Effect of Glacial Acetic Acid Treatment of Liquid-based Cytology Collections
on Performance of Cervista™ HPV HR for Detection
of High-risk Human Papillomavirus

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ABSTRACT

Inadequate cervical cytological analysis can be facilitated by glacial acetic acid (GAA)-treatment of primary liquid-based collections to remove mucus, erythrocytes, inflammatory cells, and cellular debris. In the context of a commercial HPV hybridization assay performed on 465 tandem specimens with and without GAA treatment, we show that GAA treatment significantly reduces genomic DNA content ($P < 0.0001$) and creates an increased potential for indeterminate and false-negative results. In the context of cytological workflow, laboratories should consider providing a specimen aliquot for HPV DNA detection prior to GAA treatment.
Molecular diagnostic assays specific for human papillomavirus (HPV) play a significant role in cervical cancer triage [15]. Such assays, shown to have ≥ 98.9% negative predictive value for underlying presence of cervical intraepithelial neoplasia 2+ [5, 13], can be performed on endocervical scrapings preserved in liquid transport medium. This collection, also forwarded for cytological analysis, has the potential to possess insufficient squamous cellularity, as well as abundant erythrocytes, mucus, acute inflammatory cells, and cytological debris [11]. In particular, the latter factors may compromise Papanicolaou (Pap) smear analyses that are incumbent upon filter-based processing protocols (e.g., ThinPrep®; Hologic, Marlborough, MA). As a result, glacial acetic acid (GAA)-based protocols have been developed to augment diagnostic yield, with some receiving United States Food and Drug Administration approval [4].

While this paradigm has procured a high percentage of newly-satisfactory Pap smear analyses and has increased detection of significant gynecological lesions [1, 2, 6, 11], recent data have revealed that GAA treatment increases false-positive diagnoses of atypical endocervical cells [3]. A paucity of studies, typically focused on hybrid capture technology (Hc2; Qiagen/Digene, Gaithersburg, MD), has been conflicting on the potential effect of GAA on HPV DNA detection [1, 6]. In addition to oligonucleotide probes specific for 14 high-risk HPV types (provided in three reagent mixtures), the Cervista™ HPV HR (Hologic/Third Wave Technologies, Madison, WI) possesses an oligonucleotide probe to assess relative DNA content. The presented study utilizes this internal control as one means of assessing interference potential of GAA treatment on assay performance.
In an institutional review board-approved protocol, 465 ThinPrep® specimens were collected by two laboratories. These specimens were initially defined as unsatisfactory on the basis of the 2001 Bethesda System Guidelines [12] for assessing quality of Pap smear. Laboratory A subjected unsatisfactory ThinPrep® specimens to a manufacturer-advocated protocol in which unsatisfactory specimens were initially treated with a titration of GAA and CytoLyt solution (Hologic) [2, 4]. In contrast, laboratory B validated a protocol derived from the reports of Iverson and Armour [8] and Agoff et al. [1] in which concentrated GAA was added to specimens prior to adjustment of total volume with CytoLyt. For this investigation, 2-mL aliquots were removed for Cervista™ HPV HR prior to GAA treatment (pre-wash). Following GAA treatment, 2-mL aliquots of the same specimen (post-wash) were submitted for Cervista™ HPV HR.

All Cervista™ HPV HR was performed at a single location. Assay performance, including manual Agencourt-based paramagnetic extraction [10], and results interpretation were executed per package insert. Relative genomic DNA content (gDNA), as determined by human histone-specific oligonucleotide hybridization, was required to equal or exceed a 1.5 fluorescence ratio to background for valid analysis. HPV-specific fluorescence from a given reagent mixture was required to equal or exceed a 1.525 ratio to background for a positive result. HPV-specific fluorescence equal to or exceeding a 1.93 ratio to background from all three reagent mixtures also yielded a detectable result. The significance test of proportions determined if changes in rates of HPV DNA screening result permutations (derived from GAA treatments) were significant between laboratory A and B. The t test for independent samples determined if gDNA differences upon GAA treatment were significant. This analysis also provided the basis for
interlaboratory comparison of gDNA recovery. The alpha level was set at 0.05 before the investigations commenced, and all $P$ values are two-tailed.

GAA treatment of ThinPrep® specimens significantly reduced fluorescence signal reflective of gDNA (Table 1). This effect was more pronounced with the processing protocol at laboratory A ($P < 0.001$). Overall mean signal reduction was 31.9%, with an average reduction of 53.0% noted from laboratory A. This reduction was observed despite initial specimen gDNA not differing between the two laboratories ($P = 0.41$). A total of 77 specimens (16.6%) produced a change in result interpretation or validity following GAA treatment (Table 2). Laboratory B experienced fewer indeterminate results upon GAA treatment ($P < 0.0002$).

Of 79 specimens yielding a positive HPV result prior to GAA treatment (Table 2), 3 (3.8%) generated an indeterminate result following treatment, while 23 (29.1%) reverted to a negative result. The 26 specimens that failed to retain positive status exhibited 53.6% and 56.4% reductions in fluorescence signal reflective of gDNA and HPV-specific nucleic acid content, respectively (Table 1).

Performance of the Cervista™ HPV HR has been shown to be largely equivalent to that of HC2, both from laboratory [7, 9, 10, 14] and clinical [5] perspectives. However, few studies have analyzed performance of these assays on ThinPrep® collections treated with GAA. In a randomized assessment, Agoff et al. [1] described 10 specimens that yielded a positive HC2 result only upon GAA treatment. Reversion to expected negative result occurred in three of five instances after sodium hydroxide-based neutralization of GAA-treated specimens. Conversely,
Feng and Husain [6] reported a trend toward decreased HPV DNA detection via Hc2 upon GAA treatment (43.9% detection in GAA-treated specimens, 50.4% in non-treated specimens; \( P = 0.21 \)). Luminescent output within HPV-positive specimens exhibited a similar trend across treatment groups (\( P = 0.26 \)). However, this study was limited to specimens with a cytological diagnosis of atypical squamous cells of undetermined significance (ASCUS) and, more importantly, by the fact that specimens were not analyzed in tandem. In our study, a large dataset of consecutive, GAA-treated and non-treated correlates of the same ThinPrep® collection were subjected to the Cervista™ HPV HR. Reversion of HPV-positive status and decreased gDNA may extend preliminary findings from limited Hc2 experimentation [6].

