

Correlation between histological criteria and human papillomavirus presence based on PCR assay in cervical biopsies

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The aim was to correlate histological findings in cervix lesions to human papillomavirus (HPV), as detected by polymerase chain reaction (PCR). One hundred and seven women with atypical Pap smear were submitted to colposcopic examination, and suspicious images were biopsied. The PCR assay was performed with the primers MY09/11 and GP05/06+ and, as control, the beta-globin gene was amplified. The morphological findings were correlated to HPV positivity: parakeratosis, acanthosis, koilocytotic atypia (KA), binucleation, dyskeratosis, and number of mitoses. From 107 patients, 61 biopsies were taken: 11 chronic cervicitis (CC), 36 cervical intraepithelial neoplasia (CIN) (13 CIN I; 10 CIN II; 13 CIN III), and 14 suggestive for HPV (SHPV). DNA extraction was not possible in eight cases. HPV was found in 35% CC, 77% CIN, and 64% SHPV. The analysis did not indicate any morphological criteria strongly related to HPV. The findings with highest sensitivity for HPV were KA (88.89%) and binucleation (75%), but with low specificity of 29.41 and 52.94%, respectively. The higher predictive positive values (PV⁺) for HPV were also KA (72.73%) and binucleation (77.14%). Considering KA, dyskeratosis and binucleation together, PV⁺ was 72.41%. Conclusion: Although indicative, none of the studied morphological criteria was always related to PCR virus detection, denoting some limitations for histological diagnosis.

KEYWORDS: cervix uteri, diagnosis, histopathology, HPV, PCR.

Human papillomavirus (HPV) is associated to cervical squamous intraepithelial lesions (SIL) which can evolve to cervical cancer^(1,2). Cohort studies have

demonstrated that the persistence of HPV DNA is necessary for the development of cervical neoplasms and its disappearance predicts regression of the neoplastic cells⁽³⁻⁵⁾. Most lesions arise from squamous epithelium or squamous metaplastic endocervical epithelium. When restricted to the epithelium they are named SILs, cervical intraepithelial neoplasia (CIN) or dysplasia-carcinoma *in situ*, in accordance with World Health Organization (WHO)⁽⁶⁾. Grades

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CIN I, II, and III are recognized depending on extent and severity. Several studies have shown that HPV is related to these precursor lesions as well as to advanced cancer⁽⁷⁻¹²⁾. The natural history of most cervical cancers starts from an infectious disease associated with HPV which leads to intraepithelial cellular abnormalities. As HPV detection methods developed, the prevalence of HPV DNA in low-(LSIL) and high-grade squamous intraepithelial lesions (HSIL) has increased to 80–90%⁽¹³⁾.

Most countries use Papanicolaou test as a screening to detect CIN. Positive patients are referred to colposcopy and biopsy of the suspected area. Histological diagnosis of CIN is based on morphological criteria described by WHO⁽⁶⁾. Furthermore, some histological aspects are considered suggestive of HPV (SHPV) infection in epithelial lesions. However, as the latter are liable to subjectivity in evaluation, some disagreement in diagnosis occurs among pathologists.

After the development of primers for HPV detection by the polymerase chain reaction (PCR) assay, it has become clear that HPV has a major role in cervical cancer because almost every sample contains HPV DNA^(14,15). This has led to the widespread use of HPV DNA detection tests in patients with SIL or atypical squamous cells of undetermined significance as a screening method in lieu of colposcopy, a policy that has been questioned⁽¹⁶⁾.

The aim of this study was to evaluate the morphological criteria used in biopsies to diagnose CIN and HPV from cervical samples and to compare them to the presence of HPV DNA by PCR with generic primers MY09/11 and GP05+/06+. It is hoped that this study may improve the reproducibility of morphological HPV diagnosis.

Patients and methods

This study was approved by local Institutional Ethical Committee, and women were enrolled after their written informed consent. One hundred and seven women with atypical Pap smear from a Public Health Center of Campinas, Brazil, were submitted to colposcopic examination. A cervical swab collected with a cytobrush was immersed in a sterile flask containing 1 ml DNAzol (InvitrogenTM) and sent to PCR laboratory. Biopsies were taken from suspicious colposcopic areas.

PCR

The DNAzol buffer was transferred to a 1.5 ml microcentrifuge tube with 500 µl of ethanol 95%. The solu-

tion was centrifuged at 14,000 rpm for 5 min. The supernatant was discarded; a new aliquot of 500 µl of ethanol 95% was added, followed by a centrifugation step. This procedure was done twice. The DNA was left drying for 15 min at room temperature, and resuspended in 50 µl of Tris-EDTA (10 mM/1 mM, pH 8.0).

