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# THE CYTOLOGY AND DNA DETECTION BY THE PapilloCheck® TEST IN THE DIAGNOSIS OF HUMAN PAPILLOMAVIRUS INFECTION

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The aim of this study was to make a comparison between the results obtained by cytologies and by the detection and genotyping of human papillomavirus (HPV) DNA in the screening of cervical cancer. In this study, there were 994 samples used from human females. These were obtained from liquid-based preparations. The samples were analyzed by cytological technique and by the detection of HPV DNA using the PapilloCheck® Test. The HPV was detected in 28% of the samples. Most of the cytology lesions appeared in HPV positive samples and, within these, the most serious injuries occurred mostly in samples with multiple HPV infections. The results indicate that, in general, there is a correlation between the detection of HPV DNA and cytology. However, there were some cases that emphasize the limitations of both diagnosis methods (27% cases with viral HPV DNA positive and normal cytologies and about 2% of cytological lesions detected in samples HPV negatives). It is possible to conclude that none of the two techniques is enough by itself and should be applied together in order to increase the accuracy of cervical cancer screening.

**Keywords:** human papillomavirus, cervical cancer, cytology, PapilloCheck® Test Kit

## Introduction

The presence of human papillomavirus (HPV) DNA has been detected in nearly 100% of cervical cancer, but its presence is not enough for developing the disease. There are a lot of women that have the virus but never get cancer [1–3]. Therefore, its progression may depend on the type of virus, the coinfection with more than one type of HPV or the association of the virus with other risk factors, like age or sexual behavior [2, 4].

Cervical cancer is the second most common type of cancer in the world [1, 5]. The prevalence of HPV in the world population is estimated on a range varying between 6.1% and 35.5% [6]. In Portugal, the prevalence of HPV infection is 19.4% [7].

The cytology is the first screening method to detect cervical cancer, whose abnormalities are classified by the Bethesda System [8]. However, there are a lot of false negatives caused by human error during the collection and/or the microscopic observation what makes the cytological method not 100% truthful. The overvaluation of the cytological findings leads to ambiguous diagnosis, possible overtreatment, or inadequate management of patients [9].

The molecular techniques appeared to restrict the limitations of cytological methods. They allow the direct detection of the virus, the study of the progression of the lesions, and the appropriate monitoring of infected patients, resulting in less false results [9, 10]. The association of findings from conventional cytological testing with those of newer molecular techniques is of great importance and helps to better understand the evolution of HPV infection in different epidemiological settings [11]. However, nowadays, there are a lot of molecular commercial kits with different sensitivities and specificities. This fact makes it difficult to select the molecular method to an appropriate HPV detection. Besides the Food and Drug Administration (FDA)-approved tests for HPV clinical use, laboratories may choose to use non-approved tests [12, 13]. Among the FDA-approved tests for Europe, the PapilloCheck HPV-Screening Test is one of the most frequently used [12, 14, 15]. However, there is no information about studies comparing results of HPV DNA detection obtained by this kit with the cytological results. There are, though, some studies that have showed a strong correlation with other molecular methods, like hybrid capture and GP5_/6_-PCR–EIA [11, 16, 17].

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The aim of this study was to evaluate the potential of the PapilloCheck HPV-Screening Test in the screening of the cervical cancer. The PapilloCheck HPV-Screening Test amplifies a region of the viral gene E1, detecting and identifying 24 HPV types, including 18 high-risk/probable high-risk HPV and 6 low-risk HPV [17, 18].

## Methods

*Population studied*

In this study, there were 994 samples used from human cervical smears of Portuguese females (median age, 39 years; age range, 18 to 76 years). These were obtained from liquid-based preparation, collected in health centers and gynecologists offices from all over the country. The samples, after collected, were preserved in a specimen bag for transport and received at the Microdiag Laboratory (Portugal) from February 2009 to January 2011. These samples were analyzed by cytological technique which was performed in the Microdiag Laboratory and molecular techniques that were performed in Grupo Beatriz Godinho using the PapilloCheck® Test Kit. The screening in Portugal includes only cervical cytology [19]. The HPV genotyping is only considered in selected cases: when the cytology detects lesions with undetermined significance or when the results are unsatisfactory for evaluation [5, 13].

*Slide preparation for cytological analysis*

After sampling, the cells were suspended in a liquid medium used to prepare the slides and to detect the HPV DNA by the PapilloCheck® Test.

