HC2 test and the Cervista test received FDA approval in 2003 and 2009 respectively, for triage of women with equivocal cytology graded as Atypical Squamous Cells of Undetermined Significance and for use in women 30+ years of age in addition to routine cytology screening. Cobas 4800 HPV test, which tests for high-risk HPV and genotypes concurrently for HPV 16/18, received FDA approval in 2011 for use as an adjunct test to cytology in women >30 years of age, in women 21 years or older with Atypical Squamous Cells of Undetermined Significance and in women 21 years and older for the assessment of the presence of HPV genotypes 16 or 18 (Poljak et al., 2012). Each of these HPV tests uses a different quantity of starting specimen, uses different chemistry and gives different information on high-risk HPV infection, depending on the assay format. Thus, it is necessary to assess the performance of a given HPV assay in its intended test setting and on the study population for which it is intended before it can be used clinically.

Recent guidelines developed for primary screening in women >30 years and for the evaluation of new HPV tests have recommended that the performance of all HPV assays is assessed relative to HC2 and that in a triage setting HPV DNA tests should be evaluated in combination with adjunctive biomarker tests (eg. viral E6/E7, genotyping, p16 immunostaining or promoter methylation status) and compared

to histological outcome to aid risk stratification of HPV positive women with normal cytology or identify women with higher grades of cytology who will only ever develop a lesion that is minimally invasive (Meijer et al., 2009). It has also been suggested that in a colposcopy setting, in order to achieve a balance between sensitivity and specificity, HPV testing should be evaluated in terms of target assayed, age of the women who are tested and the assay cut-off for a positive result (Cuschieri et al., 2014)

In this study, the performance of three commercially available HPV DNA tests were compared in a colposcopy referred cohort of women: HC2 for detection of 13 high-risk HPV types, Full-Spectrum HPV (FS-HPV) for 49 high and low-risk types and Molecular Beacon Real-Time HPV assay (MBRT-HPV) for 16 high and low-risk types. The FS-HPV test is a PCR based assay for the HPV L1 gene, followed by an ELISA based 96 well hybridisation assay to a cocktail of probes for the detection of either high-risk HPV, low-risk HPV or a panel of HPV genotypes whose risk is not yet determined (Jeney et al., 2007). The MBRT-HPV assay is a molecular beacon real-time multiplex PCR assay that detects HPV using combined probes as either HPV 16 or HPV 18, one of a panel of 12 high-risk HPV types or as low-risk HPV (HPV 6 or HPV 11), (Takacs et al., 2008). While both of these assays carry the CE-IVD approval mark, few studies are available which compare the performance of the FS-HPV or the MBRT-HPV, (Jeney et al., 2007 and Takacs et al., 2008). HPV genotyping was performed on all specimens using the Linear Array HPV Genotyping Test (LA-HPV), for 37 ano-genital HPV types, to determine if the performance difference of a particular HPV DNA test is attributable to a) HPV genotypes present in this population that are included in one HPV test but not the others, b) HPV genotypes that are detected at different levels of sensitivity by each of the tests c) HPV genotypes that are outside the screening range of the HPV DNA tests chosen for analysis of this colposcopy study population.

The objective of this study was to compare the performance of the HC2 test, the FS-HPV and the MBRT-HPV assay in the Irish colposcopy setting. Non-concordant results were discriminated using the LA-HPV and a “HPV genotype snapshot” of the women attending colposcopy at Coombe Women and Infants University Hospital, prior to the introduction of HPV DNA testing was taken. This is the first time that a full HPV genotyping analysis of women attending colposcopy clinics of Coombe Women and Infants University Hospital has been performed.

2. Materials and methods

2.1. Patient recruitment and clinical specimens

Colposcopy referred women who had two or more persistently abnormal smears (N=241) were recruited through the colposcopy clinics at the Coombe Women and Infants University Hospital Dublin (CWIUH) as part of CERVIVA studies (Irish Cervical Screening Research Consortium, www.cerviva.ie) between the years 2008 and 2010. All patients gave informed consent, and the study was approved by the Coombe Women and Infants University Hospital Ethics Committee. Each patient had a cervical smear specimen taken into PreservCyt on their first visit to the colposcopy clinic for cytological diagnosis, and the residual cytology specimen was used for HPV testing. The patients ranged in age from 20-60 years, and the average age was 33 years. Cytological diagnoses were performed by specialised cytotechnologists and cytopathologists at CWIUH and diagnosis was consistent with British Society for Clinical Cytology guidelines for classification of abnormal smears (Evans et al., 1986). These samples were diagnosed cytologically as either Negative, Borderline Nuclear Abnormalities, Cervical Intraepithelial Neoplasia Grade 1, 2 or 3. In Bethesda Terminology, “Atypical Squamous Cells of Undetermined Significance” is the equivalent of Borderline Nuclear Abnormalities (BSCC terminology), “Low-Grade Squamous Intraepithelial Lesions” is the equivalent of Cervical Intraepithelial Neoplasia Grade 1 and “High-Grade Squamous Intraepithelial Lesions” is the equivalent of Cervical Intraepithelial Neoplasia Grade 2 or 3. Bethesda Terminology will be used to describe the cytological classification of study specimens from this point forward. A sub-population (N=185), of women had a cervical punch biopsy or “Large Loop Excision of the Transformation Zone”, and the histology result was available for these patients. Histology results were classified according to the guidelines of the British Society for Colposcopy and Cervical Pathology as Normal, Cervical Intraepithelial Neoplasia Grade 1 or Cervical Intraepithelial Neoplasia Grade 2 or 3 (High-Grade Cervical Intraepithelial Neoplasia). Histology specimens with High-Grade Cervical Intraepithelial Neoplasia (grades 2 or 3), were grouped together for this study and classified as Cervical Intraepithelial Neoplasia Grade 2+.