To amplify the viral DNA, the generic primers MY09/11 were used^(17,18). As control of the reaction and to confirm a negative result for HPV, the beta-globin gene was amplified with RS42 and KM29 primers^(17,18). The reaction was performed in 50 µl total volume containing 1.5 µl of resuspended DNA, 10 mM dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 20 pmol for each primer, 1.5 U of Taq DNA polymerase (InvitrogenTM). The amplification consisted of an initial 5 min denaturation step followed by 40 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C. The PCR product was electrophoresed on 1.2% agarose gel, stained with ethidium bromide (0.5 mg/ml) and examined under UV light.

The result was considered positive for viral DNA when two bands were visualized, one with 450 bp, corresponding to HPV DNA and the other with 550 bp, from the beta-globin gene. If only the 550 bp band was visualized, the result was considered negative.

HPV was typed by restriction fragment length polymorphism, with seven restriction enzymes: *Bam* H1, *Dde* I, *Hae* III, *Hinf* I, *Pst* I, *Rsa* I, and *Sau*3AI as described previously by Bernard *et al.*⁽¹⁹⁾.

Negative HPV samples with the MY09/11 primers were subjected to amplification with primers GP05+/06+, which are internal to MY09/11 sequence and generated a product of 142 bp. PCR was performed as described by Walboomers *et al.*^(19,20).

We elected both MY and GP-PCR systems together because PCR is the most sensitive method for the detection of HPV DNA and because some previous studies showed that there are differences in the sensitivities of these two PCR systems^(21,22).

Biopsy

The fragment was processed and stained with hematoxylin and eosin (H&E). The diagnosis was established by two pathologists who took into consideration the following six morphologic criteria: parakeratosis, acanthosis, koilocytotic atypia (KA), binucleation, dyskeratosis, and number of mitosis by ten high power fields. When there was strong evidence for true KA defined as enlarged, hyperchromatic, wrinkled nuclei, present in large cells, with

clear cytoplasm and thick cell membranes, the diagnosis was at least CIN I, as proposed by WHO. Mild KA was defined as hyperchromatic nuclei with slight irregularities in shape and outline of the nuclear membrane and perinuclear clear cytoplasm. When there was only mild KA, without binucleated or dyskeratotic cells, the case was classified as SHPV. We considered acanthosis when the epithelium had more than ten squamous layers.

Statistical methodology

The PCR test was considered the gold standard. To compare the histological diagnosis and the PCR results to the morphological criteria listed above, and to verify whether there is association among them, the Chi-square test was used. When the number of cases was less than five, Fisher's exact test was used. To compare the number of mitosis and the histological diagnosis, Mann-Whitney exact non-parametric test (Wilcoxon) was used. To verify the agreement between the diagnosis and the criteria with PCR, *Kappa* coefficient was calculated, which can assume the values from -1 to $+1$. Values close to $+1$ indicate total agreement between the methods, while values close to -1 indicate total disagreement. Values higher than 0.75 show strong agreement; values less than 0.40 show weak, and those between 0.40 and 0.75 show intermediate agreement.

Results

Biopsy diagnoses were separated into three groups: CC, CIN, and SHPV, to verify any association between each specific group and the PCR results.

From 107 patients, 61 biopsies were taken which yielded the following diagnoses: 11 CC, 36 CIN (13 CIN I; 10 CIN II; 13 CIN III), and 14 SHPV. DNA could not be extracted from eight cases, so 53 cases were analyzed.

Evaluating the relation between PCR and biopsy diagnoses, we found HPV positivity in 37.50% of CC, 64.29% of SHPV, and 77.42% of CIN (Table 1). The comparison between morphological criteria and the PCR results did not point to any data strongly related to the HPV DNA presence. The criteria with higher sensitivity for the histological diagnosis of HPV were KA (88.89%) and binucleation (75%), but with low specificity (29.41 and 52.94%, respectively; Tables 2 and 3). The higher predictive positive value (PV^+) for HPV diagnosis was also KA (PV^+ 72.73%) and binucleation (PV^+ 77.14%).

Table 1. Correlation between histological diagnosis and PCR test

Diagnosis	PCR test		Total	Kappa
	HPV positive (%)	HPV negative (%)		
CC	3 (37.50)	5 (62.50)	8	
SHPV	9 (64.29)	5 (35.71)	14	0.2542
CIN	24 (77.42)	7 (22.58)	31	0.3367
Total	36	17	53*	

CC, chronic cervicitis; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; PCR, polymerase chain reaction; SHPV, suggestive for HPV.

*In eight cases DNA could not be extracted.

