Quality of Sample in the Molecular Determination of Human Papillomavirus in Breast Tissue: Pre-Analytical and Analytical Error-Based Approach

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Abstract

Introduction: High-risk Human Papillomavirus (HR-HPV) can play an important role in the development of breast cancer. The investigations that attempt to clarify this purpose require quality in their results, which constitute the success of each study and the assurance of each result in both clinical practice and scientific research.

Objective: to evaluate the quality of breast tissue during the analysis of HR-HPV in samples of patients with breast cancer, in order to establish a quality control of pre-analytical and analytical processes in the laboratory of molecular biology useful for clinical diagnosis and for scientific research.

Materials and methods: Twenty-five samples of mammary carcinoma (12 surgical specimens and 13 biopsies) were included with prior informed consent at INEN in Peru, which had a diagnostic pathologic report with breast receptors (RE, RP and HER2) and were analyzed for HR-HPV with the My09/My11 system. The quality evaluation was performed during extraction and amplification (β-globin, positive control (HPV 16) and negative), and the sample quality analysis under CLSI guidelines MM13-A and CLSI MM06-A2.

Results: All samples were negative for HR-HPV and one had no β-globin amplification. An overall DNA concentration of ≤ 45 ng/μL was determined. Biopsies performed better than surgical specimens did (p=0.001). No association was found between samples with ≥ 1 positive breast marker with sample quality (p=0.588), or with molecular result (p=0.778).

Conclusion: Verification of breast tissue quality during the determination of HR-HPV in breast cancer showed poor quality, with low levels of DNA concentration and significant differences between sample types.

Introduction

Human papillomavirus (HPV) infection is the most common sexually transmitted infection worldwide [1]. The HPV infection is the necessary but not sufficient cause for the development of Cervical Cancer (CC), the main cause of female mortality and a public health problem in low-and-middle income countries [2,3]. There are more than 150 types of HPV, each with a particular tropism for specific anatomical sites, where High-Risk genotypes (HR-HPV), mainly genotype HPV 16 and HPV 18, cause ≥70% CC [4,5].

Recently several studies have cataloged that HPV-AR can play an important role in the development of breast cancer assuming a cosmopolitan distribution among communities [6,7]. In fact, this association has been identified in more than 17 countries in the five continents [6-11]. These findings and their future discoveries are of great scientific interest worldwide. The development of these investigations requires quality in their results, which constitute the success of each study and the assurance of each result both in clinical practice and in scientific research [12].

The molecular methods used for the identification of HPV in breast tissue and breast cancer were INNO-Lipa [9], the next Generation Sequencing, the Line Probe Assay reverse Hybridization system, In Situ Polymerase Chain Reaction (PCR), the Restriction Fragment Length Polymorphism [13-16], semi-nested PCR, and Real-Time PCR (RT-PCR) [6].

A prior all these evaluation methods depend on the pre-analytical phase. In this phase where the specimens are obtained or part of them, and they are stored, transported and processed analytically, the greatest number of technical errors have been reported [3,17]. Both the pre-analytical phase and the selection of the method of analysis are key factors in the detection of HPV due to final sensitivity and specificity of the methods [18].

In this sense, we aimed to verify the quality of breast tissue during the determination of the Human Papilloma Virus in breast cancer tissues. This objective was given in the framework of the establishment of a quality protocol for pre-analytical and analytical processes in the molecular biology laboratory useful for clinical diagnosis and for scientific research.

Materials and Methods

Samples

The selected samples were collected by biopsy and surgical specimens, with prior informed consent authorized by the ethics committee of the National Institute of Neoplastic Diseases (INEN) of Peru, as part of the objectives of the Cancer Research-Circle. The samples were collected in outpatient Oncology Care offices, which were referred to the Tumor Bank for storage in vials of 3ml at -60 °C in the freezing system until they were processed together. A standardized code for each sample was assigned.