2.2. Hybrid capture HR-HPV DNA detection

DNA was extracted from an aliquot of 4mL PreservCyt™ specimen using the Sample Conversion Kit (Qiagen, Hilden, Germany) for high-risk HPV testing by Hybrid Capture (HC2; Qiagen, Hilden Germany). 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 the manufacturer’s guidelines. As per manufacturer’s guidelines a cutoff of RLU/CO >1 indicated a positive result.

2.3. Full-Spectrum HPV DNA detection

Nucleic acids were extracted from PreservCyt™ specimen (5mL) for the Full-Spectrum HPV assay (FS-HPV) and for the Molecular Beacon Based Real-Time PCR (MBRT-HPV) assay using the M48 BioRobot (Qiagen, Hilden, Germany) and the MagAttract RNA Cell Mini Kit (Qiagen, Hilden, Germany), and a custom designed protocol for PreservCyt™ specimens following lysis of the washed cell pellet in 400uL Buffer RLT (Qiagen, Hilden, Germany). Nucleic acids were eluted in 50uL Buffer TE (Qiagen, Hilden, Germany) and the eluate was divided into two aliquots: one for the FS-HPV the other for the MBRT-HPV. The FS-HPV assay was performed according to manufacturer's instructions. Briefly, the L1 region of a range of 49 HPV genotypes was amplified in a non-competitive multiplex PCR. The biotinylated amplicons were detected by solid phase hybridisation on a streptavidin coated microplate to a low-risk, a high-risk or a general HPV (un-classified risk) fluorescein conjugated probe which reacts with anti-fluorescein-horse-radish peroxidase antibody and HRPO chromogenic substrate (trimethylbenzidine) to yield a blue product in the case of a positive reaction. Each specimen was hybridised to an internal control which was prepared in parallel with the specimen. The HPV types detected by the low-risk probe included HPV 6, 11, 42, 43, 44/45, 55; by the high-risk probe: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68. Besides the low-risk and high-risk HPV types, the FS-HPV assay also detects the following HPV types whose risk is unclassified: 2, 3, 7, 10, 13, 26, 27, 28, 29, 30, 34, 40, 53, 54, 57, 61, 67, 70, 72, 73, 74, 81, 82, 83, 84, 85, 89, 90 and 91.

2.4. Molecular beacon based real-time HPV detection

The MBRT-HPV is a real-time molecular beacon based multiplex assay performed in a multiwell plate. It has the ability to distinguish HPV positivity as HPV 16 or 18 using a 5'-JOE labelled molecular beacon, as high-risk HPV positive for one or more of 12 HPV types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) using a 5'-TET labelled molecular beacon or as low-risk positive for HPV types 6 and/or 11, using a 5'-Texas-Red-(T) 6-FAM (wavelength shifted) labelled molecular beacon, while the internal control is detected by a 5'-FAM labelled molecular beacon. These four reactions occur in the one well with all the primers and probes contained in the MBRT-HPV mastermix (GenoID, Budapest, Hungary). The MBRT-HPV assay was performed on a second aliquot of the extracted nucleic acid according to manufacturer's instructions. Briefly, 3.5uL of nucleic acid was added to MBRT-HPV mastermix containing a final volume of 11uL per reaction and cycling was performed on an AB7900 (Life Technologies, Carlsbad, CA, USA) with thermo-cycling conditions as follows: 95°C for 10 min, 55°C for 5 min, (95°C 15 s, 30°C 20s, 95°C 15s, 50°C for 1 min) x25. Results were interpreted according to the manufacturer's instructions.

2.5. HPV genotyping using Linear Array HPV Test

Nucleic acids were extracted from 250uL of PreservCyt™ for the Linear Array HPV genotyping assay using the Amplilute® Liquid Media Extraction kit, (Roche, Mannheim, Germany) as described by the manufacturer's instructions. Briefly, PCR amplification and line blot colorimetric detection were performed for HPV types 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39 and CP6108.

