Technical Issues Associated With Estimating the Prevalence of HPV-52 in Cervical Intraepithelial Neoplasia

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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

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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 cancers[4,5] 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 to 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 al[12] 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.[17,18] The sensitivity and specificity of molecular detection of individual HPV genotypes are known to vary between HPV assays.[19–21] 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 amplicon, primer sequence), detection probes, and amplicon detection method. A comprehensive description of these can be found in a recent review,[22] and examples of the most commonly used assay types are also shown in Table 1. The most common amplification targets for alpha papillomaviruses include the L1, E6, and E7 genes. Amplification primer sets comprise generic primers, consensus primer sets, or type-specific primer pairs. Detection platforms include reverse hybridisation/line probe assays, microarray, quantitative polymerase chain reaction (qPCR), and bead-based flow cytometry.

Primer and probe sequences in particular have the potential to introduce genotype-specific detection bias. Consensus or degenerate primer sets (ie, a set of multiple primers designed to collectively amplify multiple genotypes in a single pool) and generic primers (ie, a single pair of primers also designed to amplify multiple genotypes) are more prone to bias than genotype-specific primer sets, where each pair of primers has been optimised to specifically amplify only a single genotype. This is primarily because the target sequences of particular genotypes will match more closely to consensus or generic primer sequences than others and will therefore amplify more efficiently.[51,52] Specifically, for HPV-52, none of the SPF1/2, SPF10, or PGMY09/11 consensus primer sets contain primer pairs that are a perfect match for the L1 target sequence of HPV-52.[52–54] 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.[52,55,56] GP5+/6+ 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.[57] 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.[52] 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.[58] 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.[59] 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 of 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.[51] 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.[21,60]

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 PGMY-primer–based Linear Array HPV Genotyping Test (Roche Molecular Diagnostics, Pleasanton, CA, USA) nor the SPF10-primer–based INNO-LiPA HPV version 2

Table 1. Characteristics of some of the most common HPV genotype tests.

TEST NAME	GENE REGION	PRIMER SET	DETECTION METHODS	HPV-52 DETECTION	REFERENCES
RHA-LiPA25 V1	L1	SPF10	Reverse line blot	Yes	van der Marel et al,[23] van Hamont et al[24]
INNO-LiPA V2	L1	SPF10	Reverse line blot	Shared probe[a]	Pretet et al[25]
INNO-LiPA Extra CE test	L1	SPF10	Reverse line blot	Yes	Kovanda et al[26]
Roche Linear Array	L1	PGMY	Reverse line blot	Shared probe[b]	Resende et al,[27] van Hamont et al[24]
CLART Human papillomavirus 2	L1		Microarray	Yes	Pista et al[28]
EUROArray	E6/E7		Microarray	Yes	Cornall et al[29]
PCR and line blot–based detection (in-house)[c]	L1	PGMY, GP5+/GP6+, SPF, others	Reverse line blot	Yes	Azuma et al,[30] Kim et al,[31] van den Brule et al[32]
Pyrosequencing/Sanger sequencing	Various		Sequencing	Yes	Sanger et al,[33] Antonishyn et al,[34] Chen et al[35]
INFINITI HPV Genotyping Assay	E1		Microarray	Yes	Erali et al[36]
HybriBio HPV GenoArray (GA) genotyping assay	L1	PGMY and GP5+/GP6+	Microarray	Yes	Ding et al,[37] Hou et al[38]
HPV DNA Chip Biomedlab	L1		Microarray	Yes	Zhao et al[39]
HPV 9G DNA chip	L1		Microarray	Yes	Sung et al[40]
MyHPV chip	L1	GP5+/GP6+	Microarray	Yes	Kang et al[41]
Multiplex PCR using Luminex XMAP	L1	GP5+/GP6+	Microsphere beads	Yes	García et al,[42] Schmitt et al[43]
Amplisens HPV[d]	E region		Multiplex/real-time PCR assays	Yes	Agodi et al[44]
Multiplex E7 PCR/APEX assay	E7		Multiplex/real-time PCR assays	Yes	Gheit et al,[45] Deodhar et al[46]
AnyplexTM II HPV-28	L1/L2/E6/E7		Multiplex/real-time PCR assays	Yes	So et al[47]
BD Onclarity HPV	E6/E7		Multiplex/real-time PCR assays	Yes	Wright et al,[48] Schiffman et al[49]
CervicGen HPV RT-qDx assay	E6/E7		Multiplex/real-time PCR assays	Yes	Wang et al[50]

Abbreviation: PCR, polymerase chain reaction.
[a]Shared probe with 31, 33, 40, 53, and 58.
[b]Shared probe with 33, 35, and 58.
[c]Some laboratories still use these techniques for research purposes.
[d]There are different Amplisens kits produced in different companies in Italy and Moscow.