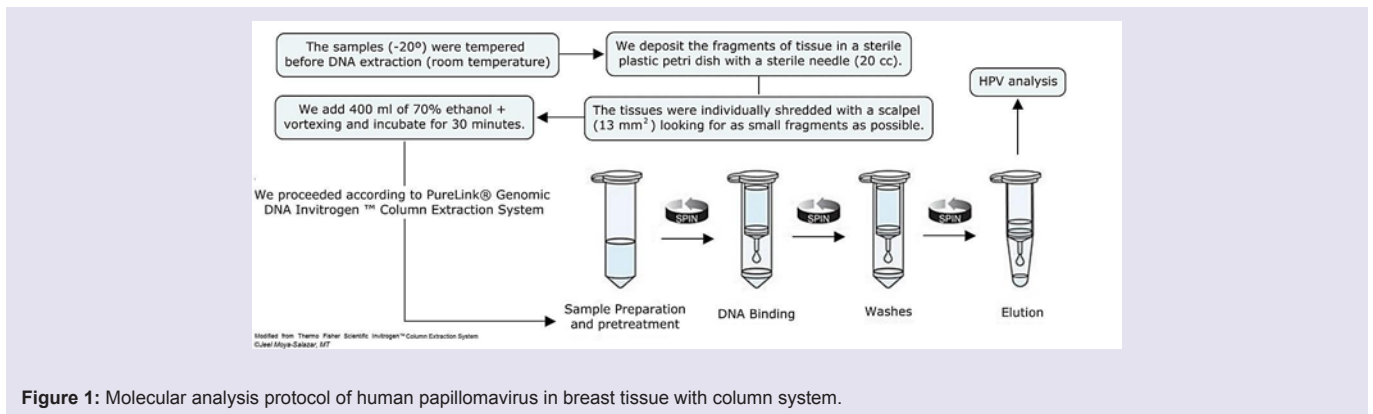The clinical records were collected to identify the main

Figure 1: Molecular analysis protocol of human papillomavirus in breast tissue with column system.

components of interest of the Cancer Research Circle:

- Date of receipt of sample
- Type of sample collected
- Patient's ID
- Pathological report
- Results of the previous Immunohistochemical.

Pathological report

Based on the clinical and pathological results, we have established the diagnosis of lobular, infiltrating, papillary breast carcinoma, etc. were established. We performed the evaluation of breast-hormonal recipients (Estrogen Receptor (ER), Progesterone Receptor (PR), and (human epidermal growth factor receptor 2 (C-Erb2 or HER2)) by Immunohistochemistry (Dako, Glostrup, Denmark) [19]. We included 25 samples of breast tissue for the evaluation of HPV infection and its immunological component.

Molecular test for HPV detection

All the vials containing breast tissues were transported from the Tumor Bank to the area of Molecular Biology of the "Maes Heller" Center. We used the PureLink® Genomic DNA Invitrogen™ Column Extraction System (Thermo Fisher Scientific, Carlsbad, CA) extraction kit [20,21], as described in Figure 1.

The DNA extracts were quantified with the Qubit™ fluorometer (Thermo Fisher Scientific, Carlsbad, CA) with a cut-off value of 70 ul of DNA. The HPV's DNA detection from breast tissue was performed with the HPV Consensus PCR and Genotyping system, which uses the My09/My11 primers that pair with the L1 region of the HPV genome (band 450 bp). In all analyzes (run of 5 samples) a pair of primers for the β-globin gene was included as an internal quality control.

The protocol indicated 2 cycles at 94 ˚C (30 sec), 3 cycles at 56 ˚C (35 sec), 4 cycles at 68 ˚C (35 sec), then 2 cycles at 94 ˚C (30 sec), 3 cycles at 56 ˚C (35 sec), and lastly 4 cycles at 68 ˚C (18 sec). Genotyping was performed with reverse line hybridization technique and chemiluminescence as described previously [21].

The amplified products were run by horizontal electrophoresis using agarose gel (3% at 112 V for 40 minutes) then colored with SYBR Safe (for 15 minutes) and visualized in the UV transilluminator.