The slides were prepared by the automated system Thin Prep [20]. The sample vial was placed into the ThinPrep 2000 and a gentle dispersion step to break up blood, mucus, and non-diagnostic debris was done. A series of negative pressure pulses was generated, which drew fluid though a ThinPrep 2000 filter to collect a thin layer cellular material on a glass slide. The slide was then ejected into a cell fixative bath, stained by Pap staining (using hematoxylin, orange G and EA50 [20]), and evaluated according to the Bethesda System classification [8].

*Detection of HPV DNA by PapilloCheck® Test Kit*

The PapilloCheck® Test Kit was used according to manufacturer instructions (PapilloCheck; Greiner Bio-One GmbH, Frickenhausen, Germany). PapilloCheck® Test Kit detects and differentiates 24 types of human papillomavirus, including 18 high-risk/probable high-risk HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, 82) and 6 low-risk HPV (HPV6, 11, 40, 42, 43, 44/55) by DNA chip technology. The test cannot distinguish between HPV-55 and HPV-44 due to cross-reaction, and the manufacturer instructions does not differentiate the high-risk HPV of the probable high-risk HPV [18].

*DNA extraction*

DNA was extracted according to PapilloCheck® DNA Extraction Kit. Two hundred and fifty microliters of each sample was added to 80 µl of buffer L1, 2.4 µl of carrier RNA solution, and 20 µl of proteinase K solution. Samples were briefly vortexed and incubated at 56 °C for 30 min. The samples were briefly centrifuged, added of 250 µl of buffer L4, briefly vortexed, and incubated at 70 °C for 15 min, briefly centrifuged and the pellet was resuspended in 300 µl of ethanol. The lysate was transferred to the columns and centrifuged at 11,000 *g* for 1 min twices. The columns were washed by centrifugation (11,000 *g* for 1 min at room temperature), using in a first centrifugation 500 µl of buffer W1 and in a second centrifugation 600 µl of buffer W2. The columns were centrifuged again at 11,000 *g* for 1 min to dry the membrane completely. The DNA was eluted with 100 µl of pre-warmed (70 °C) elution buffer E that was incubated at room temperature for 1 min and centrifuged at 11,000 *g* for 1 min [21].

*PCR HPV amplification*

For each reaction, the following were used: 19.8 µl of PapilloCheck® MasterMix, 0.2 µl of HotStarTaq DNA polymerase, 1 µl of Uracil-N-DNA glycosylase, and 5 µl of DNA sample. The amplification was carried out on the GeneAmp® PCR system 9700 (PE Applied Biosystems) using the following thermal profile: 20 min at 37 °C, 15 min at 95 °C; 30 s at 95 °C, 25 s at 55 °C, 45 s at 72 °C (40 cycles), followed by 30 s at 95 °C and 45 s at 72 °C (15 cycles). The samples were stored at 4 °C until analysis [18].

*Hybridization*

Five microliters of the PCR product was mixed with 30 µl of the PapilloCheck® Hybridization buffer at room temperature. This preparation was briefly spun down, and 25 µl of the mix was transferred into each compartment of the chip that was incubated for 15 min at room temperature in a humid atmosphere. The washing solution, 140 ml double-distilled water with 14 ml PapilloCheck® Buffer A and 1.75 ml PapilloCheck® Buffer B, was divided in three equal parts into three reaction tubes and marked as I, II, and III. The washing solution II was preheated to 50 °C before use. The chip was washed in three steps: at room temperature with washing solution I for 10 s, at 50 °C with washing solution II for 60 s, and at room temperature in washing solution III for 10 s. After wash, the chip was dried by centrifugation [18].

*Scanning and analysis*

Scanning and analysis of the data were performed with the Greiner Bio-One CheckScannerTM that was linked to a computer where the CheckReportTM Software presents the results [18].

*Statistical methods*

Data analysis was carried out using Microsoft Excel 2010.

## Results

*Sample characterization*

The age of the 994 females varied between 18 and 76 years old. The women with positive HPV DNA varied between 19 and 67 years old. Thirty-three percent of the positive cases were observed in women with ages between 19 and 30, 38% in women with 31 to 40, 19% in women with 41 to 50, 7% in women with 51 to 60, and finally 2% in women with 61 to 67 years old.

*Cytological findings*

From the 994 women tested, 464 (47%) presented a normal cytology, 238 (24%) had inflammation, 7 (0.7%) had changes related with intrauterine contraceptive device (ICD), 27 (2.7%) had atrophy, 22 (2.2%) presented changes related with the presence of *Candida* spp., 113 (11%) had atypical squamous cells of undetermined significance (ASC-US), 5 (0.5%) had atypical squamous cells not excluding high-grade squamous intraepithelial lesion (ASC-H), 81 (8%) presented low-grade squamous intraepithelial lesion (LSIL), 19 (1.9%) had high-grade squamous intraepithelial lesion (HSIL), and 1 (0.1%) had squamous cell carcinoma *(Table 1)*. Twelve samples were unsatisfactory for evaluation.

