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Comparison of a Human Papillomavirus Test with the Hybrid Capture 2 and a Real-Time PCR Method for 18 High-Risk and Low-Risk Human Papillomavirus Infections in Women

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Abstract

Squamous intraepithelial lesions (SILs), which are a kind of cancer, and HUMAN papillomavirus (HPV) viral load have frequently been investigated as a risk factor in findings that were in dispute. These studies made use of several HPV viral quantitation assays [including the commercially available hybrid capture 2 (HC2) assays], which vary in their capacity to consider variations in cervical cell collection, linear dynamic range of viral load quantitation, and identified type-specific compared to cumulative viral load measures. Real-time PCR assays were used to quantify the viral loads of HPV 16 and HPV 18 to see if they differed from HC2 quantity (which does not account for cellularity or multiple infection) in terms of the relationship between viral loadand prevalent the SIL and malignancies. In general population, such as the real time PCR measurement of HPV-16, or viral load rose linearly according to the increasing grade of SIL, but themeasurement of HPV-18 used equivalent method increased via low grade SIL (LSIL), with high grade SIL experiencing a higher viral load of HPV-18 and malignancies occurring at a rate equivalent to that of cytologically healthy woman. Utilizing the clinical cut point of 1.0 pg/ml, HC2 viral load distinguished between woman with any level of cytological abnormalities (i.e., normal verses >LSIL) and woman who had cytologically normal tissue, but did the alter as the severity of the lesion rose. HC2 did not appear to plateau at large copy number, and there was no indication of considerable variation in the overall cellularity of the specimens. HC2 estimation of the total viral load, however, overstated the total viral load caused by everyone kind of coinfection.

Keywords: Human Papillomavirus (HPV), Hybrid Capture 2 (HC2), Real-Time PCR, High-Risk HPV, Cervical Cancer Detection.

INTRODUCTION

The virus known as HPV causes human papilloma. There are more than 200 different members of the HPV family, some of which infect the skin and others which affect the mucous membranes in the oral and genital regions of people. Most HPV kinds are benign, and the infection either goes away on its own or, rarely, the virus may cause papilloma or warts that closely related papilloma viruses can also cause in animals [1]. At the basal epithelium, HPV infection happens. Though infections occur frequently, most illnesses go away on their own in a year or two. The most significant risk factor for the development of cervical cancer is persistent infection, which affects only a tiny percentage of infected people. Human Papillomavirus-infected women and men can have cancer. Low-risk HPV viruses cause genital warts and high-risk HPV

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viruses cause cervical and vaginal cancer. The term "high-risk" (HR) HPV types refer to HPV types that have a higher risk of cancer development. According to reports, cervical malignancies caused by HPV 16 and/or HPV 18 account for between 68% and 82% of all HPV-associated invasive cancers. HPV 18 has been identified as the second most common cause of these cancers. Anogenital malignancies, including cervicovaginal and other types of head and neck cancers, can be brought on by HR-HPVs [2, 3]. Clinical manifestations of HPV infection include anogenital warts, recurrent respiratory papillomatosis, cervical cancer precursors (cervical intraepithelial neoplasia), and cancers, including cervical, anal, vaginal, vulvar, penile, and oropharyngeal cancer. The universally accepted screening approach for cervical cancer smear staining with Papanicolaou staining (pap smear). The pap smear stain enables for the detection of precancerous or cancerous tumors. A well- organized, cytology-based cervical cancer screening programmed has been effective in lowering cancer incidence and preventing premature death. Cervical cancer has not been eradicated in some countries [4]. Human papillomaviruses are made up of an icosahedral viral particle (virion) that is encased in a protein capsid that is made up of 72 pentameric capsomers and contains an 8000 base pair double-stranded circular DNA molecule with a diameter of 52–55 nm. When epithelial cells are infected, viral DNA spreads across the full thickness of the epithelium, but complete virions are only present in the tissue's upper layers. Accordingly, depending on the kind and severity of the lesion, viral DNA can be detected either in virions or as episomal or integrated HPV sequences. There are about 100 different kinds of HPV, some of which can cause warts on your hands, feet, and face. Numerous HPV strains, including those that harm your vulva, vagina, cervix, penis, and scrotum, are capable of genital harm. More women might be protected from cervical cancer thanks to the human papillomavirus (HPV) testing's better sensitivity for high-grade cervical intraepithelial neoplasia (CIN) compared to cytology [5].

MATERIALS AND METHOD

Materials and Method of Hybrid Capture Technique and Real-Time PCR

Purpose: Procedure for performing hybrid capture or real-time polymerase chain reaction for detection of HPV DNA, high-risk and low-risk from cervical scrapings, fresh tissue specimens in specimen transport medium.

Specimen Collection and Handling.

