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Comparison of Real-Time Multiplex Human Papillomavirus (HPV) PCR Assays with the Linear Array HPV Genotyping PCR Assay and Influence of DNA Extraction Method on HPV Detection▽‡

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Real-time human papillomavirus (HPV) type-specific multiplex PCR assays were developed to detect HPV DNA in specimens collected for the efficacy determination of the quadrivalent HPV (type 6, 11, 16, and 18) L1 virus-like particle (VLP) vaccine (Gardasil). We evaluated the concordance between type-specific multiplex HPV PCR and the widely used, commercially available Roche Linear Array genotyping PCR assay. Female genital swab specimens were tested for the presence of L1, E6, and E7 sequences of HPV type 6 (HPV6), HPV11, HPV16, HPV18, HPV31, HPV45, HPV52, and HPV58 and E6 and E7 sequences of HPV33, HPV35, HPV39, HPV51, HPV56, and HPV59 in type- and gene-specific real-time multiplex PCR assays. Specimens were also tested for the presence of L1 sequences using two versions of the Roche Linear Array genotyping assay. Measures of concordance of a modified version of the Linear Array and the standard Linear Array PCR assay were evaluated. With specimen DNA extraction using the Qiagen Spin blood kit held as the constant, multiplex PCR assays detect more HPV-positive specimens for the 14 HPV types common to both than either version of the Linear Array HPV genotyping assay. Type-specific agreements between the assays were good, at least 0.838, but were often driven by negative agreement in HPV types with low prevalence, as evidenced by reduced proportions of positive agreement. Overall HPV status agreements ranged from 0.615 for multiplex PCR and standard Linear Array to 0.881 for multiplex PCR and modified Linear Array. An alternate DNA extraction technique, that used by the Qiagen MinElute kit, impacted subsequent HPV detection in both the multiplex PCR and Linear Array assays.

The second most common type of cancer among women worldwide is cervical cancer, with approximately 493,000 new cases and 27,400 deaths in 2002 (30). Human papillomavirus (HPV) infection is a necessary risk factor for cervical cancer (29). A pooled analysis of 12 studies conducted in 25 countries showed that HPV DNA was present in 95% of cervical cancer subjects, and the 15 most common types were the following, in descending order of frequency: HPV type 16 (HPV16), HPV18, HPV45, HPV31, HPV33, HPV52, HPV58, HPV35, HPV59, HPV56, HPV39, HPV51, HPV73, HPV68, and HPV66 (16).

Diagnosis of HPV infection relies on the detection of the viral DNA in clinical samples; thus, accurate detection and genotyping of human HPV are of critical importance for determining the prevalence of HPVs in a given population and for determining the risks associated with infections of a particular type. In addition, accurate molecular diagnostic tools are necessary for determining the long-term efficacy of HPV vaccines. The two methodologies most widely used for HPV detection in epidemiological studies are target amplification, as used in PCR assays using degenerate or consensus primers, and signal amplification, as used in the Hybrid Capture 2 assay (17). PCR-based assays utilize amplification of HPV DNA, which is directed by primers that bind to specific regions, most commonly to the L1 open reading frame (ORF), of genital HPV genomes. Analysis of amplification products is commonly performed by enzyme immunoassays or reverse line blot assays. In addition, multiplex HPV genotyping methods based on hybridization to fluorescently labeled beads have been reported (17).

The quadrivalent HPV type 6, 11, 16, and 18 L1 vaccine (Gardasil) was approved for use in women aged 9 to 26 for prevention of infection and disease with cervical cancer, for prevention of cervical, vulvar, and vaginal precancerous lesions and genital warts caused by HPV6, -11, -16, or -18, and recently, for use in males in the same age range for the prevention of infection and genital warts caused by HPV types 6 or 11 (8, 28). Real-time HPV type-specific multiplex HPV PCR assays were used in all phase III studies for the efficacy determination of the quadrivalent HPV vaccine (27, 28). These assays simultaneously detect two (E6 and E7) or three (L1, E6, and E7) ORFs of a particular HPV type. All assays are HPV type and ORF specific. Several studies have evaluated the methodological variation of HPV DNA detection (4, 9, 18–21, 26). In addition, recent studies have evaluated a commercialized version of the line blot assay, the Linear Array (Roche Molecular Systems, Alameda, CA) (3, 31). Here, we compare the detection of 14 HPV types by the real-time HPV multiplex HPV PCR assays with that by the Linear Array assay.