Laboratory A utilized a GAA protocol that was based on a 9:1 ratio of CytoLyt solution (Hologic) to GAA. 30 mL of this solution was added to centrifuged ThinPrep® contents. Following a second centrifugation step, pelleted material was resuspended in 20 mL of CytoLyt for subsequent reprocessing. In contrast, the protocol utilized by laboratory B involved a 2-minute treatment of residual ThinPrep® contents with 3 mL of concentrated GAA and adjustment of specimen volume to 50 mL with CytoLyt prior to centrifugation. Pelleted material was resuspended in 5 mL CytoLyt prior to reprocessing. Depending upon cellularity associated with initial specimen collection, residual ThinPrep® volume following initial specimen processing may be variable. With this residual volume having the potential to be quite low (i.e., < 5 mL), it is possible to have a CytoLyt:GAA ratio in the laboratory B protocol [1, 8] exceeding the 9:1 ratio utilized in the laboratory A protocol. As a result, mean gDNA reduction experienced by laboratory B would be less severe than that determined from laboratory A. This can be further inferred from gDNA comparisons of the 26 initially HPV-positive specimens that reverted to
HPV-negative status upon GAA treatment ($P = 0.04$; Table 1). However, it should not be understated that both laboratories experienced overall similar rates of HPV-positive pre-wash specimens reverting to negative status upon GAA treatment ($P = 0.74$; Table 2).

In conclusion, utilization of the Cervista™ HPV HR, particularly the histone-based internal control component, resulted in the finding that GAA treatment of ThinPrep® specimens can significantly alter fluorescence signal reflective of gDNA. Moreover, GAA treatment of ThinPrep® specimens has a proclivity for false-negative HPV result generation. ThinPrep® specimens that are initially unsatisfactory for Pap smear analysis should be aliquoted for HPV DNA detection prior to GAA treatment. Furthermore, laboratories that routinely treat all specimens with GAA prior to cytological processing should reconsider this practice on the basis of potential ASCUS-reflex molecular HPV detection. These steps should enhance the overall clinical pathology contribution to cervical cancer triage.

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REFERENCES


TABLE 1: Relative genomic DNA content (gDNA) of ThinPrep® collections processed by two laboratories prior to and following glacial acetic acid treatment. Mean changes in high-risk HPV and gDNA fluorescence within a subset of 26 ThinPrep® collections with initially-detectable HPV-specific DNA that did not retain positive status following glacial acetic acid treatment are also provided.
<table>
<thead>
<tr>
<th>Facility</th>
<th>n</th>
<th>Mean gDNA Pre-wash</th>
<th>Mean gDNA Post-wash</th>
<th>Mean percentage gDNA reduction per specimen pair</th>
<th>n</th>
<th>Mean Percentage Fluorescence Reduction</th>
<th>gDNA</th>
<th>High-risk HPV-specific DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory A</td>
<td>207</td>
<td>10.22$^*$</td>
<td>4.58$^*$</td>
<td>&lt;0.0001</td>
<td>53.0$^*$</td>
<td>13</td>
<td>62.7$^*$</td>
<td>55.8$^*$</td>
</tr>
<tr>
<td>Laboratory B</td>
<td>258</td>
<td>9.72$^*$</td>
<td>7.94$^*$</td>
<td>0.002</td>
<td>15.0$^*$</td>
<td>13</td>
<td>44.6</td>
<td>57.5</td>
</tr>
<tr>
<td>Cumulative</td>
<td>465</td>
<td>9.94</td>
<td>6.45</td>
<td>&lt;0.0001</td>
<td>31.9</td>
<td>26</td>
<td>53.6</td>
<td>56.4</td>
</tr>
</tbody>
</table>

$^*$P = 0.41 for laboratory-specific pre-wash gDNA

$^*$P < 0.0001 for laboratory-specific post-wash gDNA

$^*$P < 0.0001 for laboratory-specific percentage gDNA reduction

$^*$P = 0.04 versus reduction demonstrated by laboratory B

$^*$P = 0.84 versus reduction demonstrated by laboratory B
TABLE 2: Results of Cervista™ HPV HR testing of ThinPrep® collections prior to and following glacial acetic acid treatment.
<table>
<thead>
<tr>
<th>Pre-wash/post-wash Result</th>
<th>Interlaboratory Comparison</th>
<th>Cumulative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Laboratory A (%)</td>
<td>Laboratory B (%)</td>
</tr>
<tr>
<td>negative/negative</td>
<td>139 (67.1)</td>
<td>196 (76.0)</td>
</tr>
<tr>
<td>negative/indeterminate(^a)</td>
<td>37 (17.9)</td>
<td>14 (5.4)</td>
</tr>
<tr>
<td>positive/positive</td>
<td>18 (8.7)</td>
<td>35 (13.6)</td>
</tr>
<tr>
<td>positive/indeterminate</td>
<td>2 (1.0)</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>positive/negative</td>
<td>11 (5.3)</td>
<td>12 (4.7)</td>
</tr>
</tbody>
</table>

\(^a\)Indeterminate due to genomic DNA to background fluorescence ratio $< 1.50$

\(^b\)Numbers too small for valid statistical comparison