2.6. Statistical analysis

Proportional agreement between tests was assessed using Cohen's Kappa (k) and a McNemar's Test was used to assess statistical significance. Sensitivity and positive predictive values were assessed using histology confirmed Cervical Intraepithelial Neoplasia Grade 2+ as the gold standard and the 0.95 confidence interval.

3. Results

3.1 Cytological and histological characteristics of the study patients

In total, 14.5% (35/241) of cytology specimens were diagnosed as Negative for Intraepithelial Lesions or Malignancy, 9.1% (22/241) were diagnosed as having Atypical Squamous Cells of Undetermined Significance, 24% (58/241) had Low-Grade Squamous Intraepithelial Lesions and 52.3% (126/241) had High-Grade Squamous Intraepithelial Lesions. Histological diagnoses were available for N=185 women. Of these women, 5.9% (11/185) had Normal histology, 23.2% (43/185) had Cervical Intraepithelial Neoplasia Grade 1 and 70.8% (131/185) had Cervical Intraepithelial Neoplasia Grade 2+. The average age of the women in the study was 33 years ranging between 20 and 60 years. When women were divided into <30 (N=106) and 30+ years of age (N=135), Low-Grade Squamous Intraepithelial Lesions were more common in women <30 years (31.13% , (0.95CI 27.1% to 35.2%)) than in women 30+ years (17.8% , (0.95CI 15.3% to 20.2%)), and High-Grade Squamous Intraepithelial Lesions were more common in women 30+ (59.3%, (0.95CI 55.2% to 63.3%)) than in women <30 (43.4%, (0.95CI 38.7% to 48.1%)). The Negative for Intraepithelial Lesion or Malignancy rate was 16% in women <30 and 14.1% in women 30+, while the rate of Atypical Squamous Cells of Undetermined Significance was 9.4% versus 8.9% in these age groups with overlapping 0.95 confidence intervals, indicating that there was no significant difference.

3.2 HPV prevalence

PreservCyt specimens (N=241) were tested for HPV prevalence by each of the three assays: HC2, Full-Spectrum HPV (FS-HPV) and Molecular Beacon Based Real-Time HPV assay (MBRT-HPV). The same 13 high-risk HPV genotypes were included by each of the three detection assays, with the exception of HPV 66, which was detected as the 14th high-risk HPV type by the FS-HPV and the MBRT-HPV only and was not included in the HC2 test. The number of valid test results varied for each test from 87.6% (211/241) for the MBRT-HPV to 98% (236/241) for the FS-HPV and 234/234 (100%) for HC2. The overall prevalence of HPV as determined by each of these three tests was 91.9% (217/236) for FS-HPV, 80.1% (169/211) for MBRT-HPV and 83.3% (195/234) for HC2. When positivity by the general or the low-risk HPV probe only was excluded from the calculation of high-risk HPV prevalence using the FS-HPV test, and when HPV positivity for low-risk HPV types only was excluded from the calculation of high-risk HPV prevalence by the MBRT-HPV assay, then the prevalence of high-risk HPV across each of the three tests was 78.8% (186/236) for FS-HPV, 83.3% (195/234) for HC2, and 78.7% (166/211) for MBRT-HPV. There were 198 cytology specimens which had valid HPV test results by each of the three HPV detection assays (Table 1). In this subset, the concordance of the FS-HPV and the MBRT-HPV for detection of high-risk HPV types by comparison to HC2 was 94.6% ($k=0.792$) and 87.4% ($k=0.532$). There was no statistical difference between the HC2 test and the FS-HPV for the detection of high-risk HPV overall, however this was not the case for MBRT-HPV where the difference in detection of high-risk HPV genotypes was statistically significant (McNemar's Test, $P=0.0164$). When analysed by age group (<30 years and 30+ years of age), there was a statistically significant difference in the prevalence of HPV by both the FS-HPV test (<30 years, 95.5% (0.95 CI 94.6% to 96.4%), 30+ years 91.8% (0.95CI 90.4% to 93.2%)) and the HC2 (<30 years, 89.8% (0.95 CI 87.9% to 91.7%), 30+ years 85.5% (0.95CI 83.1% to 87.8%)) in women <30 years of age by comparison to those who were 30+ years of age. The MBRT-HPV assay did not show any difference in HPV prevalence according to age group.

3.3 HPV prevalence across cytological categories

The overall prevalence of HPV in each of the cytological categories is presented in Table 1. The HC2 and the FS-HPV performed consistently well across all grades of cytology, while the MBRT-PCR had a lower rate of HPV detection across all grades of cytology. In specimens with High-Grade Squamous Intraepithelial Lesions, the detection rate for HPV was equally high for both the FS-HPV and HC2 assays at 99.1% and 100% respectively. For Low-Grade Squamous Intraepithelial Lesions, the FS-HPV had a statistically significantly higher rate of overall HPV detection than the HC2 at (98.0% v's 85.7%, $P=.04$) while for Atypical Squamous Cells of Undetermined Significance, the rate for the two tests was the same at 86.7%. If the detection of non high-risk HPV types by the FS-HPV assay is