Quality evaluation

In principle, we developed an amplification and extraction control process (primers for the human β-globin gene (GH20 (forward) - GAAGAGCCAAGGACAGGTAC, and corresponding PCO4 (reverse) -CAACTTCATCCACGTTC ACC) with a band of 268 bp). Moreover, a positive control (biopsy corresponding to HPV type 16 of 310 bp) and a blank as a negative control were included [21]. For the analysis of sample quality and storage, the evaluation parameters referred to in the CLSI guide MM13-A, MM06-A2, and the requirements for breast tissue from the American College of Pathologists were used [22-24].

Data analysis

The data analysis was performed with descriptive statistics, obtaining percentages, means and standard deviation values. We evaluated the associations between receptor negativity, type of sample, and type of breast carcinoma, and DNA quantification. In addition, we analyzed the variability between surgical specimen and biopsy related to the DNA quantification. The Pearson test was used considering a p value < 0.05 as statistically significant. The technique used for the statistical verification of the results was through the analyzer IBM SPSS v21.0 (Armonk, USA). This study received the approval of the INEN ethics committee as part of the objectives of the Cancer Research Circle (204-2015-FONDECYT).

Results

We analyzed 25 samples, of which 12 (48%) were surgical pieces, and 13 (52%) were biopsies with Core-Needle Aspiration (CNA). All these samples were extracted and analyzed in accordance with clinical requirements and pathological reports, all of which were negative for HPV infection.

The CNA had better performance than the surgical pieces (p = 0.001), obtaining 30% more nucleic acids (Table 1). We was determined an overall concentration of DNA ≤ 45 ng/ μL; DNA was extracted from 100% of samples.

Table 1 shows the main quality components of the study. We showed fragments of different sizes that depended on the tissue collection method. Twenty-eight percent (7 samples) of CNA and surgical pieces had therapeutic margins. We found an association between tissues obtained with therapeutic margins and a low amount

Table 1: Major baseline components evaluated during the analysis of Human Papillomavirus in breast tissue of patients diagnosed with breast carcinoma.

Type of sample¶	DNA concentration‡	Immunohistochemical analysis†
Surgical specimen (n=11)	30.1±4 ng/µL	4 (HER2-), 1(HER2-/ER-),2(ER-/PR-/HER2-)
Biopsy (CNA) (n=13)	42.3±3 ng/µL	6 (HER2-), 3(HER2-/PR-),1(ER-/PR-/HER2-)