**Table 1.** Cytological results in the 994 samples

| Cytological results | |
|---|---|
| Normal | 464 (46.68%) |
| *Candida* spp. | 22 (2.21%) |
| Inflammation | 238 (23.9%) |
| ICD | 7 (0.70%) |
| Atrophy | 27 (2.72%) |
| ASC-US | 113 (11.37%) |
| ASC-H | 5 (0.50%) |
| LSIL | 81 (8.14%) |
| HSIL | 19 (1.91%) |
| Squamous cell carcinoma | 1 (0.10%) |
| Unsatisfactory | 17 (1.71%) |

ICD: intrauterine contraceptive device; ASC-US: atypical squamous cells of undetermined significance; ASC-H: atypical squamous cells not excluding high-grade squamous intraepithelial lesion; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion

*Detection of HPV DNA*

From the 994 women tested, 701 were negative for HPV DNA, 285 were positive and 8 samples showed unsatisfactory result (due to failures on the controls tests [18]) *(Table 2)*. From all the positives results, 258 women (91%) presented high-risk/probable high-risk HPV and 63 had low-risk HPV (22%). Of all these women, 36 (13%) had both high-risk/probable high-risk HPV and low-risk HPV *(Table 2)*. Of the 258 women with high-risk/probable high-risk HPV, the HPV16 was the most frequent, followed by HPV31, HPV51, and HPV56 *(Table 3)*. Of the 63 women with low-risk HPV, the HPV42 was the most frequent *(Table 4)*.

**Table 2.** Detection of HPV DNA results in the 994 samples

| Detection of HPV DNA | | |
|---|---|---|
| Positive | Negative | Unsatisfactory |
| 285 (28.67%) | 701 (70.52%) | 8 (0.80%) |
| Positive HPV* | | |
| High-risk/probable high-risk HPV | Low-risk HPV | More than one genotype of risk |
| 258 (90.53%) | 63 (22.11%) | 36 (12.63%) |

*The sum of percentages is higher than 100% because women can be infected with more than one HPV type

*Comparison of cytology analysis results and HPV DNA detection results*

The largest percentage of women (54.9%) with a normal cytology corresponded to negative samples for HPV DNA. Thirty-three percent of the women negative for HPV had other non-neoplastic findings (*Candida* species, inflammation, ICD, or atrophy). About 9% of the women with a negative result for the detection of HPV DNA had ASC-US or ASC-H, 1.3% had LSIL, and 0.14% had HSIL *(Table 5)*.

Around 28% of the women positive for HPV presented a normal cytology. Twenty percent of women positive for HPV DNA had other non-neoplastic findings, *Candida* species, inflammation, ICD, or atrophy. About 19% of the samples contaminated with HPV showed ASC-US or ASC-H, 25% had LSIL, and 6% had HSIL. It was also found one case of squamous cell carcinoma *(Table 5)*.

Almost all of the samples from the HSIL group were positive to high-risk/probable high-risk HPV. Twenty-four

**Table 3.** Prevalence of the different types of high-risk/probable high-risk HPV in the 994 samples

| | High-risk/probable high-risk HPV | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| HPV genotype | 53 | 56 | 58 | 59 | 66 | 68 | 70 | 73 | 82 |
| Number of samples | 29 | 36 | 27 | 15 | 18 | 23 | 7 | 7 | 9 |
| % | 11.20 | 13.90 | 10.42 | 5.79 | 6.95 | 8.88 | 2.70 | 2.70 | 3.47 |
| HPV genotype | 16 | 18 | 31 | 33 | 35 | 39 | 45 | 51 | 52 |
| Number of samples | 54 | 10 | 43 | 13 | 8 | 19 | 7 | 37 | 17 |
| % | 20.85 | 3.86 | 16.60 | 5.02 | 3.09 | 7.34 | 2.70 | 14.29 | 6.56 |

**Table 4.** Prevalence of the different types of low-risk HPV in the 994 samples

| | Low-risk HPV | | | | | |
|---|---|---|---|---|---|---|
| HPV genotype | 6 | 11 | 40 | 42 | 43 | 44/55 |
| Number of samples | 9 | 4 | 5 | 32 | 11 | 12 |
| % | 14.29 | 6.35 | 7.94 | 50.79 | 17.46 | 19.05 |

