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Comparison of the Performance of the Abbott Realtime High-Risk (HR) HPV Test, the Digene Hybrid Capture 2 Test and an 'In House' Multiplex HPV PCR Test in Anal Specimens

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Abstract

This study aimed to investigate the performance of three assays for detecting high-risk human papillomavirus (HrHPV). We compared the performance of Abbott *Realtime* PCR (ART) and the Hybrid Capture 2(HC2) methods for the detection of HrHPV in a total of 120 patients referred to a sexual health clinic for suspected anal disease or known high risk patients being routinely followed. Assay results from ART and HC2 were compared to a customised in-house multiplex PCR (MPX) that also included sequencing for 20 genotypes. There was strong agreement between the ART and HC2 for detecting HrHPV (k=0.70). There was excellent agreement between ART and the MPX for HPV 16 ± 18 detection (κ=0.82, $CI_{95\%}$: 0.72-0.93), for HPV 16 alone (κ=0.86, $CI_{95\%}$: 0.76-0.95), and for HPV 18 alone (κ=0.84, $CI_{95\%}$ 0.72-0.96). Genotyping profiles demonstrated a high proportion of subjects with multiple HrHPV genotypes at the time of testing. A key clinical outcome of analytical molecular methods that detect HrHPV in anal specimens is the opportunity for optimal management of sub-

jects at risk of developing high-grade anal intraepithelial neoplasia (HGAIN) or anal squamous cell carcinoma (ASCC). Moreover, methods that distinguish HrHPV types 16, 18 and other HrHPV types provide information relevant to understanding the biology of HPV related anal disease including recognition of local HrHPV profiles, consideration of alternative management in cases with other HPV infections and correlation with additional clinical symptoms that may influence the progress of anogenital disease.

Keywords: HPV Testing Methods; Anal Cancer; Anoscopy; HPV Genotyping

Abbreviations: HrHPV- High-risk human papillomavirus; PCR- Polymerase chain reaction; ART- Abbott RealTime PCR; HC2- Hybrid Capture 2; MPX-Multiplex; HPV- Human papillomavirus; HGAIN- High-grade anal; intraepithelial neoplasia; ASCC- Anal squamous cell carcinoma; MSM- Men who have sex with men; HSIL- high-grade squamous intraepithelial lesion; IR- Incidence rate; PY- Person-years; HIV- Human immunodeficiency Virus; HAART- highly active antiretroviral therapy; AIN- Anal intraepithelial neoplasia; HRA- High resolution anoscopy; STD- Sexually Transmitted Disease; TP-ThinPrep; NG- Neisseria gonorrhoeae; CT- Chlamydia trachomatis; DNA- deoxyribonucleic acid; RNA- ribonucleic acid; RLU- relative light unit; Bp- base pair; Hr- High-risk; LBC- Liquid-based cytology; MSW- Men who have sex with women

Introduction

A causal role for ASCC and its precursor lesion, HGAIN has been linked to persistent HrHPV infection [1]. Epidemiological studies have identified high risk groups who include men who have sex with men (MSM), women with a history of cervical high-grade squamous iintraepithelial lesions (HSIL) or HPV related gynaecological cancer and other immunocompromised subjects, notably solid organ transplant recipients [2]. In MSM who are HIV positive and who have anal HPV infection, the rate of anal cancer is 131/100,000 compared to 31/100,000 in HIV negative MSM [3]. Within these groups the likelihood of acquiring HPV and developing AIN has been associated with at risk sexual behaviours such as increasing numbers of sexual partners and a range of cofactors including smoking habits and HAART and STI's [4]. Despite the availability of HPV vaccines that could prevent up to 80% of anal cancers, recent epidemiological evidence reveals a rising incidence of ASCC and associated mortality in women and males in high risk groups [5]. HIV positive MSM comprise the largest and most disproportionately affected group for anal disease among these at risk categories and have been the focus of many studies seeking to unravel the biology, diagnosis and management of anal cancer and precursor lesions. When compared with the incidence rate (IR) of ASCC in the general population of around 1-2 cases /100,00 person years (py), the IR of anal cancer in HIV positive MSM is 85/100,000 py and 19/100,000 py in HIV negative MSM [3]. Despite the availability of HPV vaccines that could prevent up to 80% of anal cancers, recent epidemiological evidence reveals a rising incidence of ASCC and associated mortality in women and males in high risk groups [5].

