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Erik Munson

University of Wisconsin - Milwaukee

Elizabeth R. Schroeder

Wheaton Franciscan Laboratory

Kevin C. Ross

Wheaton Franciscan Laboratory

Connie Yauck

Wheaton Franciscan Laboratory

Theresa Bieganski

Wheaton Franciscan Laboratory

See next page for additional authors

Authors

Erik Munson, Elizabeth R. Schroeder, Kevin C. Ross, Connie Yauck, Theresa Bieganski, Robert D. Amrhein, Maureen Napierala, and April L. Harkins

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Erik Munson,^{a,b} Elizabeth R. Schroeder,^a Kevin C. Ross,^a Connie Yauck,^a Theresa Bieganski,^a Robert D. Amrhein,^a Maureen Napierala,^a April L. Harkins^c

Wheaton Franciscan Laboratory, Milwaukee, Wisconsin, USA^a; College of Health Sciences, University of Wisconsin–Milwaukee, Milwaukee, Wisconsin, USA^b; College of Health Sciences, Marquette University, Milwaukee, Wisconsin, USA^c

Two important preanalytical protocols performed on liquid-based cytological specimens, namely, automated cytology processing and glacial acetic acid (GAA) treatment, may occur prior to the arrival of specimens in a molecular diagnostics laboratory. Ninety-two ThinPrep vials previously positive for high-risk human papillomavirus (HPV) via the Cervista HPV HR test were preselected and alternated with 92 previously negative ThinPrep vials. The specimen set was processed in a consecutive fashion by an automated cytology processor without fastidious decontamination precautions. Carryover potential was subsequently assessed by performance of the Aptima HPV assay on aliquots from reprocessed ThinPrep vials. All previously negative ThinPrep vials yielded a negative result following routine automated cytology processing, despite close proximity to known-positive ThinPrep vials. In separate experiments, aliquots from 236 ThinPrep vials were forwarded for tandem analysis with and without GAA treatment. Data from GAA- and mock-treated specimens generated by Aptima HPV were compared to correlate data generated by Cervista. A 99.2% concordance of Aptima HPV results from GAA-treated and mock-treated specimens was noted. This result differed from the concordance result derived from Cervista (91.5%; $P < 0.0002$). Of the initially positive Cervista results, 21.9% reverted to negative following GAA treatment; the correlate value was 2.7% for Aptima HPV ($P = 0.01$). While deleterious effects of GAA treatment on genomic DNA were noted with Cervista ($P = 0.0015$), GAA treatment had no significant effects on Aptima HPV specimen signal/cutoff ratios or amplification of internal control RNA ($P \geq 0.07$). The validity of an Aptima HPV result is independent of GAA treatment and routine automated cytology processing.

Detection of high-risk human papillomavirus (HPV) E6/E7 transcripts is a recent diagnostic advancement in the context of cervical cancer triage. Transcription-mediated amplification (TMA) of these targets (Aptima HPV; Hologic/Gen-Probe, San Diego, CA) performs in an equivalent fashion to high-risk HPV DNA hybridization assays, such as the Digene HC2 high-risk HPV DNA test (Qiagen, Gaithersburg, MD) (1–4) and the Cervista HPV HR (Cervista; Hologic, Madison, WI) (5), for the detection of indolent cervical intraepithelial neoplasia (CIN) 2+. The aforementioned studies also document improved specificity of Aptima HPV over that of DNA hybridization in the context of atypical squamous cells of undetermined significance (ASC-US) or non-significant cytology diagnoses and in cases of non-CIN 2+ biopsy results.

Aptima HPV is an FDA-cleared assay for the analysis of liquid-based cytology collections (ThinPrep; Hologic, Marlborough, MA) on high-throughput instrumentation such as the Tigris DTS system (6). Past package insert guidelines have referred to meticulous and costly cross-contamination mitigation steps required when the ThinPrep vials are subjected to automated cytology processing prior to HPV detection (7). In addition, specimen carryover (potentiated by either form of automation) may be of concern to both laboratorians and cytopathologists. Moreover, previous data (8–10) suggest that performance characteristics of DNA-based HPV modalities may be affected by glacial acetic acid (GAA) treatment of liquid-based cytology collections. The purpose of this investigation was to assess such preanalytical concerns inherent to the performance of the Aptima HPV.

(Results of this work were previously presented, in part, at the 113th General Meeting of the American Society for Microbiology, Denver, CO, 18 to 21 May, 2013.)

