Environmental Performance of Xylene, Hydrochloric Acid and Ammonia Solution During Pap Stain for Diagnosing Cervical Cancer

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Introduction

In countries with a low degree of development, cervical cancer is a very frequent neoplasia, with a high number of deaths in women during a time in their lives when they play an important role in the family.\textsuperscript{1,2,3} Cervical cancer is also a reflection of inequality and poverty, as it affects poor and rich countries differently. At least 80% of deaths caused by cervical cancer occur in countries with a low degree of development.\textsuperscript{3,4} Peru evidences a similar inequality, with many patients with cervical abnormalities in very poor populations, mainly with HSIL (high squamous intraepithelial lesion) and carcinomas, due to low levels of healthcare and social support, and as a consequence, there are high levels of mortality for this disease.\textsuperscript{1,5} The most commonly used tests in cervical cancer diagnosis include the Papanicolaou (Pap) test and cervicoscopy, along with testing and genotyping for HPV, a common sexually transmitted infection and causal agent of cervical cancer.\textsuperscript{6,7,8}

Background. Little importance has been placed on sustainability of the Papanicolaou (Pap) stain, the gold standard for the diagnosis of cervical cancer, for global environmental health. The standard Pap stain uses environmentally toxic and carcinogenic reactants such as xylene, hydrochloric acid and ammonia solution.

Objectives. To eradicate the use of environmentally toxic and carcinogenic reactants through the validation of the Ecologic Papanicolaou (Eco-Pap) test.

Methods. Reagent handling strategies were divided in three phases: used Harris’ progressive hematoxilin, polychromatic solution and direct mounting that were analyzed by PEED-Cytology, Staining Quality Index (ICT) and the Bethesda system 2014.

Results. A total of 52,319 Pap smears stained with Eco-Pap were admitted (ICT=0.91). Validation of the Eco-Pap versus conventional staining was optimal (Kappa =0.89) and the sensitivity and specificity of the method were 57% and 98%, respectively. Eco-Pap reduced the environmental contamination produced by xylene (66 liters), hydrochloric acid and ammonia (5.5 liters each) over nine months, and all diagnoses coincided with the cytological details.

Conclusions. The Eco-Pap is an innovative method that transforms Pap testing into a sustainable and supportable technology.

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Ecological-Papanicolaou (Eco-Pap). This method:

1. Reduces and eliminates the use of environmentally toxic reagents (xylene, hydrochloric acid and ammonia),
2. Reduces operator occupational risk,
3. Decreases the time and economic costs, and
4. Is an environmentally sensitive method for cervical cancer diagnosis.

The research objective was to compare this Eco-Pap method to the conventional Pap stain method in terms of yield diagnosis, as well as operational parameters, including time and cost.

Methods

An experimental, prospective, cross-sectional cytology study was performed in the pathological anatomy service of the diagnosis assistance department of the Hospital Nacional Docente Madre Niño “San Bartolomé” (HONADOMANI SB) in Lima, Peru. Sample size was calculated using EPIDAT 4.1 (Xunta de Galicia, España), considering a sensitivity of 0.95, a heterogeneity of 50% and a margin of error of 0.04, and obtaining a sample size of 2,500 cervical smears.

Only cervical smears that met the requirements for technique competence and normalized procedures in the manual of the Health National Institute of Peru, standard quality control measures according the Bethesda System 2014, and standardize operational procedures for each test were considered. Samples were prepared in a conventional preparation and sent to the laboratory for cervical-uterine screening, among five health networks and micro-networks in five districts (Los Olivos, Rímac, San Martín de Porres, Lima and Tupac Amaru).

Analytical Procedure

The method used for screening the cervical smears was ecological modification of the Pap test, as described in Table 1.

| Hydration          | Tap water          | 10 dips | QC Microscopial  |
|--------------------|--------------------|---------|------------------|
| Harri’s progressive|                    |         |                  |
| Hematoxilyn        |                    | 1 to 3 minutes* |                |
| Nuclear Staining   | Tap water          | 10 dips |                  |
| Alcohol 96%        | 1 minute           |         |                  |
| Cytoplasmic Staining| Polychromatic solution | 30 secs to 1 minute* | QC Microscopial |
| Alcohol 96%        | 1 minute           |         |                  |
| Clearing and Mount | Absolute alcohol   | 1 minute |                  |
| Entellan or New Entellan | —            |         | QC Microscopial  |

