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RESEARCH ARTICLE

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Analytical performance of a low-cost multiplex polymerase chain reaction human papillomavirus genotyping assay for use in Sub-Saharan Africa

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We have tested a multiplex polymerase chain reaction (PCR) human papillomavirus (HPV) genotyping assay to fill the need for rapid and low-cost HPV detection in Sub-Saharan Africa. This method allows high throughput genotyping and simultaneous detection of 14 high-risk and two low-risk HPV types, by PCR amplification of HPV DNAs in a single reaction tube. In this study, we describe stepwise experiments to validate the multiplex HPV PCR assay for determination of HPV genotypes from 104 cervical brush samples from Tanzanian women. Assay performance was evaluated by determination of intra-laboratory reproducibility, sensitivity, and specificity. Further performance was assessed by comparison with the widely accepted and validated HPV My09/My11 amplification and hybridization assay. Statistics; the Cohen kappa (κ) and McNemar *P* values were used to analyze interobserver and intermethod agreement. Overall concordance between the multiplex and line blot hybridization assays was 99% (per sample) with a κ value equal to 0.95; and 96.49% (per detection event) with a κ value of 0.92. Interobserver reproducibility of the assay per sample was 95.76% with κ of 0.91. These results demonstrate that the multiplex HPV PCR assay has high analytical sensitivity and specificity in detecting as many as 16 different HPV genotypes and that its simplicity and low cost makes it well suited for sub-Saharan Africa.

KEYWORDS
detection, high-risk human papillomavirus, human papillomavirus genotypes, human papillomavirus, low-risk human papillomavirus, multiplex polymerase chain reaction, performance, Sub-Saharan Africa, validation

1 | INTRODUCTION

Cervical cancer is the fourth most common cancer in women worldwide (528 000 new cases), and the second most common cancer in developing countries (445 000 new cases) in 2012.[1] The disease burden is greatest among women from low- and middle-income countries (LMIC) and with limited medical service resources.[1,2] In Sub-Saharan Africa, the age-standardized rate for cervical cancer is approximately 35 per 100 000 women.[1] Due to lack of cervical cancer screening services, African women are more likely to present with late-stage cancers, thus causing significant treatment expense to families and governments.[3] Human papillomavirus (HPV) is one of the most common sexually transmitted infections (STIs) in the world. A majority of epidemiological studies have established that HPV is the primary cause of cervical cancer and genital warts.[4-6] Persistent HPV infection with additional exposure to tobacco, oral contraceptives and parity,[7] lead to an increased risk of cervical dysplasia, accumulation of mutations, and integration of HPV

genomes into the host genome; which finally leads to the progression toward high-grade dysplasia and cervical cancer.

Current approaches to reduce the incidence of cervical cancer rely upon cervical cancer screening methods and prophylactic HPV vaccines.[8-12] The screening methods include either visual inspection, cytology evaluation, and HPV tests. The visual inspection test consists of the naked eye inspection of the cervix after the application of 3% to 5% acetic acid (VIA) or the Lugol iodine (VILI) using a cotton swab. The visible changes in tissue pigmentation after solution application are classified as positive (lesion) or negative (normal) results. The Papanicolaou (Pap) test detects precancerous lesions at the cellular level by identification of abnormal or large nuclei. In this method, a small sample of cells is collected from the cervix and examined under an optical microscope by a pathologist. However, HPV DNA testing is considered the most objective, sensitive, and highly reproducible cervical screening approach to date. These assays test for the presence of DNA or RNA from high-risk (HR) HPV types in cervical cells and are used in conjunction with cervical screening particularly when the Pap or VIA results are inconclusive.[13] HPV testing is still considered a cotest,[14-16] yet has been used as primary cervical screening in some European countries.[17] Some limitations in using HPV testing in developing countries are the cost, the laboratory infrastructure needed, and the need for trained laboratory technicians. There are several HPV testing methods available. Multiplex HPV PCR is a common method which relies on simultaneous amplification of target DNAs of different molecular weights, each corresponding to a different HPV genotype.

In this study, results from an HPV multiplex PCR genotyping assay were compared with that of the My9/My11 hybridization assay as the "gold standard." In low-income countries, often cost and availability make more sophisticated HPV genotyping assays[18] unobtainable. In addition, although there are several HPV test kits available in the market, their reliability and validity still need to be evaluated.[19,20] HR HPVs are associated with cervical cancer whereas the LR HPV types such as 6 and 11 are associated with benign genital warts, hence both are included in the multiplex PCR assay.[1,21] In this study we adapted and validated a multiplex HPV PCR assay which detects 14 HR HPV genotypes (16, 18, 30, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66); and two low-risk (LR) HPV genotypes (6 and 11) in a single reaction.[22] Our performance analysis showed that the HPV multiplex PCR genotyping assay is a reliable low-cost alternative to commercial methods.

