Title: Benefit of double reading cytology smears as a triage strategy among high-risk human papillomavirus (HPV) positive women in Mexico

Running Title: cytology smears as triage for high-risk HPV

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Author Contributions
ELP and JS obtained funding; ELP, JC, and JS developed the study design; ELP, LTI, and JS coordinated the study, BRP, DAE, IRMP, JML, LLM, LTI, RHL, PMH, and PRP collected and managed the study data; EC, FMC, and HF reviewed the cytology slides; AC, BRP, CMC, DAE, LLM, LTI, PRP, and RHL conducted statistical analyses; AC, BRP, CMC, DAE, EC, FMC, HF, JC, JR, JS, LLM, LTI, PRP, RHL, and YNF, interpreted the study results; AC, CMC, DAE, and YNF wrote and revised the manuscript based on the co-authors suggestions.

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Precis
Over 36,212 FRIDA Study participants provided two cervical samples during the same clinic visit to test for high-risk human papillomavirus (hrHPV), and liquid-based cytology (LBC) to triage the women with a hrHPV positive result. The detection of histologically confirmed cases of cervical high-grade squamous intraepithelial lesions or worse (HSIL+) was increased nearly 21% by having each LBC slide read by two cytotechnologists as part of the routine work of the cytological laboratory.

Abstract

Background: To determine if the detection of histologically confirmed cases of cervical high-grade squamous intraepithelial lesions or worse (HSIL+) can be increased by having each liquid-based cytology (LBC) slide read by two cytotechnologists as part of the routine work of the cytological laboratory.

Methods: Over 36,212 women aged 30 to 64 years participated in the FRIDA Study in Tlaxcala, Mexico, between 2013 and 2016. For each participant, two cervical samples were collected at the same clinic visit to test for high-risk human papillomavirus (hrHPV), and LBC to triage those with a hrHPV positive result. LBC slides were distributed among seven cytotechnologists, with each slide read independently by two blinded cytotechnologists. All women with an atypical cells of undetermined significance (ASCUS+) or worse result were referred to colposcopy for further evaluation and diagnosis. Three pathologists evaluated the biopsy specimens to confirm the final HSIL+ diagnosis. The HSIL+ detection rates for the single reading versus double reading were estimated and compared.

Results: A total of 3,914 women with a positive hrHPV test result were followed up with LBC. The first and second cytology readings resulted in 43 HSIL+ cases detected. The double reading strategy detected 9 additional HSIL+ cases, resulting in a total of 52 HSIL+ cases. The HSIL+ detection rate increased from 10.99/1,000 with a single reading to 13.29/1,000 with a double reading strategy (p-value=0.004).

Conclusions: A 20.9% increase in HSIL+ cases detected was achieved with a double reading of the LBC slides in this sample of hrHPV positive women.

Keywords: cervical cancer, double reading, high-risk HPV, liquid-based cytology, screening,

Mexico

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Introduction

Globally, cervical cancer is the fourth most common cancer among women, and the second most frequent cancer in less developed regions.[1] In 2018, there were an estimated 570,000 new cases of cervical cancer, representing 6.6% of all female cancers worldwide.[2] Approximately 90% of cervical cancer deaths occur in low- and middle-income countries,[2] where mortality from this disease can be up to 18 times higher than in high-income countries.[3] In Mexico, cervical cancer is the second leading cause of death due to cancer and the second most common cancer among all women, but first among women aged 15 to 44 years.[4]

Organized screening programs began using the cytology-based Papanicolaou (Pap) test during the mid-20th Century. Despite lack of evidence from randomized control trials, an abundance of observational data has proven the effectiveness of organized cytology screening in reducing the incidence of, and mortality from, cervical cancer in developed countries.[5-8] However, the performance of cervical cytology-based screening programs in many less-developed countries is suboptimal.[9-11] For instance, although a nationwide cytology-based cervical cancer screening program (CCSP) was implemented in Mexico in 1974, the resulting decreases in incidence and mortality have been modest [12]. In 2012, there were 13,960 new cervical cancer cases diagnosed and 4,769 cervical cancer-related deaths.[4]