*Time according to preparation and use of reagents based on QC

Table 1—Protocol for Pap Stain Ecological Modification

Abbreviations

- **Eco-pap**: Ecologic Papanicolaou
- **HONADOMANI SB**: Hospital Nacional Docente Madre Niño “San Bartolomé”
- **HSIL**: High-grade squamous intraepithelial lesion
- **SCC**: Squamous cell carcinoma
- **LSIL**: Low-grade squamous intraepithelial lesion
- **Pap**: Papanicolaou
- **AIS**: Adecarcinoma in situ
- **ASCUS**: Atypical squamous cells of undetermined significance
- **CIN**: Cervical intraepithelial neoplasia
- **CIS**: Carcinoma in situ
- **NLIM**: Negative for intraepithelial lesion or malignancy
Control strategies

Reagent handling strategies were divided into three phases. The first was performed during nuclear staining, where in addition to the use of Harris’s progressive hematoxylin, which eliminates the use of hydrochloric acid and ammonia by staining only the nucleus (progressive hematoxylin formulation: lower concentration of hemalum: 2–4 gm/L, glacial acetic acid: 20 mL, aluminum sulfate: 18.3 gm, among others), a control system was developed that eliminates mercury oxide, the oxidant component of hematoxylin that causes water contamination in cytopathology laboratories. The process consisted of filtering the hematoxylin with a membrane filter grid, GN-6 Metricel® 0.45 μm, 47 mm (PALL, NY, USA), before its final use, then pulling away the filtered fraction to gauge the preparation for the new reagent, achieving a complete recycling of the reagent. The process of filtering is performed 10 times. In the last step, the reagent is stored inside a 2 L dark container for a year, and during this time it can be reused for staining, due to the slow maturation of hematoxylin.

The second phase consists of a single alcohol bath, followed by polychromatic staining that is a combination of conventional EA-36, preferentially with conventional Orange G-6. This reduces the number of alcohol baths used in both steps for cleaning excess stain.

Lastly, in the clearing phase, two or three baths in xylene were eliminated, leading directly to mounting in anhydrous resin Entellan® or new Entellan® (Merck, Darmstadt, Germany), which contain fewer toxic substances and in lower amounts in parts-per million (ppm) –100 ppm average over an eight-hour workday and retain the same function for obtaining optimal microscopic visibility under the same optimal conditions as gold standard staining, as demonstrated photographically.26-25

Validation Study

The method was subject to a validation study before its application to determine staining quality in comparison to the conventional gold standard Pap testing. This validation was evaluated following the manual of technique competence and proceedings of the National Institute of Health of Peru and the inter-observational results showed that the refraction index and cellular transparency were optimal.26-30 All cytotechnologists and cytopathologists were evaluated and certified by the National Institute of Health of Peru and had an average level of diagnosis agreement of kappa = 0.81. All had over 12 years of experience in cytopathology.26

Quality control and statistical analysis

The quality of the smears was evaluated based on the staining quality index (SQI) and the Bethesda 2014 guide for micro and macroscopic quality control. The data analysis was performed with SPSS version 20.0 (IBM, Armonk, USA) using inferential statistics. Non-parametric tests included Cohen's kappa, and diagnostic tests and ROC curve were evaluated during the validation phase. The evaluation method was performed with 3,906 smears evaluated with both methods. All slides were analyzed by seven cytotechnologists and five cytopathologists.

Ethics

This research was approved by the Ethics and Research Committee of HONADOMANI SB.

Results

A total of 52,319 Pap tests stained with Eco-Pap were included in the present study. The global SQI was 0.91, with valuations for chromatic pattern (SQI=0.90), cytoplasmic morphology, background parameters and bacterial flora (SQI=0.89). Total reagent volumes were 66 L of xylene, and 5.5 L each of HCl and NH₃, with US $11,505 in costs saved.
Method Validation

Double-blind experiment validation of Eco-Pap versus conventional staining was performed in 5% of cervical smears 30 days before the beginning of implementation in HONADOMANI SB (December 2014), obtaining a Kappa correlation index of 0.89 between methods. The cytological validation results are shown in Figure 1 and 2.