2 | MATERIALS AND METHODS

2.1 | Study population and specimen collection

The study protocol was reviewed and approved by the Institutional Review Boards of Ocean Road Cancer Institute (ORCI), Dar es Salaam, Tanzania and the University of Nebraska-Lincoln. The participant women were recruited from ORCI, Bagamoyo and Chalinze screening clinics, and informed consent was obtained from the women before sample collection. Women had a gynecological examination, including visual inspection with acetic acid and a conventional Pap test. Pap smear collection was performed using the concave end of an Ayer's spatula, samples were evenly spread on a glass slide and sprayed with fixative. Pap smear results were determined by three blinded cytologists according to Bethesda classification system 2001.[23] For HPV DNA genotyping, cervical cells were collected from the opening of the cervix using a cytobrush. Each brush was put into a cryotube and stored at 4°C until DNA extraction.

2.2 | DNA extraction of cervical samples

Cervical DNA was extracted from cytobrushes with 200 µL lysate solution according to the Qiagen DNeasy Blood & Tissue Kit (Qiagen Inc, Valencia, CA; cat no. 69506). The DNA concentrations of the samples was determined using a NanoDrop Spectrophotometer. Cervical DNA samples were stored at −20°C until PCR analysis.

2.3 | Multiplex PCR assay

Sixteen HPV genotypes (6, 11, 16, 18, 30, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66) were obtained from Karolinska Institute (International HPV Reference Center, Sweden). These plasmids were used as controls and test amplicons in the analytical experiments. The cloned PGEMT was obtained from Promega (Madison, WI). The aminolevulinate synthase 1 (ALAS1) gene was cloned into the PGEMT vector and the construct was used in the analytical, sensitivity, and specificity experiments. All plasmids were confirmed by restriction digest before HPV genotyping experiments. PCRs were performed using a multiplex PCR kit (Qiagen Inc; Redwood City, CA), according to manufacturer's instructions. A previously developed protocol, with minor modifications, was followed.[22] At least 50 ng of DNA sample solution (HPV DNA plasmid or clinical sample) was used as a template for PCR amplification. Samples were incubated at 95°C for 15 minutes, followed by 40 cycles of denaturation at 94°C (30 seconds), annealing at 70°C (90 seconds), and extension at 72°C (60 seconds). PCR products were analyzed on a 6% polyacrylamide gel electrophoresis (PAGE) in 1XTBE and stained with ethidium bromide. Gel images were captured with a ChemiDoc MP Imaging System (Bio-Rad; Hercules, CA). A positive genotyping result was called if a clear band was visualized on the gel. All HPV genotypes were detected by a single band except for HPV types 16 and 58, which were detected by two separate bands.[22]

End-point detection limits of HPV genotype-specific PCR was achieved by serial dilutions of each respective HPV template DNA, plus 1000 ng of Salmon sperm DNA as a carrier in each reaction tube. This experiment was used to optimize each HPV genotype primer set. End-point detection limits were performed for individual or multiple HPV genotypes present in a single reaction tube. Reproducibility experiments were repeated twice within 2 to 3 weeks, by two blinded observers who read identical gel images. For the purposes of the analytical performance comparisons, and for training of Tanzanian lab personnel, the described genotyping experiments were done in the United States.

2.4 | HPV hybridization method

PCR was performed using biotin-labeled MY09/MY11 consensus HPV L1 primers, in addition to biotin-labeled human β-globin primers, which were used as an indicator of DNA quality as previously described.[24] About 50 ng of DNA was added to each 100 μL PCR reaction and subjected to 40 amplification cycles. One hundred and three samples were interrogated by this method. Products were first hybridized against the cellular control DNA, β-globin, then against membrane bound arrays of HPV standard DNAs. Standard DNAs included 38 different HPV types: 6/11, 16, 18, 26/69, 30, 31, 32/42, 33, 34, 35, 39, 45, 51, 52, 53, 54, 56, 57/2/27, 58, 59, 61, 62, 66, 67, 68, 70, 71, 72, 73, 81, 82, 83, 84, 85, 86/87, 90/106, 97, and 102/89. There were two separate mixtures, mix-1 contained 7, 13, 40, 43, 44, 55, 74, and 91, while mix-2 contained 3, 10, 28, 29, 77, 78, and 94. Negative specimens for β-globin gene amplification were excluded from the analysis. PCR results were recorded on a 0 to 5 scale based on the signal intensity of dot-blots. For comparison purposes, a sample genotyped by hybridization method was considered positive only if one or more of the sixteen HPV genotypes present in the multiplex HPV PCR assay was detected; otherwise the sample was considered negative.

