The Cellient System for Cytohistology to Analyze p16 Positive Dyskeratocytes in Paraffin Sections of HPV-Positive Cervical Scrapes

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Abstract: The Cellient™ Automated Cell Block System (Hologic) can be used to analyze cells of HPV-positive cervical scrapes staining positive with the biomarker p16. For this study fourteen cervical scrapes of Tanzanian women infected with HPV testing positive for HPV were selected. The paraffin Cellient sections were stained with the Papanicolaou method, with hematoxylin eosin (HE), and with the biomarker p16. This pilot study was limited to cases classified as atypical squamous lesion of unknown significance (ASCUS) and high-grade squamous lesion (HSIL) as diagnosed in the ThinPrep slide. The Cellient paraffin sections (cut from paraffin blocks prepared from the residual cervical sample) were classified into negative, atypical, CIN 1, CIN 2, and CIN 3. Multiple HPV genotypes were encountered in 79% of the scrapes. HPV16 was found in six scrapes and HPV52 in four. In the Papanicolaou sections, it was easy to detect dyskeratotic cells. Eleven of the 14 cases were p16 positive and five contained p16 positive dyskeratocytes. Of the 10 ASCUS scrapes, two contained p16 positive CIN 1 epithelial fragments. All four HSIL cases contained p16 positive CIN 3 epithelial fragments. In HPV-positive HPV-positive women, the Cellient system resulted in high quality histology sections with perfect p16 images of dyskeratocytes.

Keywords: Cellient, HPV, dyskeratosis, p16, HIV, BoonFix.

INTRODUCTION

In the current decade in which human papillomavirus (HPV) testing is becoming a national screening requirement in many countries, cytologists must develop skills in multidiagnostic techniques that include cervical morphology and immunocytochemistry.

Various methods are being introduced to detect high-risk HPV, but the Cellient™ Automated Cell Block System (Hologic), producing paraffin blocks could provide an ideal method for cytohistologists as it compares favorably with traditional cell block sectioning [1]. Although such automated techniques have an up front cost, they could prove to be cost-effective by saving preparation time for the cytologist, and thereby allowing more samples to be assessed in the required timeframe of clinical practice. In the paraffin sections, we can analyze p16 staining of dyskeratocytes [2] in cases classified as ASCUS (atypical squamous cells of unknown significance) and as HSIL (high-grade squamous intraepithelial lesion) [3].

In Africa, the HIV/AIDS epidemic has significantly affected health, economy, social infrastructure, and education. Women in particular are bearing the brunt of the disease through having to care for infected spouses and children.

The establishment of a community kitchen, at which local women produce yogurt containing probiotic Lactobacillus rhamnosus GR-1 [4], provided an opportunity to assess the effect of daily yogurt intake on the health of women.

Participants for the yogurt study, as published in the International Dairy Journal [5], were recruited from women attending the HIV treatment clinic of Sekou-Toure regional hospital or surrounding hospitals in Mwanza, Tanzania. Eligible subjects were non-pregnant, HIV-positive females, over 18 years of age who had used anti-retroviral therapy (ART) for at least 6 months. Subjects were excluded if breastfeeding, intolerant to lactose or fermented milk. The Medical Research Coordinating Committee of the National Institute for Medical Research, Tanzania, approved the study on the effects of including yogurt into the diet of HIV-positive women. Participants were informed of the purpose of the trial and gave their signed or thumb-printed informed consent before participation. Women waiting to be interviewed and for their cervical scrape are shown in Figure 1.

Because HPV infection is seen as a HIV-defining illness, it should be managed as such. The majority of the attending patients cannot afford their own medical...
care, therefore it was decided to perform Pap smears and investigate this at a wider level and report the clinical relevant data back for care and treatment. The results and samples collected were of such significance that we decided to elaborate on it and publish the results. In the context of this yogurt study, a cervical scrape was taken from each participating woman.

The aim of the present pilot study was to test the Cellient™ system on HPV-positive cervical scrapes collected in Tanzania.