The division includes the samples with histopathological diagnosis of invasive carcinoma of the breast, infiltrating carcinoma of breast subtype Nos / NST, infiltrating carcinoma of the breast, infiltrating mammary carcinoma subtype NOS, breast tissue with the presence of focal atypical ductal hyperplasia, breast parenchyma with focal changes fibriadenomatous, chronic granulomatous mastitis, and fibroepithelial lesions related to fibroadenoma with the presence of ductal hyperplasia.
‡ Average concentration of DNA quantified with the fluorometer Qubit™
† Positive results: ≥ 70% of cells with reactivity for the marker compared to the control.
Abbreviations: ER: Estrogen Receptor; PR: Progesterone Receptor; HER2: Human Epidermal Growth Factor Receptor 2; n: Number of samples.
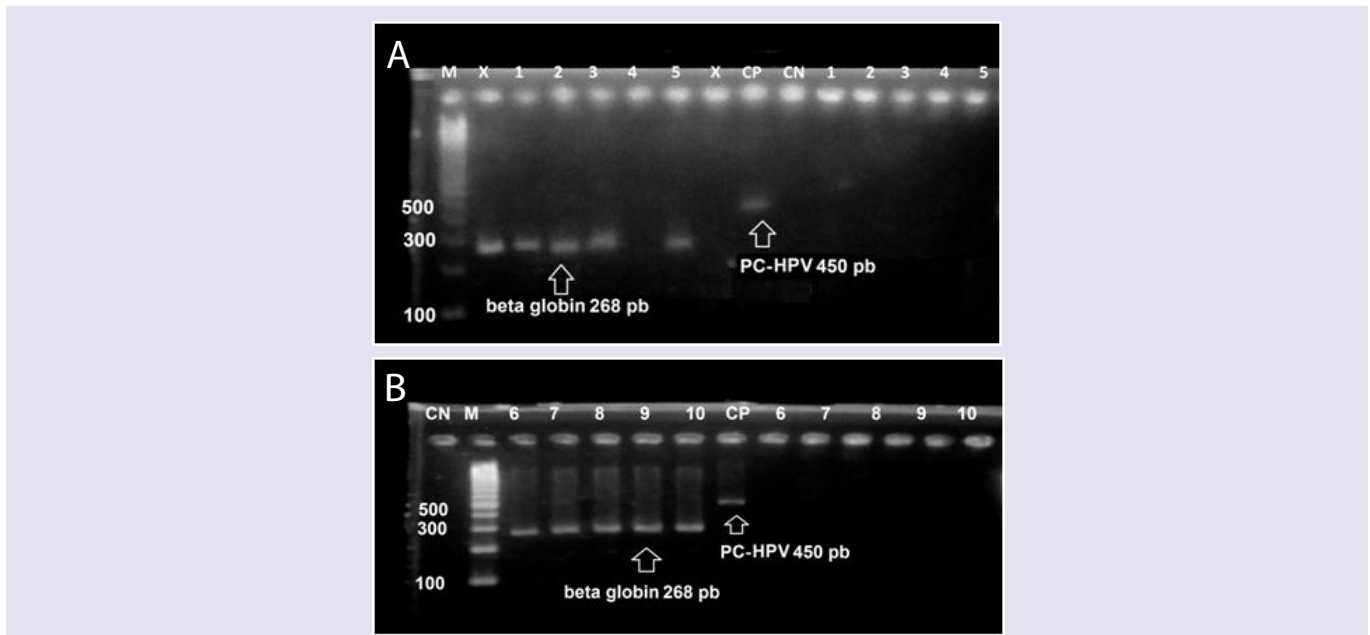

Figure 2: Results of the Polymerase Chain Reaction (PCR) for Human Papillomavirus (HPV). A. Evaluation of five patients (Nº4: surgical piece, diagnosis of infiltrative carcinoma of the breast, double negative (RE +, RP +) B. Evaluation of five mammary biopsies CP: Positive control, CN: Negative control M: leader X: Cervical biopsy sample (not included in the study) CP-HPV: HPV's consensus primer.

of DNA (rho=0.758, p=0.015).

All positive controls had amplification at the level of the 450 band, with the exception of sample ID-4 that did not show amplification of the control gene (β-globin) being excluded (Figure 2).

Four (16%) results were considered doubtful in their Immunohistochemical evaluation. In total, 3 triple-receptor-negative samples were reported, 4 (16%) did not present pathological evaluations and 3 (12%) presented dubious results for the immunohistochemical markers.

No association was found between the positivity of ≥1 breast-hormonal-receptor marker with the sample quality (p=0.588), nor with the molecular result (p=0.778). No significant differences were found between the types of samples (p = 0.779) but in the clinical-pathological diagnosis (p = 0.017).

Discussion

In this study we showed that all tissue samples from breast cancer were negative for HPV. We determined that a sample did not demonstrate DNA extraction and amplification quality with the β-globin control, that 44% samples had low DNA levels (≤ 34 ng/µL)

and that these characteristics were related to the type of sampling.

Molecular analysis in pathology is characterized by the dynamic change of technologies and markers, where the quality and quantity of nucleic acids are greatly affected by the type of collection, sample storage, manual processing and extraction method. There are real and very important differences between the analysis of HPV in cell brushes and breast tissue obtained by surgical procedures. In previous studies of cervical HPV-analysis, no great limitations have been found in the quantification acid nucleic [21], however, tissue and cellular evaluations depend on the number of altered cells present in the test sample, obtaining better results (reduction of false-negative results rates) with ≥ 200 cells per test sample [25].

