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ABSTRACT: HPV52 is one of the most commonly detected genotypes in women with cervical intraepithelial neoplasia (CIN). Some methods for genotyping HPV are, however, biased against detection of HPV52. Current literature on this topic primarily focuses on the earliest consensus primer sets MY09/11 and GP5+/6+, or derivatives thereof. There are now many more genotyping assays in use. Given the importance of HPV52 and CIN, and the recent approval of the nine-valent HPV vaccine including HPV52, we undertook an updated discussion and analysis of HPV52 detection in women with CIN2+ by assay type and geographical region from cohorts published between 2006 and 2016. Little difference in HPV52 prevalence was observed by assay type, except sequencing and restriction fragment length polymorphism methods. The most commonly used genotyping methods in the past decade appear to be consistent for detection of HPV52. However, in longitudinal studies the same assay system should be used where possible.

KEYWORDS: HPV-52, genotyping, CIN, assays, PCR

Why Is Prevalence of HPV-52 Important?
Cervical cancer is the fourth most common cancer in women across the world, accounting for an estimated 266 000 deaths in 2012. The burden of cervical cancer is greater in less developed areas, with 87% of cervical cancer deaths occurring in these regions, although this is probably underreported in developing nations.1 Persistent infection with human papillomavirus (HPV) types can result in transformation of normal cervical epithelium into high-grade cervical intraepithelial neoplasia (CIN II and III) which may progress to cervical cancer. In 2005 and 2009, the International Agency for Research on Cancer listed 14 high-risk HPV (HR-HPV) types as carcinogenic or probably carcinogenic to humans (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), although HPV-66 was downgraded to possibly carcinogenic at the 2009 meeting.2,3 Although the attribution of specific HPV types to cervical cancer varies geographically, HPV types 16 and 18 are the most common genotypes identified in cervical cancers across the world, accounting for approximately 70% of cervical cancers4,5 and upwards of 50% of CIN II/III.6

The introduction of the quadrivalent (4vHPV) vaccine, which specifically targets HPV-16 and HPV-18 (as well as low-risk HPV types 6 and 11), has resulted in significant reductions in the prevalence of all 4 targeted HPV types in the general population.7 A 9-valent (9vHPV) vaccine has recently been approved for use in several countries and offers protection against HPV-31, HPV-33, HPV-45, HPV-52, and HPV-58 in addition to the 4 genotypes included in the 4vHPV vaccine. These additional genotypes account for approximately 19% of cervical cancers worldwide, and introduction of these types in the 9vHPV vaccine has potential to protect against approximately 90% of cervical cancers.8,9 In the context of the 9vHPV vaccine, defining an accurate prevalence estimate for HPV-52 is important to inform decisions about which vaccine is most appropriate for use for a specific population and for postvaccination monitoring.

The global prevalence of HPV-52 in cervical cancers has been reported to be between 2.8% and 3.8%,4–6,10 and approximately 12% in CIN II/III.6,10 However, HPV-52 prevalence estimates in women vary greatly across differing geographical regions, and several studies from Eastern Asia report it as being the second or third most common HPV type detected in cervical neoplasia.11–16 A recent meta-analysis reported the relative prevalence of HPV-52 to be 16.5% in women with high-grade cervical disease (CIN II/III) and 5.7% in cervical cancers of unspecified histology in Eastern Asia, compared with only 8.1% and 1.8% in Europe.10 Studies using laser capture microdissection conducted by our group in Australian women estimated the prevalence of HPV-52 in CIN III and cervical cancers to be 4.5% and 2.3%, respectively (manuscripts in preparation). Furthermore, several studies have shown HPV-52 prevalence in CIN to vary by age. For example, Chao et al12 described the HPV type prevalence in a...
population of Taiwanese women with CIN II/III and reported a higher prevalence of HPV-52 in older women (aged 50 years and above) compared with younger women (24.7% and 17.8%, respectively).

The variation reported in HPV-52 prevalence estimates is likely to vary based on the age and location of women studied. However, prevalence estimates of HPV-52 could also be affected by the HPV assay used. During recent years, a variety of HPV genotyping assays have been developed and used to estimate HPV genotype prevalence in women with cervical lesions worldwide. In this commentary, we explore the potential influence of the techniques used for HPV genotyping on estimates of HPV-52 prevalence in women with high-grade cervical lesions. We also discuss technical issues surrounding the HPV-52 testing with the aim of informing readers of the potential limitations of specific tests, how these limitations may impact prevalence estimates, and recommendations on how to minimise the potential effects of these technical limitations. To achieve this aim, we conducted a literature review of studies reporting HPV-52 prevalence in women with CIN II/III and performed a comparison of HPV-52 prevalence stratified by geographical region and by HPV assay type. Due to the large range of HPV primer sets and detection methods available, this is not a comprehensive review of all methods, but rather a discussion of potential issues using some of the most commonly used methods as examples.