Morphological criteria versus diagnosis

Koilocytotic atypia occurred in all cases diagnosed as CIN, 92.86% of SHPV and has not been seen in CC. Binucleation appeared in 86.11% of CIN, 57.14% of SHPV and was absent in CC. Dyskeratosis was present in 80.56% of CIN, 50% of SHPV, and 9.09% of CC. Mitoses appeared in 58.33% of CIN, 35.71% of SHPV, and 9.09% of CC but no association was found between number of mitoses and diagnosis. Binucleation, dyskeratosis, and mitoses showed a significant positive association with CIN and negative association with CC (P -values = 0.0001, 0.0001, and 0.0024, respectively). There was no association between acanthosis and parakeratosis with any of the three diagnoses.

Presence of HPV by PCR versus biopsy diagnosis

CIN versus PCR

Sensitivity = 88.89%, specificity = 41.67%, PV^+ = 77.42 and predictive negative value = 62.50. It means that 77.42% of CIN will be HPV DNA positive, according to PCR test. However, *kappa* coefficient was 0.3367, indicating low agreement between the positive PCR test and the diagnosis of CIN (Table 1).

SHPV versus PCR

Sensitivity = 75.00%, specificity = 50.00%, PV^+ = 64.29, predictive negative value = 62.50. It means that 64.29% of SHPV will be HPV DNA positive, but the *kappa* coefficient was 0.2542 (Table 1).

Presence of HPV by PCR versus morphological criteria (Tables 2 and 3)

Two morphologic criteria were particularly associated to PCR positive results:

- 1 Koilocytotic atypia with high sensitivity of 88.89%, but low specificity of 29.41%; PV^+ was 72.73 and *Kappa* coefficient 0.2090.
- 2 Binucleation with sensitivity of 75.00%, specificity of 52.94%; PV^+ was 77.14 and *Kappa* coefficient 0.2751.

Both criteria showed an accuracy of about 70%; however, the agreement *kappa* coefficient for these morphological data was very low.

The other criteria: number of mitoses, dyskeratosis, acanthosis, parakeratosis had no agreement with PCR result (*kappa* = -0.0220, 0.0484, 0.0236, and -0.0015, respectively).

Discussion

Morphological criteria versus diagnosis

Koilocytotic atypia was present in all cases diagnosed as CIN. Its presence was expected, mainly in CIN I because it was used to define this lesion. Acanthosis, parakeratosis, and the number of mitoses did not have association with any diagnosis.

PCR versus biopsy diagnosis

PCR versus CIN

The majority of CIN showed HPV positivity (77.42%) and a high sensitivity of 88.89%. It means that among all HPV positive cases, 88.89% were CIN and out of all CIN, 77.42% were HPV positive. These data are in accordance with previous studies. Liaw *et al.*⁽²³⁾ studied HPV and cervical neoplasia in a case-control group. HPV DNA was found in 92% of HGSIL and invasive cancer, 54% of LGSIL, and in 9% of controls. Herrero *et al.*⁽¹¹⁾ in Costa Rica, screened 9175 women to obtain a standard final diagnosis, and tested 3024 women for more than 40 types of HPV with a PCR-based system. Seventy-three percent of LGSIL were HPV positive, with HPV16 being the predominant type (16% of positive subjects). HPV was found in 89% of HGSIL and 88% of cancers, with HPV16

being strongly predominant (51 and 53% of positive subjects). Virtually all HGSIL and cancers had associated HPV types, with high odds ratios (OR) and attributable fractions around 80%. However, our data demonstrated that there was a weak agreement between presence of HPV and CIN diagnosis, with *kappa* coefficient of 0.3367, that means HPV positive does not always mean CIN.

PCR versus SHPV

The histological diagnosis of SHPV was related to 64.29% of positivity for PCR test. But, the *kappa* value of 0.2542 indicates a weak agreement between the virus positivity and histological diagnosis of SHPV. So, we should not diagnose 'suggestive of HPV' based only on the presence of slight, doubtful KA because the probability of misdiagnosis is 35.71%. Instead, we should suggest that the chance of HPV infection is small.

PCR versus morphological criteria

PCR versus KA (Table 2)

When KA is present in the sample, there is a good chance for an HPV infection. However, the low *kappa* coefficient (0.2090) shows that KA does not always mean HPV infection, based on PCR results. We can see KA with negative PCR in 27.23% of the cases. According to our data, if we diagnose HPV exclusively based on this criterion we will be mistaken in 27.23% of the cases. Considering KA, dyskeratosis, and binucleation together, a similar PV^+ for the presence of HPV was obtained (72.41%). It is known that KA is currently considered the major morphological criterion used by pathologists to diagnose HPV infection. However, this finding is not completely trustworthy according to our results and as previously demonstrated by Nuovo⁽²⁴⁾. These authors detected HPV DNA sequences in 63 and 56% of colposcopically visible vaginal and cervical lesions, respectively, that were diagnosed as condyloma or

Table 2. Correlation between KA and PCR test

KA	PCR test		Total
	HPV positive (%)	HPV negative (%)	
Present	32	12	44 (83.02%)
Absent	4	5	9 (16.98%)
Total	36 (67.92%)	17 (32.08%)	53 (100%)

HPV, human papillomavirus; KA, koilocytotic atypia; PCR, polymerase chain reaction.