Some explanations for the unsatisfactory performance of the CCSPs in Mexico and other less developed countries include low coverage rates, poor quality of the cervical specimen obtained, inadequate training of cytotechnologists, and lack of quality control assurance.[8,9] Sampling errors can occur when clinicians fail to effectively collect and transfer abnormal cervical cells onto the Pap slide, whereas detection errors often result from missing abnormal cells on the Pap slide or misinterpretation. Both sampling and diagnostic errors, along with the

lack of quality assurance mechanisms, contribute to the relatively low sensitivity of the Pap test.[13] Several studies in Mexico have reported that the sensitivity of the Pap test to detect histologically confirmed cases of cervical high-grade squamous intraepithelial lesions (HSIL) or cancer ranges from 40-59%, with corresponding specificities ranging from 87-98%.[14-16]

To increase diagnostic accuracy, organized CCSPs often incorporate various quality control mechanisms. As a standard measure in Mexico, a cytopathologist routinely reexamines abnormal cervical slides to report a final interpretation, in addition to randomly re-screening 10% of samples reported as normal by cytotechnologists. However, this approach has been criticized for its ineffectiveness in reducing the rates of false negatives due to the small number of normal results that are reviewed.[17] Other rescreening alternatives such as rapid review and rapid prescreening have also evolved. Rapid screening involves a quick review of all the negative cervical specimens, while in rapid prescreening all samples undergo a quick prescreening prior to routine screening. Studies have found that both strategies are more effective quality control measures than the 10% rescreening strategy.[18-22]

Another alternative to increase diagnostic accuracy could be to introduce an additional blind reading of all cytology smears. This strategy has never been examined in an organized CCSP, since reading a large number of cytology slides twice could be inefficient in terms of time, labor, and costs. However, with the increased interest in human papillomavirus (HPV) DNA testing as a primary screening method, and the use of liquid-based cytology (LBC) as a triage procedure to refer high-risk HPV (hrHPV) positive women to colposcopy,[23-25] it could be feasible to incorporate a double cytology reading without introducing a heavier workload. The purpose of this study was to evaluate if the detection of histologically confirmed cases of HSIL or worse could be increased by having each LBC slide read by two cytotechnologists. This

research project was conducted as part of the Forwarding Research for Improved Detection and Access for Cervical Cancer Screening and Triage (FRIDA) Study,[26] which is evaluating different triage alternatives for hrHPV positive women to determine the best practices for cervical cancer detection in Mexico.

Methods

Study population and screening procedures

This study was part of the larger FRIDA Study, which recruited 36,212 women who underwent primary HPV screening using the Cobas4800 test and LBC triage for those with a hrHPV positive result, in the state of Tlaxcala, Mexico from 2013 to 2016. The study design, methods, and baseline characteristics of the participants are reported elsewhere.[26,27] Briefly, the FRIDA Study was conducted within the Sanitary Jurisdiction No. 1, which includes 32 of Tlaxcala's 60 municipalities and involves 100 primary health care centers that provide cervical cancer screening services. Trained health care providers enrolled women aged 30 to 64 years. Women who were pregnant at the time of recruitment, had a hysterectomy, or were unable to provide informed consent were excluded. The FRIDA Study protocol and procedures were approved by the Institutional Review Boards (IRBs) of the participating institutions, which included the National Institute of Public Health of Mexico, the Tlaxcala State Ministry of Health, and the Mexican Institute of Social Security, as well as the Mexican regulatory agency COFEPRIS. The FRIDA Study is registered in ClinicalTrials.gov (NCT02510027).

Sample preparation and cytology interpretation

During a single clinic visit, trained nurses or physicians performed a pelvic exam and collected two separate cervical samples using a Cervex-Brush (Rovers) for each participant. The

first cervical sample was collected and placed in a SurePath vial for the LBC test and the second sample was stored in a ThinPrep vial for hrHPV DNA testing. The LBC samples were only processed and evaluated to triage the hrHPV positive results. The LBC slides were processed at the Central Cytology Laboratory of Tlaxcala using a semi-automated BD PrepStain™ Slide Processor. Slides were distributed among seven cytotechnologists and each slide was evaluated twice by two different cytotechnologists, blind to each other's interpretation. If there was a discordance between two readings, a cytopathologist reexamined the slide to determine the final result. As a quality control measure required by the Mexican CCSP, the cytopathologist reviewed all cases of atypical cells of undetermined significance or worse (ASCUS+) reported by the cytotechnologists to determine the final result. Additionally, 8% of negatives were also reviewed by the cytopathologist. The additional ASCUS+ cases detected from this subset of negative results were not considered in this analysis of the utility of double reading LBC slides. The cytology interpretation was based on the Bethesda 2001 criteria.[28]