Technical Issues Around Testing for HPV-52

Known or suspected systematic issues with HPV-52 detection have been reported in several studies. The regularly conducted HPV LabNet Global proficiency panels have repeatedly reported poor detection of HPV-52, with particularly high levels of false negatives or false positives reported for specific assays. The sensitivity and specificity of molecular detection of individual HPV genotypes are known to vary between HPV assays. All currently available full HPV genotyping assays use polymerase chain reaction (PCR)–based target amplification. The primary differences between genotyping assays are the target amplicon (gene region targeted, size of amplification), the presence of high-copy-number HPV-16 DNA and/or low-copy-number, non–HPV-16 high-risk genotypes in the presence of low-viral-load HPV-16. Conversely, we attempted to artificially generate genotype bias by masking low-copy-number, non–HPV-16 high-risk genotypes in the presence of high-copy-number HPV-16 DNA and/or an additional high-risk genotype on the Linear Array HPV Test, which is based on the MY09/11 primer set. Although masking of several genotypes was observed, HPV-52 detection was not affected. In contrast to consensus primer pairs or sets, assays based on multiplexed genotype-specific primer pairs are designed to reduce genotype bias by eliminating competition for primer binding.

All capture and detection probes for genotype identification, regardless of assay type, are designed to be as genotype specific as possible. Some of the earliest and most widely used commercial assays, reverse line probe assays that target part of the L1 gene sequence, experience cross-reactivity in the probe target region of HPV-52. Specifically, neither the PGMY09/11 consensus primer sets contain primer pairs that are a perfect match for the L1 target sequence of HPV-52. Thus, HPV-52 may in theory be less efficiently amplified and less likely to be detected at a lower copy number and may also potentially be outcompeted in the presence of better-matched genotypes. The generic primer pairs MY09/11 and GP5+/6+ similarly contain mismatches to the HPV-52 L1 target sequence and are less sensitive for the detection of HPV-52 at lower viral DNA copy number. This has also been reported to have very low sensitivity for HPV-52 in a Chinese cohort of cervical cancer specimens due to a particular sequence variation in the target sequence that is overrepresented outside of Europe. The PGMY09/11 consensus primer set was based on the MY09/11 generic primer pair and has improved detection of several genotypes, including HPV-52, due to a decreased number of primer mismatches. MY09/11 and GP5+/6+ primers have been experimentally shown to amplify HPV-52 molecular clones much less efficiently than HPV-16, even in single-genotype reactions, and even less efficiently in the presence of high-copy-number HPV-16. A recent study investigated whether HPV-52 was masked in the presence of HPV-16 when samples were tested using L1 PGMY09/11 or MY09/11 consensus primer-based genotype assays. This was determined by performing HPV-52–specific real-time PCR on samples that had originally tested negative for HPV-52, with half testing positive for HPV-16 and half negative for HPV-16. The results suggested that some masking may have occurred in the presence of high-viral-load HPV-16. Conversely, we attempted to artificially generate genotype bias by masking low-copy-number, non–HPV-16 high-risk genotypes in the presence of high-copy-number HPV-16 DNA and/or an additional high-risk genotype on the Linear Array HPV Test, which is based on the PGMY09/11 primer set. Although masking of several genotypes was observed, HPV-52 detection was not affected. In contrast to consensus primer pairs or sets, assays based on multiplexed genotype-specific primer pairs are designed to reduce genotype bias by eliminating competition for primer binding.