MATERIALS AND METHODS

Cytology specimens. In a study approved by the Wheaton Franciscan Healthcare Institutional Review Board, residual fluid from ThinPrep vials was subjected to molecular analysis. A ThinPrep 2000 (Hologic) was utilized for automated processing of all primary ThinPrep collections. The procedure was executed per manufacturer guidance (11) and without sodium hypochlorite-based postprocessing decontamination protocols devoted to each processing event. Cytotechnologists did not change gloves each time a new ThinPrep vial was introduced to the ThinPrep 2000.

GAA treatment of primary ThinPrep collections. A protocol derived from previous reports (8, 12) was validated for GAA treatment of ThinPrep collections initially observed to contain excessive erythrocytes, cervical mucus, acute inflammatory cells, or cytological debris. In brief, residual ThinPrep contents were treated for 2 min with 3 ml concentrated GAA. Final specimen volume was adjusted to 50 ml with CytoLyt solution (Hologic). Upon centrifugation, pelleted material was resuspended in 5 ml CytoLyt prior to automated reprocessing.

Aptima HPV. For analysis by Aptima HPV on the Tigris DTS system, 1-ml ThinPrep aliquots were dispensed into tubes containing lysis medium. Paradigms of oligonucleotide-specific target capture, TMA, and hybridization protection (13) were utilized to generate a luminescence-based result. The assay detects E6/E7 mRNA from high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. Internal control

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Address correspondence to Erik Munson, erik.munson@wfhc.org.

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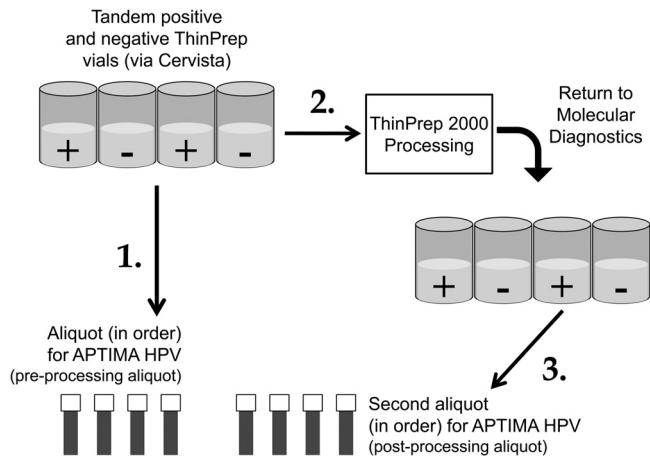


FIG 1 Experimentation to assess the effect of automated cytology processor carryover potential on Aptima HPV performance.

material (provided within assay reagents and subjected to the same methodology as specimen aliquots) was used to assess the presence of endogenous specimen inhibitors, with these data expressed as relative light units (RLU). An analyte signal-to-cutoff ratio (*s/co*) represented the relationship between the specimen RLU value and a calibrator-derived analyte cutoff value. An *s/co* of ≥ 0.50 denoted a positive result.

Cervista. Two-milliliter ThinPrep aliquots were subjected to a previously described paramagnetic-based DNA extraction and HPV-specific Invader-based hybridization (14). In brief, luminescence-labeled oligonucleotides specific for DNA of high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 were incubated with DNA extracts for 4 h at 62°C. Relative genomic DNA (gDNA) content was assessed by a luminescence-labeled human histone-specific oligonucleotide and was subsequently expressed as a ratio to background luminescence. gDNA-specific luminescence equaling or exceeding a 1.50 ratio to background constituted sufficient DNA extraction for a valid patient result. HPV-specific luminescence from a given oligonucleotide mixture was required to equal or exceed a 1.525 ratio to background for a positive result.

Assessment of carryover potential relative to automation. Ninety-two tandem pairs of ThinPrep vials, constituting both a positive and negative result via previous clinical Cervista performance, were subjected to Aptima HPV both before (preprocessing aliquot) and after ThinPrep 2000 processing (postprocessing aliquot) (Fig. 1). Pre- and postprocessing aliquots of a given specimen were analyzed in the same batch for Aptima HPV testing. Operation of the automated cytology processor was shared within a pool of four cytotechnologists. Forty randomly selected ThinPrep vials were subjected to gDNA content determination by Cervista both before and after cytology processing.