Colposcopy and histological confirmation

All women with a positive hrHPV result and an ASCUS+ LBC result were referred to colposcopy for further evaluation and treatment if needed. The colposcopists were informed of the triage results and medical history of the referred patients. The colposcopy findings were reported according to the 2011 International Federation of Cervical Pathology and Colposcopy guidelines.[29] Before biopsy collection, endocervical samples were obtained for all women using an Endocervex Brush and treated as one biopsy sample. Biopsies were systematically collected, one from each quadrant from the most abnormal, acetowhite areas of the squamocolumnar junction for each patient and placed in one paraffin block.

Histological interpretation was based on Mexico's CCSP's criteria.[30] Participants were classified as normal if they were negative for intraepithelial neoplasia, had acute or chronic cervicitis, or low-grade squamous intraepithelial lesions (LSIL). Participants with a histopathology diagnosis of high-grade intraepithelial lesion, cancer in situ, or invasive cervical cancer were classified as HSIL+.[30] A final diagnosis was determined if two pathologists agreed on the result. In case of a disagreement, a third pathologist conducted an additional evaluation.

Statistical analysis

We conducted descriptive analyses to determine demographic and reproductive characteristics, as well as the prevalence of ASCUS+ among the hrHPV positive participants. Our analyses were restricted to participants with a double reading of their LBC results. Kappa statistics were computed to evaluate pairwise inter-observer agreement among the seven cytotechnologists who performed the first and second single reading of all LBC slides (n=3,914). A weighted average of the kappa values was calculated using the percentage of cytology samples read by each pair of cytotechnologists as weights.[31] We also determined a kappa statistic and the percent agreement between the first and second single readings of the discordant or ASCUS+ diagnoses (n=648). The combined results of the two readings by cytotechnologists were classified in two mutually exclusive scenarios: (1) ASCUS+ concordant (an ASCUS+ result on both the first and second reading) and (2) discordant. Cytopathology and histology results were obtained for each scenario, and the total number of HSIL+ cases detected was determined from the ASCUS+ women diagnosed by the cytopathologists who attended colposcopy.

The HSIL+ detection rate was calculated by dividing the total number of HSIL+ cases that were histologically confirmed by the number of hrHPV positive women who attended colposcopy. Approximate 95% confidence intervals (CI) for the detection rates were calculated

using the Agresti method.[32] McNemar's test for correlated proportions was used to compare the HSIL+ detection rate in a single reading to that in a double reading, taking into account the matched-pair study design. An additional analysis was conducted to estimate the number of colposcopies needed to detect one HSIL+ case: the number of women with a colposcopy and histopathological result was divided by the number of HSIL+ cases detected by (1) the first single reading, (2) the second single reading, (3) the average of the two single readings, and (4) the double reading. We adjusted our estimates to account for non-verification bias.[33] We assumed that the HSIL+ detection rate among the 146 women with an ASCUS+ result who did not return for a colposcopy and biopsy collection would have been the same as the observed detection rate among the 310 women who did receive the gold standard confirmation, i.e., that the colposcopy loss-to-follow-up was missing completely at random.

P-values are two-sided and a p-value less than $\alpha = 0.05$ was considered significant. Statistical analysis was performed using Stata 12 (StataCorp LP, College Station, Texas, USA).

Results

From a total of 36,212 women who participated in the FRIDA Study, 4,051 (11.2%) women were found to be hrHPV positive. An additional 127 observations with pending cytology results and 10 inadequate cytology smears were excluded from these analyses. Our final sample size included 3,914 hrHPV positive women who underwent LBC triage. A total of 648 (16.6%) women were diagnosed with ASCUS+ by at least one cytotechnologist, of which 456 (70.4%) were confirmed by the cytopathologist, and 52 women received a histologic diagnosis of HSIL or cervical cancer (16.8%). For the "first" single reading by a cytotechnologist, 473 (12.1%) of the LBC smears were reported as ASCUS+, of which 375 (79.3%) were confirmed by the cytopathologist as ASCUS+, with 43 (17.3%) cases being histologically diagnosed as HSIL+

among the women with available histological result. For the "second" single reading by a cytotechnologist, 524 (13.4%) ASCUS+ cases were reported, from which 398 (76.0%) were confirmed by the cytopathologist, with 43 (16.0%) cases receiving a final histological diagnosis of HSIL+. (Figure 1)