The goal was to determine how the internally developed multiplex HPV PCR system of HPV detection performed relative to the widely used Linear Array assay. However, differences in DNA input volumes and DNA extraction techniques

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between the recommended protocols complicated the ability to perform a direct comparison. Initially, the DNA extraction technique was held as a constant, with DNA isolated from swab specimens using the Qiagen Spin blood kit, the preferred process for use with multiplex HPV PCR. With this specimen set, multiplex HPV PCR was performed as per its standard real-time amplification/detection protocol, Linear Array was performed as per its standard amplification and detection protocols, and Linear Array was performed per a modified amplification and detection protocol, such that DNA input would be equal to that of multiplex HPV PCR. Subsequently, an additional DNA extraction technique was used with specimens extracted by the Qiagen MinElute kit, the preferred process cited in the manufacturer's protocol for the Linear Array. With this specimen set, both the multiplex HPV PCR and Linear Array assays were run as per their standard amplification and detection protocols. Comparative analyses between these data sets were performed to bridge the gaps to enable a direct one-to-one system comparison of the two HPV detection assay systems executed under optimal performance conditions for each.

MATERIALS AND METHODS

Specimen collection and processing. Female genital swab specimens were collected as part of ongoing clinical trials to evaluate the efficacy of the quadrivalent HPV vaccine (28). As part of the clinical trials, institutional review boards at each center approved the protocols, and written informed consent was obtained from all subjects. Endo-Ecto cervical swabs and labial/vulvar/perineal/perianal swabs were collected from each patient, and each swab was placed in 1 ml of digene specimen transport medium (STM). Each specimen was divided into three pristine aliquots and frozen. Retained aliquots of specimens previously tested for vaccine trial purposes were evaluated here for the purpose of assay comparisons. Previous HPV results generated via Qiagen QIAamp 96 DNA blood kit sample DNA extraction, and multiplex HPV PCR testing was used as a screen to identify positive specimens and ensure adequate representation for each of the 14 HPV types evaluated in this study.

DNA isolation using the Qiagen QIAamp 96 DNA blood kit. DNA was extracted from 200 μl of each of the 444 swab specimens using the Qiagen QIAamp 96 DNA blood kit in a 96-well format. The purified DNA was eluted in 200 μl of Qiagen AE buffer. HPV-negative MRC5 cells (500,000 cells/well) and HPV16-positive SiHa cells (2,500 cells/well) were included in the processing of each plate to serve as positive controls for DNA isolation and HPV detection. Negative-control wells included in plate processing contained 200 μl of STM buffer.

DNA isolation using the Qiagen MinElute media kit. DNA was purified from 166 additional swab specimens using the Qiagen MinElute media kit in accordance with the manufacturer's protocol, with no optional kit reagents utilized. DNA was extracted from 200 μl of each sample and eluted in 200 μl of Qiagen AVE buffer. The controls described above were processed with each set of specimens.

Real-time multiplex HPV PCR assay. Samples were tested in human β-globin and individual type-specific and gene-specific real-time multiplex HPV PCR assays for the L1, E6, and E7 open reading frames (ORFs) of HPV6, HPV11, HPV16, HPV18, HPV31, HPV45, HPV52, and HPV58 and the E6 and E7 ORFs of HPV33, HPV35, HPV39, HPV51, HPV56, and HPV59 (12, 22, 23). The assays were performed in a 96-well format with 50-μl/well reaction volumes. Each well contained 4 μl of sample DNA (regardless of DNA concentration), QuantiTect PCR master mix (Qiagen, Inc.), uracil-DNA glycosylase (0.5 U/reaction; Invitrogen), HPV type- and ORF-specific fluorescent oligonucleotide probes, and the HPV type- and ORF-specific primers (see Table S1 in the supplemental material). The cycling conditions used for the multiplex PCR assays were 50°C for 2 min, 95°C for 15 min, followed by 45 cycles of 94°C for 15 s and 60°C for 1 min, with the exception of the HPV11 PCR assay, whose conditions were 50°C for 2 min, 95°C for 15 min, followed by 50 cycles of 94°C for 30 s and 60°C for 1 min. After thermal cycling and data collection, preestablished thresholds were set, and the data were exported to a Microsoft Excel workbook for compilation and analysis. A sample was considered PCR positive for a specific HPV type if a minimum of 2 ORFs were PCR positive and PCR negative for a specific HPV type if all ORFs were PCR negative. A negative HPV result was considered valid only if a portion of the human β-globin ORF was amplified in a separate reaction to verify that adequate DNA was present in the specimen. Under Merck Research Laboratory's standard HPV PCR clinical testing rules, samples that result in a single-ORF-positive result would be retested from a fresh aliquot of the specimen for verification. In this analysis, single-ORF-positive results were not independently verified due to limited sample availability and were considered negative.

Roche Linear Array HPV genotyping test, standard protocol (s-LA). Linear Array HPV PCRs were performed in accordance with the manufacturer's recommendations (February 2005 version). Each PCR mixture (100 μl) utilized 50 μl sample DNA (regardless of concentration). Colorimetric detection was performed in accordance with the manufacturer's recommendations. Color-developed strips were evaluated by aligning the sample strips with the Linear Array HPV genotyping reference guide and visually determining if the bands were visible for a particular genotype. Both high β-globin and low β-globin controls must have been visible for the strip results to be considered valid for that sample.