1. *Sample type:* Specimen transport medium-stored cervical cells that were obtained during a cervical brushing.
2. Specimen transport cervical cells collected through a cervical brushing and stored in the specimen.
3. *Specimen preparation:* Prior to use, vortex the samples for 5–10 seconds at room temperature once they have completely defrosted. Allow reagents to reach ambient temperature before proceeding.

Reagent

1. Indicator dye.
2. Denaturation buffer.
3. Probe diluent.
4. Mixing the high- and low-risk HPV probes involves briefly centrifuging the vial containing the diluent to force the liquid to the vial's bottom. gentle mixing (tap) [6].

Preparation

The preparation of the diluted probs is shown in Table 1.

Table 1. Preparation of volume probe diluent and take a number of strips.

Number of Tests/Strips	The Volume Probe Diluent		Probe for Volume
96/12	4.0 ml	(4000 ul)	160 ul
72/9	3.0 ml	(3000 ul)	120.0 ul
48/6	2.0ml	(2000 ul)	80.0 ul
24/3	1.0 ml	(1000 ul)	40.0 ul
1	0.045 ml	(45 ul)	1.8 ul

RESULT

Principle of Hybrid Capture 2 Technique

The HC2 high-risk /low-risk HPV DNA Test is a diagnostic method that employs the hybrid capture technology. The test is designed to quality detect. There are 13 high-risk subtypes of HPV DNA and four low-risk types in cervical specimens. The specific HPV detected by the assays by the assay includes high-risk types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 68, as well as low-risk types 6, 11, and 42-44. The procedure involves the hybridization of the target DNA in the specialized HPV RNA probe cocktail. This hybridization results in the formation of RNA: DNA hybrids. RNA: DNA hybrids are then captured on the surface of a microplate well, which has been coated with antibodies specific to RNA: DNA hybrids. To detect the captured hybrids, alkaline phosphatase-conjugated antibodies that specificallybind to RNA: DNA hybrids are added. Multiple alkaline phosphatase molecules are attached to each antibody. Consequently, multiple conjugated antibodies can bind to each captured hybrid, leading to significant signal amplification. When the bound alkaline phosphatase cleaves a chemiluminescent substrate, light is emitted measured as relative light units (RLU) using a luminometer. DNA tests can determine the specific HPV types of present. Additional testing may be required to determine the specific HPV types when the tests indicate the presence of the HPV DNA.

Principal of Real-Time PCR

With excellent specificity, the diagnostic test is an in vitro assay that makes use of real-time PCR technology to identify HPV (human papillomavirus). Each sequence-specific probe used in the test has a 5' fluorophore and a 3' quencher. The test uses probe-based qPCR technology [7].

The fluorophore and quencher on the probe interact in such a way that, in the absence of target DNA the quencher effectively absorbs the fluorescence emitted by the fluorophore, resulting in no detectable net fluorescence. The probe binds to its target template during the PCR amplification procedure. The PCR Taq Polymerase's 5' to 3' exonuclease activity hydrolyzes the probe when the DNA sample is duplicated, freeing the 5' fluorophore for floating in the reaction mixture. The qPCR equipment can detect this emitted fluorophore and transform it into an amplification plot that shows the presence and amount of the target HPV. The HPV one qualitative PCR kit includes human ACTIN control in addition to 14 high-risk HPV strains for identification. [8]

The cycling results demonstrated distinct control setups: Figure 1 (Cycling A, RED) served as the internal control, ensuring the consistency of the assay; Figure 2 (cycling A, yellow) acted as the positive control, confirming the assay's sensitivity; and Figure 3 (cycling A, orange) was utilized as the negative control, validating the absence of non-specific amplification. Interpretation of the specimen depicts in Table 2.

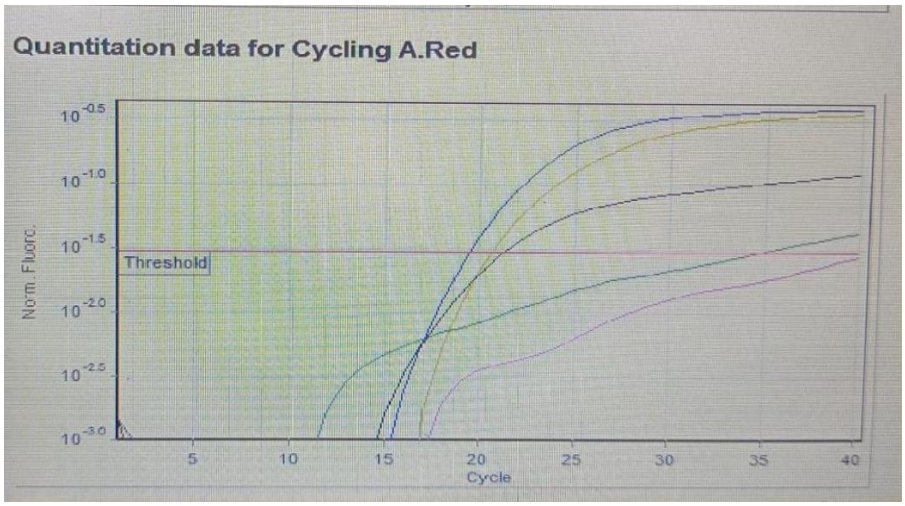

Figure 1. This cycling A. RED is an internal control.