Table 1 reports the characteristics of the 3,914 hrHPV positive FRIDA Study participants. The prevalence of ASCUS+ was 11.7%, and was highest among women aged 40 to 49 years (12.5%). The mean age of the hrHPV positive participants was 41.0 years (SD=8.8) and 40.3 years (SD=8.3) for women with an ASCUS+ result. Table 2 presents the kappa statistics that measured pairwise interrater agreement between the seven cytotechnologists who performed the first and second single readings of all LBC slides. The results ranged from 0.47 to 0.82 with the weighted average of the kappa statistics of 0.68 (SD=0.10). Table 3 shows the percent agreement between the first and second single readings of the discordant or ASCUS+ diagnoses (Kappa=0.067, 95% CI= 0.010-0.123). Exact agreement was observed in 247 of the 648 slides that were read twice (38%), and agreement within one category was 62%. No agreement was observed for the inadequate, negative, and atypical glandular cells of undetermined significance (AGUS) diagnoses.

Cytology and histology results are summarized in Table 4. From the 374 ASCUS+ cases that were identified concordantly in both cytology readings, 319 (85.3%) were confirmed by the cytopathologist, and 34 cases received a histological diagnosis of HSIL+ (16.3%). The cytopathologist reviewed the 99 discordant cases that were reported as ASCUS+ on the first reading and negative or inadequate on the second reading. He identified 56 ASCUS+ cases, of which 9 were histologically diagnosed as HSIL+. Similarly, 79 of the 150 discordant results that were reported as negative in the first reading but ASCUS+ in the second were identified as

ASCUS+ by the cytopathologist. Nine women in each group received a HSIL+ diagnosis. As part of the double reading strategy, the cytopathologist identified a total of 456 women with an ASCUS+ diagnosis, and 52 cases were histologically confirmed as HSIL+. (Table 4)

Table 5 reports the number of HSIL+ cases detected using a single reading strategy compared to the double reading strategy. A total of 43 histologically confirmed cases of HSIL+ were detected on average from both single cytology readings. This corresponds to a HSIL+ detection rate of 10.99 (95% CI: 8.13, 14.80) per thousand women screened. The double reading resulted in a total of 52 cases of HSIL+, with a detection rate of 13.29 (95% CI: 10.11, 17.41) per thousand women. This represents an additional 9 cases detected, 2.30 more HSIL+ cases diagnosed per thousand, and an increase of 20.9%, compared to the single reading strategy (p-value = 0.0039). After accounting for non-verification bias, a total of 65 histologically confirmed cases of HSIL+ would be detected from the single cytology reading, with a HSIL+ detection rate of 16.61 (95% CI: 13.02, 21.14) per thousand. The adjusted double reading strategy would identify 77 cases of HSIL+, with a detection rate of 19.67 (95% CI: 15.74, 24.54) per thousand. This represents an additional 12 cases detected, 3.07 more HSIL+ cases diagnosed per thousand, and an increase of 18.5%, compared to the single reading strategy (p-value = 0.0005).

Table 6 compares the performance of the single cytology reading to the double reading strategy to detect HSIL+ cases, and the number of colposcopies needed to detect one HSIL+ case. The estimated number of colposcopies needed to detect a case of HSIL+ for the average of the single reading was 6.01, which is similar to the number needed for the double reading strategy, 5.96. We observed no differences even after adjusting to account for non-verification bias.

Discussion

We investigated if the detection of histologically confirmed HSIL or worse can be improved by having LBC slides read by two cytotechnologists, within the real-life operating conditions of a cervical cancer screening program in Mexico. Our findings indicate that the performance of LBC to detect HSIL+ cases is improved by a double cytology reading and suggest that it could be implemented as a feasible strategy to improve of the current CCSP in Mexico. Although the double reading of cervical cytology slides is likely to be more costly and time-consuming in the context of a Pap-based screening program, this would not be the case if HPV testing was used as the initial screening test, which is becoming more common in some countries.[26,34] Of the 36,212 women who participated in the FRIDA Study, a total of 3,914 (11%) were hrHPV+ and underwent triage with LBC. Thus, instead of having to review nearly 40,000 LBC slides, the cytotechnologists were able to conduct a double reading of a substantially smaller number of slides.