2.5 | Statistical analyses

All statistical analyses were carried out using SAS software, version 9.4 (Cary, NC) and Excel 2016 (Microsoft, Seattle, WA). For purposes of the assay performance analyses, we chose a sample size of 104. This initial sample size was based on resource practicality in the absence of reliable data on the expected performance of both assays. Agreement assessment, between methods (multiplex HPV PCR and hybridization) and observers, was assessed by Cohen's kappa test, which is a standard statistical tool for assay performance comparison. The Cohen kappa coefficient (κ) varies from 0 to 1, where 0 to 0.20 indicates slight agreement, 0.21 to 0.40 fair agreement, 0.41 to 0.60 moderate agreement, 0.61 to 0.80 substantial agreement and 0.81 to 1 near perfect agreement. The Cohen kappa tests were performed per sample or per event, where an event is considered to be a specific HPV genotyping call, including a negative call. Contingency tables were used to calculate sensitivity and specificity values with 95% confidence intervals (CIs). The McNemar statistical test is a second tool for assessment of different assay methods. A McNemar test with a *P* value of 0.05 is considered significant, however when there is perfect agreement, and there are zero discordances, then a *P* value of 1 is still considered significant.[25] Clinical sensitivity was calculated as the proportion of women with high-grade lesions (ASC-H and HSIL) tested as positive by mPCR or the hybridization method. Clinical specificity was calculated as the proportion of women who tested negative among those without high-grade lesions (NILM-LSIL).

3 | RESULTS

The multiplex HPV PCR assay utilizes amplified products from 16 different HPV genotypes which map to different regions in the linear

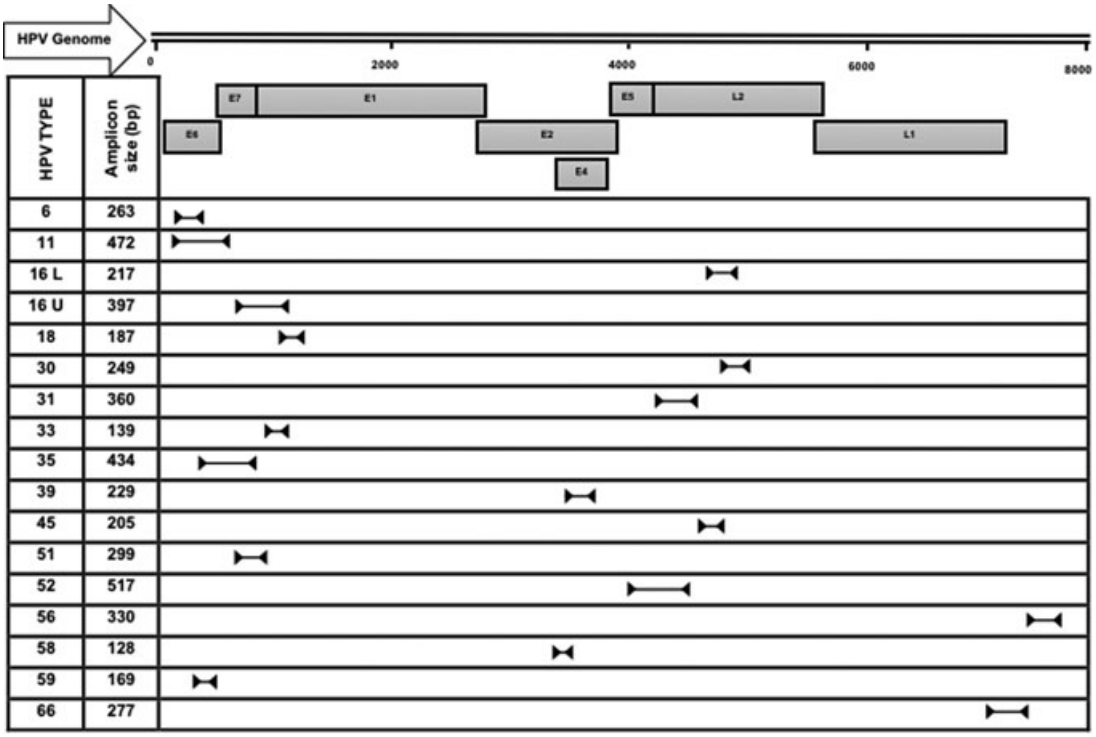

FIGURE 1 Schematic diagram of PCR products mapped on HPV genome. Each of the 16 PCR products of the different HPV genotypes are shown mapped to different open-reading frames on the linear representation of HPV genome. The black arrows indicate the size of each amplicon. HPV, human papillomavirus; PCR, polymerase chain reaction