The biology of the development and progression of anal disease is presumed to follow the same steps as the natural history of cervical carcinoma - acquisition of and persistence of HrHPV infection, an intraepithelial phase followed by invasion [6].The natural history of anal HPV infection includes the possibility of infection clearance, therefore making the identification of additional cofactors that lead to permanent establishment of infection and the temporal development of HGAIN an important milestone especially for the effective management of the disease [7]. Clinical examination, HRA, biopsy, and or cytology of suspected lesions go to form the algorithm of tests to establish an accurate diagnosis of AIN or ASCC. HPV assay on collected cellular material is an important adjunct test especially when evaluating management of high-risk patients or if cytology and biopsy results are negative or inconclusive [8]. Approximately 20 HrHPV genotypes infect genital epithelia. Of these, HPV types 16 and/or 18 are reported as the most oncogenic types but may coexist with or be absent when other HrHPV types are detected in cases of high-grade anal disease [1].

The rate of HGAIN in HIV-positive MSM has been shown to be more than 3 times that of HIV negative MSM [5]. Comparative data from meta-analyses showed a lack of consensus on the prevalence of HrHPV infection or the associated changes of AIN and progression of disease to anal cancer [9]. Reasons for this are drawn from studies of anal disease that have demonstrated variation in the precision of sampling methods and the interpretation of cytology and biopsy findings for predicting the degree of abnormality [10, 11]. Despite the heterogeneity of study findings for significant predictors for HGAIN across all vulnerable populations it is recognised that the most at-risk group are MSM with HIV immunosuppression and HrHPV infection especially HPV 16 [12]. To further our understanding of the relationship between clinico-pathological findings and the presence of HrHPV genotypes in anal specimens collected during the HRA procedure, we compared three molecular methods- two commercial platforms and an in-house multiplex method, with a view to investigate the utility and agreement of each for the detection of HrHPV. The investigation would validate an optimal testing platform for our practice, provide insight into the spectrum of HPV genotypes in the targeted cohort and allow subsequent investigation of the other contributing diagnostic factors in the biology and management of anal disease.

Research into anal cancer and its precursors is evolving rapidly especially since treatment of precursors has been shown to reduce the incidence of anal cancer [13].The genotypes associated with HGAIN are not fully established, nor the number of genotypes associated with the disease and progression. The use of a genotyping test appears to be important for anal disease monitoring and research.

Materials and Methods

Study population

The study was approved by the Royal Perth Hospital Ethics Committee (2010-057) and informed consent was obtained from each patient before participating in the study. A total of 120 patients were recruited between June 9th 2010 and February 22nd 2011 for whom there was cellular material for HPV assessment and either cytology or biopsy for assessing any cellular abnormality. Six patients were examined twice over this period. Demographic data relating to sex, HIV status and sexual preference were collected.

The patient population included referrals to the sexual health clinic for suspected anal disease, or high-risk patients with anal disease or abnormal cytology being routinely followed in the clinic. Patients underwent high resolution anoscopy with specimens collected for sexually transmitted disease (STD) studies, diagnostic cytology by conventional and ThinPrep (TP) methods and HPV assay. Tests for STD's included rectal culture and sensitivity studies for *Neisseria gonorrhoeae* (NG) and PCR testing for *Chlamydia trachomatis* (CT). Cytology specimens were collected using a cervical brush device through a proctoscope. The brush was rotated from the anal canal to the anal verge as the proctoscope was withdrawn. A conventional smear was made first, and immediately fixed in 95% ethanol. To preserve the remaining cellular material the brush was vigorously rinsed in a collection vial for liquid based cytology and adjunct testing. An aliquot of this specimen was removed for high-risk HPV assay by the HC2, ART and MPX methods.

HPV DNA Assay Methods

The HC2 (Qiagen) test was performed according to manufacturer's protocol for 13 high risk mucosal genotypes (i.e., genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). It included denaturation of HPV DNA, and incubation with an RNA probe B to form RNA/DNA hybrids. The hybrids were captured in a solid phase with antibodies specific for RNA DNA hybrids and were attached by antibodies conjugated to alkaline phosphatase. The chemi-luminescence of the conjugated antibody hybrid complex was measured as relative light units (RLU's) by a luminometer. A sample was classified positive when the RLUs were equal to or greater than 1.