excluded, then the detection rate of the FS-HPV in High-Grade Squamous Intraepithelial Lesions (97.3%) and Low-Grade Squamous Intraepithelial Lesions (83.7%) was slightly lower than that of HC2 (High-Grade Squamous Intraepithelial Lesions (100%) and Low-Grade Squamous Intraepithelial Lesions (85.7%)), but with no statistical significance (Table 1). In the Negative for Intraepithelial Lesion or Malignancy cytological group, the FS-HPV assay detected HPV (high or low-risk) in a greater percentage (60.9%) of specimens than either the HC2 (30.4%) or the MBRT-HPV (30.4%) and this was statistically significant $P=.046$ (Table 1). If the detection of non high-risk HPV genotypes is excluded, then the detection rate of the FS-HPV in the Negative for Intraepithelial Lesion or Malignancy group was 26.1% (Table 1). This was similar to the high-risk detection rate of the MBRT-HPV assay (30.4%) and the HC2 (30.4%), and there was no statistical significance in the rate of high-risk HPV detection by any of the three assays in the Negative for Intraepithelial Lesion or Malignancy cytology cases (Table 1). The HPV positivity rate overall ranged from 81.8% to 95.5% in women <30 years of age and from, 82.7% to 91.8% in women 30+ depending on the HPV test analysed. The only test which detected more HPV in one age group over another was the FS-HPV which detected more positives in the <30 years group (95.5%, 0.95CI 94.6% to 96.4%) than in the 30+ group (91.8%, 0.95CI 90.4% to 93.2%).

3.4 Sensitivity and positive predictive value of HPV tests

To assess the clinical performance of the different HPV testing technologies, metrics such as sensitivity and positive predictive value (PPV) were used, to determine the clinical utility of the various HPV detection approaches. Histological confirmation of the disease (Cervical Intraepithelial Neoplasia Grade 2+) was used as the primary end point for calculating sensitivity and PPV. In this specific cohort of 241 women, histological diagnosis was available for N=185 women: 5.9% (11/185) had Normal histology, 23.2% (43/185) had Cervical Intraepithelial Neoplasia Grade 1 and 70.8% (131/185) had Cervical Intraepithelial Neoplasia Grade 2+. Based on specimens that had a valid result by each of the three assays and a histology result of either Normal or Cervical Intraepithelial Neoplasia Grade 2+ (N=122), the sensitivity of the HC2, FS-HPV and MBRT-HPV tests were 98.3%, 97.4% and 93.9% respectively, while the PPV for HC2, FS-HPV and MBRT-HPV was 94.1%, 94.1% and 97.3% respectively (Table 2). For calculation of sensitivity, and PPV, specimens that had an histology result of Cervical Intraepithelial Neoplasia Grade 1 were not included in this analysis.

3.5 Genotyping by HPV Linear Array

HPV genotyping was performed on the same cohort of 241 patients using the Linear Array HPV Genotyping Test (LA). This test screens for 37 different HPV types in a single sample. Briefly, the majority of specimens 99.2% (239/241) had valid results on the LA, as indicated by the amplification of the beta-globin controls for each specimen. Of the valid results, 95.4% (228/239) of specimens genotyped were positive for HPV. The ten most common HPV genotypes in order of decreasing prevalence were: HPV 16 (50.6%), HPV 31 (14.1%), HPV 33 (12.0%), HPV 58 (10.4%), HPV 42 (9.1%), HPV 61 (7.9%), HPV 53 (7.9%), HPV 18 (6.6%), HPV 62 (6.6%) and HPV 45 (6.6%), (Table 3). Of these top ten HPV genotypes, HPV 42, HPV 61, HPV 53, and HPV 62 are not classified as high-risk HPV genotypes. All cytology specimens positive for these 4 genotypes, that had High-Grade Squamous Intraepithelial Lesions, were co-infected with high-risk HPV types. The ten most common high-risk HPV genotypes were 16, 31, 33, 58, 18, 45, 59, 51, 56 and 39. The prevalence of high-risk HPV type 52 could not be determined as the LA test does not test specifically for HPV 52. Multiple HPV genotypes were detected in 138/239 (57.7%) of all specimens.