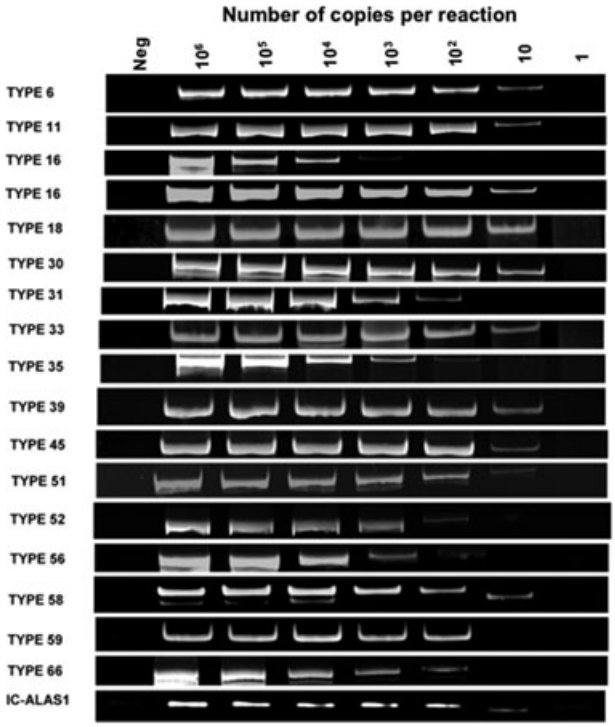

FIGURE 2 Determination of the end-point detection limit of each of the 16 HPV genotypes using the multiplex PCR assay. Ten-fold serial dilutions of the internal control (IC: aminolevulinate synthase 1, ALAS1) or each of the HPV DNAs were subjected to amplification. HPV genotypes −6, 11, 16(L), 18, 30, 31, 33, 39, 45, and 58 were detected at 1 to 10 copies per reaction, whereas HPV genotypes −16 (U), 35, 52, 56, 59, and 66 were detected at 10 to 100 copies per reaction. The dilutions of PCR templates is indicated above each lane (10^8 to 1 viral copy per reaction). *Neg*, indicates a reaction without HPV DNA added. ALAS1, aminolevulinate synthase 1; HPV, human papillomavirus; PCR, polymerase chain reaction

HPV genome (Figure 1). HPV16 has two PCR products, a lower HPV16 band (L) (217 bp), and an upper HPV16 band (U) (397 bp). The analytical sensitivity of detection of each of the 16 different HPV genotypes was analyzed by PAGE, as shown in Figure 2. The end-point detection limit of HPV genotypes 6, 11, 16(L), 18, 30, 31, 33, 39, 45 and 58 ranged from 1 to 10 copies; and 10 to 100 copies for HPV genotypes 16(U), 35, 52, 56, 59, and 66. The number of copies per reaction determined by the multiplex HPV PCR assay were comparable to that obtained from real-time PCR and capillary electrophoresis (CE) methods and gel electrophoresis (Table 1). Real-time PCR had the lowest detection limits when compared with the other two methods. Overall, the end-point detection limits obtained with the multiplex assay were comparable to the ones obtained by CE. The end-point detection limit assays of the multiplex PCR assay containing six different HPV genotypes (6, 16, 31, 33, and 52) is shown in Figure 3. This figure demonstrates that the number of copies detected when using the mixture were similar as those detected for their respective individual HPV genotypes; 1 to 10 copies for 6, 16(L), 31, and 33 and 10 to 100 copies for HPV genotypes 16(U) and 52 (Figure 2). In addition, these results suggest that the multiplex HPV PCR assay possesses high specificity in detecting each of the six HPV genotypes with no exhaustion of PCR reagents.