Figure 2. Cycling A. yellow is a positive control.

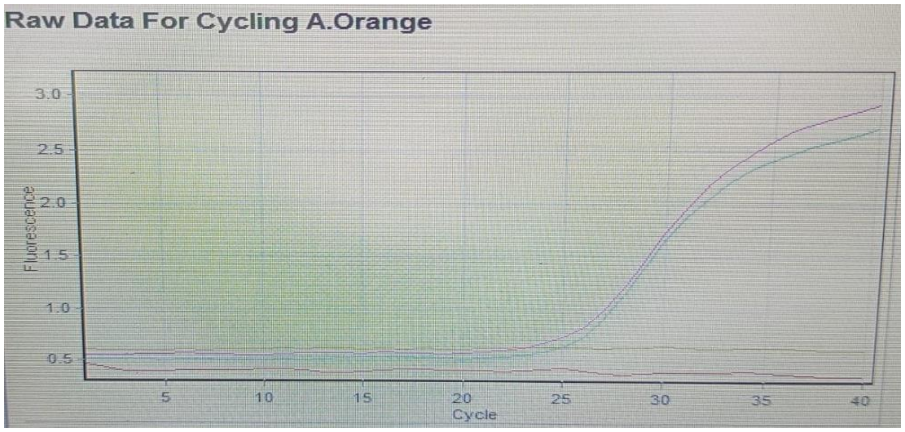

Figure 3. Cycling orange is a negative control.

Table 2. Interpretation of specimen result.

RLU/CORatio	HC2High-RiskHPV DNATest Result	Result Report	Interpretation
<1.0	Positive	HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 not detected	PAL LSIL reduced likelihood that CIN 2–3 or cancer will be found at colposcopy compared with HC2 high-risk HPV DNA test positive LSIL.
			PAP HSIL is expected to be the uncommon result, repressing possible errors in HC2 high-risk HPV
≥1.0	Negative	HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, or 68 detected.	PSA WNL There is a low possibility that a high- grade CIN is underlying the HPV infection, which may be temporary and resolved or permanent.
			LSIL and PAP Colposcopy have a low but slightly enhanced risk of picking up an underlying high- grade CIN. High-grade illness progression is conceivable, according to medical research.
			AP HSIL A colposcopy is most likely to reveal CIN 2-3 or malignancy.
			PR WNL It is unlikely that an HPV infection is caused by a high-grade CIN, which might either be permanent or transient.
			The LSIL and PAP The likelihood of finding an underlying high-grade CIN during colposcopy is modest but somewhat elevated. Medical study suggests that the progression of a high-grade sickness is possible.
			AP HSIL The likelihood of finding CIN 2–3 or cancer during a colposcopy is high.

Table 3. Comparison between HC2 and real time PCR in HPV (total sample 30).

Technique	Positive	Negative	Total sample	Interpretation
HC2	4 samples	26 samples	30	In this case, out of 26 samples, 4 samples are positive.
Real-Time PCR	5 sample	25 samples	30	In the case of real-time PCR, out of 26 samples, 5 samples are positive.

- In HC2 positive results show that the cervical sample may contain any HPV type out of 18 types.
- In Real-Time PCR 4 results were matched from HC2, and 1 extra positive showed that DNA from cervical sample contains 16 types.
- PCR is more sensitive than HC2 because for HC2 5000cp/µl HPV is needed for screening, but PCR can risk minimal DNA and amplify (Table 3).

Table-4. Comparison between HC2 and real-time PCR in HPV.

Technique	Positive	Negative	Total sample	Interpretation
HC2	13	27	40	In this case, out of 40 samples, 13 samples are positive.
Real-Time PCR	10	30	40	In real-time PCR 10 samples, are positive.

Note: Total sample 40.

- Positive results in HC2 show that out of 40 cervical samples 13 samples appear positive, which means they may contain any HPV type out of 18 types of HPV.
- In PCR 3 samples were not matched from HC2 because the PCR master mix has primer and probe for 16 and 18 types.
- HC2 covers 18 types of HPV but is unable to identify which type is present (Table 4).

Table 5. Comparison between HC2 and real-time PCR in HPV.

Technique	Positive	Negative	Total Sample	Interpretation
HC2	2	23	25	Out of 25 samples, 2 samples are positive.
Real-Time PCR	2	23	25	Out of 25 samples, 2 samples are positive.