Roche Linear Array HPV genotyping test, modified protocol (m-LA). The Linear Array HPV genotyping PCR mixture volume was modified to match that of the multiplex HPV PCR assay specimen template DNA inputs. Total PCR volume was reduced to 50 μl, with 4 μl sample DNA (regardless of concentration) used. For subsequent colorimetric detection, 50 μl (instead of 100 μl) of denaturation buffer was added to each PCR, and then 75 μl was hybridized to the strip. Hybridization, washing, conjugate binding, and colorimetric detection were performed in accordance with the manufacturer's protocol. Color-developed strips were evaluated as described above.

Statistical analysis. HPV positivity results from matched swab specimen data sets from the two methodologies were merged, cross-tabulated, and analyzed by HPV type for agreement rate, proportion of positive agreement (P_{pos}), which is calculated as twice the number of agreed positives/(total number of specimens + number of agreed positives − number of agreed negatives), proportion of negative agreement (P_{neg}), which is calculated as twice the number of agreed negatives/(total number of specimens − number of agreed positives + number of agreed negatives), and McNemar's P value (a measure of the degree of imbalance of discordant pairs).

RESULTS

Multiplex HPV PCR versus m-LA with Qiagen Spin blood kit DNA extraction. Because there are significant differences in the volumes of sample evaluated in the multiplex HPV PCR assay and the Linear Array (LA) assay, (4 μl DNA sample per PCR versus 50 μl DNA sample, respectively), we modified the LA assay reaction protocol, restricting the total PCR volume to 50 μl and reducing the DNA sample input volume to 4 μl of sample DNA, as in the multiplex HPV PCR assay testing protocol. In 444 of the specimens isolated with the Qiagen Spin blood kit and evaluated by these two HPV detection methods, the total number of specimens valid for β-globin in multiplex HPV PCR and m-LA was 440 and 406, respectively. As shown in Table 1, only one specimen that was valid for β-globin in m-LA was not valid in multiplex HPV PCR; therefore, 405 commonly valid specimens were used for direct assay comparisons. As seen in Table 1 in the row titled "any of the 14 HPVs," 279 specimens were positive using the multiplex HPV PCR assay and 249 were HPV positive using the m-LA. The multiplex HPV PCR assay identified 39 specimens as positive that the m-LA classified as negative, while the m-LA identified only 9 specimens as positive that the multiplex HPV PCR assay classified as negative. The agreement rate of HPV status of a specimen for any of the 14 HPV types evaluated was 0.881, with rates of 0.909 and 0.830 when considering the proportions of positive and negative agreement, respectively. When considering individual HPV types, the agreement rate between the two methods ranged from 0.943 (HPV31) to 0.998 (HPV11). In all individual type analyses, however, the proportion of

TABLE 1. Concordance of HPV detection with Merck multiplex HPV PCR and modified Linear Array following DNA extraction with the Qiagen Spin blood kit

Assay	No. of specimens with multiplex PCR/modified Linear Array result of:				Agreement rate	P_{pos}	P_{neg}	P value[a]
	+/+	+/−	−/+	−/−				
HPV6	30	6	2	367	0.980	0.882	0.989	0.288
HPV11	9	1	0	395	0.998	0.947	0.999	1.000
HPV16	52	8	5	340	0.968	0.889	0.981	0.579
HPV18	25	6	1	373	0.983	0.877	0.991	0.131
HPV31	29	22	1	353	0.943	0.716	0.968	**<0.001**
HPV33	14	7	0	384	0.983	0.800	0.991	**0.023**
HPV35	19	5	2	379	0.983	0.844	0.991	0.450
HPV39	43	8	2	352	0.975	0.896	0.986	0.114
HPV45	13	3	1	388	0.990	0.867	0.995	0.617
HPV51	46	11	5	343	0.960	0.852	0.977	0.211
HPV52	32	15	0	358	0.963	0.810	0.979	**<0.001**
HPV56	29	17	0	359	0.958	0.773	0.977	**<0.001**
HPV58	25	6	0	374	0.985	0.893	0.992	**0.041**
HPV59	27	3	1	374	0.990	0.931	0.995	0.617
Any of the 14 HPVs	240	39	9	117	0.881	0.909	0.830	**<0.001**
β-globin	405	35	1	3	0.919	0.957	0.143	**<0.001**

[a] *P* value as determined by McNemar's test. *P* values of <0.05 indicate significant differences (in boldface).

positive agreements was slightly lower, ranging from 0.716 in HPV31 to 0.947 in HPV11, than the proportion of negative agreements. Statistically significant differences in the detection of HPV in clinical samples (McNemar's P value < 0.05) were noted for HPV31, HPV33, HPV52, and HPV56 when considering overall positivity with any of the 14 HPV types (Table 1).