The American Society for Clinical Pathology recognizes the value of a second opinion in pathology and cytopathology to reduce diagnostic errors and improve patient outcomes, especially when medical interventions are mostly based on pathology interpretations.[35] This recognition of the benefits associated with having two diagnostic opinions instead of one can also apply to the utility of double reading LBC slides, especially in the context of an HPV-based CCSP. In a country like Mexico, where cervical cancer continues to be a public health problem, the double reading of cytology slides could be a promising strategy to reduce false negatives.[36] Although a double reading strategy could potentially reduce specificity and increase the costs and morbidity of finding false positives, our findings indicate that the single and double reading strategies have a similar percentage of women with an ASCUS+ who received a negative histology result.

The seven cytotechnologists reached substantial agreement in reading LBC slides, with a weighted average Kappa statistic of 0.68, which is greater than other studies.[37,38] However, this level of agreement is not homogeneous between the different pairs of cytotechnologists, since interpretation is highly dependent on the level of experience of the cytotechnologist. Cytology reading is known to be subjective with poor reproducibility [38],[39] and the findings of our study were no exception. The percent agreement between the first and second single reading of the discordant or ASCUS+ diagnoses was 0.067, which is considered "slight" agreement. Of the 259 negative slides that were reread by the cytopathologist, 14 were found to have an ASCUS+ diagnosis (5.4%). These 14 diagnoses included: 9 ASCUS, 1 LSIL, 2 HSIL, and 2 Atypical Glandular Cells. The additional ASCUS+ cases detected from this subset of negative results were not considered in this analysis of the utility of double reading LBC slides. These results highlight the major problems with cytology: that significant lesions can still be missed and the accuracy of cervical cytology diagnoses must be improved.

This study has some limitations. The cytotechnologists and cytopathologist were aware of the HPV DNA results of each slide they reviewed because only hrHPV positive samples were processed. Studies have shown that prior knowledge of hrHPV status can increase the ASCUS+ detection rate among cytotechnologists.[40,41] However, all slides evaluated were among HPV positive women so we do not expect the possibility of internal biases. Discrepancies in cytology interpretation could be due to the condition of the cytology laboratory, the education and training of the cytotechnicians, etc. However, since this observational study took place in what could be considered a "real-world" healthcare setting, the results and clinical implications generated from the study are possibly more informative than the finding obtained from a controlled study. Another limitation was the number of participants who were lost to follow-up because they did

not return for a colposcopy (31%). We estimated the HSIL+ detection rate for all 456 women with an ASCUS+ result correcting for verification bias. Despite these limitations, to our knowledge, this is the first study to evaluate the utility of a blind, independent double reading of cytology slides strategy within an HPV-based screening program triaged with LBC. Our finding that the number of colposcopies needed to detect a case of HSIL+ does not increase by using a double reading strategy is particularly relevant in the context of a population-based screening program whose objective is to identify the largest number of cases while optimizing the use of resources. By simultaneously collecting two separate cervical samples (first the specimen for LBC and then the sample for hrHPV DNA testing), we reduced the probability of a poor quality specimen for the LBC test and eliminated the need for an additional clinic visit to provide another cervical specimen in case of a positive hrHPV result.

In conclusion, our study suggests that, in the context of an HPV-based primary CCSP, the double reading of LBC slides among hrHPV positive women can significantly increase the detection rate of HSIL+ cases. While the success of cytology in reducing the incidence and mortality of cervical cancer is undeniable, the suboptimal inter-observer reproducibility and relatively high false-negative rate are significant limitations that need to be addressed. The findings of our study indicate that having two separate cytotechnologists review each LBC slide may help attenuate these limitations with beneficial clinical implications. The early detection of precancerous lesions in more women can facilitate the timely delivery of patient care, better utilization of health care resources, more appropriate patient management, and a reduction in health care costs. The specific costs and benefits of this strategy need to be evaluated in future studies to determine its value for different CCSPs.

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