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Evaluation of the Novaplex II HPV28 Detection Assay for HPV Typing in Formalin-Fixed Paraffin-Embedded Tissues

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Abstract

Prophylactic human papillomavirus (HPV) vaccines are recommended for prevention of HPV-associated cancers. Type-specific detection of HPV in formalin-fixed paraffin-embedded (FFPE) tissues retrieved from diagnostic pathology laboratories is important in monitoring the impact of HPV vaccines. However, few typing assays have been validated for testing FFPE samples. We compared results of the Novaplex II HPV28 Detection (Novaplex) assay with those from our reference assay (Linear Array with reflex Line Probe Assay) on 708 FFPE samples from cervical lesions. Novaplex showed high type-specific concordance with the reference method for HPV16/18, 9 types targeted by the Gardasil 9 vaccine, 14 high-risk types, and 21 types covered by comparison assays. The rate of inadequate samples was low in both approaches (3.4% reference and 1.7% Novaplex). The proportion of discrepant types was less than 3.5% and positive concordance was greater than 75.0%. Furthermore, the type-specific positive agreement (92.0% to 98.0%), negative agreement (96.0% to 99.0%), and accuracy (97.0% to 99.0%) was high. Cohen's kappa ranged from 0.86 to 0.89, indicating excellent agreement between Novaplex and reference assays. Our results show that Novaplex is a suitable method for detection of HPV in FFPE tissues.

Introduction

Human papillomavirus (HPV) infection is an extremely common sexually transmitted infection in the world with most sexually active adults infected with HPV at some point in their lifetime.[1] More than 200 HPV types have been identified to date. High-risk HPV types cause cancers of cervix, vagina, vulva, penis, anus, and oropharynx whereas other HPV types cause low-grade cellular changes, anogenital warts, and respiratory papillomatosis.[1] Monitoring type-specific detection of HPV in the general population and in cervical precancers has been used to document real-world effectiveness of primary prevention.[2, 3] Continued use of HPV typing assays are essential to identify potential disparities in vaccine impact as well as the potential for type-replacement that will inform public health decisions.

The Novaplex II HPV28 Detection Assay (hereafter referred to as Novaplex) recently became available in the United States (U.S.) for research use only. The kit is available internationally as the Anyplex II HPV28 Detection assay (hereafter referred to as Anyplex)

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and is approved by the European Union for *in vitro* diagnostic use. Novaplex/Anyplex is a real-time multiplex PCR-based assay that identifies 28 HPV types including 14 high-risk and an additional 14 anogenital HPV types along with a human control gene.[4] Anyplex has been demonstrated to be sensitive and specific for HPV typing with performance comparable to commonly used HPV tests.[4–8] Manufacturer-approved sample types do not include formalin-fixed, paraffin-embedded (FFPE) tissues and few studies[9–11] have evaluated the performance of the Novaplex/Anyplex assay with this sample type. FFPE tissues are a valuable resource for type-specific monitoring of HPV vaccine impact in precancers and cancers. Although the DNA in FFPE tissues typically is stable during long-term storage, DNA fragmentation and cross-linking with proteins during fixation can have negative effects on HPV detection.[12] Therefore, more HPV typing assays must be validated using FFPE tissues to ensure accurate reporting of HPV prevalence in this sample type.

In this study, we evaluated the performance of the Novaplex assay for HPV detection using FFPE tissues collected from women with cervical intraepithelial neoplasia (CIN) grade 2+ lesions by comparing typing results with our reference assay, Roche Linear Array (LA, no longer manufactured) with reflex to the RHA kit SPF10-Line Probe Assay 25 (LiPA) assay for negative or inadequate specimens.