Determination of GAA effect on Aptima HPV. Residual material from 236 ThinPrep vials deemed unsatisfactory for initial cytology screening was forwarded for tandem Aptima HPV and Cervista analyses following treatment by GAA (Fig. 2). In brief, an ~3.5-ml aliquot of ThinPrep contents was removed for subsequent molecular HPV screening (mock treatment). The remainder of the ThinPrep collection was subjected to the aforementioned GAA treatment protocol. Aliquots (GAA treatment) were then delivered to the Aptima HPV and Cervista testing systems, along with appropriate aliquot volumes of the corresponding mock treatment. GAA and mock treatments of a given specimen were analyzed in the same batch for Aptima HPV and Cervista testing.

Data analysis. The significance test of proportions was used to determine if differences in result distributions were significant. Kappa and agreement statistics were calculated with respect to results generated with and without GAA treatment. The *t* test for independent samples was used to determine if differences in luminescent data were significant between

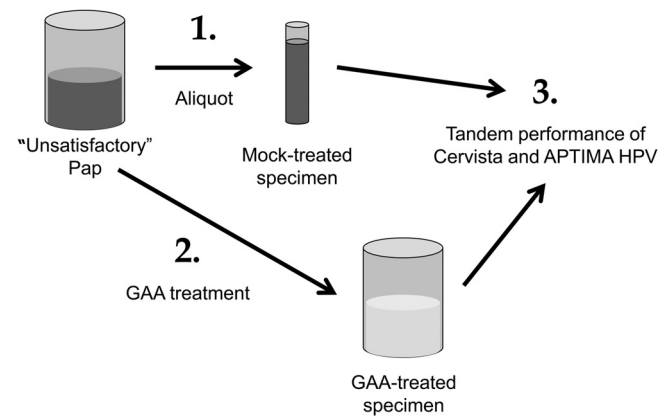


FIG 2 Experimentation to assess effects of glacial acetic acid (GAA) treatment of ThinPrep vials on Cervista and Aptima HPV performance.

treatment groups. The alpha level was set at 0.05 before the investigations commenced, and all *P* values are two-tailed.

RESULTS

Characterization of antecedent Cervista-positive and -negative ThinPrep contents by Aptima HPV. Eighty-nine (96.7%) of 92 preselected Cervista-positive or -negative ThinPrep vials generated expected results when a preprocessing aliquot was subjected to Aptima HPV (Fig. 3A). The same concordance rate was derived from Aptima HPV analysis of aliquots of the ThinPrep study set following automated cytology processing (Fig. 3B).

One specimen yielded positive Aptima HPV results from both preprocessing and postprocessing aliquots of the antecedent Cervista-negative ThinPrep vial. An additional two specimens generated negative Aptima HPV results when the expected antecedent Cervista results were positive. As such, these three tandem pairs were excluded from automated cytology processing carryover analysis.

Effect of automated cytology processing on subsequent Aptima HPV performance. Forty randomly selected ThinPrep vials

A.

| Pre-processing | | APTIMA HPV Result | |
|----------------------------|----------|-------------------|----------------|
| | | Positive | Negative |
| Antecedent Cervista Result | Positive | 45 | 1 ^a |
| | Negative | 2 ^b | 44 |

B.

| Post-processing | | APTIMA HPV Result | |
|----------------------------|----------|-------------------|------------------|
| | | Positive | Negative |
| Antecedent Cervista Result | Positive | 44 | 2 ^{a,c} |
| | Negative | 1 ^{b,c} | 45 |

^aOne specimen yielded positive APTIMA HPV results from both pre-processing and post-processing aliquots of an antecedent Cervista-negative ThinPrep vial.

^bOne specimen yielded negative APTIMA HPV results from both pre-processing and post-processing aliquots of an antecedent Cervista-positive ThinPrep vial.

^cThree tandem sets of ThinPrep vials eliminated from carryover analysis.

FIG 3 Results from Aptima HPV assessment of preprocessing (A) and postprocessing (B) aliquots of 92 preselected Cervista-positive and -negative ThinPrep vials.