Note: Total sample 25.

- In HC2, 2 samples were positive out of 25 samples.
- In Real-Time PCR 2 samples (16 and 18) were positive out of 25 samples.
- In This case study we find all the cervical samples were matched because both samples are 16 and 18 type as HC2 and PCR both covers 16 and 18 type (Table 5).

Table 6. Comparison between HC2 and real time PCR in HPV.

Technique	Positive	Negative	Total sample	Interpretation
HC2	9	21	30	Out of 30 samples, 9 samples are positive.
Real Time PCR	6	24	30	Out of 30 samples, 6 samples are positive.

Note: Total sample 30.

- In HC2 9 samples appear positive out of 30 (6 positive samples had more than 100 values and 3 samples were lesser than 10 values.
- In PCR 6 samples were positive out of 30 (3 samples from HC2 were not matched).
- HC2 can sometimes pick up similar homologs that give false positive results, which is why the PCR 3 sample was not matched (Table 6).

INTERPRETATION

Note: The digene HC2 HPV DNA test cutoff of 1pg/ml translates to 100, 000 copies of HPV per milli litre or 5,000 copies per assay.

1. Low risk HPV probe only for STM specimens with RLU/cutoff value ratios 1.0 regarded as "positive" for at least one of HPV types 6, 11, 42, 43, or 44.
2. STM specimens with RLU/cutoff value ratios of 1.0 or less with the high-risk HPV probe alone is regarded as "positive" for at least one of the following HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68.
3. Retesting the material is advised by QIAGEN if the RLU/CO ratio of a PreservCyt specimen is between 1.0 and 2.5. If the preliminary retest outcome is favorable (1.0 RLU/CO), There is no need for additional testing because the specimen can be declared positive. To produce a result, a second retest (third result) must be performed if the first retest result is negative (1.0). The outcome of the second retest is taken into the end outcome, which needs to be recorded.
4. If a specimen's RLU/Cutoff ratio is nearly 1.0 but less than 1.0 and high-risk HPV infection is suspected, alternative testing procedures and/or a repeat specimen should be considered.
5. STM specimens are deemed "positive" for one or more HPV types from each group of probes if the RLU/cutoff value ratios for both the low-risk HPV pprobe and the high-risk HPV probe is 1.0.
6. Using the combined-probe cocktail, STM specimens with RLU/cutoff values ratios less than 1.0 are HPV types 6, 11, 16, 18, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 56, 58, 59, and 68 are all regarded as positive. [9, 10]

DISCUSSION

This study focused on specimens with normal or nonspecific cytological findings (NILM, reactive changes, atrophy), unlike prior studies, which made it difficult to predict HPV presence through cytological or pathological analysis. Despite this, real-time PCR of HPV-16 viral load showed a significant association with increasing cervical neoplasia severity, even when accounting for specimen cellularity. Among cervical scraps with unclear cytological diagnoses, the viral load reduced notably with higher concentration. Contrary to other studies, the mean cell concentration did not vary significantly by lesion grade. These results might differ due to the standardization of sample collection by the same doctors, which minimized cell collection variability, unlike in multisite or self-collection studies. In addition, the viral load sample was obtained using a cervical brush after a Pap smear, reducing sample variability. Costa Rican women in the study exhibited significant cervical inflammation, suggesting that human DNA from inflammatory cells may have influenced the results, leading to a null correlation. The variability in cell collection should be assessed and corrected, with future studies focusing on counting epithelial cells and analyzing infected vs. uninfected cells for a deeper understanding of HPV's role in cervical carcinogenesis.

HPV-18 viral load showed a minimal increase in malignancies and HSIL compared to normal cytological samples, which persisted even after accounting for sample variability. This contrasts with previous research suggesting a strong association between HPV load and disease incidence. The HPV-18 viral load's log-normal distribution, like HPV-16, indicates that the lack of a correlation with neoplasia is not due to assay misclassification. Previous studies using fewer sensitive tests could not capture all HPV-positive individuals, potentially affecting results.

Real-time PCR, despite its high sensitivity and risk of contamination, remains the gold standard for diagnosing HPV, though it requires careful review, especially when amplification exceeds C_T values. HC2, another HPV testing method, showed a good correlation with PCR for HPV-16 infections but had limitations in detecting lower viral loads, especially in the 1.0 pg/ml range. HC2 technology has a higher detection threshold than PCR, limiting its ability to detect low viral loads but remains reliable in identifying high-risk HPV types with clinical relevance.

CONCLUSIONS

Women with CCs seldom have a negative HC2 test result, and roughly half of these tumors are found to include high-risk HPV strains that are included in the HC2 test. when examined with more accurate

PCR-based methods. Adenocarcinomas are more common in uncommon CCs with verified HPV negativity and are linked to a worse DFS.

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