**MATERIAL AND METHODS**

**Subjects and Sampling**

Cervical scrapes of the first 14 HIV-positive women with a cytology diagnosis ASCUS or HSIL were selected for this study. Participants provided written or thumb-print informed consent as part of a randomized controlled trial approved by the Ethical Review Board in Tanzania (ClinicalTrials.gov, Identifier: NCT01258556) [5].

A cervical scrape was taken as follows: the tip of the Cervex-Brush® Combi (Rovers Medical Devices, Oss, the Netherlands) was placed in the endocervical canal and rotated. The Cervex-Brush Combi has the shape of a broom. These brooms were placed in a vial with the coagulant formalin-free fixative BoonFix®, with polyethylene glycol (PEG) as one of its four components (Denteck, Zoetermeer, the Netherlands). Finally the vials with the samples were transported to the laboratory in Leiden, the Netherlands. In the Leiden laboratory each vial was placed in a commercial paint shaker and by its rigorous shaking all tissue fragments collected by the Cervex-Brush Combi emerged into the BoonFix solution.

**The ThinPrep Slides and Cytology Classification**

From each sample six ThinPrep® slides (Hologic) were prepared in the T3000. The remaining material in the vial was stored. The cytology ThinPrep slides were scored according to the Bethesda system [3].

**The HPV PCR Method**

The archived samples were analyzed for 25 HPV genotypes using a highly sensitive PCR-reverse hybridization Line Probe Assay, the so-called INNO-LiPA HPV genotyping extra system (Innogenetics, Belgium). The SPF10 primer set amplifies a 65-bp region in the L1 open reading frame [6]. Detection after PCR is based on the principle of reverse hybridization. Amplification products are subsequently hybridized using specific oligonucleotide probes in a single typing strip [7]. For the current study, HPV-positive scrapes were included.

**The Cellient Method**

All the remaining material in the vial was used for the Cellient method. This was collected into a cassette...
and loaded into the instrument. Eosin was applied and vacuum-drawn through the sample. Alcohol was similarly applied and drawn through the sample for dehydration. To clear the alcohol, the procedure was repeated with xylene. The sample was then embedded in paraffin and finally embedded in an additional layer of paraffin during processing in the finishing station.

From the Cellient paraffin block eight serial sections were cut, two for the Papanicolaou staining, one for the hematoxylin eosin (HE) staining, and five for the p16 immunostainings.

**Morphologic Criteria in ThinPrep and Cellient Slides**

**Metaplastic Cells**

Metaplastic cells are defined as cells with a vesicular nucleus and a nuclear-cytoplasm ratio of around 0.5 [8]. In the p16 stain, the vesicular nature of the nucleus is still visible.

**Koilocytotic Cells**

Koilocytotic cells display a clear zone well demarcated around the nucleus [8]. Particularly in the ThinPrep cytology slides it is easy to identify koilocytes.

**Squamous Pearls**

Squamous pearls are whirls of squamous cells. In the ThinPrep slides these pearls display small pyknotic nuclei. The cytoplasmic staining is either turquoise or red. In the Papanicolaou-stained paraffin Cellient sections, the cytoplasm is either bright red or dark green (Figure 2), nuclei are not always in the section.

![Figure 2: Squamous pearl in a Papanicolaou-stained paraffin Cellient section. The cytoplasm is either bright red or dark green. In this section, the nuclei are not cut.](image)

**Dyskeratotic Cells**

In the ThinPrep slides, dyskeratotic cells have a pyknotic nucleus, a nuclear cytoplasmic ratio between 2 and 6, and orange or green staining cytoplasm. In the Papanicolaou-stained Cellient paraffin sections, the cytoplasm is green or orange-red (Figures 3 and 4). In these sections, the pyknotic nuclei of the dyskeratotic cells can be relatively large (Figure 5). In the HE stain, dyskeratotic cells have bright red cytoplasm. In the p16 Cellient paraffin sections, dyskeratotic cells can display brown cytoplasm and brown-blue nuclei (Figure 6) or intensely brown pyknotic nuclei in which the blue (hematoxylin) staining is hidden by the brown color (Figure 7). In the same field of view of the paraffin section, a positive p16 staining parakeratotic cells can be encountered next to a dyskeratotic cell without p16 staining and a (relatively large) blue pyknotic nucleus. Also, a p16 positive dyskeratotic cell can be detected in the Cellient section next to a p16 positive metaplastic cell with only little brown staining of its vesicular nucleus.