Multiplex HPV PCR versus s-LA with Qiagen Spin blood kit DNA extraction. DNA was prepared using the Qiagen Spin blood kit, and both the multiplex HPV PCR and s-LA assays were performed on 432 swab specimens. Thus, 4 μl of input DNA was used in the 50-μl multiplex HPV PCRs, while 50 μl of input DNA was used in the 100-μl s-LA reaction. Human β-globin and HPV testing results are shown in Table 2. The total number of specimens valid for β-globin in multiplex HPV PCR and s-LA was 428 and 379, respectively. All 379 specimens valid by s-LA were also valid by multiplex HPV PCR, and only these valid specimens were used for direct assay comparisons. Of these, 263 tested positive for any of the 14 HPV types evaluated using the multiplex HPV PCR assay, and 146 specimens tested HPV positive using the s-LA. As shown in the "any of the 14 HPVs" row in Table 2, of the 263 multiplex HPV PCR-positive specimens, 145 were s-LA classified as HPV negative, while the s-LA identified only one positive specimen that the multiplex HPV PCR assay classified as negative. The agreement rate of positivity status of a specimen for any of the 14 HPV types evaluated was moderate at 0.615, with similar agreement rates of 0.618 and 0.612 when considering proportions of positive and negative agreement, respectively. When considering individual HPV types, the agreement rate between the two methods ranged from 0.894 (HPV56) to 0.982

TABLE 2. Concordance of HPV detection with Merck multiplex HPV PCR and standard Linear Array following DNA extraction with the Qiagen Spin blood kit

Assay	No. of specimens with multiplex PCR/standard Linear Array result of:				Agreement rate	P_{pos}	P_{neg}	P value[a]
	+/+	+/−	−/+	−/−				
HPV6	13	19	0	347	0.950	0.578	0.973	**<0.001**
HPV11	3	7	0	369	0.982	0.462	0.991	**<0.001**
HPV16	28	26	1	324	0.929	0.675	0.960	**0.023**
HPV18	9	20	0	350	0.947	0.474	0.972	**<0.001**
HPV31	6	39	0	334	0.897	0.235	0.945	**<0.001**
HPV33	1	18	0	360	0.953	0.100	0.976	**<0.001**
HPV35	9	14	0	356	0.963	0.563	0.981	**<0.001**
HPV39	9	39	0	331	0.897	0.316	0.944	**<0.001**
HPV45	7	6	3	363	0.976	0.609	0.988	0.505
HPV51	21	33	1	324	0.910	0.553	0.950	**<0.001**
HPV52	17	25	0	337	0.934	0.576	0.964	**<0.001**
HPV56	3	40	0	336	0.894	0.130	0.944	**<0.001**
HPV58	13	16	0	350	0.958	0.619	0.978	**<0.001**
HPV59	16	11	1	351	0.968	0.727	0.983	**0.009**
Any of the 14 HPVs	118	145	1	115	0.615	0.618	0.612	**<0.001**
β-globin	379	49	0	4	0.887	0.939	0.140	**<0.001**

[a] *P* value as determined by McNemar's. *P* values of <0.05 indicate significant differences (in boldface).

TABLE 3. Concordance of HPV detection with standard and modified Linear Array assays following DNA extraction with the Qiagen Spin blood kit