The sampling is a critical process to ensure the integrity and accuracy of the quality of nucleic acids, since any inappropriate process results in the degradation of nucleic acids [22-24]. In the complexity of a high-performance pre-analytical phase, the validation and verification of the tests must be performed based on the requirement of a number of samples, which include the evaluation of the type and complexity of the test, the prevalence of the study's goal in the community, data analysis requirements, etc. [26].

The type of study sample (smear, Formalin-Fixed Paraffin-Embedded (FFPE) tissue), anatomical location and population distribution affect the analysis of nucleic acids, such as HPV DNA [27,28]. An inadequate DNA quality (degradation or fractionation) has been demonstrated in the analysis of HPV samples in tissues by PCR [29]. For breast tissue, the extraction methods are diverse (lupectomy, CNA, mastectomies, biopsies, surgical pieces with/without margins, re-excision pieces, sentinel lymph node, etc.). All of these must ensure the correct pathological diagnosis, the estimation of prognostic factors, and response to treatment. The type of sample depends mainly on the previous clinical considerations and on the protocols that are followed before their diagnostic analysis; this represents a key point for the viral genomic analysis in breast tissue [30].

In the Peruvian clinical practice, the CNA and the surgical specimens with margins are the most frequent samples. For that reason, we need the importance of the correct fixation in neutral buffered formalin that avoids the false negatives and allows the visualization of the histological grade, the evaluation of vascular invasion, and other cellular components. Hence, we propose as an interference role the ink used to delimit the surgical margins, further investigation is also required to determine how the dye/ink impacts the analysis of subsequent nucleic acids.

Consideration should be given to the tissue fixation solution used during sampling since these usually induce DNA degradation [31]. Although the tissues can be stored for ≤ 2 years at ≤ 70 ℃ in liquid nitrogen, as was done in this study, not all health centers have these storage tools, so it is recommended to send the tissue immediately to both the pathological and research laboratory.

Due to the institution where the study was conducted presents strict work protocols, we could not perform pre-treatments to the breast tissues analyzed. These pre-treatments eliminate the inherently large amount of endogenous nucleases in tumor tissues, and the bloody components prior to analytical processing, as well as improve storage and processing time controls after thawing [22].

During the last decade, mainly the tissue biobanks have elaborated protocols for the maintenance of the integrity of the tissues and the preservation of the nucleic acids and proteins. The possibility of error results may be due to the low concentration of viral DNA in clinical samples, mainly in tissues or also, in the presence of endogenous PCR inhibitors in the sample [32]. In this study, we determined average viral DNA concentrations below the cut-off. This could explain the final result of the molecular analysis (Table 1). Other errors include the use of inadequate/inefficient nucleic acid extraction methods.

The type of nucleic acid extraction system will depend on the objectives of the molecular analysis, since an extraction for molecular sequencing, will not be the same as for the qualitative diagnosis of nucleic acids. In this study, we used a column extraction system that has high performance, although its limitations in the extraction of DNA from FFPE tissues (column capture method) have been demonstrated [21,33]. According to quality recommendations, each molecular tissue processing must perform repetitions of positive and negative controls per run [34].

The quality assurance strategies during the nucleic acid extraction

and amplification of this study were the addition of the negative control (ultra-pure water) to ensure that there is no contamination during the analysis, and the amplification of β-globin in the same reaction for controlling adaptation of the sample. In each molecular evaluation, it is necessary to use reference genes (beta-actin, HLA, GAPDH, β-globin, HPRT1, among others) that generate validation data and stability of the analysis together for each experimental milieu. As all these have a certain degree of variation, the validation of the selected gene for each molecular process is necessary.

Besides for the purpose of eliminating errors in the quantification of nucleic acids and avoiding variations in the efficiency of RT-PCR derived in the MIQE guidelines, which currently allows the pertinent selection of endogenous controls for these analyze, we also consider that during conventional PCR this selection is vital and necessary [35]. There is software (geNorm, RefGenes, Genevestigator, etc.), spreadsheets (Bestkeeper, REST, etc.), among other tools that allow carrying out statistical evaluations of a panel of reference genes for the process of validation and standardization of the protocols with the samples together [36].