![Figure 3: Dyskeratotic cell in a Papanicolaou-stained Cellient paraffin section. The cytoplasm is orange-red (see Figure 6).](image)

![Figure 4: Two dyskeratotic cells in a Papanicolaou-stained Cellient paraffin section. The cytoplasm of these cells is orange-red.](image)
Figure 5: Dyskeratotic cells in a Papanicolaou-stained Cellient paraffin section. Here, the cytoplasm stains green. The pyknotic nuclei can be relatively large.

Figure 6: Dyskeratotic cell in a p16 Cellient paraffin section with brown cytoplasm and brown-blue nuclei.

Figure 7: Dyskeratotic cell in a p16 Cellient paraffin section with a rather large (pyknotic) nucleus.

RESULTS

In total, 11 HPV genotypes were found, two low risk (lr) (HPV44 and HPV70) and nine high risk (HPV16, HPV26, HPV51, HPV52, HPV53, HPV56, HPV58, HPV66, HPV69). All 14 scrapes were positive for hrHPV (Table 1), and in addition scrapes 2, 4, 12, and 13 were positive for lrHPV. Multiple HPV genotypes were encountered in 11/14 (79%) cases.

<table>
<thead>
<tr>
<th>Case</th>
<th>Low-risk HPV</th>
<th>High-risk HPV</th>
<th>p16 positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>52, 69</td>
<td>Dyskeratotic cells</td>
</tr>
<tr>
<td>2</td>
<td>70</td>
<td>52, 69</td>
<td>Dyskeratotic cells</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>51</td>
<td>No p16 staining</td>
</tr>
<tr>
<td>4</td>
<td>70</td>
<td>66</td>
<td>No p16 staining</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>51, 66</td>
<td>No p16 staining</td>
</tr>
<tr>
<td>6</td>
<td>16, 56</td>
<td></td>
<td>CIN 1 cells</td>
</tr>
<tr>
<td>7</td>
<td>16, 56</td>
<td></td>
<td>CIN 1 cells and dyskeratotic cells</td>
</tr>
<tr>
<td>8</td>
<td>16, 51, 53</td>
<td></td>
<td>Metaplastic cells</td>
</tr>
<tr>
<td>9</td>
<td>16, 53, 66</td>
<td></td>
<td>Koliocytotic cells</td>
</tr>
<tr>
<td>10</td>
<td>16, 53</td>
<td></td>
<td>Metaplastic cells</td>
</tr>
<tr>
<td>11</td>
<td>16</td>
<td></td>
<td>CIN 3 cells and dyskeratotic cells</td>
</tr>
<tr>
<td>12</td>
<td>44</td>
<td>26, 52</td>
<td>CIN 3 cells</td>
</tr>
<tr>
<td>13</td>
<td>70</td>
<td>52</td>
<td>CIN 3 cells</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>58</td>
<td>CIN 3 cells and dyskeratotic cells</td>
</tr>
</tbody>
</table>

In Table 2 the cytologic diagnoses of the Papanicolaou-stained ThinPrep slide classified as ASCUS and as HSIL and the histology diagnoses of the Papanicolaou- and HE-stained Cellient paraffin slides are presented.

In three cases, metaplastic cells were observed in the paraffin Cellient sections, in seven cases koliocytosis, in one squamous pearls, and in seven dyskeratosis.

As many as five cases (1, 2, 7, 11, and 14) had p16 positive dyskeratocytes. Only in case 3, 4, and 5 no p16 staining cells were observed in the Cellient paraffin sections.