**Table 5.** Cytological results in negative and positive samples for HPV DNA in the 994 samples

| Cytological results | Negative HPV | Positive HPV | High-risk/probable high-risk HPV | Low-risk HPV |
|---|---|---|---|---|
| Normal | 385 (54.92%) | 79 (27.72%) | 70 (27.13%) | 13 (20.63%) |
| *Candida* spp. | 18 (2.57%) | 2 (0.70%) | 1 (0.39%) | 1 (1.59%) |
| Inflammation | 185 (26.39%) | 49 (17.19%) | 46 (17.82%) | 8 (12.70%) |
| ICD | 4 (0.57%) | 3 (1.05%) | 2 (0.78%) | 1 (1.59%) |
| Atrophy | 25 (3.57%) | 2 (0.70%) | 2 (0.78%) | 0 |
| ASC-US | 61 (8.70%) | 51 (17.89%) | 48 (18.60%) | 14 (22.22%) |
| ASC-H | 1 (0.14%) | 4 (1.40%) | 4 (1.55%) | 1 (1.59%) |
| LSIL | 9 (1.28%) | 71 (24.91%) | 61 (23.64%) | 22 (34.92%) |
| HSIL | 1 (0.14%) | 18 (6.32%) | 18 (6.98%) | 1 (1.59%) |
| Squamous cell carcinoma | 0 | 1 (0.35%) | 1 (0.39%) | 0 |
| Unsatisfactory | 12 (1.71%) | 5 (1.75%) | 5 (1.94%) | 2 (3.17%) |

percent of the women with high-risk/probable high-risk HPV had LSIL, 19% had ASC-US and 1.6% had ASC-H. The samples with low-risk HPV were more frequent in LSIL women with 35%. Twenty-two percent of the women with low-risk HPV had ASC-US, 1.6% had ASC-H, and 1.6% had HSIL *(Table 5)*.

Concerning to the multiple infections results *(Table 6)*, the normal cytologies were more frequent in the women with only one HPV type with 35%. Fifteen percent of the women with only one HPV type had ASC-US, 1.1% had ASC-H, 18.99% had LSIL, and 5.6% had HSIL. The women with more than one HPV type had more smears with lesions. About 26% of these samples showed the presence of ASC-US, nearly 2% had ASC-H, about 35% had LSIL, and around 5% had HSIL.

## Discussion

Several studies have confirmed the presence of HPV DNA in nearly 100% of invasive carcinomas of the cervical epithelium, leading to the widely accepted thesis that HPV infection is a “necessary cause, but not sufficient, for the development of cervical cancer” since virtually only a fraction of female carriers of the virus develops the disease [1–3]. It is estimated that about 75% of sexually active population is in contact with one or more HPV types in their lifetime. The majority of these infections is, however, eliminated by the immune system, and the patients do not develop symptoms [22].

Of the 994 women tested in this study, about 70% were negative for HPV and 28% were HPV positive. Compared with the literature, these values fall within the expected

**Table 6.** Cytological results of HPV-positive samples according to the type of risks and the presence of multiple infections in the 994 samples

| Cytological results | HPV positive samples with HPV of more than one type of risk | HPV positive samples with more than one HPV, independently of the type of risk | HPV positive samples with only one HPV, independently of the type of risk |
|---|---|---|---|
| Normal | 3 (8.33%) | 15 (14.42%) | 63 (35.20%) |
| *Candida* spp. | 0 | 0 | 2 (1.12%) |
| Inflammation | 6 (16.66%) | 15 (14.42%) | 33 (18.44%) |
| ICD | 0 | 0 | 3 (1.68%) |
| Atrophy | 0 | 1 (0.96%) | 1 (0.55%) |
| ASC-US | 11 (30.55%) | 24 (23.08%) | 27 (15.08%) |
| ASC-H | 1 (2.77%) | 2 (1.92%) | 2 (1.12%) |
| LSIL | 12 (33.33%) | 37 (35.58%) | 34 (18.99%) |
| HSIL | 1 (2.77%) | 8 (7.69%) | 10 (5.59%) |
| Squamous cell carcinoma | 0 | 0 | 1 (0.55%) |

range of HPV prevalence in the world population that varies between 6.1% and 35.5% [6, 23], but is higher than the Portuguese estimated prevalence, which is, according with the CLEOPATRE Portugal study, 19.4% [7]. From the positive samples for HPV, the vast majority (about 90%) had HPV considered high-risk/probable high-risk, which may be due to the fact that a large number of viruses are considered high-risk or to the fact that the technique used detect more high-risk HPV than low-risk HPV. As observed in other studies [5–7, 24–26], from the women infected with high-risk/probable high-risk HPV, the HPV16 was the most frequent virus, followed by HPV31, HPV51, and HPV56. Also, as shown previously [25], from the women infected with low-risk HPV, HPV42 was the most prevalent.