Assay[b]	No. of specimens with standard Linear Array/modified Linear Array result of:				Agreement rate	P_{pos}	P_{neg}	P value[a]
	+/+	+/−	−/+	−/−				
HPV06	15	0	16	346	0.958	0.652	0.977	**<0.001**
HPV11	3	0	6	368	0.984	0.500	0.992	**0.041**
HPV16	31	0	28	318	0.926	0.689	0.958	**<0.001**
HPV18	9	0	15	353	0.960	0.545	0.979	**<0.001**
HPV26	1	0	1	375	0.997	0.667	0.999	1.000
HPV31	6	0	22	349	0.942	0.353	0.969	**<0.001**
HPV33	1	0	12	364	0.968	0.143	0.984	**0.002**
HPV35	7	0	16	354	0.958	0.467	0.978	**<0.001**
HPV39	10	0	36	331	0.905	0.357	0.948	**<0.001**
HPV40	0	0	10	367	0.973	0.000	0.987	**0.004**
HPV42	3	0	28	346	0.926	0.176	0.961	**<0.001**
HPV45	9	1	4	363	0.987	0.783	0.993	0.371
HPV51	25	0	25	327	0.934	0.667	0.963	**<0.001**
HPV52	34	0	39	304	0.897	0.636	0.940	**<0.001**
HPV53	9	0	14	354	0.963	0.563	0.981	**<0.001**
HPV54	6	0	2	369	0.995	0.857	0.997	0.476
HPV55	4	0	5	368	0.987	0.615	0.993	0.074
HPV56	4	0	24	349	0.936	0.250	0.967	**<0.001**
HPV58	13	0	14	350	0.963	0.650	0.980	**<0.001**
HPV59	18	0	10	349	0.973	0.783	0.986	**0.004**
HPV61	20	0	10	347	0.973	0.800	0.986	**0.004**
HPV62	25	0	8	344	0.979	0.862	0.989	**0.013**
HPV66	10	0	6	361	0.984	0.769	0.992	**0.041**
HPV67	1	0	10	366	0.973	0.167	0.987	**0.004**
HPV68	3	0	10	364	0.973	0.375	0.986	**0.004**
HPV69	2	0	1	374	0.997	0.800	0.999	1.000
HPV70	1	0	1	375	0.997	0.667	0.999	1.000
HPV71	3	0	3	371	0.992	0.667	0.996	0.248
HPV72	0	0	1	376	0.997	0.000	0.999	1.000
HPV73	4	0	11	362	0.971	0.421	0.985	**0.003**
HPV81	4	0	3	370	0.992	0.727	0.996	0.248
HPV82	1	0	4	372	0.989	0.333	0.995	0.134
HPV83	6	1	2	368	0.992	0.800	0.996	1.000
HPV84	12	0	17	348	0.955	0.585	0.976	**<0.001**
IS39	1	0	2	374	0.995	0.500	0.997	0.480
HPV CP6108	17	0	10	350	0.973	0.773	0.986	**0.004**
Any of the 37 HPVs	159	0	106	112	0.719	0.750	0.679	**<0.001**
β-globin	377	2	19	34	0.951	0.973	0.764	**<0.001**

[a] P value as determined by McNemar's test. P values of <0.05 indicate significant differences (in boldface).
[b] HPV64 was omitted, as there were no positive samples in either assay.

(HPV11). In all individual type analyses, however, the proportion of positive agreements are much lower, ranging from 0.100 in HPV33 to 0.727 in HPV59, than the proportion of negative agreements. Statistically significant differences in the detection of HPV in clinical samples (McNemar's P value < 0.05) were noted for all types except HPV45 (Table 2).

s-LA versus m-LA with Qiagen Spin blood kit DNA extraction. We evaluated the overall impact of the altered m-LA compared with that of the s-LA protocol in the detection of any of the 37 HPV types detected by the LA assay. Results for 36 of 37 HPV types are shown in Table 3. Because no positive results were obtained for HPV64 in either format of the Linear Array assay, it was omitted. In 432 of the specimens isolated with the Qiagen Spin blood kit and evaluated by both versions of the Linear Array detection methods, the total number of specimens valid for β-globin in s-LA and m-LA was 379 and 396, respectively. Two specimens were valid for β-globin in s-LA that were not valid in m-LA. Inversely, 19 were valid in m-LA that were not in s-LA. Therefore, the 377 specimens commonly valid for β-globin were used for direct assay comparisons. Overall, 159 specimens were positive for any of the 37 HPV types evaluated using the s-LA, and 265 were HPV positive using the m-LA. The m-LA identified 106 specimens as positive that the s-LA classified as negative, while the s-LA identified none that the m-LA classified as negative. The agreement rate for HPV status of a specimen for any of the 36 HPV types analyzed was 0.719, with rates of 0.750 and 0.679 when considering proportions of positive and negative agreement, respectively. When considering individual HPV types, the agreement rate between the two methods ranged from 0.897 (HPV52) to 0.997 (HPV69, HPV70, and HPV72). The proportion of positive agreement was highly variable by type, ranging from 0.000 in HPV72 to 0.862 in HPV62. Statistically significant differences in the detection of HPV in clinical samples (McNemar's P value < 0.05) were noted for all types except the following 12 types: HPV26, HPV45, HPV54, HPV55, HPV69, HPV70, HPV71, HPV72, HPV81, HPV82, HPV83 and IS39, of which only HPV45 was a member of the

TABLE 4. Concordance of HPV detection with Merck multiplex HPV PCR and standard Linear Array following DNA extraction with the Qiagen MinElute media kit