(Innogenetics 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 positivity[61,62] (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] A more recent SPF10 line probe assay, RHA kit HPV SPF10-LiPA25, version 1 (Labo Bio-medical Products, Rijswijk, The Netherlands), has a single unambiguous probe that only reacts with HPV-52.

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[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+ (Hybrid Capture 2 or GP5+/6+) or another assay lacking full genotyping such as Roche Cobas 4800 or Amplicor. Our aim was to assess whether there was any evidence for systematic bias for or against HPV-52 detection based on commonly used genotyping assays and to provide commentary on the findings. To this end, we conducted a literature review of studies assessing the prevalence of HPV-52 in women with cervical lesions that were published between 2006 and October 2016. Articles were retrieved from the PubMed database using the following search terms: (HPV[All Fields] AND ('genotype'[MeSH Terms] OR 'genotype'[All Fields])) AND (('cervical'[MeSH Terms] OR 'CIN'[All Fields] OR 'cervical'[All Fields]) AND ('neoplasms'[MeSH Terms] OR 'neoplasms'[All Fields] OR 'neoplasia'[All Fields])). Only papers published in English were reviewed. Study selection was performed in 2 phases: (1) screening for eligibility based on title and abstract (performed by E.P.), and (2) final inclusion based on full-text assessment (E.P., M.M., and A.C.). Uncertainties regarding eligibility were resolved by discussion between all the authors. Studies were excluded if they were not original studies, no histological diagnosis was available, 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. Data were extracted from each study for HPV-52 prevalence for histologically confirmed diagnosis of 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 is presented in Supplementary Table 2.

Data including testing methods, genotypes, DNA region targeted for detection, geographical source of sample, sample size, participant ages, HPV vaccination status, sample type, 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.

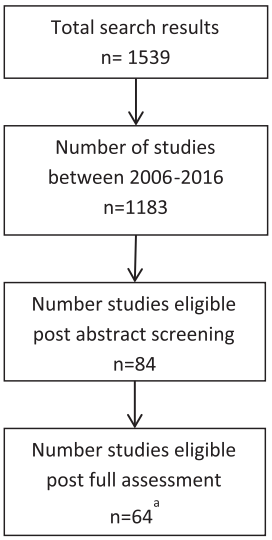

Figure 1. Literature search strategy.
[a]One study compared 2 different assays, and a second study compared 2 cohorts of different ages; thus, a total of 66 published data sets were included in the analysis.

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.[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.[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.

Table 2. HPV-52 prevalence by test (high-grade lesions, ie, CIN2+).

ASSAY	N (TOTAL SAMPLE SIZE)	N (DATA SETS)	PREVALENCE, %	
			MEDIAN	IQR
PCR reverse hybridisation methods				
SPF10 RLB	1715	6	7.5	6–19.6
Linear Array or RLB	18 918	23	11.2	7.9–15.1
Multiplex and real-time PCR assays	5961	18	11.1	7.2–13.1
Microarray-based test	4922	16	8.7	7.2–9.4
Sequence or enzymatic digestion	1056	7	2.3	0–5.3

Abbreviations: IQR, interquartile range; PCR, polymerase chain reaction; RLB, reverse line blot.
Includes all specimen types.

Table 3. HPV-52 prevalence by region (high-grade lesions, ie, CIN2+).

REGION	N (TOTAL SAMPLE SIZE)	N (DATA SETS)	PREVALENCE, %	
			MEDIAN	IQR
Africa	13	1	38.5	
Asia	8255	27	7.9	5.3–15.9
Oceania	2491	7	8.9	7.6–13.9
Europe	5283	16	9.1	6.7–13.1
North America	15 888	13	9.4	7.9–13.5
South America	422	5	9.2	6.1–12.2
South Africa	220	1	6.8	

Abbreviation: IQR: interquartile range.
Includes all specimen types.

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+).

SPECIMEN TYPE	N (TOTAL SAMPLE SIZE)	N (DATA SETS)	PREVALENCE, %	
			MEDIAN	IQR
Cervical cells and biopsy	1235	4	9.8	4.2–14.9
Cervical cells	21 307	50	9.2	6.8–14.8
Biopsy	863	4	8.4	3.8–10.7
Biopsy FFPE	9167	12	7.0	4.1–9.4

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. Analysed the data from the literature review: EP. Contributed to the initial drafting and writing of the manuscript: EP, MM, SP, AC. All authors reviewed and approved the final manuscript.

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