The agreement charts for comparison between observer calls using the Multiplex HPV PCR assay are shown in Figure 4. For the "per sample" comparison (Figure 4A), the chart shows an almost perfect agreement (dark gray shading), with only a small partial agreement region (light gray shading) for the negative and positive results. The proportion of agreement "per sample" analysis was 99% (103/104 samples), and the Cohen kappa coefficient was 0.978 (95% CI, 0.934-1.000). The McNemar *P* value was 0.3173. Regarding the "per event" (per genotype) comparison, the proportion of agreement was 97.4% (114 of 117 events), with a κ coefficient value of 0.946 (95% CI, 0.885-1.000) (Figure 4B). The corresponding McNemar *P* value was 0.5637. The exact agreement regions, shown in Figure 4B, are slightly smaller than those in the "per sample" graph (Figure 4A) due to an increased number of "events" counted. These results indicate that there is excellent agreement between the detection methods. The results of this comparison is summarized in Table 2.

Figure 5 shows the comparison of detection of 103 cervical samples between multiplex HPV PCR (filled circles) and hybridization (open triangles) methods. In this analysis, one of the samples was not considered because there was a disagreement between observers' calls for the multiplex assay. Our results showed that 4 out of 103 samples were discordant. Specifically, two cervical samples, which were HPV negative by the hybridization method, were found to have HPV types 33 and 66 by the multiplex assay. These corresponded to samples number 5 and 73, respectively. Furthermore, two additional HPV genotypes were detected in samples number 20 and 54, which were not detected by the hybridization method: HPV types 11, 18, and 66 (mPCR); and HPV 33 and 66 (mPCR), respectively (Figure 5). In essence, these results suggest the HPV mPCR method has superior sensitivity.

Figure 6 shows the agreement charts for the comparison between the two genotyping detection methods. The proportion of agreement per sample was 98% (101 of 103 samples), and the Cohen kappa coefficient obtained was 0.955 (95% CI, 0.891-1.000) (Figure 6A). The corresponding McNemar score was $P = 0.3173$. The proportion of agreement of the per-event analysis was 96.5% (109 of 113 events), while the κ coefficient was 0.923 (95% CI, 0.849-0.997) (Figure 6B). The McNemar score was $P = 0.5637$. Overall, results in both cases indicate almost perfect agreement between the Multiplex HPV PCR and HPV hybridization assays. Each of the McNemar scores is significant up to a value of 1. These results indicate excellent agreement between the methods. The results of this comparison is summarized in Table 2.

Of the 104 cervical samples used, 12.50% were negative for intraepithelial lesion or malignancy (NILM) while the remaining had atypical squamous cells of higher pathological categories (ASCUS-HSIL). Thirty-three samples out of 104 samples tested positive by multiplex HPV PCR assay, none of the samples were excluded from the analysis as all samples had sufficient human DNA, as indicated by the internal control. In testing the reproducibility of the multiplex

TABLE 1 Comparison of end-point detection limits obtained by real-time PCR, capillary electrophoresis, and gel electrophoresis

			Detection limit (no. of molecules/reaction)		
HPV type	Amplicon size, bp	Amplified region	Real-time PCR	Capillary electrophoresis	Multiplex HPV PCR
6	263	E6	1	10-100	1-10
11	472	E7	1	1-10	1-10
16L	217	L2	1	1-10	1-10
16U	397	E1	1	10-100	10-100
18	187	E1	1	1-10	1-10
30	249	L2	1	1-10	1-10
31	360	L2	1	1-10	1-10
33	139	E1	1	1	1-10
35	434	E6-E7-E1	1	10-100	10-100
39	229	E2	1	1-10	1-10
45	205	L2	1	1-10	1-10
51	299	E7-E1	1	1-10	1-10
52	517	E5-L2	1	1-10	10-100
56	330	LCR	1	10-100	10-100
58	128	E2	1	1-10	1-10
59	169	E6-E7	1	10-100	10-100
66	277	L1-LCR	1	10-100	10-100
IC	100	ALAS1	1	1	1-10

Abbreviations: ALAS1, aminolevulinate synthase 1; HPV, human papilloma virus; PCR, polymerase chain reaction.

PCR assay, the samples were retested 2 to 3 weeks from the initial test, gel analyses for genotype calls were analyzed by two independent observers.

The analytical sensitivity and specificity of the multiplex HPV PCR assay were 100% and 94.26%, respectively. The clinical sensitivity is defined as the ability of the test to correctly identify those patients with disease, in this case the patients who had pap smear results of ASC-H to HSIL. While the clinical specificity refers to the ability of the test to correctly identify those patients without the disease (LSIL-NILM).[35] The clinical sensitivity and specificity of the multiplex HPV PCR and Line Blot hybridization using the Bethesda classification system 2001 were comparable.