Assay	No. of specimens with multiplex PCR/standard Linear Array result of:				Agreement rate	P_{pos}	P_{neg}	P value[a]
	+/+	+/−	−/+	−/−				
HPV6	24	3	0	140	0.982	0.941	0.989	0.248
HPV11	11	1	0	155	0.994	0.957	0.997	1.000
HPV16	44	3	1	119	0.976	0.957	0.983	0.617
HPV18	19	4	3	141	0.958	0.844	0.976	1.000
HPV31	18	4	2	143	0.964	0.857	0.979	0.683
HPV33	3	6	4	154	0.940	0.375	0.969	0.752
HPV35	2	1	9	155	0.940	0.286	0.969	**0.027**
HPV39	4	1	14	148	0.910	0.348	0.952	0.371
HPV45	10	3	4	150	0.958	0.741	0.977	1.000
HPV51	8	1	26	132	0.838	0.372	0.907	**<0.001**
HPV52	14	4	13	136	0.898	0.622	0.941	0.052
HPV56	3	1	12	151	0.922	0.316	0.959	**0.006**
HPV58	3	6	1	157	0.958	0.462	0.978	0.131
HPV59	0	0	18	149	0.892	0.000	0.943	**<0.001**
Any of the 14 HPVs	122	5	35	5	0.760	0.859	0.200	**<0.001**
β-globin	167	0	1	0	0.994	0.997	0.000	1.000

[a] P value as determined by McNemar's test. P values of <0.05 indicate significant differences (in boldface).

subset of HPV types evaluated in the previous multiplex HPV PCR/LA comparisons (significant differences are show in boldface in Table 3).

Multiplex HPV PCR versus the standard Linear Array with Qiagen MinElute media kit DNA extraction. Utilizing DNA extracted with the Qiagen MinElute kit, the preferred process cited in the manufacturer's protocol for the Linear Array, both the multiplex HPV PCR and s-LA were run as per their standard amplification and detection protocols. In the 168 specimens isolated with the Qiagen MinElute media kit and evaluated by these two HPV detection methods, the total number of specimens valid for β-globin in multiplex HPV PCR and s-LA was 167 and 168, respectively. Only one specimen was valid for β-globin in s-LA that was not valid in the multiplex HPV PCR; therefore, 167 commonly valid specimens were used for direct assay comparisons. As shown in the "any of the 14 HPVs" row of Table 4, 127 specimens were positive for any of the 14 HPV types evaluated using the multiplex HPV PCR assay, and 157 were HPV positive using the s-LA. The multiplex HPV PCR assay identified 5 positive specimens that the s-LA classified as negative, while the s-LA identified 35 positive specimens that the multiplex HPV PCR assay classified as negative. The agreement rate for HPV status of a specimen for any of the 14 HPV types evaluated was 0.760, with rates of 0.859 and 0.200 when considering proportions of positive and negative agreement, respectively. When considering individual HPV types, the agreement rate between the two methods ranged from 0.838 (HPV51) to 0.994 (HPV11). The proportion of positive agreements was variable by type, ranging from 0.000 in HPV59 to 0.957 in HPV11 and HPV16. Statistically significant differences in the detection of HPV in clinical samples (McNemar's P value < 0.05) were observed for HPV35, HPV51, HPV56, and HPV59 and when considering overall positivity with any of the 14 HPV types (significant differences are indicated in boldface in Table 4).

Specimen positivity for multiple HPV types. A comparison of the abilities of each assay to detect multiple HPV type infections is shown in Table 5. As shown in Table 5, a comparison was conducted on the largest data set generated in this study and, therefore, utilized the DNA extracted by the Qiagen

TABLE 5. Evaluation of multiple-HPV type-positive specimens

Characteristic	Qiagen Spin blood DNA isolation[a]					MinElute DNA isolation[b]		
	Multiplex HPV PCR with 14 types	s-LA		m-LA		Multiplex HPV PCR with 14 types	s-LA	
		14 types	37 types	14 types	37 types		14 types	37 types
No. of HPV-negative specimens	116	260	218	143	112	42	10	4
No. of HPV-positive specimens	261	117	159	234	265	126	158	164
% of single-type positives	48.7	68.4	47.2	53.8	27.2	57.9	46.8	22.6
% of multiple-type positives	51.3	31.6	52.8	46.2	72.8	42.1	53.2	77.4
% positive for:								
Two types	31.8	18.8	23.9	30.3	25.7	28.6	35.4	26.2
Three types	11.9	10.3	17.6	11.1	20.0	10.3	11.4	23.2
Four types	3.8	2.6	7.5	3.4	12.5	2.4	4.4	11.0
Five types or more	3.8	0.0	3.8	1.3	14.7	0.8	1.9	17.1

[a] n = 377 specimens.
[b] n = 168 specimens.

Spin blood kit in the multiplex HPV PCR, the m-LA, and the s-LA assays. The 377 specimens with valid β-globin results in all three assay formats were assessed for HPV status, and positive specimens were evaluated for the number of types detected in each specimen. When considering positive specimens for the 14 common HPV types only, multiplex HPV PCR was able to detect the most multiple-type positives (51.3%), followed by m-LA (46.2%) and then s-LA (31.6%). Comparison of all 37 HPV types in the s-LA and m-LA protocols shows that the m-LA version is more capable of detecting multiple-type positives (72.8%) than the s-LA version (52.8%). Also shown in Table 5, a comparison was conducted on the subset of specimens from which DNA was extracted by the Qiagen MinElute kit in the multiplex HPV PCR and s-LA assays. When combined with this DNA extraction system, multiple-type HPV detection was improved in s-LA and reduced in multiplex HPV PCR.