Although, the validation can be an embarrassing process, it will allow obtaining guarantor quality results that imply avoiding repetitions, because working with results from erroneously analyzed data (due to poor selection of housekeeping genes) can be more expensive in the long term. For example, conventional analysis of β-globin are a determining factor for the inclusion of participants in studies of HPV prevalence [37,38]. We propose that reference guides should be established (such as the MIQE guide) and the use of software for conventional PCR analysis (mainly Open Source).

In addition to the previous pre-analytical considerations for the detection of viral DNA, the selection and adequate use of analysis methods are required. The ability of the methods to amplify different sizes and types of DNA fragments of specific genotypes of HPV is one of the main limitations of molecular tests. For example, when HPV DNA detection was compared by PCR it was shown that the MY09/MY11 method (15% detection) had less detection than the GP5+/GP6+ method (L1 region amplification) [39].

Zhebe and Wilander demonstrated that both methods have a similar sensitivity in the detection of HPV in cervical biopsies, but point out that MY09/MY11 presents less performance (≤ 3%), like the results of Remmerbach et al., in oral mucosal preparations [40,41]. In part this could explain our negative results. However, to demonstrate the efficiency of the method, external quality control and verification of the protocols used is required [22,23].

The main goal of the study was not the comparative analysis of PCR techniques with My09/My11 primers against other methods (RT-PCR, in situ PCR, GP5+/GP6+method, etc.) for the detection of HPV in breast tissue, thus, we cannot explain the performance of the test. In general, if the detection method has limitations, analytical errors. Analytical errors (false results) of detection will be generated that will affect the quality of the study, and the clinical management of the patients with negative results. These limitations must be considered to ensure high-quality results that allow their correct interpretation.

Recently, the findings of HPV in breast tissue have been validated

with the simultaneous analysis of ≥ 3 molecular methods [6,42-44]. This should have been considered in the current Peruvian report on HPV in breast tissue to ensure the quality of its findings [45].

To evaluate all these quality components, there are international regulatory organizations that provide a basic system for the evaluation of clinical laboratories. However, molecular tests are not specified under the general requirements of the CLIA'88 guide where the quality ranges for each procedure are established.

The quality requirements establish a channel between the practical specific quality requirements of the main organisms (CLIA, FDA, CMS, etc.) with the accuracy in the diagnostic tests. But to establish these requirements are needed, among other things, acceptable types of samples for analysis. About breast tissue and HPV viral DNA standard guidelines are not yet established. Each laboratory should establish its own policies and procedures for the method under evaluation, in coordination with accredited agencies [46].

Finally, the null frequency of HPV reported in this study may also be due to the limited etiological distribution of the virus as the cause of this neoplasm, and the inclusion of patients without previous reports of cervical lesion or HR-HPV infection [6,42,43,47]. Although, several international groups have demonstrated the presence of HPV in different non-epithelial or mucosal organs [6-11,13-15,18,48-50], and their possible genetic role for the development of breast cancer [7,51], a rigorous causal-association has not yet been found that supports its clinical evaluation and explains its mechanism of breast infection [36,52].

More studies are required to evaluate the oncogenic role of HPV in breast tissue, explaining its possible non-sexual dissemination and the tropism of viral subtypes. Likewise, if a diligent association is established between HPV and breast carcinoma, its routine diagnostic application and its importance for health should be argued, because if HPV analysis for CC has not yet been seriously applied in Peru, we believe that its application to be far away for breast cancer.

Conclusion

Verification of breast tissue quality during the analysis of HPV in breast cancer showed poor quality, with low levels of DNA concentration and significant differences between sample types.

This same quality system should be evaluated in a greater number of samples and confronted with other DNA extraction systems, since there are technologies in development that allow improving the performance of the studies. All these elements are significant for the development of the current molecular pathology in both diagnosis and research.

We consider that with each quality assessment activity, the analysis procedures will be enhanced through various activities of continuous improvement, looking for quality to be assured in each phase based on the protocols organized to establish a quality management of pre-analytical and analytical processes in the laboratory of molecular biology useful for clinical diagnosis and for scientific research.

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