Evaluation of contaminant diminution rates

The processes for reducing institutional contamination were evaluated quarterly for each eliminated toxic or carcinogenic reagent. Additionally, the quarterly savings was estimated for each reagent (Figures 3 and 4). For the 52,319 Pap tests, Eco-Pap used 66 fewer L of xylene, 5.5 fewer L of HCl, and 5.5 fewer L of NH₃ compared to the conventional Pap testing method (Figures 5 and 6). Thus, Eco-Pap significantly reduced the biocontamination hazard generated by use of toxic or carcinogenic reagents, reduced the bioaccumulation of xylene (resulting from accumulation of used xylene bottles in the cytology area) and proved to be an environmentally protective method.
Cytological valuation

The cytological staining was homogenous, and the refraction index and cellular transparency were optimal for the tridimensional cellular disposition. The sensitivity and specificity of the method was 57% and 98%, respectively.

The cellular details for diagnosis of normal and neoplastic cytology coincided with the specific details of each lesion. The characteristics for atypical squamous cells of undetermined significance (ASCUS), low grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), cervical intraepithelial neoplasia, squamous cell carcinoma and adenocarcinoma in situ were clearly appreciable (hyperchromasia, altered nucleus:cytoplasm (N:C) ratio, nuclear irregularities, etc.) (Figures 7, 8, 9, 14).

The cytological characteristics for infective/inflammatory processes (suggestive of changes due to infection by HPV, Gardnerella vaginalis, Trichomonas vaginalis, Herpes virus, etc.) reparative cytology and non-neoplastic findings maintained the distinctive characteristics for each process (Figures 10, 11, 12, 13).

Figure 7—Squamous cell carcinoma (SCC) (20x)
Figure 8—Low–grade squamous intraepithelial lesion, cannot exclude HSIL (LSIL-H) (40x)
Figure 9—Adenocarcinoma in situ (AIS) (40x)
Figure 10—Reactive endocervical cells (40x)
Figure 11—Shift in flora suggestive of bacterial vaginosis (40x)
Figure 12—Cellular changes consistent with herpes simplex virus (40x)
Figure 13—Cells with diagnostic koilocytic (HPV) features of low–grade squamous intraepithelial lesion have a sharply defined perinuclear cavity, condensation of cytoplasm around the periphery, and abnormal nuclear features including enlargement and nuclear membrane irregularity (40x)
Figure 14—Atrophy (20x)
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Discussion

Eco-Pap reduced the environmental contamination by xylene, hydrochloric acid and ammonia, achieving a reduction (prevention) of 66 L of xylene, and 5.5 L each HCl and NH₃ over three quarters, compared to what is conventionally used during Pap testing (Figure 3), and at the same time, allowed for the diagnosis of cervical malignancies (LSIL, HSIL, carcinoma in situ, adenocarcinoma in situ, etc.) (Figures 7-14), leading to environmental, social and health benefits for the community. In addition, the Eco-pap method had a total quarterly cost savings of US $11,505.00.

Eco-Pap uses progressive hematoxylin, polychromic solution and direct mounting, which eliminates and reduces the use of environmentally toxic and carcinogenic reagents, reducing the impact of occupational exposure, given that adequate protection materials are not routinely used in cytology laboratories in Peru. Inhalation of xylene can cause irritation in the mucous membranes of the nose and throat, and in high concentrations can produce nausea, vomiting, headache, serious respiratory difficulties, cough, heart anomalies, proteinuria and hematuria after excessive inhalation. It can induce neurological disorders, and xylene and hydrochloric acid are associated with the development of cancer, leukemia and lymphocytic brain tumors in cases of chronic exposure to high concentrations over long periods of time. Similarly, hydrochloric acid and ammonia produce intoxication and environmental problems due to the pollution they produce. Prolonged exposure to both reagents, both by direct contact and inhalation, can be damaging to the health of people exposed to them. Manifestations of intoxication by these reagents are heterogeneous, but can affect the respiratory system (cough, dyspnea, wheezing, bronchopulmonary problems), the nervous system (stupor, confusion, lack of coordination, difficulty in walking), skin and mucous membranes (burns, glottis edema, sight loss, temporary blindness), and the circulatory system (weak and rapid pulse, fever, decrease in blood pressure), among others.