TABLE 1 Cervista-derived gDNA assessment of 40 ThinPrep vial aliquots prior to and following automated cytology processing

| Cervista result | <i>n</i> | Mean gDNA content in: | | <i>P</i> |
|-----------------|----------|-----------------------|------------------------|----------|
| | | Preprocessing aliquot | Postprocessing aliquot | |
| Positive | 20 | 15.877 | 13.774 | 0.22 |
| Negative | 20 | 13.728 | 14.017 | 0.84 |
| Cumulative | 40 | 14.802 | 13.896 | 0.41 |

yielded equivalent gDNA results via Cervista before and after reprocessing ($P = 0.41$) (Table 1), suggesting that reprocessing would not bias a postprocessing Aptima HPV result on the basis of insufficient amounts of nucleic acid. When results were stratified by individual cytotechnologists, no significant differences in pre- and postprocessing gDNA content were observed ($P \geq 0.15$) (data not illustrated). When tested by Aptima HPV, all 45 evaluable negative postprocessing aliquots retained a negative result, and 97.7% of 44 evaluable positive postprocessing aliquots retained a positive result.

Effect of GAA on Aptima HPV. Of 236 prospective ThinPrep vials requiring GAA treatment, 37 were initially positive via Aptima HPV, while 41 were initially positive via Cervista. Less than 1% of all Aptima HPV results varied upon GAA treatment (Table 2), while 8.5% of initial Cervista results changed under similar circumstances ($P < 0.0002$). Just 2.7% of initially positive Aptima HPV results failed to retain this status following GAA treatment, whereas 21.9% of initially positive specimens via Cervista reverted to a negative result upon GAA treatment ($P = 0.01$). Aptima HPV measures related to high-risk HPV RNA detection (s/co) and inhibitor assessment (internal control RLU) did not vary post-GAA treatment ($P \geq 0.07$) (Table 3). In contrast, gDNA content (as measured by Cervista) was significantly reduced following GAA treatment (Table 2).

DISCUSSION

Laboratories exploring automation prospects for both ThinPrep vial processing and molecular HPV performance have been confronted with recommendations for meticulous decontamination of such instrumentation, particularly with respect to automated cytology processors. Past package insert material for delivery of processed ThinPrep contents to specimen aliquot tubes for Aptima HPV (7) has described an in-house-validated procedure to mitigate the potential for cross-contamination during ThinPrep 2000 processing. Two important components of this procedure included (i) soaking the filter cap in 0.5% sodium hypochlorite

TABLE 3 Data output specific to Cervista and Aptima HPV assays with respect to mock and glacial acetic acid treatment of 236 ThinPrep specimens

| Background parameter | Mean data for: | | <i>P</i> |
|-----------------------------------|----------------|---------------|----------|
| | Mock treatment | GAA treatment | |
| Cervista gDNA content | 11.05 | 9.27 | 0.0015 |
| Aptima HPV signal/cutoff | 1.881 | 1.673 | 0.64 |
| Aptima HPV internal control value | 218,394 | 227,264 | 0.07 |

solution for 1 min between samples and (ii) mandating that the operator change gloves between each sample.

When these recommendations first surfaced, alternative strategies for primary specimen processing were entertained by laboratories dealing with significant cytology and/or HPV testing volume. Prealiquoting for HPV screening from a cytology specimen with low cellularity could further reduce volume or render the specimen unusable for assessment by cytological analysis. Furthermore, aliquoting of every specimen for HPV screening may not be necessary if the cytology result yields a result other than ASC-US (15). This strategy could also be limited by fiscal, staffing, and consumable storage considerations. Some of these ancillary factors could also influence the decision of a laboratory to implement strict decontamination practices surrounding each ThinPrep input through an automated processor. Moreover, turnaround time to final HPV screening result would be predicated based on the length of time necessary for initial cytology processing.

A final alternative strategy entails extensive verification of a routine automated cytology processing protocol (as described in Materials and Methods), with subsequent Aptima HPV screening designated a laboratory-modified test (16). When this study was initiated, Cervista was the HPV screening modality employed by our laboratory. As such, when we attempted to preselect positive and negative specimens for a carryover analysis investigation (Fig. 1), the possibility existed for discordance between the Cervista and Aptima HPV data. Explanations for this have ranged from increased sensitivity of TMA-based testing versus that of DNA hybridization (17) to a recent characterization of false-positive results inherent to Cervista (5). Furthermore, specimens may have contained DNA or RNA, but not both. Indeed, three tandem pairs of data were excluded from analysis (Fig. 3B) because two initially positive Cervista results did not translate into positive Aptima HPV results, while one initially negative Cervista specimen was Aptima HPV positive.