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(Iinnogenetics N.V., Ghent, Belgium) have a unique detection probe for HPV-52. The Linear Array HPV-52 probe also detects HPV-33, HPV-35, and HPV-58. The single INNO-LiPA probe for HPV-52 also cross-reacts with HPV-31, HPV-33, HPV-40, HPV-53, and HPV-58. Therefore, in the presence of any of these other genotypes, there is a need for an additional confirmatory test to determine HPV-52 positivity61,62 (Supplementary Table 1). This confirmatory test is not always performed and potentially causes underestimation of HPV-52 prevalence.63–67 Conversely, a sample can be falsely called HPV-52 positive if the additional genotype probes needed to identify HPV-31, HPV-33, HPV-35, HPV-40, HPV-53, or HPV-58 do not bind efficiently or if the user assumes HPV-52 positivity without performing a confirmatory test. In the HPV LabNet International proficiency studies, HPV-52 is systematically reported as being present in samples only containing HPV-35 or HPV-58 DNA by some testing laboratories using Linear Array or INNO-LiPA.17,68

<table>
<thead>
<tr>
<th>TEST NAME</th>
<th>GENE REGION</th>
<th>PRIMER SET</th>
<th>DETECTION METHODS</th>
<th>HPV-52 DETECTION</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHA-LiPA25 V1</td>
<td>L1</td>
<td>SPF10</td>
<td>Reverse line blot</td>
<td>Yes</td>
<td>van der Marel et al,23 van Hamont et al²⁴</td>
</tr>
<tr>
<td>INNO-LiPA V2</td>
<td>L1</td>
<td>SPF10</td>
<td>Reverse line blot</td>
<td>Shared probe³⁶</td>
<td>Pretet et al²⁵</td>
</tr>
<tr>
<td>INNO-LiPA Extra CE test</td>
<td>L1</td>
<td>SPF10</td>
<td>Reverse line blot</td>
<td>Yes</td>
<td>Kovanda et al²⁶</td>
</tr>
<tr>
<td>Roche Linear Array</td>
<td>L1</td>
<td>PGMY</td>
<td>Reverse line blot</td>
<td>Shared probe³⁶</td>
<td>Resende et al,²⁷ van Hamont et al²⁴</td>
</tr>
<tr>
<td>CLART Human papillomavirus 2</td>
<td>L1</td>
<td></td>
<td>Microarray</td>
<td>Yes</td>
<td>Pista et al²⁸</td>
</tr>
<tr>
<td>EUROArray</td>
<td>E6/E7</td>
<td>Microarray</td>
<td></td>
<td>Yes</td>
<td>Cornell et al²⁹</td>
</tr>
<tr>
<td>PCR and line blot–based detection (in-house)²⁶</td>
<td>L1</td>
<td>PGMY, GP5+/ GP6+, SPF, others</td>
<td>Reverse line blot</td>
<td>Yes</td>
<td>Azuma et al,³⁰ Kim et al,³¹ van den Brule et al³²</td>
</tr>
<tr>
<td>Pyrosequencing/Sanger sequencing</td>
<td>Various</td>
<td></td>
<td>Sequencing</td>
<td>Yes</td>
<td>Sanger et al,²³ Antonishyn et al,²⁴ Chen et al²⁶</td>
</tr>
<tr>
<td>INFINITI HPV Genotyping Assay</td>
<td>E1</td>
<td>Microarray</td>
<td></td>
<td>Yes</td>
<td>Erali et al³⁶</td>
</tr>
<tr>
<td>HybriBio HPV GenoArray (GA) genotyping assay</td>
<td>L1</td>
<td>PGMY and GP5+/GP6+</td>
<td>Microarray</td>
<td>Yes</td>
<td>Ding et al,³⁷ Hou et al³⁸</td>
</tr>
<tr>
<td>HPV DNA Chip Biomedlab</td>
<td>L1</td>
<td>Microarray</td>
<td></td>
<td>Yes</td>
<td>Zhao et al³⁹</td>
</tr>
<tr>
<td>HPV 9G DNA chip</td>
<td>L1</td>
<td>Microarray</td>
<td></td>
<td>Yes</td>
<td>Sung et al⁴⁰</td>
</tr>
<tr>
<td>MyHPV chip</td>
<td>L1</td>
<td>GP5+/GP6+</td>
<td>Microarray</td>
<td>Yes</td>
<td>Kang et al⁴¹</td>
</tr>
<tr>
<td>Multiplex PCR using Luminex XMAP</td>
<td>L1</td>
<td>GP5+/GP6+</td>
<td>Microsphere beads</td>
<td>Yes</td>
<td>Garcia et al,⁴² Schmitt et al⁴³</td>
</tr>
<tr>
<td>Amplisens HPV⁴</td>
<td>E region</td>
<td></td>
<td>Multiplex/real-time PCR assays</td>
<td>Yes</td>
<td>Agodi et al⁴⁴</td>
</tr>
<tr>
<td>Multiplex E7 PCR/APEX assay</td>
<td>E7</td>
<td></td>
<td>Multiplex/real-time PCR assays</td>
<td>Yes</td>
<td>Gheit et al,⁴⁶ Deodhar et al⁴⁸</td>
</tr>
<tr>
<td>AnyplexTM II HPV-28</td>
<td>L1/L2/E6/ E7</td>
<td></td>
<td>Multiplex/real-time PCR assays</td>
<td>Yes</td>
<td>So et al⁴⁷</td>
</tr>
<tr>
<td>BD Onclarity HPV</td>
<td>E6/E7</td>
<td></td>
<td>Multiplex/real-time PCR assays</td>
<td>Yes</td>
<td>Wright et al,⁴⁸ Schiffman et al⁴⁹</td>
</tr>
<tr>
<td>CervicGen HPV RT-qDx assay</td>
<td>E6/E7</td>
<td></td>
<td>Multiplex/real-time PCR assays</td>
<td>Yes</td>
<td>Wang et al⁵⁰</td>
</tr>
</tbody>
</table>