The ART (Abbott Molecular GmbH & Co KG Wiesbaden Germany) is a qualitative multiplex real time test for the detection of 14 HrHPV types. The method tests separately for HPV16 and HPV18 while concurrently detecting the 12 remaining HrHPV types as a group (31, 33, 35, 39, 45, 51, 52, 56 58, 59 ,66, 68). The PCR is performed with modified gp5+/6+ primers amplifying a short fragment (approximately 150bp) of the L1 region. The master mix comprises four probes: a VIC labelled probe to identify HPV16, a NED labelled probe specific to HPV 18, a FAM labelled probe to detect the "other" types and a quasar labelled probe for the internal control.

Internal validation for the method is via an endogenous human beta globin sequence. This was the control checkpoint for cell adequacy, sample extraction and amplification efficiency. The analytical sensitivity is 500 copies per assay for HPV types 16, 18, 35, 39, 45, 51, 59, 66 and 68, 2000 copies for HPV types 31, 33, 52, 56, and 5000 copies per assay for HPV 58. A threshold cycle number of 32 established by the manufacturer was considered the assay cut off for positivity [14].

The MPX PCR detects 20 HPV genotypes, namely low risk types 6, 11, 26, 53, 73, 82 and high-risk types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68. The nested PCR uses L1 gene primers with external primers MY09/MY11 amplifying a 450bp product and the internal primers GP5+/GP6+ amplifying a 150bp product. Both first and second round products are sequenced to obtain the genotype. The method incorporates an internal human beta globin control and equine herpes inhibition control. This method was considered the gold standard where discordant results between the two commercial assays were observed.

Results

An HPV assay result from at least one sample was available for 111 male (96% MSM), and 9 female patients. Fifty six percent of the male subjects were HIV-positive. None of the female patients were HIV-positive. The median age was 46 (range 19-83) years.

The HC2 test was positive in 84% of 116 tests whereas the ART test was positive in 78% (54% genotypes 16 ± 18; 46% other Hr genotypes) of 123 tests. The MPX test detected Hr genotypes in 98% of 116 tests (59% genotypes 16 ± 18; 41% other Hr genotypes). The agreement varied between the platforms when matched. It was strong between the two commercial platforms (k=0.70, $CI_{95\%}$: 0.53 – 0.86) and for the ART vs MPX (k=0.66 CI95%: 0.43-0.89) (Table 1); there was moderate agreement between the HC2 and MPX methods alone (κ=0.48, $CI_{95\%}$: 0.19 - 0.77).There was strong agreement between HPV 16 ± 18 detection by the ART and MPX (κ=0.82, $CI_{95\%}$: 0.72-0.93) as there was for HPV 16 alone (κ=0.86, $CI_{95\%}$: 0.76-0.95) and for HPV 18 alone (κ=0.84, $CI_{95\%}$ 0.72-0.96).

Table 1: Comparison of the agreement between the three assay methods

ART vs HC2, ART vs MPX and HC2 vs MPX for the detection of HPV and the agreement between ART and MPX for detection of hrHPV 16 or hrHPV 18

	Contrast		
Measure of agreement	ART vs. HC2	ART vs. MPX	HC2 vs. MPX
Detection of any high-risk HPV			
Sensitivity (CI95%)	97.8 (92.2-99.7)	100 (95.8-100)	96.5 (90.1-99.3)
Specificity (CI95%)	65.4 (44.3-82.8)	53.3 (26.6-78.7)	45.5 (16.7-76.6)
Kappa (CI95%)	0.70 (0.53-0.86)	0.66 (0.43-0.89)	0.48 (0.19-0.77)
N	116	102	100
Detection of HPV16 or HPV18			
Sensitivity (CI95%)		98.1 (90.1-100)	
Specificity (CI95%)		83.7 (70.3-92.7)	
Kappa (CI95%)		0.82 (0.72-0.93)	
N		102	

Sensitivity and Specificity for Each Method

The sensitivity of each of the testing platforms for the detection of HrHPV DNA is very good (Table 1- kappa 0.82). Despite the good overall agreement between the ART and HC2 tests discrepant results were observed for six cases (Figure 1). The HC2 detected HrDNA in twenty-five cases that were not detected by the ART or the MPX method.