3.6 HPV genotype and cytology distribution

HPV 16 was the most common HPV genotype across all cytological disease categories (Table 3). In High-Grade Squamous Intraepithelial Lesions, HPV 16 (31.7%), was the most prevalent followed by HPV 31 (9.8%), HPV 33 (8.7%), HPV 58 (4.5%) and HPV 62 (3.4%). In the low-grade cytological category, the prevalence of the top 4 high-risk HPV types were; HPV 16 (34.5%), HPV 58 (12.1%), HPV 59 (10.3%), HPV 45 (10.3%) followed by HPV 31, HPV 33, and HPV 62 at 8.6%, respectively, (Supplemental Table 1). In the Negative for Intraepithelial Lesion or Malignancy cytological

category, 21/39 (53.8%) of HPV infections were high-risk, the rest were either low-risk or infections with HPV genotypes whose risk is not yet known. The percentage of specimens with high-risk HPV infections increased with grade of cytology from 51.4% in cytology specimens which were Negative for Intraepithelial Lesion or Malignancy and 81.8% in Atypical Squamous Cells of Undetermined Significance to 84.5% in Low-Grade Squamous Intraepithelial Lesions and 100% in High-Grade Squamous Intraepithelial Lesions (Figure 1). The percentage of specimens with multiple type infections was lowest in the Negative for Intraepithelial Lesion or Malignancy cytological category (28.6%) and High-Grade Squamous Intraepithelial Lesions (60.0%) categories and increased from 63.6% in the to 67.2% in Low-Grade Squamous Intraepithelial Lesions (Figure 1).

3.7 HPV genotype and histology status

In cytology specimens with a matched histology specimen which was Cervical Intraepithelial Neoplasia Grade 2+, HPV 16 (66.4%) was the most prevalent followed by HPV 31 (22.1%), HPV 33 (18.32%), HPV 58 (9.2%), HPV 62 (8.4%), HPV 45 (7.6%), HPV 18 (6.1%), HPV 51 (6.1%), HPV 59 (6.1%) and HPV 39 (4.6%). The most commonly detected low-risk HPV genotype in this cohort was HPV 42 (7.6%). HPV 42 was the 6th most commonly detected genotype overall in this study. The multiple infection rate in this cohort was 58.8%.

3.8 HPV genotyping to resolve discrepant HPV detection results

HPV genotyping was performed to evaluate specimens with a discrepant result for high-risk HPV using the other 3 technologies i.e. specimens that were concordant by 2 or more of either FS-HPV (high-risk), MBRT-HPV (16/18 or high-risk), HC2 or LA-HPV genotyping test for high-risk HPV (Supplemental Table 2). Where a sample was positive by 2 tests and one of those was the LA-HPV genotyping assay, the genotype assigned to that specimen was the LA-HPV result. A negative LA-HPV result was used to determine a false positive HPV detection result in situations where a cytology specimen was positive for HPV by one technology only but was negative by LA-HPV for any of the HPV genotypes tested by that technology. A cytology specimen had a false negative result for a particular technology, if it was positive by the other two HPV detection technologies or by one technology and was positive by LA-HPV for one or more of the genotypes detected by that technology.

As no one test was 100% sensitive, there were false positive HPV results and false negative HPV results using each test method. The HC2 high-risk HPV, which screens for 13 high-risk HPV genotypes, gave 4 false positive high-risk HPV and 4 false negative high-risk HPV in this population, when compared to LA-HPV, FS-HPV assay, and MBRT-HPV. Two of the 4 false positives were

falsely positive due to cross reaction with low-risk HPV types and the remaining two were HPV negative by all other assays. The 4 HC2 false negative samples contained high-risk HPV genotypes 16, 39, 56, 51 and 52 and were positive by either the FS-HPV or the MBRT-HPV for high-risk HPV. Three of the four samples contained HPV 16.

For the MBRT-HPV, which screens for 14 high-risk HPV and 2 low-risk, 16 false negatives and 3 false positives were detected. The 16 false negatives contained a mixture of high-risk HPV genotypes 35, 52, 16, 68, 39, 51, 56, 31, 58. The high-risk HPV genotypes that occurred most frequently in specimens that were false negative by MBRT-HPV were HPV 16 (9/16) and HPV 51 (4/16). The 3 false positives by the MBRT-HPV assay contained low-risk HPV genotypes 53, 73, 42, and 54, and were negative by the FS-HPV and HC2 for high-risk HPV types.

For the FS-HPV assay, which screens for 49 HPV genotypes of high, low and undetermined risk, there were no false positives and 5 false negatives. The 5 false negatives contained high-risk HPV genotypes 16, 18, 31, 33, 52. Two of the 5 specimens contained HPV 16. Four of the 5 false negatives were positive by both the MBRT-HPV and the HC2, while the other was negative by MBRT-HPV and HC2 and was genotype 52 by LA-HPV.

4.0 Discussion

This study compared the performance of three commercially available HPV detection methods (HC2, FS-HPV and MBRT-HPV), in cervical cytology specimens of women attending the colposcopy clinics of Coombe Women and Infants University Hospital, Dublin. The infecting HPV genotypes in these women were identified by LA-HPV. This is the first study which examines the performance of the FS-HPV and the MBRT-HPV for HPV detection in colposcopy patients and is the first review of HPV genotypes in an Irish colposcopy setting.