Abbreviation: PCR, polymerase chain reaction.
²Shared probe with 31, 33, 40, 53, and 58.
²²Shared probe with 33, 35, and 58.
²³Some laboratories still use these techniques for research purposes.
²⁴There are different Amplisens kits produced in different companies in Italy and Moscow.
What Are the Actual Impacts of These Technical Issues?

A meta-analysis of global distribution of HPV-52 in cases of cervical neoplasia has been conducted previously\(^\text{10}\); it was not our intention to repeat this analysis. Numerous comparisons between assays for HPV detection have been published; however, these generally report detection of any HR-HPV, measured against the criterion standard HPV test for CIN II+. Nevertheless, these generally report detection of any HR-HPV, measured against the criterion standard HPV test for CIN II+. In addition to the 64 published studies, we included unpublished data from our group which compared detection of HPV genotypes by 4 different assays to make a total of 70 data sets. A list of data sets included in the analysis.

In all of the 70 data sets, HPV-52 prevalence estimates could not be accurately determined, data from multiple assays were presented and HPV-52 prevalence estimates for each individual assay could not be determined, only CIN I or CIN III HPV-52 prevalence estimates were provided, published earlier than 2006, study subjects were infected with human immunodeficiency virus or otherwise immunocompromised, and repeated reports derived from the original cohort and tested with the same assay. A flow chart of the study selection process is depicted in Figure 1. The electronic search strategy retrieved 1539 records in total, of which 1183 were published after 2006. After screening on title and abstract, 84 publications met our initial selection criteria. After full-text assessment, 64 publications were included. One study compared 2 different assays, and a second study compared 2 cohorts of different ages; thus, a total of 66 published data sets were included in the analysis.

HPV-52 prevalence, diagnosis, study period, and references were recorded in a database. Similar assay types were grouped into SPF10 reverse line blot assay, Linear Array or in-house PGMY reverse line blot assay, multiplex and real-time PCR, microarray-based detection, Sanger sequencing, or enzymatic digestion (including restriction fragment length polymorphism [RFLP]). Overall prevalence (median percentage and interquartile range [IQR]) of HPV-52 stratified by assay, geographical distribution and sample type was described.

Participant age data were not presented consistently, and therefore, analyses were not able to be adjusted for age. Most references did not report vaccination status, and those that did indicated very low or no vaccination. Given the publication time frame, we assumed universally low or no vaccination.

The overall pooled estimate of HPV-52 prevalence across the 70 data sets included in this study was 10.3% (binomial 95% confidence interval [CI]: 9.9–10.6), which is only marginally lower than the recent estimate of 11.0% (95% CI: 10.7–11.3) reported by Bruni et al.\(^\text{69}\) The slight discrepancy in HPV-52 prevalence estimates is likely a result of different literature search strategies and different study inclusion criteria imposed by Bruni et al.\(^\text{69}\)

Little difference was observed between most of the assay types investigated (Table 2 and Supplementary Figure 1A). Linear Array and multiplexed/real-time PCR assays had the highest median prevalence of HPV-52 at around 11%, whereas median detection by SPF10 or microarray was slightly lower at approximately 8%. The IQR overlapped for all 4 of these assay types. Detection of HPV-52 was, however, substantially lower for sequencing or enzymatic digestion methods, with a median prevalence of only 2.3%. Closer inspection of these 7 data sets shows that most used older primer sets, such as MY09/11, GP5+6+, or SPF1/2, which have established bias against HPV-52 amplification as described above.
There was no systematic difference between geographical regions reported, with all regions at approximately 8% to 9%, with the exception of 2 disparate African studies (Table 3 and Supplementary Figure 1B).