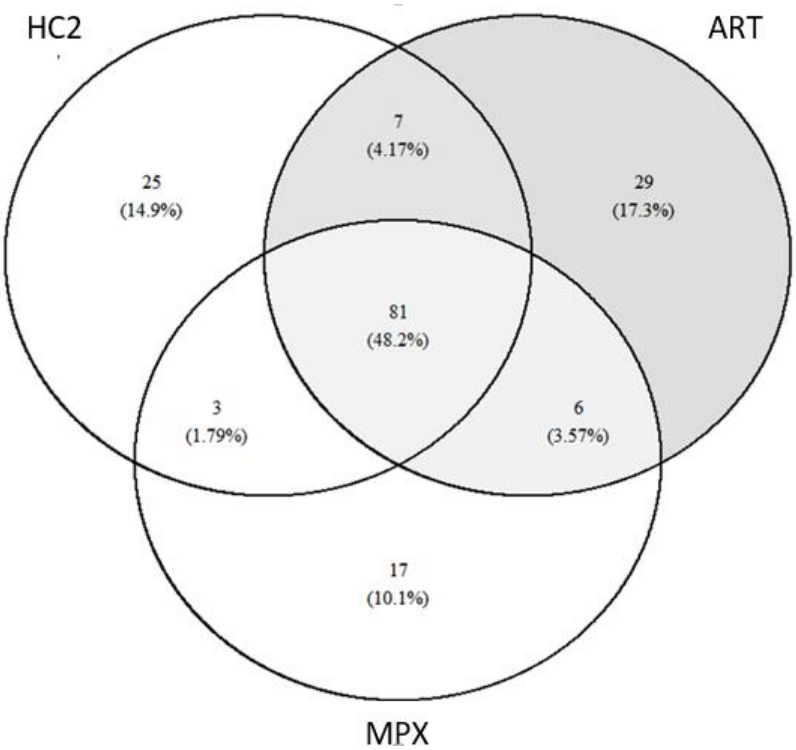

Figure 1: Venn diagram showing the agreement between the detection of HrHPV by HC2, ART and MPX. All methods showed concordance in (48.2%) 81 cases

The ART assay demonstrated similar sensitivity to the MPX method. There was one case where the Abbott test detected HrHPV DNA (not 16 or 18) whilst MPX identified type 16 among six other high- risk types detected in the specimen.

When comparing genotype detection for HPV 16, 18 on LBC samples ART vs MPX, agreement was substantial (sensitivity 98.1%, kappa 0.82). Outlying specimens that tested positive for HC2 were more likely to be negative for ART and MPX.

A majority of subjects were identified with multiple genotypes as detected by sequencing samples tested by the MPX method (range 0-12, median 2) (Figure 2).

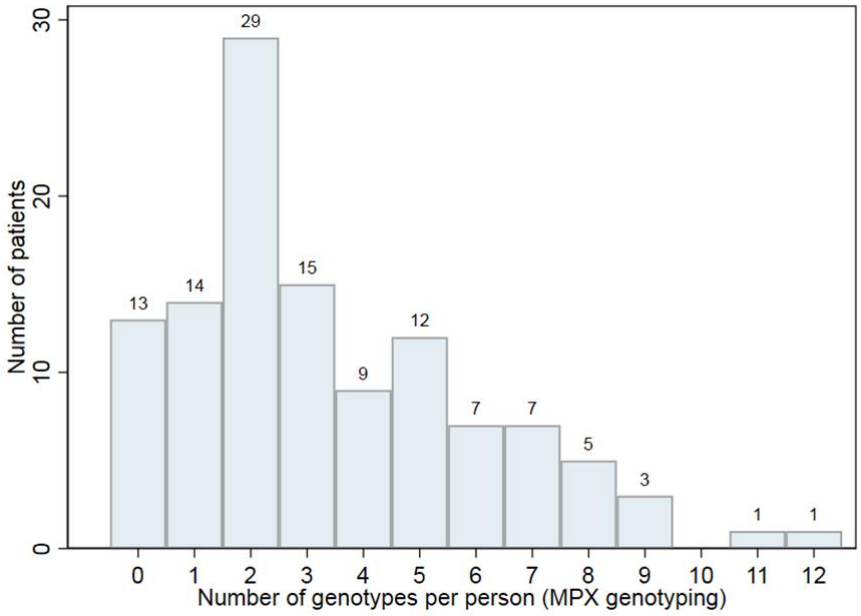

Figure 2: Number of genotypes of HPV per subject that were detected by sequencing samples tested by the MPX method