Table 3 shows the HPV genotyping results of both methods according to the pap smears results. For the case of normal pathology (NILM) both methods detected the same number of positive and negative results. Regarding the abnormal pathology (ASCUS-HSIL)

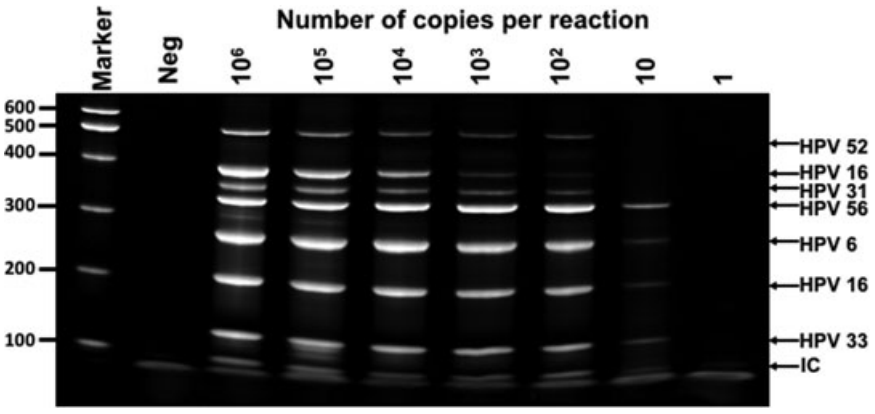

FIGURE 3 The end-point detection limit of multiple HPV templates using the multiplex PCR assay. HPV genotypes: 6, 16, 31, 33, 52, and 56 were diluted by 10-fold serial dilution and subjected to multiplex PCR. The relative detection limit is indicated by the copy number per reaction above each lane (10^6 to 1 copies per reaction). HPV, human papilloma virus; Neg, negative control (without HPV DNA); Marker, 1-kb DNA marker; PCR, polymerase chain reaction

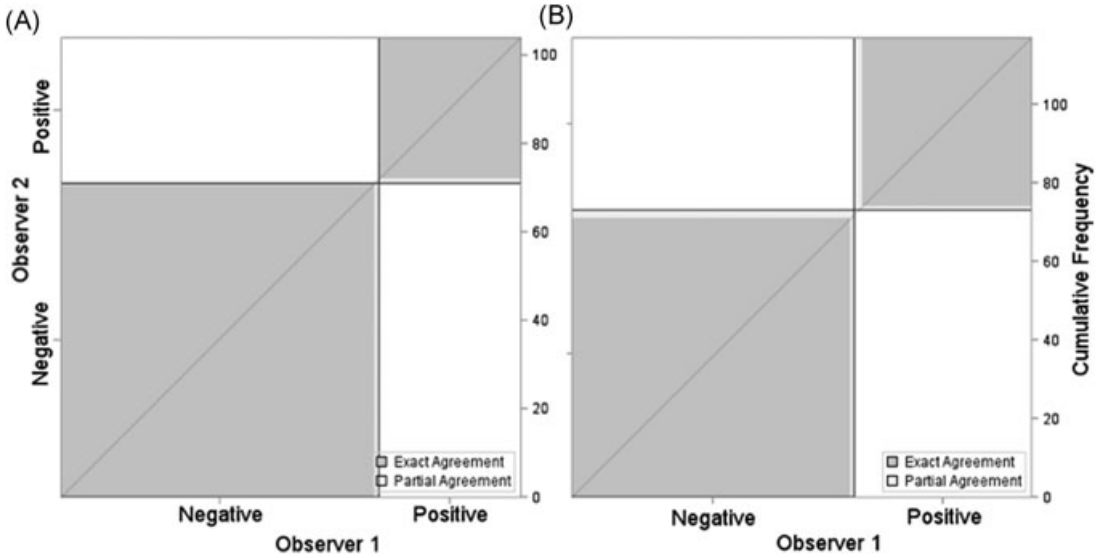

FIGURE 4 Agreement chart for intra-observer calls by multiplex HPV PCR assay. A, Agreement between observers per sample. B, Agreement between observers per event. Shaded squares indicate exact agreement (dark gray), and partial agreement (light gray). HPV, human papilloma virus; PCR, polymerase chain reaction

classification, it is shown that the mPCR assay is more sensitive than the hybridization assay, since the HPV mPCR assay detected two more positive samples than the hybridization assay.