The largest number of data sets was from Asian populations, and this group had the widest range of reported prevalence of HPV-52, but the lowest median overall at 7.8%. This result is in contrast to several previous reports that indicated parts of Asia had higher rates of HPV-52 associated with CIN II/III than other regions of the world.10–16 This apparent discrepancy may have several explanations. The 2014 meta-analysis used a different literature search strategy, including studies in Chinese, and calculated the prevalence of HPV-52 as a proportion of HPV-positive cases only, which may have resulted in differing prevalence estimates.10 We observed wide variation in prevalence estimates by country in our literature sample, and therefore, individual studies conducted in particular regions may have reported higher prevalence of CIN-associated HPV-52. Finally, a high proportion (41%) of reports from Asia were conducted using microarray-based tests, which in our analysis had slightly lower rates of HPV-52 detection than some other assay types.

Overall, most studies reported testing of cervical cytology samples. There was little difference in overall prevalence of HPV-52 by sample type; however, biopsy samples had slightly lower HPV-52 prevalence than cytology samples, as would be expected (Table 4 and Supplementary Figure 1C).

### Recommendations for HPV-52 Genotype Testing of Women With CIN II/III

Over the past couple of decades, there have been many reports of bias against detection of HPV-52 in women with high-grade cervical disease. The results of our review of studies published in the past decade are reassuring. With the exception of reports using sequencing or RFLP, none of the other assays demonstrated systematic bias with respect to HPV-52 prevalence. The low prevalence of HPV-52 detected by sequencing/RFLP can likely be explained using generic primer pairs that have known bias against HPV-52. Sanger sequencing and RFLP also have the inherent disadvantage of poorly discriminating mixed genotype samples and are not ideal for HPV genotyping. Most of the remaining studies used newer assay systems or updated primer sets as described above.
Table 4. Breakdown of HPV-52 prevalence by specimen type (high-grade lesions, ie, CIN2+).

<table>
<thead>
<tr>
<th>SPECIMEN TYPE</th>
<th>N (TOTAL SAMPLE SIZE)</th>
<th>N (DATA SETS)</th>
<th>PREVALENCE, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cervical cells and biopsy</td>
<td>1235</td>
<td>4</td>
<td>9.8</td>
</tr>
<tr>
<td>Cervical cells</td>
<td>21,307</td>
<td>50</td>
<td>9.2</td>
</tr>
<tr>
<td>Biopsy</td>
<td>863</td>
<td>4</td>
<td>8.4</td>
</tr>
<tr>
<td>Biopsy FFPE</td>
<td>9167</td>
<td>12</td>
<td>7.0</td>
</tr>
</tbody>
</table>

Abbreviations: FFPE: formalin-fixed paraffin-embedded; IQR, interquartile range.

The data from this review, and our own research (in preparation), identify reverse line blot assays based on amplification of an L1 target using PGMY-based consensus primer sets as the most sensitive for HPV-52 detection. Despite the known bias against HPV-52 by these primer sets, factors which may counter this include recent modifications to the primer sets to improve HPV-52 detection and the relatively large template input volume for the Linear Array assay (50 µL). For reverse line blot assays with a shared HPV-52 probe, a confirmatory HPV-52-specific test should be used for all ambiguous results. There are at least 2 published qPCR protocols for confirmatory HPV-52 testing, and both are simple, rapid, and cost-effective.61,62

All HPV assays perform slightly differently to other assays. In general, the advice should be to use the same assay system where possible to compare longitudinal samples, to perform confirmatory testing where indicated, and to investigate further any results that appear unusually low or high.

Author Contributions

This work was carried out in collaboration with all authors. Developed the structure of the paper: AC. Performed the initial literature search: EP. Decided on final eligibility of manuscripts for inclusion in literature review: EP, MM, AC. All HPV assays performed slightly differently to other assays. In general, the advice should be to use the same assay system where possible to compare longitudinal samples, to perform confirmatory testing where indicated, and to investigate further any results that appear unusually low or high.

REFERENCES