No HPV assay detected 100% of HPV infections, and there were false positive and false negative HPV results by each of the assays (Supplemental Table 2), by comparison to the other tests. As expected in this cohort of women with persistently abnormal cervical cytology smears, the prevalence of HPV was high by each of the assays at 83.3% for HC2, 91.9% for FS-HPV and 80.1% for MBRT-HPV, with adjusted high-risk only HPV detection rates of 61.4% for FS-HPV and 78.7% for MBRT-HPV (Table 1). Other studies which have examined prevalence of high-risk HPV infections in mixed colposcopy referral populations have noted high HPV prevalence rates regardless of the HPV DNA detection assay used (Cuschieri et al, 2014; Szarewski et al., 2012), indicating that most commercially available assays are sufficiently sensitive for HPV detection in this setting. The HC2 test and the FS-HPV had similar HPV detection rates across all grades of cytology whereas the MBRT-HPV had a much lower rate, which was probably associated with its lower analytical sensitivity for HPV 16, combined with the fact that HPV 16 was the most dominant HPV genotype overall. It has been suggested that the performance of HPV assays in a triage setting should be assessed with relevance to age among other factors (Cuschieri et al, 2014). Reflecting the natural history of HPV infections, Low-Grade Squamous Intraepithelial Lesions were associated with younger age (<30 years) while High-Grade Squamous Intraepithelial Lesions were statistically more common in women 30+ years of age. In this study, high-risk HPV DNA prevalence was highest for each assay in women under 30 years of age, indicating that a second triage test in addition to HPV DNA in younger women may be necessary for further risk stratification.

The GenoID FS-HPV assay and the MBRT-HPV were first described in 2007 and 2008, and while they are CE marked they are mainly only in use throughout Hungary thus far. More studies such as this one are warranted to assess the performance of these two assays in different test settings and in different populations. In a previous publication, FS-HPV was compared to HC2 for detection of HPV in 81 women with Atypical Squamous Cells of Undetermined Significance or Squamous Intraepithelial Lesions giving a concordance rate of 83.9%, which compares well to this study where concordance was 93.7% in the same cytological category (Jeney et al., 2007). In the study which first describes the MBRT-HPV assay, its concordance to FS-HPV for detection of HPV was 89.44% (Takacs et al., 2008), comparing well to this study (83.8%). Meijer et al., have suggested that new

HPV screening tests are assessed relative to HC2 (Meijer et al., 2009). When compared to HC2, the FS-HPV showed greater concordance (94.6% ($k=0.792$)) than the MBRT-HPV (87.4% ($k=0.532$)) for detection of high-risk HPV types and there was no statistical difference between the HC2 test and the FS-HPV for the detection of high-risk HPV overall.

The HC2 assay was the most sensitive for high-risk HPV detection in Cervical Intraepithelial Neoplasia Grade 2+ disease (98.3%), followed closely by the FS-HPV (97.4%) and then the MBRT-HPV (93.9%). It was not possible to determine the specificities of the assays as there were not enough women with Normal histology results and therefore not enough true negatives. While the MBRT-HPV assay did not detect as many HPV infections as the HC2 or the FS-HPV, it did not detect any false positive HPV results when concurrent Cervical Intraepithelial Neoplasia Grade 2+ histology results and HPV positivity was used as the true positive. The MBRT-HPV test had a higher PPV (97.3%), than the other tests: (94.1%) for HC2 and (94.1%) for FS-HPV, and this was statistically significant. PPV is a critical measure of the performance of the test, as it reflects the probability that a positive test is predictive of high grade cervical disease. However, this is highly dependent on the prevalence of disease and the study patients in this case were highly biased towards disease, therefore diagnostic performance should be considered in this context. Other studies have also demonstrated that HC2 has high sensitivity for detection of High-Grade Cervical Intraepithelial Neoplasia. A recent study, which compared the performance of a number of different HPV detection technologies for detection of Cervical Intraepithelial Neoplasia Grade 3+ in women referred with one or more borderline or mildly abnormal smears, showed that the sensitivity of HC2, Abbott RealTime, BD HPV and Cobas 4800 was 100% and the sensitivity of HC2 for Cervical Intraepithelial Neoplasia Grade 2+ was 96% (Mesher et al., 2013). In a European retrospective study by Denton et al, HC2 had a sensitivity of 90.1% for the identification of biopsy confirmed high-grade Cervical Intraepithelial Neoplasia at 6 months in women presenting with Atypical Squamous Cells of Undetermined Significance cytology and 95.7% in women with Low-Grade Squamous Intraepithelial Lesions (Denton et al., 2010). In a previous study performed in a colposcopy referred population in another Dublin maternity hospital, HC2 had a sensitivity of 90.5% for detection of CIN2+, which is slightly lower than reported in this study, however that study was of women referred for persistently low-grade cytology only and unlike this study there were no women who had high-grade cytology (White et al., 2013).

The LA-HPV assay was used in this study to provide a snapshot of the HPV genotypes present in a colposcopy referred cohort of women and to assess whether the HPV genotype coverage of each of the detection assays affected their clinical performance. Only for the MBRT-HPV assay did the genotyping analysis reveal reduced positivity that could be attributed to failed detection of a particular HPV genotype (HPV 16) which was included in the assay. The MBRT-HPV test has a lower

analytical sensitivity than the other tests for HPV 16 which is demonstrated by a lower clinical sensitivity than either the HC2 or the FS-HPV tests (Table 2).