Half of the HPV-DNA negative samples presented normal cytology finding. There was also a large number of cases of cytological inflammation, which was classified as non-neoplastic finding [8], and it is quite natural that it appears on HPV negative samples, because an inflammation may be due to various factors including the presence of other infectious agents, the menstrual cycle at the time of harvest, or simply the lack of hygiene. The existence of cellular changes (non-neoplastic) associated with the presence of *Candida* spp. has also become evident, although to a lesser extent (2.6%). Even though there was no viral DNA detected in these samples, such cases should be kept under surveillance, since the presence of other organisms (e.g. *Candida* spp., *Gardnerella vaginalis*, *Trichomonas vaginalis*, or *Chlamydia trachomatis*) has been associated with HPV infection and they may act as co-factors [27, 28]. Cellular changes (non-neoplastic) associated with the use of the ICD or atrophy was also observed, but in small quantities. More than 8% showed ASC-US, and 0.14% had ASC-H. These two categories correspond to an inexact diagnosis, because, although it seems to be cytological changes suggestive of squamous intraepithelial lesion, these are qualitatively or quantitatively insufficient to make sure that in fact this is the correct diagnosis [29, 30]. However, the fact that this classification is not associated with the presence of HPV does not mean that it can be ignored. Previous studies had demonstrated that the chance of intraepithelial lesions appearing in subsequent tests is very high [8]. This doubt may be due to the existing lesions are still very weak. The negative detection of the viral DNA does not mean that the HPV is not present. In fact, the cause of these lesions can be a non-detected virus, since the technique does not detect all of the existing HPV and, moreover, can be also a failure in the extraction process, leading to insufficient DNA extraction. The same suppositions may also explain the observation of visible lesions consistent with LSIL or HSIL in 1.3% and 0.14% of cases, respectively, even when the detection of viral DNA is negative. These inconsistent cases must be evaluated considering the history of each individual that can help to clarify the diagnosis.

In the case of samples contaminated with viral HPV DNA, the number of normal cytological findings decreases considerably, but is still about 27%. This value opposes to the about 2% of cytological intraepithelial lesions detected in the samples HPV negative. This may be an indicator that the molecular technique is more reliable. There are, however, some reasons that can justify the difference, which makes it difficult to get a definitive conclusion. In some cases, women can have the viral infection but not develop cancer symptoms [1–3, 22]. In other cases these normal cytologies may correspond to the presence of low concentration of viruses that are detected at an early stage of the disease, before the lesions appear. So it is important to maintain surveillance in order to clarify whether the host is able to eliminate the virus or not [31]. Other possible reason for these values is the presence of false negatives in the cytological results that may be due to failures occurring during the observation, since this is a manual technique that may be affected by human error, which undermines the effectiveness of this method. Inflamma-

tion, *Candida* spp., ICD, or atrophy were detected, but in low quantities. The significant increase of the presence of ASC-US (17.9%), ASC-H (1.4%), LSIL (24.9%), and HSIL (6.3%) was notorious in women with HPV positive comparatively to the HPV negative. A case of carcinoma was also observed. These are the expected results, consistent with the proven relationship between HPV infection and this type of injury [1–3, 7]. The percentage of cases of ASC-US, LSIL, and ASC-H does not differ much among the samples contaminated with different degrees of risk. The amount of HSIL samples was higher in the samples with high-risk/probable high risk HPV infection, which is in agreement with what has been described previously [1, 2, 7].

The HPV DNA-positive women with more than one HPV type correspond to a higher percentage of LSIL and ASC-US cases than samples containing only one HPV type. This tendency was also observed in women that have more than one HPV of more than one type of risk. The presence of multiple HPV types and their relationship with a greater probability of causing disease or increasing the severity of symptoms has been poorly studied. Recent studies advocate the association between infections caused by multiple HPV types and risk of developing disease [4, 7, 9, 32–34].

The range of ages of infected women (19 to 67 years) proves that there is no age limit for the presence of HPV. Despite a higher incidence of HPV-positive cases in women with less than 40 years, these results corroborate the information that any woman presents a high risk of infection [2, 35].

The main conclusion of this study is that both methods are reliable, but not enough by itself, being advisable to use them together rather than separately. Thus, molecular techniques are not effective enough to replace the cytological screening techniques, but shall be used to complement them.

This study contributes to reinforce the idea that there is an urgent need for good screening programs, including the cytological screening techniques and the HPV-DNA detection, such as the PapilloCheck® Test Kit, to a better understanding of the evolution of HPV infection in the different epidemiological settings.

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