Materials and Methods

Study Samples

Residual anonymized specimens from HPV surveillance projects were used in this analysis. These specimens were determined exempt from human subjects research by the Institutional Review Board of the Centers for Disease Control and Prevention. Sections were cut from archived FFPE samples (n=708) of cervical precancers using precautions to prevent cross-contamination and were stored at room temperature until extraction as previously described.[12] The archived samples were at various ages when received from multiple sites and was stored at our facility for 54 to 1336 (mean = 345.3) days before extracting DNA. Of the 708 samples included in this analysis, 201 previously typed by reference assay were selected to ensure representation of all 28 HPV types detected by Novaplex and 507 were sequentially received surveillance samples.

DNA Extraction from FFPE tissues

Total DNA for each sample was extracted from a 10-μm section of FFPE tissue as previously described[12] with the following modifications: high-heat treatment at 120°C occurred in 180 μL of Lysis Buffer Tissue (catalog # CMG-805; Perkin Elmer, Waltham, MA) for 20 min. After 5 minutes of incubation, paraffin was melted from FFPE samples, and the contents of the tube were mixed by finger-flicking to ensure the tissues are submerged in the Lysis Buffer Tissue. The sample tubes were removed, allowed to sit on bench for 3 min and then centrifuged briefly to collect the condensate. The tissues were further lysed by adding 20 μL of Proteinase K solution (16 μg/μL), samples vortexed for 3-5 seconds and then incubated overnight at 65°C for 16 hours. The sample tubes were centrifuged briefly to collect the condensate, and the lysate from each tube was transferred to individual well of deep-well plate containing 10 μL of 1% Tween-20. The total DNA from lysate was purified

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using the Perkin Elmer Viral NA/gDNA kit and Chemagic 360 Automatic Nucleic Acid Extractor (Perkin Elmer) by eluting DNA in 120 μL of Elution Buffer. To monitor for DNA contamination, four nuclease-free water aliquots were processed in parallel with FFPE samples in 96-well plates.

HPV Reference Assay

All samples were tested with LA (Roche Molecular Systems, Branchburg, NJ) as previously described.[12] LA is a qualitative HPV typing test using *L1* gene consensus PGMY primers yielding a 450-bp amplicon detected by reverse line blot hybridization for identification of 37 HPV types (HPV6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52(XR), 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, 89, and IS39). Ambiguous results from the XR probe, which detects HPV52 and cross-reacts with HPV33, 35, and 58, were resolved with a HPV52-specific real-time TaqMan PCR assay.[13] Samples with negative or inadequate (HPV and beta-globin negative) results by LA were further tested in the LiPA (Labo Bio-medical Products B.V., Netherlands). LiPA[14] is a reverse hybridization assay for qualitative detection of 25 HPV types (HPV6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70, and 74). The assay uses SPF10 primers[15] for amplification and reverse line blot hybridization for detection of the 65-bp *L1* region. The assay was performed following manufacturer's instructions. Interpretation of certain LiPA probes is ambiguous for determination of a specific HPV type without additional probes to resolve ambiguity. When a sample's probe results could not definitively classify positivity for a least one HPV type, the sample was classified as HPV negative for analysis. As the LiPA assay does not include a human gene internal control, samples with no HPV detected were considered inadequate. The typing results from reference assay (LA with reflex to LiPA) were interpretated using LA & LiPA Testing Algorithm shown in Table S1. Due to constrains in workflow, the reference assay was always performed before the Novaplex assay.

Novaplex II HPV28 Detection (Novaplex) Assay

The Novaplex (Seegene Technologies, Walnut Creek, California) is a multiplex fluorescent-based PCR assay that allows for simultaneous amplification, detection, and differentiation of 28 HPV types (HPV6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 66, 68, 69, 70, 73, and 82). The assay uses tagging oligonucleotide cleavage and extension technology (TOCE) and Seegene's proprietary primer and probe sequences to distinguish 28 HPV types. It uses two separate PCR reactions to amplify 28 HPV types and an internal control. Amplicons are differentiated by fluorophores and melt-curve analysis. TOCE PCR for Novaplex was done using 10 μL of DNA extract or control samples by following the manufacturer's protocol. Data from melt-curve analysis after 50 cycles was used and analyzed automatically using Seegene Viewer software to identify HPV types. The HPV typing results from Novaplex were interpreted following manufacturer's recommendation with the exception that detection of any of the 28 HPV types was sufficient to consider the samples HPV-positive regardless of the detection status of internal control. A positive for internal control was required to report HPV negative results and samples negative for both HPV and internal controls were designated inadequate.