Not surprisingly, HPV 16 was the most common genotype across all grades of cytology, after which HPV 18 was the 8th most common HPV genotype overall and the 6th most common HPV genotype (6.1%) in cytology specimens with confirmed Cervical Intraepithelial Neoplasia grade 2+. The HPV18 prevalence in this study was low by comparison to the ASCUS LSIL Triage Study, where genotyping of 608 smears from women with histological confirmed Cervical Intraepithelial Neoplasia grade 3+ at enrollment revealed an average HPV 18 prevalence of 13.2% by either line blot or linear array and that HPV 18 was the 5th most common HPV genotype in that cohort (Castle et al., 2010). The low HPV 18 prevalence in this study might be explained by a lower than average overall HPV 18 prevalence as genotyping data from the Irish cervical screening population indicate that HPV 18 is the 4th most common genotype overall and present in only 6.1% of all high-grade cytology cases (unpublished results). In a separate study, genotyping of archival formalin fixed material from High-Grade Cervical Intraepithelial Neoplasia, between the years 2001-2008 revealed that only 6% of histology cases were HPV 18 positive (Tjalma et al., 2013). Worldwide, HPV 16 and HPV 18 are present in ~71% of all cervical cancers, however the prevalence of HPV 18 is known to vary widely geographically from 7% of all cervical cancer cases in Europe and North America to 20% in Oceania and 23% in Africa (de Sanjose et al., 2010). It has been suggested in a previous study, that precancerous lesions harbouring HPV 18 or HPV 45 may be underrepresented as only 57% of cancers containing HPV 18 or 45 were diagnosed at colposcopy by comparison to 84% of HPV 16 associated cancers (Safaeien, et al., 2009). It is possible that cytological sampling of glandular lesions containing HPV 18 may be ineffective or that HPV 18 viral activity may be altered in such a way that HPV 18 positive cells are under-represented in a cytological smear sample. The only way to correctly determine the burden of HPV 18 associated cervical cancers would be to genotype the cervical tissue of women with cervical carcinoma in-situ (Tjalma et al., 2013).

In this study, 4 of the 10 HPV genotypes detected most commonly were low-risk. Analysis of HPV genotypes across the cytology categories showed that low-risk HPV genotypes were identified in all cytology cases, in particular genotypes HPV 42, HPV 61, HPV 53 and HPV 62, and in High-Grade Cervical Intraepithelial Neoplasia Grade 2+ cytology, the low-risk HPV genotypes were always accompanied by one or more high-risk HPV genotypes. Interestingly, after identification of a common HPV 16 etiology, the top 5 HPV genotypes in the Low-Grade Squamous Intraepithelial Lesions and High-Grade Squamous Intraepithelial Lesions categories differed. HPV genotypes were present at high prevalence in the women with high-grade disease that were not as prevalent in the women with low-grade disease and *vice versa* in particular for HPV types 31, 33, 59 and 45. The advantage of HPV genotype identification is highlighted here, as not only are HPV genotypes that may have caused the abnormality identified, but newly acquired HPV infections, infections with HPV genotypes that

clear rapidly or infections with emerging HPV genotypes that may eventually become the dominant persistent genotype within a population may also be identified.

While not explored in this study, the FS-HPV test can also be used to identify specific HPV genotypes using type specific HPV hybridization probes rather than pooled HPV probes following amplification of a general HPV amplicon using the L1F/L1R primer set (Jeney et al., 2007). Such type specific identification alongside the general detection of HPVs whose risk classification is not yet known using the “HPV with unclassified risk” probe set, may permit interrogation of a sample for specific recurrent HPV genotypes while monitoring for a shift in the prevalence of certain HPV genotypes following the introduction of HPV vaccination programmes. The extended coverage of the FS-HPV assay may be useful for detection of HPV genotypes other than the high-risk genital types which may be associated with dermatological conditions, head and neck cancers or it may be useful to a laboratory that wishes to extend HPV testing outside of the genital space.