4 | DISCUSSION

The main goal of this study was to validate the analytical detection of HPV genotypes by the multiplex HPV PCR assay comparison with the clinically validated, WHO-approved, HPV (My09/My11) hybridization method. Overall, this comparison is highly concordant, consistent, and reproducible. We chose to calculate the efficiency of HPV genotype detection per sample as well as per event (per genotype), so that we could determine if differing multiplicities of HPVs affected detection efficiency. The assay demonstrated high analytical sensitivity in detecting HPV DNA at very low copy number (between 10 and 100 copies per cell), which is crucial to studying the natural history of HPV pathogenesis and disease diagnosis.[26,27] The downstream analyses using either PAGE or CE gives the researcher choices depending on resource availability, while still using the same simple PCR method.

Agreement between observers was 97.4% with a κ coefficient value of 0.946 (113 of 118 events), and 99% with κ coefficient equal to 0.978 (103 of 104 samples), with a McNemar score of $P = 0.3173$, signifying almost perfect agreement. As a matter of routine, we believe that it is more reliable to have two independent observers do genotype calls. Table 2 shows the results of all performance assays. The HPV mPCR assay offers high reliability for detection of HPV genotypes present in a single reaction, without exhaustion of PCR reagents (shown in Table 1). This result shows that the assay can be reliable when simultaneous detection of multiple HPV genotypes is required.[28] Several studies suggest that infection of multiple HPV genotypes in the cervical epithelium is associated with development of cervical neoplasia.[29,30] Like most of the HPV DNA amplification tests, mPCR showed high analytical sensitivity. Because of the extreme sensitivity of the assay, we prefer to premix reagents in PCR tubes under in a PCR clean room under an isolation hood. We clean all work surfaces, pipettes and gloves with 10% bleach before mixing reagents to avoid cross contamination.

TABLE 2 Summary of statistical tests of the HPV mPCR assay performance in comparison to the My9/My11 HPV hybridization assay

	Percent agreement	Cohen kappa	McNemar	Sensitivity	Specificity
mPCR vs My9/My11 blot					
Per sample	98%	0.955	$P = 0.3173$	100%	94%
Per event (genotype)	96.50%	0.923	$P = 0.0455$		
mPCR Intraobserver comparison					
per sample	99%	0.978	$P = 0.3173$		
Per event (genotype)	97.40%	0.946	$P = 0.5637$		

Abbreviations: HPV, human papilloma virus; PCR, polymerase chain reaction.

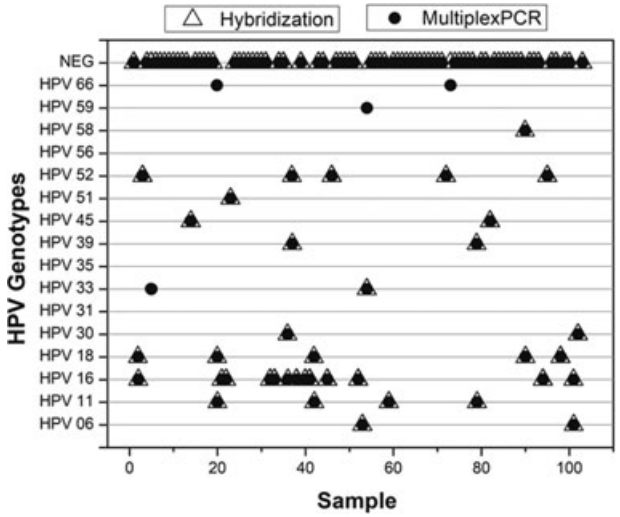

FIGURE 5 Comparison of the HPV genotype detection efficiency between multiplex HPV PCR and HPV hybridization methods. The different HPV genotypes detected are represented as filled circles (multiplex PCR) and open triangles (hybridization method). The results from 103 samples were compared. HPV negative results are also shown in the plot. HPV, human papilloma virus; PCR, polymerase chain reaction

Comparing the HPV mPCR assay with the hybridization method demonstrates almost perfect agreement for the sixteen HPV genotypes tested (see Table 2). The selection of HR HPV genotypes 16, 51, 35, and 18 in this assay makes it useful in Sub-Saharan African countries,[31] where these genotypes are highly prevalent. Since the multiplex PCR assay detects most of the relevant HR-HPVs as well as the LR-HPV which cause condylomas (HPV 6 and 11), it provides the most disease-relevant information. The multiplex HPV PCR proved an efficient use of resources since it has a reagent cost of only $3 per sample, including US to Tanzanian shipping costs for 1000 premixed reactions, the final reagent cost rises to about $3.10 per sample. Commercial HPV genotyping kits often require expensive assay detection equipment such as real-time PCR machines or proprietary detectors. For example, the Panatyper real-time PCR kit (Panagene; Deajeon, South Korea), which detects 20 HR-HPVs, costs approximately $33 per sample. But this also requires a four-color real-time PCR machine. A similar real-time PCR kit (Biotivate, Cincinnati, OH) to detect 21 HPVs, costs about $28 per sample. The more popular HPV linear array kits cost more than $40 per sample to run. Such assays are too expensive for sustainable clinical use in Sub-Saharan Africa. The advantages of the HPV multiplex PCR assay are that it requires minimal reagents and is performed in a single reaction tube. From PCR reaction to genotype determination takes about 4 hours.