DISCUSSION

It is important to understand the benefits and limitations of the different HPV detection methodologies in common use for appropriate interpretation of epidemiologic and clinical research on HPV and HPV-related disease (1, 2, 11, 15). In the multiplex HPV PCR assays, the detection of multiple open reading frames for each HPV type decreases the chances for false-negative results due to the presence of genetic variants of a particular type or due to integration of the viral DNA into the host genome. The risk of false-positive results is also decreased based on the requirement for the multiplex HPV PCR assay that at least two of the ORFs amplify and are simultaneously detected to be considered a positive sample. Type- and ORF-specific PCR primers are coupled with type- and ORF-specific fluorescently labeled probes in each of these assays to allow simultaneous amplification and detection in real time without post-PCR processing. Detection of multiple ORFs of a single HPV type through assay-incorporated redundancy allows for high sensitivity and specificity.

Several consensus primer PCR-based systems have been developed and are widely used to detect and genotype HPV types in clinical samples (5, 9, 10, 13, 25). These systems have the ability to amplify multiple HPV types simultaneously and subsequently genotype the amplimers with type-specific probes. Using consensus primer PCR amplification and colorimetric line blot style detection systems, however, can result in inaccuracies. For example, in mixed-type infections, competition for reagents can result in a loss of sensitivity and detection of viral types present at lower copy numbers (14). An additional limitation is that these detection systems, including the Linear Array, are based upon detection of a portion of a single open reading frame (ORF) of HPV. Should there be a disruption due to mutation, the presence of type variants, or a loss of that open reading frame due to integration in a given infection, the detection of the presence of HPV may be unsuccessful.

We evaluated the concordance of HPV detection with multiplex HPV PCR and two versions of the Linear Array, m-LA and s-LA, for the 14 types common to both assays following specimen DNA extraction with the Qiagen Spin blood kit, the standard extraction method used in our laboratory. The m-LA was initially utilized for comparison because it equalized the amount of input specimen DNA in the amplification reaction to that used in the multiplex HPV PCR assays and utilized the entire m-LA amplification reaction in the subsequent detection steps to better equate the amplicon-available detection to the real-time detection in the multiplex HPV PCR. The multiplex HPV PCR assay results were also compared to the Linear Array results produced using the standard assay protocol, regardless of the discordance of the quantities of input DNA between the assays.

A comparison of the 14 HPV types of multiplex HPV PCR with those of the m-LA and s-LA for detecting multiple HPV type infections is shown in Table 5. Multiplex HPV PCR detected multiple types in 51.3% of HPV-positive specimens, whereas m-LA and s-LA detected multiple types in 46.2% and in only 31.6%, respectively. It has been proposed that consensus primer-based HPV detection assays suffer from lower sensitivities in the presence of multiple infections due to competition for assay reagents. A recent study (24) of another broad-spectrum HPV detection method, INNO-LiPA, followed by type-specific HPV testing, resulted in the identification of additional HPV positives with type-specific PCR, consistent with the results we have shown in this study. The m-LA assay's ability to detect multiple HPV types in a specimen increased relative to the s-LA assay's ability (Table 5). The m-LA PCR resulted in a dramatic increase in the ability to detect HPV DNA and increased concordance with type-specific multiplex HPV PCR assays. In addition to the potential for competition to impact s-LA results, it is also possible that the consensus L1 PCR amplification reactions were impacted by DNA isolation method-associated factors. While multiplex HPV PCR assays were optimized and validated for use with the Qiagen Spin blood extraction system, it was not the recommended system for use with the s-LA, and the recommended volumes of extracted DNA were used in the respective amplification reactions without regard for DNA concentration. It is possible that the yield of specimen DNA, potential residual PCR inhibitors, and even the composition of the DNA elution buffer used in the Qiagen Spin blood DNA isolation could influence the dynamics of the s-LA PCR. Because specimen DNA accounts for 50% of the total s-LA PCR, these factors may have a greater influence on the dynamics of that amplification reaction. Although we did not specifically examine which factors were responsible for the change in HPV detection with the change in m-LA PCR, it is possible that the reduction in the volume of specimen DNA influenced the outcome through the change in the final concentration of DNA, the effect of potential residual inhibitors, and/or the impact of chemical components of the DNA elution buffer. Because overall and type-specific concordance to multiplex HPV PCR assays (with built-in redundancy in the detection of multiple type-specific ORFs) was increased with m-LA as opposed to s-LA, there appears to have been no loss in specificity with the increased sensitivity gained in our LA assay optimization. Our results are very similar to those reported by Dunn et al. (7), where different results were obtained in the Linear Array HPV testing when volumes of extracted DNA were varied in the LA PCR for optimization of the LA assay in their laboratory.