TABLE 2 Summary of intra-assay comparisons of Cervista and Aptima HPV prior to and following glacial acetic acid treatment of 236 specimens

| Result parameter | Results (%) for: | | <i>P</i> | Agreement (95% CI) ^a | Kappa (95% CI) |
|---|------------------|------------|----------|---------------------------------|-----------------|
| | Cervista | Aptima HPV | | | |
| Remain positive following GAA treatment | 78.1 | 97.3 | 0.01 | 0.903 (0.731–0.975) | 0.367 (0–0.894) |
| Remain negative following GAA treatment | 95.8 | 99.5 | 0.02 | 0.963 (0.922–0.984) | 0.215 (0–0.568) |
| Mock, ^b GAA treatment result concordance | 91.5 | 99.2 | <0.0002 | 0.955 (0.916–0.977) | 0.274 (0–0.577) |

^a CI, confidence interval.^b No treatment with glacial acetic acid.

As a result, 89 tandem sets of antecedent Cervista-positive and -negative ThinPrep vials were evaluated for carryover. The mean HPV-specific luminescence-to-background ratio of the HPV-positive vials (5.209) was approximately 240% greater than the cutoff value required for a positive result (data not illustrated). The aforementioned 96.7% concordance rate between Aptima HPV/Cervista results in the carryover portion of this investigation was higher than the 88.7% concordance rate between the two assays previously described in a study set of 4,056 specimens (5). In addition to the smaller study set utilized in the current investigation, preselection of (Cervista) strongly HPV-positive ThinPrep vials would inherently lend itself to a higher concordance rate than would a prospective analysis.

In spite of the strongly HPV-positive ThinPrep vial directly preceding a negative ThinPrep vial during cytology reprocessing, the subsequent performance of Aptima HPV did not result in reversion of expected negative results to positive. These observations imply that carryover risk ascribed to the ThinPrep 2000 is negligible in the context of TMA-based HPV screening. Moreover, the high rate of concordance experienced in Aptima HPV performance of the preprocessing aliquots (Fig. 3A), apart from analytical variance related to the Cervista assay, suggests that the risk for cross-contamination related to the Tigris DTS itself is also very minimal. Chernesky et al. (18) reported >98% agreement of Aptima Combo 2 (Hologic/Gen-Probe) *Chlamydia trachomatis* results in precytology and postcytology fractions of 394 SurePath (BD Diagnostics-TriPath, Burlington, NC) collections. The data in our study not only were generated on the basis of a different microbe and an alternative liquid-based cytology medium but also were assessed with an automated platform for nucleic acid amplification testing and an automated means of cytology processing. Similar findings are reported.

We previously demonstrated that Cervista is greatly affected by GAA treatment (10). Analysis of 465 specimens revealed significant decreases in gDNA content and rates of positive HPV results upon GAA treatment. This effect was independently documented for two divergent GAA treatment protocols. These findings were consistent with a less-rigorous study (9) but were a departure from those of another report describing no deleterious effects of GAA (8). To our knowledge, one assessment of GAA effect on Aptima HPV has been reported. Dockter et al. (19) added known concentrations of GAA to ThinPrep contents and demonstrated negligible effects on Aptima HPV. In our report, we present a second large independent data set to confirm the deleterious effects of GAA on Cervista. Furthermore, a prospectively designed investigation of primary ThinPrep specimens directly demonstrated less of an effect of GAA treatment on Aptima HPV than on Cervista (Table 2). Whereas the gDNA content of Cervista was significantly affected by GAA treatment, GAA did not significantly affect parameters associated with mRNA-based Aptima HPV ($P \geq 0.07$) (Table 3). Additional studies may be warranted to determine if RNA is less susceptible to the effect of GAA or if the target capture system inherent to Aptima assays plays a role in mitigating the potentially deleterious effect of this treatment.

In conclusion, specimen carryover emanating from the function of an automated cytology processor was not evident upon the subsequent performance of Aptima HPV. Although past guidelines for meticulous decontamination protocols have recently been superseded by internal manufacturer studies and package insert revisions (20), protocols described in this report can prove

useful to cytopathologists and molecular diagnosticians in the context of quality assurance programs. In particular, the schematic illustrated in Fig. 1 can address College of American Pathologists Accreditation Program standards, such as checklist requirement MOL.34875, which deal with carryover in the molecular diagnostics laboratory (21). GAA treatment of ThinPrep vials has less deleterious effects on Aptima HPV performance than on Cervista. Taken together, preanalytical factors are of minimal concern with respect to Aptima HPV performance, ultimately allowing for appropriate contributions of adjunctive HPV screening data to overall cervical cancer triage.

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