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Data Analysis

Statistical analysis was performed using R Studio software.[16] Novaplex results were compared to reference data. Type-specific concordance and sample concordance between reference assays and Novaplex were determined for four groups (HPV 16/18, 9v, 14HR and 21HPV). 9v corresponds to 9 HPV types (HPV6, 11, 16, 18, 31, 33, 45, 52, and 58) targeted by the Gardasil 9 HPV vaccine, 14HR corresponds to 14 high-risk HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), and 21HPV denotes 21 HPV types (HPV6, 11, 16, 18, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, and 70) common among LA, LiPA and Novaplex assays. The type-specific concordance was determined by calculating discrepant types, positive agreement, negative agreement and positive concordance values using formulae shown in Table S2. The agreement from Cohen's kappa (κ) score was interpreted as follows[7]: < 0.20, poor, 0.21-0.40, fair; 0.41-0.60, moderate; 0.61-0.80, strong; 0.81-1.0, excellent. The McNemar's test was used to evaluate if one assay is more likely to be positive than the other and a p-value < 0.05 was considered to be statistically significant. For type-specific concordance analysis, inadequate samples from the reference and Novaplex assays were considered to be negative for all types in order to keep the denominator consistent between the type-specific and sample concordance analyses. For sample concordance analysis, type agreement (restricted to 21 types) is considered full concordance when reference data and Novaplex results agree for all HPV types, partial concordance when assays agree on detection for at least one but not all HPV types, and full discordance when typing results do not match for detection of any HPV types.

Results

HPV typing results from reference and Novaplex Assays

As shown in Table 1, the reference assay detected HPV in 635 (89.7%) and 24 (3.4%) were inadequate whereas Novaplex detected HPV in 639 (90.3%) and only 12 (1.7%) were inadequate.

Type-specific concordance between Novaplex and reference assays

The type-specific concordance between the Novaplex and reference assays, shown in Table 2, indicates excellent agreement, Cohen's kappa for all three HPV comparison groups ranged from 0.86 to 0.89. These values did not differ significantly between groupings; HPV 16/18 (κ = 0.89; 95% CI, 0.84 – 0.94, 9v (κ = 0.89; 95% CI, 0.86 – 0.91), 14HR (κ = 0.87; 95% CI, 0.85 – 0.89), and 21HPV (κ = 0.86; 95% CI, 0.85 – 0.88). The proportion of discrepant types was low (1.4% to 3.3%) and positive agreement was high (92.0- 98.0%). Negative agreement was 96.0 to 99.0% and accuracy ranged from 97.0% to 99.0%. The positive concordance values ranged from 77.0% to 84.0% and were comparable among all four HPV groups analyzed in this study. The Novaplex assay detected more HPV types than the reference assay regardless of the HPV groups analyzed; McNemar's p-value was less than 0.05 for all foure HPV groups.

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Overall HPV concordance by sample between Novaplex and reference assays

The concordance for overall HPV detection by sample between the Novaplex and reference assays (Table 3) was high, >89.0% for all four HPV groups analyzed: HPV 16/18 (90.5%), 9v (89.9%), 14HR (91.7 %) and 21HPV (92.2 %). Full concordance for type agreement decreased from 94.0% to 75.0% as additional HPV types were included.