We evaluated the overall impact of the altered m-LA compared with that of the s-LA protocol in the detection of any of the 36 HPV types of the 37 HPV types detected by the LA

assay. HPV64 was omitted from Table 3 because no positive results were obtained for that type in either formats of the Linear Array assay. Overall and for all individual HPV types evaluated, more HPV positives were detected through the use of the m-LA than through that of s-LA, and the difference was significant for any of the 36 HPVs and individually for 24 of the 36 types. Consistent with the results for the 14 HPV types common to the Linear Array and multiplex HPV PCR assays, the m-LA has increased sensitivity and is able to detect more multiple-type-positive specimens than the s-LA protocol when considering 36 of the 37 detectable HPV types (Table 5).

Few assessments of DNA extraction methodology on the performance of HPV detection systems have been published to date. The aforementioned Dunn et al. study (7), in addition to evaluating alternate volumes of DNA specimen tested, examined the impact of alternate extraction systems (Qiagen DNA blood and Qiagen MinElute media kits) on Linear Array performance. Their experiments showed that both the volume and extraction system used greatly impacted the detection of weak signal bands (weak signals suggest lower starting copy numbers) and had a much lower impact on the detection of strong signal (and presumably higher-copy-number) viral bands. To determine if the relatively poor performance of the s-LA protocol was a result of the use of the Qiagen Spin blood DNA extraction kit with final DNA elution in Qiagen AE buffer, we performed additional testing of 168 swab specimens after DNA isolation with the recommended Qiagen MinElute media kit and final DNA elution with the kit's AVE buffer. As shown in Table 4, agreement rates remained high (0.760 overall and ranging from 0.838 to 0.994 for individual HPV types) and were improved compared to those of s-LA executed on specimens with DNA isolated via the Qiagen Spin blood kit. However, a shift occurred when specimen DNA was isolated with the MinElute kit for many HPV types toward numerically increased detection by the s-LA protocol as opposed to by multiplex HPV PCR, though the differences were only statistically significant for 4 individual types and in the "any of the 14 types" analysis. The specimens chosen for this evaluation of DNA extraction were selected based on HPV results from prior multiplex HPV PCR testing to ensure representation by all 14 HPV types being evaluated. Although all standard controls for the assay, such as type-specific plasmids in a background of human DNA and HPV16-positive SiHa cells, continued to be successfully detected following MinElute DNA extraction, the change in the DNA extraction method appeared to negatively impact multiplex HPV PCR detection for some types. HPV59 was most dramatically affected, with none of the previously screened HPV59-positive specimens testing positive following Qiagen MinElute DNA extraction and multiplex HPV PCR type-specific testing. HPV35, HPV51, and HPV56 were similarly affected, and the differences between multiplex HPV PCR and s-LA for these types were statistically significant. Additionally, as shown in Table 5 under "MinElute DNA isolation," the rate of detection of multiple HPV types was now higher in s-LA and lower in multiplex HPV PCR. This supports the notion that DNA extraction methods and elution or suspension buffers need to be considered for the impact on the reaction chemistries of any PCR-based detection system. In a recent evaluation of oral HPV detection methods, D'Souza et al. (6) demonstrated the differences in HPV detection with Linear Array among four different DNA isolation methods. In that study, however, three of the four extraction methods evaluated utilized the same buffer, LoTE (3 mM Tris, 0.2 mM EDTA), to resuspend purified DNA, and the method they found to be superior used a different resuspension buffer (Puregene DNA hydration buffer). Although the components of the kit buffers in which the final DNA product is suspended are often proprietary and not widely shared, one can speculate that there are potential differences in concentrations of substances such as Tris-EDTA which could impact the efficiency of a downstream PCR if used in a large enough proportion in the final reaction. The impact of the DNA extraction methods used should be examined and carefully considered in the design and interpretation of PCR-based HPV detection studies.

The results of this assay comparison study of HPV detection in clinical swab specimens with Merck's HPV type-specific real-time multiplex HPV PCR assays and the Roche HPV Linear Array assay demonstrate the following: multiplex HPV PCR assays are able to detect more positive specimens than both s-LA and m-LA when clinical specimen DNA is isolated using the Qiagen Spin blood kit, modification of the Linear Array protocol increased the ability of the Linear Array assay to detect single and multiple HPV positives and greatly increased concordance with the multiplex HPV PCR assays, and use of an alternate method of DNA isolation altered the performance of the multiplex HPV PCR and s-LA typing assays.

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