We found that the HPV multiplex PCR assay had the ability to detect HPV16 in African samples, despite the fact that there are known to be Africa-specific HPV16 variants from the region.[32] Some further optimization of this assay for African-specific variants may improve detection of HPV16 and perhaps other genotypes. Because of the sensitivity, this assay may also be useful to assess the role of HPV in Adenocarcinomas. We did a small-scale analysis of the HPV multiplex assay for its ability to predict clinical disease determined by pap smear. Clearly, a thorough clinical analysis would be needed with a much larger sample size to assess the clinical value of the assay.[33] High sensitivity and specificity in clinical samples would suggest that the assay has potential use particularly in low-resource clinical settings. Cervical cancer is highest in low income countries, thus, simple low-cost solutions to assess HPV related disease are essential.[1] The WHO has now suggested that HR HPV DNA testing should be prioritized over VIA. This will require well-validated and inexpensive HPV DNA detection assays.[34] The HPV multiplex assay described here would be a good candidate for clinical use in Sub-Saharan Africa.

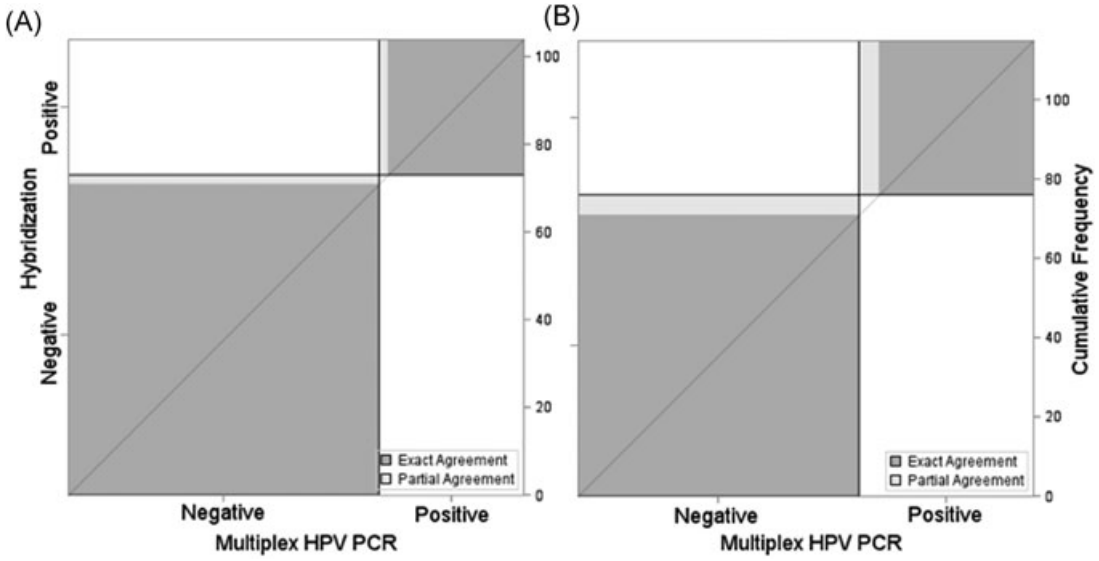

FIGURE 6 Agreement charts for comparison of the HPV genotyping detection methods. A, Agreement chart of detection methods per sample. B, Agreement chart of detection methods per event. Shaded squares indicate exact agreement (dark gray) and partial agreement (light gray). HPV, human papilloma virus; PCR, polymerase chain reaction

TABLE 3 Comparison of Multiplex HPV PCR and hybridization methods in relation to the pap smear results

		Hybridization		
Cytology	Multiplex	Positive	Negative	Total
Normal (NILM)	Positive	5	0	5
	Negative	0	8	8
	Subtotal	5	8	13
Abnormal (ASCUS-HSIL)	Positive	25	2	27
	Negative	0	63	63
	Subtotal	25	65	90
		30	73	103

Abbreviations: ASCUS-HSIL, atypical squamous cells of higher pathological categories; NILM, negative for intraepithelial lesion or malignancy.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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