Discussion

Over the past few decades, several hundred HPV tests have been developed but most were designed for use with exfoliated cells. Few tests have been validated for use with FFPE tissues.[10,17] HPV detection in FFPE tissues is important for monitoring the impact of vaccines on type-distribution in the most significant public health and clinical outcomes of HPV infection, specifically cancer and precancer. Our findings, in agreement with several earlier studies, indicate Anyplex/Novaplex is suitable for HPV detection in FFPE specimens.[9–11] Lillsunde-Larsson and colleagues found higher rate of HPV detection in 99 FFPE samples of lesions clinically suspected to be HPV associated with Anyplex compared with their in-house real time PCR protocol (82% versus 68%)[9]. Another study by Rollo and colleagues with 160 FFPE samples from oropharyngeal squamous cell carcinomas (OPSCCs), showed 61.3% HPV positivity rate by Anyplex compared to 49.4% by INNO-LiPA (Fujirebio Inc. Tokyo, Japan) and 50.6% by Xpert typing assays.[10] Similarly, the study by Veyer and colleagues with 55 FFPE samples from head and neck squamous cell carcinoma showed 60.0% HPV positive by Anyplex compared to 67.2% by INNO-LiPA.[11]

Our study examined a larger number of samples (n=708) and included samples representing all 28 types in the Anyplex assay as well as unselected samples from routine surveillance. In addition, the surveillance samples originate from 5 areas of the U.S., each including multiple submitting pathology laboratories allowing our results to be more generalizable, as variations in tissue processing that could impact fixation and assay sensitivity, are included. Compared with our reference assay, Novaplex detected a similar number of HPV-positive samples (90.3% vs. 89.7%) and had fewer invalid samples (1.7% vs. 3.4%). The similar detection rate differs from the previous Anyplex validation studies.[9–11] We used a high-temperature extraction method to optimize DNA yield from FFPE samples.[18] In addition, taking into account the potential impact of cross-linking on DNA fragmentation, the reference method uses reflex testing by LiPA that relies on a 65 bp amplicon when samples fail to yield the 450 bp amplicon of the LA.[19] The size of the HPV DNA region targeted by Novaplex for detection of 28 HPV types is not known as the information is proprietary.

We used the reference assay for determining type-specific prevalence in HPV-associated cancers and monitoring HPV vaccine impact in cervical pre-cancer for more than 10 years.[20] With discontinuation of the LA assay, an alternative typing assay was needed. The 28 types included in the Novaplex assay covered more than 95% of the types detected in this ongoing surveillance and the assay is streamlined compared to the reference method, so it is regarded as a reasonable alternative. A goal of this validation study was to determine the effect that shifting to a new typing assay would have on efforts to monitor impact of the HPV vaccination program. Therefore, we examined concordance for HPV types grouped in

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relation to HPV16 and 18, the types most frequently detected in HPV-associated cervical cancers, types targeted by Gardasil 9 vaccine (9v), 14HR types targeted by clinical HPV tests, and all 21 types targeted by both Novaplex and reference methods. High type-specific (positive, negative agreement and Cohen's kappa) and sample concordance (overall HPV status) for all four HPV groups analyzed suggest excellent agreement between Novaplex and reference assays. When compared to the reference method, Novaplex detected more HPV types within each of the HPV groups (McNemar $p<0.0001$), nonetheless high concordance with reference assay indicates that shifting to Novaplex will have minimal impact on monitoring results.

This study has several limitations. The analysis was restricted to cervical lesions, however, the similar distribution of the same high-risk types in lesions at all anatomic sites suggests findings should be applicable to other anatomic sites. Type-specific and sample concordance analysis was not performed for individual HPV types as our study was not powered for individual type analysis. DNA purified from high temperature extraction were used and other methods of DNA extraction have not been evaluated. DNA yield and integrity can vary depending on the method of extraction. This may impact HPV typing results particularly for assays relying on large amplicons, such as LA (450 bp amplicon). In addition, variables of storage conditions such as time and temperature that affect the integrity of nucleic acids extracted from FFPE tissues[21] have not been evaluated. False positive or false negative results with either assay cannot be excluded, however the reference assay has been extensively used in surveillance studies.

In conclusion, our results support use of the Novaplex II HPV28 Detection Assay for HPV detection in DNA extracted from FFPE tissues when used in combination with a high-temperature DNA extraction method.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The findings and conclusion in this study is of authors and do not necessarily represent the official position of the Centers for Diseases Control and Prevention. All authors declare no potential conflicts of interest.