Agreement coefficient (*kappa*) = 0.2090; predictive positive value (PV^+) = 72.73; predictive negative value (PV^-) = 55.56; sensitivity = 88.89; specificity = 29.41.

intraepithelial neoplasia. HPV DNA sequences were detected in 14 and 47% from other vaginal and cervical lesions that did not fulfill the histologic criteria for condyloma or intraepithelial neoplasia (ie, 'non-diagnostic'). In vaginal lesions, Nuovo *et al.*⁽²⁵⁾ detected HPV in 63% of the cases with KA and in 11.7% without. The presence of the virus has been also demonstrated by electron microscopy^(26,27) and HPV DNA hybridization methods^(28,29).

If the KA is considered as pathognomonic for the presence of HPV, then some factors must be interfering in the sensitivity of PCR:

- 1 There might be some virus sequences that escape from the consensus primers used in this assay or there could be deletions in the L1 sequence flanked by the consensus primers.
- 2 Variation among detection methods: Husnjak *et al.*⁽³⁰⁾ compared five different PCR methods for detection of HPV in cervical scrapes. The first group was tested with three sets of consensus primers located within the L1 region of HPV genome: MY09/MY11 (ie, MY), L1C1/L1C2-1/L1C2-2 (ie, LC), and pI-1/pI-2 (ie, pI) primer sets. The second group of samples, which were all negative with the MY primers, was tested further with the LC primers, as well as with the GP5/GP6 (ie, GP) primers. In the first group ($n = 164$), there were 76.2% positive results obtained with at least one set of consensus primers⁽³⁰⁾. In the second study group ($n = 250$), there were 8.4, 38.8 and 4% samples positive with the LC primers, the nested MY/GP, and the HPV type-specific primer sets, respectively. They conclude that the use of the MY/GP nested PCR increased significantly the positivity rate of HPV DNA detection and should be used for samples with a low copy number of HPV DNA. Evander *et al.*⁽³¹⁾ compared the results obtained with MY, GP, and nested MY/GP primers. There were 56.5% specimens negative with MY and positive only with GP primers. The possible reasons are the smaller size of the PCR product (142 bp GP product against 450 bp MY product) and the fact that GP primers

do not contain degenerated bases. Gu *et al.*⁽³²⁾ found variation among detection methods which use HPV L1 consensus sequence and HPV E6 sequence. They detected HPV L1 consensus sequences only in 29.1% of the samples while HPV E6 was found in 60.7%.

- 3 The conditions for amplification could be not excellent for all HPV sequence.

On the other hand, we found low specificity (29.41%) for the KA, meaning that cases without this criterion could be HPV DNA positives. So, we should consider KA together with other morphological markers for HPV in the biopsy to improve our diagnosis for this virus infection.

PCR versus binucleation (Table 3)

The relation between HPV and binucleation presented high sensitivity (75%) and moderate specificity (52.94%). Nevertheless, *kappa* coefficient (0.2751) indicates a weak agreement meaning that binucleation is a marker with a similar value as KA to indicate HPV infection, but it has a higher power to exclude negative cases when not present.

PCR versus mitosis, dyskeratosis, acanthosis, parakeratosis

There was no agreement between the above criteria and PCR result (*kappa* = -0.0220, 0.0484, 0.0236, and -0.0015, respectively), showing that these elements are non-specific for HPV diagnosis.

Conclusions

Histological diagnosis of CIN was highly associated with the presence of HPV. The highest PV⁺s were: KA and binucleation, with high sensibility but low specificity. Considering KA, dyskeratosis, and binucleation together, a similar positive predictive value for the presence of HPV was obtained (72.41%). According to our data, if we diagnose HPV exclusively based on

Table 3. Frequency of binucleation and its relation to PCR test

Binucleation	PCR test		Total
	HPV positive (%)	HPV negative (%)	
Present	27	8	35 (66.04%)
Absent	9	9	9 (33.96%)
Total	36 (67.92%)	17 (32.08%)	53 (100%)

HPV, human papillomavirus; PCR, polymerase chain reaction.

Agreement coefficient (*kappa*) = 0.2751; predictive positive value (PV⁺) = 77.14; predictive negative value (PV⁻) = 50.00; sensitivity = 75.00; specificity = 52.94.

these morphological criteria, we will be mistaken in 27.59% of the cases. We can conclude that histological data are a good sign of HPV presence; nevertheless, none of the morphological criteria studied was always related to the virus, stressing their limitations when compared to PCR test.

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