With regard to protective materials, cytology laboratories have little information in the form of safety data sheets and other chemical-specific information, in addition to poor controls. The Occupational Health Manual of the Ministry of Health of Peru outlines the minimum personnel, environmental protection requirements and adequate preventive measures for laboratories at each level of the healthcare system. However, only 10% of laboratories have fume hoods and regulations for their assessment, and the vast majority are in breach of compliance with regulatory standards in all phases of the diagnostic process. HONADOMANI SB does not have fume hoods for handling aromatic polycyclic hydrocarbons or for the preparation of toxic reagents. The necessary protective equipment such as gloves, scrubs and masks are also of low quality (latex gloves, cotton scrubs and simple dust masks). It is under these conditions that the process of cytological diagnosis takes place, and there are no work flowcharts to ensure a working environment free of toxicity. In fact, in the cytology manual of the National Institute of Peru, there is no mention of correct processes for the use, treatment, bioaccumulation or elimination of xylene in cytology laboratories. Unfortunately, Peru has no requirements for proper handling and disposal of laboratory wastewater containing HCl and NH₃. This study suggests the need for regional and global organizations to design manuals for better management of toxic and carcinogenic reagents in cytology laboratories.

Eco-Pap reduces the use of toxic and carcinogenic reactants and lowers acquisition costs and handling of these reactants, costs that could be distributed to other areas of institutions or to new diagnostic methods in cytology such as HPV genotyping. Furthermore, reducing the staining steps enables a faster diagnostic response (it generally takes from four to seven weeks with a loss of patients between 40 and 60%) for the treatment and monitoring of cervical cancer. The cost per Pap test could also be reduced, making it more accessible to populations with few resources in marginal urban areas that are generally far from healthcare centers. Moreover, this method makes it possible to maintain costs in periods of economic shifts, inflation and international dollarization (Figure 4).

Eco-Pap yields results that are comparable to the gold standard staining method (kappa=0.89) and has a more favorable cost-benefit ratio than commercial modifications of staining, allowing for diagnosis of cervical cancer at a lower economic cost. Moreover, if every cytology laboratory in the world ceased using xylene during clearance in Pap staining, the environmental impact caused by industrial production of xylene would be greatly reduced.

In this study, 66 fewer liters of xylenes were used with the Eco-Pap method. A great amount of pollution could be prevented if all hospitals in the world that use this solvent would decrease its use, and the production of xylene would also be decreased. Xylene is obtained through catalytic
cracking of petroleum and is highly polluting to the environment in its production. We can reduce its use by 90%, obtaining the same results in the diagnosis of cervical cancer. Therefore, we urge regional and global health organizations such as the World Health Organization (WHO) and Pan American Health Organization (PAHO) to demonstrate the benefits of the Eco-Pap method.

The majority of cytological screening is performed with the Pap test due to its low cost, accessibility and ease of use. The wide applicability and development of conventional cytology using the Papanicolaou method has increased the amount of cytological tests performed every year. For example, 3,276,045 cytological tests were performed by the Instituto Mexicano del Seguro Social during 2012, and 118,016 cytological tests were performed by the Hospital Nacional Docente Mare-Niño "San Bartolomé" from 2013-2014. This testing method requires adequate environmental management to ensure the decontamination and proper management of dangerous waste and to prevent serious health problems among the cytology staff, patients and the environment.

The Eco-Pap is a sustainable and supportable method that allows for the effective diagnosis of cervical cancer, and we propose that the name 'Eco-Pap' may be attractive to clinicians and patients.

Conclusions

Cervical cancer is an important public health issue and is associated with high morbidity and mortality in underdeveloped countries. It is primarily diagnosed by the use of the Papanicolaou test which employs highly polluting toxic and carcinogenic reagents (xylene, hydrochloric acid and ammonia), generating a harmful work environment depending on the duration of exposure.

In spite of the advent of commercial modifications of conventional Papanicolaou testing, current methods have a low cost-benefit ratio and are unable to be routinely used in the diagnosis systems of the healthcare centers in Lima and Peru. Our proposed Eco-Pap method eliminates the use of environmentally toxic and carcinogenic reagents, reduces the risks due to occupational exposure and has a better cost-benefit ratio for patients.

The Eco-Pap is an innovative, sustainable and supportable technology.

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