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Table 1

Overall HPV results for FFPE samples

Number of samples (%)		
HPV Result	**Reference**	**Novaplex**
Positive	635 (89.7)	639 (90.3)
Negative	49 (6.9)	57 (8.0)
Inadequate	24 (3.4)	12 (1.7)
Total	708 (100)	708 (100)

The data correspond to HPV types detected by each typing assay; 41 types by reference assay and 28 types by Novaplex.

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Table 2

Type-Specific Concordance between Novaplex and Reference assay

HPV type group	Novaplex/ Reference +/+, +/−, −/+, −/−	Proportion Discrepant Types (%)	Positive Agreement (%, 95% CI)	Negative Agreement (%, 95% CI)	Accuracy (%)	Positive Concordance (%)	Kappa (95% CI)	McNemar p-value
HPV 16/18	247, 41, 6, 1122	3.32	98.0 (95.0, 99.0)	96.0 (95.0, 97.0)	97.0	84.0	0.89 (0.84, 0.94)	< 0.0001
9v	491, 73, 38, 5770	1.7	93.0 (90.0, 95.0)	99.0 (98.0, 99.0)	98.0	82.0	0.89 (0.86, 0.91)	< 0.0001
14HR	655, 126, 48, 9083	1.7	93.0 (91.0, 95.0)	99.0 (98.0, 99.0)	98.0	79.0	0.87 (0.85, 0.89)	< 0.0001
21HPV	711, 154, 60, 13943	1.4	92.0 (90.0, 94.0)	99.0 (99.0, 99.0)	99.0	77.0	0.86 (0.85, 0.88)	< 0.0001

Reference assay is the Linear Array with reflex Line Probe Assay

9v, 9 HPV types (HPV6, 11, 16, 18, 31, 33, 45, 52, 58) targeted by the 9-valent HPV vaccine

14HR, 14 high-risk HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68)

21HPV, 21 HPV types (HPV6, 11, 16, 18, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70) targeted by Novaplex and reference assays

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Table 3

Overall HPV Concordance by sample between Novaplex and reference assay

HPV type group	Overall HPV status of sample by Novaplex	Overall HPV status of sample by Reference n (%)			Type Agreement n (%)		
		Positive	Negative	Inadequate	Full Concordance	Partial Concordance	Full Discordance
HPV 16/18	Positive	243 (34.3)	32 (4.5)	4 (0.6)	662 (94.0)	4 (1.0)	42 (6.0)
	Negative	6 (0.8)	384 (54.2)	25 (3.5)			
	Inadequate	0 (0.0)	0 (0.0)	14 (2.0)			
9v	Positive	457 (64.5)	28 (4.0)	5 (0.7)	603 (85.0)	50 (7.0)	55 (8.0)
	Negative	21 (3.0)	168 (23.7)	16 (2.3)			
	Inadequate	1 (0.1)	0 (0.0)	12 (1.7)			
14HR	Positive	571 (80.6)	23 (3.2)	6 (0.8)	555 (78.0)	101 (14.0)	52 (7.0)
	Negative	21 (3.0)	67 (9.5)	8 (1.1)			
	Inadequate	1 (0.1)	0 (0.0)	11 (1.6)			
21HPV	Positive	591 (83.5)	22 (3.1)	6 (0.8)	532 (75.0)	126 (18.0)	50 (7.0)
	Negative	19 (2.7)	50 (7.1)	8 (1.1)			
	Inadequate	1 (0.1)	0 (0.0)	11 (1.6)			

Reference assay is the Linear Array with reflex Line Probe Assay

9v, 9 HPV types (HPV6, 11, 16, 18, 31, 33, 45, 52, 58) targeted by the 9-valent HPV vaccine

14HR, 14 high-risk HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68)

21HPV, 21 HPV types (HPV6, 11, 16, 18, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70) targeted by Novaplex and reference assays