Conclusion

In this study we compared the results obtained by three primary screening molecular techniques for detecting HPV DNA in a series of patients attending a public sexual health clinic. The HC2 method is a clinically validated test that has been utilised as the standard method for our reporting laboratory and detects DNA from 13 HrHPV genotypes. The two PCR-based methods provide targeted genotype information - the ART detected 14 HrHPV genotypes with concurrent identification of HrHPV 16 and/or 18 and the MPX method detects 20 genotypes including 6 low risk and 14 HrHPV types.

The overall agreement between the three methods was very good when the methods were compared for the detection of HrHPV. However, the assays tested here disagreed from between 17 to 29% of the time i.e., the assays were not detecting the same infections (Figure 1). There were several discordant results which typically involved a negative result for one method in the presence of a positive result in the other two or vice versa where one method was positive in the presence of a negative outcome by the other two. There is no definite explanation for the observed differences but assay design, sample processing, virus particle detection, cross reactivity among other variables may make precise comparison between analytical methods implausible at this time. Sample variation is an unlikely cause for the differences observed in this study. Reports of the frequency and type of HPV in anal samples are few and typically comparison studies of the performance of HPV tests have been on cervical screening populations. In a review of the comparison of HC2 with ten assays approximately 30-50% of samples were discordant [15]. In that report the highest concordance was reported between HC2 and ART where assays still disagreed in one third of all positive cases.

Reported confounding influences affecting outcome (either positive or negative) of HC2 include limited material, too few viral copies in available material, infection by low-risk HPV genotypes, cross reactivity with low-risk HPV or HrHPV genotypes outside of the test range.

The collective genotyping results from the MPX platform provided novel data for this cohort for the wide range of benign and oncogenic HPV infections at the time of investigation (Figure 2). This information will be valuable in subsequent studies of correlation with demographic, clinical and current and emerging biomarker characteristics. While the influence of persistent HPV16 on cancer development in HIV-positive MSM is accepted the influence of other HrHPV types and syndemic factors requires further investigation [16].

The clearance, maintenance and acquisition of anal HPV genotypes has not been widely reported. One study recorded the acquisition, and clearance in MSM and men who have sex with women (MSW)[17]. In MSM the rate of acquisition of HPV 16 was 6.5 times greater than the rate for MSW; in MSM clearance of HPV 16 and HPV 18 occurred in 27.2% (3/11 subjects) and 62.5% (5/11 subjects) respectively while all MSW subjects with HPV 16 as the prevalent infective HPV type cleared the infection within 6 months. A recent longitudinal investigation of untreated HGAIN in MSM revealed greater rates of clearance of cytological HGAIN (AIN2) in younger subjects who showed no persistent HrHPV over 3 years and lower rates of clearance in those with persistent HPV16 or other HrHPV [7]. The emerging evidence that HGAIN can regress spontaneously presents a potential opportunity to weigh the benefits of intervention against adverse outcomes from current treatment practices. A promising approach has been reported using molecular biomarkers to allow stratification of risk in target populations with a view to improving cancer prevention and avoid overtreatment of lesions that are unlikely to progress [18].

This study provides a valuable snapshot of anal disease in a high-risk population and has demonstrated the prevalence of anal HPV infection, the frequency of multiple HPV genotypes from material obtained from single HRA episodes. The adaptation of the technology to the management of AIN in our laboratory is still to be formalized [11].

This comparison study sheds light on the clinical utility of the each of the HPV assays examined in subjects attending a sexual health clinic.

i) Each test identified the presence of HrHPV infection in the cohort with a moderate level of agreement

ii) ART and MPX confirm the prevalence of HPV 16 and HPV 18 in the study cohort

iii) Complete (MPX) genotyping of HPV separately identified the range of benign and oncogenic types in the cohort

iv) The level of agreement between the ART and MPX platforms for the detection of HrHPV validates the incorporation of these tests in the pathology investigation algorithm.

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