Multiple infections were a common feature, with 58% of women having greater than one genotype. The multiple infection rate of this colposcopy referred population was very similar to that of our routine screening population (56.5%, unpublished results). In this study, multiple infections were highest in cytology specimens of the Low-Grade Squamous Intraepithelial Lesions cytology group (66.3%) and lower in the Negative for Intraepithelial Lesion or Malignancy (28.6%) and High-Grade Squamous Intraepithelial Lesions cytology categories (60%). By comparison to the routine screening population where the multiple infection rate is 51.6% in cytology cases Negative for Intraepithelial Lesion or Malignancy (unpublished results), the lower multiple infection rate in women Negative for Intraepithelial Lesion or Malignancy at the time of colposcopy may indicate a subset of women who have a history of abnormal smears but have resolved some of their HPV infections prior to colposcopy as opposed to those women who are HPV positive at the screening stage with as yet no evidence of cytological changes. The average number of infecting genotypes was two, with a maximum number of ten. While this might seem an alarmingly high number of multiple genotypes, the intensity of the bands produced by colorimetric patterning on the line-blot varies greatly, with low visibility bands probably indicating a low viral load infection (Wentzensen et al., 2012). The propensity of intermittent infections and low viral load infections to cause cytological changes remains unclear and it has been shown repeatedly that persistently testing positive for any high-risk HPV genotype is a good surrogate for HPV genotype specific persistence, particularly in women aged 30 years and older (Castle et al, 2009). However, despite advances in HPV detection technologies and a move towards grouped HPV detection of selected high-risk HPV genotypes, there is no convenient way to identify type specific persistence other than by multiple screening rounds using type specific methods that are often very labour intensive and are performed manually.

Overall, this study describes comparable performance of the HC2 and the FS-HPV assay in colposcopy patients and provides a useful snapshot of the HPV genotypes present before commencement of HPV testing as triage, or as test of cure. While the MBRT-HPV assay is slightly less sensitive than either the HC2 or the FS-HPV, it has a higher positive predictive value and as it is a real-time PCR based technology it can be readily automated. As the FS-HPV PCR amplicon can be readily genotyped, HPV testing using the FS-HPV may be a useful alternative to the HC2 in populations which require HPV genotyping or where the prevalence of a particular genotype needs monitoring. Preliminary studies, to compare the performance of different HPV detection assays and assess the HPV genotype distribution in a given population, are warranted, prior to selecting a HPV assay for use in cervical screening.

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Table 2. Sensitivity and positive predictive values of each HPV test for detection of CIN2+ disease (N=122)

HPV test	CIN2+ Histology	
	Sensitivity	Positive Predictive Value
HC2	112/114 (98.3%)	112/119 (94.1%)
	95% CI (97.9%-98.6%)	95% CI (93.1%-95.1%)
FS-HPV (HR only)	111/114 (97.4%)	111/118 (94.1%)
	95% CI (96.9%-97.8%)	95% CI (93.1%-95.1%)
MBRT-HPV (HR only)	107/114 (93.9%)	107/110 (97.3%)
	95% CI (92.8%-94.9%)	95% CI (96.8%-97.8%)

Table 1

Overall HPV and High-risk HPV detection across the various grades of cytology by HC2, FS-HPV and MBRT-HPV

Cytological diagnosis	NILM ^a	ASCUS ^b	LSIL ^c	HSIL ^d	Total HPV positive
High-risk HPV positive by HC2 ^e	30.4% (7/23)	86.7% (13/15)	85.7% (42/49)	100% (111/111)	87.4% (173/198)
HPV positive by FS-HPV ^f	60.9% (14/23)	86.7% (13/15)	98.0% (48/49)	99.1% (110/111)	89.9% (178/198)
High-risk HPV positive by FS-HPV	26.1% (6/23)	80.0% (12/15)	83.7% (41/49)	97.3% (108/111)	84.3% (167/198)
HPV positive by MBRT-HPV ^g	30.4% (7/23)	60.0% (9/15)	85.7% (42/49)	94.6% (105/111)	85.9% (170/198)
High-risk HPV positive by MBRT-HPV	30.4% (7/23)	60.0% (9/15)	79.6% (39/49)	94.6% (105/111)	80.8% (160/198)

^aNegative for Intraepithelial Lesion or Malignancy

^bAtypical Squamous Cells of Undetermined Significance

^cLow-Grade Squamous Intraepithelial Lesion

^dHigh-Grade Squamous Intraepithelial Lesion

^eHybrid Capture detects HPV genotypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68

^fFull-Spectrum HPV Amplification and Detection System detects HPV genotypes: 6, 11, 42, 43, 44/45, 55, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 2, 3, 7, 10, 13, 26, 27, 28, 29, 30, 34, 40, 53, 54, 57, 61, 67, 70, 72, 73, 74, 81, 82, 83, 84, 85, 89, 90 and 91

^gGenoID Real-Time HPV Assay detects HPV genotypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 6 and 11

Figure 1. Prevalence of high-risk HPV and multiple type infections by Linear Array HPV Genotyping Test for detection of 16 high-risk HPV genotypes (16,18,31,33,35,39,45,51,56,58,59,66 and 68) and 21 HPV genotypes of either low or undetermined risk in a colposcopy cohort of 239 women.

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Figure 1.

Highlights

- Full-Spectrum HPV test and HC2 had similarly high sensitivity for detection of HPV in women with CIN2+.
- Molecular Beacon Real-Time HPV assay had the highest positive predictive value for CIN2+.
- The most common HPV genotypes in this Irish colposcopy clinic were HPV16, 31, 33, 58, 18, 45, 59, 51, 56 and 39.
- Multiple HPV infections were detected in 57.7% of colposcopy smears.