DISCUSSION

In the yogurt study performed, the cervical smear on which a Nugent scoring of the bacterial flora was performed (data not shown in the current paper) disclosed that eating yogurt did improve the vaginal microbiota over the study month.

Therefore, yogurt provides a safe nutritious food that can be made locally and taken daily by HIV-subjects receiving anti-retroviral therapy. It has the potential to transfer health benefits to the gut and vagina, but the extent to which a probiotic can add to
this through rectal to perineal transfer remains to be determined.

As the cervical samples of these HIV-positive women were available, HPV-PCR could be performed and HPV-positive samples could be selected for this pilot Cellient study.

The Cellient™ Automated Cell Block System was able to provide high quality histology and outstanding images obtained with the biomarkers p16, all this on cervical scrapes on which 22 HPV genotypes were established by PCR. Nuclear and cytoplasmic p16 positivity was observed in metaplastic cells, koilocytes, and dyskeratotic cells. Of the CIN 1 epithelial fragments, 10-30% of the cells was p16 positive, whilst in CIN 3 epithelial fragments over 50% stained positive. This is in accordance with our experience of p16 in Shandon cytoblock paraffin sections. Note that p16 positivity can also be observed in the cytohistology of adenocarcinoma of the endocervix [9].

It is well-known that HPV infection can induce a disturbance of the keratinization process leading to dyskeratosis. The Papanicolaou method (in which dye competition between light green and eosin is exploited) is well-suited to detect these dyskeratotic cells by their strikingly orange color (Figures 3 and 4), whilst others stain intensely green (Figure 5). To the best of our knowledge, this is the first report on the positive staining of benign dyskeratotic cells with the biomarker p16 (Figures 6 and 7).

It should be noted that the pyknotic nuclei of dyskeratotic cells can be rather large suggesting polyploidy as illustrated in Figure 5. Such dyskeratotic cells can have relatively large nuclei and accordingly be designated as atypical (see case 1, 2, and 3 and Figure 5). The biomarker p16 is used to detect over-expression of p16INK4a protein in cervical premalignant and malignant lesions [10-12]. Galgano et al. [13] conclude that immunocytochemical staining for p16 is a useful and reliable adjunct for the identification of CIN 2+. We can add that in the Cellient paraffin sections of cervical scrapes, the p16 positive staining dyskeratotic cells can be identified and classified according to their nuclear size and nuclear cytoplasmic ratio.

The cervical scrapes were fixed in the coagulant fixative BoonFix, of which polyethylene glycol (PEG) is an important component, mixed with ethyl alcohol. This fixative can be used for cervical scrapes [14] and for biopsies processed by microwave technology [15]. Note that the Cellient method is based on vacuum techniques and not on microwave technology as it is used on a large scale in the LCPL since 1987 [16,17]. This fixative does not contain formalin nor methanol, the latter being an important component of Preserve of Hologic. In the Hologic system using Preserve, it is advised not to do PCR within one month of cervical sampling. In contrast, when the cervical scrape is

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Table 2: Cytology of the ThinPrep Slide and Cytohistology of the Cellient Paraffin Sections

<table>
<thead>
<tr>
<th>Case</th>
<th>Cytology</th>
<th>Cytohistology</th>
<th>Cell types</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ASCUS</td>
<td>Atypia</td>
<td>Dyskeratosis and koilocytosis</td>
</tr>
<tr>
<td>2</td>
<td>ASCUS</td>
<td>Atypia</td>
<td>Dyskeratosis and koilocytosis</td>
</tr>
<tr>
<td>3</td>
<td>ASCUS</td>
<td>Atypia</td>
<td>Dyskeratosis and koilocytosis</td>
</tr>
<tr>
<td>4</td>
<td>ASCUS</td>
<td>Negative</td>
<td>Koilocytosis</td>
</tr>
<tr>
<td>5</td>
<td>ASCUS</td>
<td>Negative</td>
<td>Squamous pearls</td>
</tr>
<tr>
<td>6</td>
<td>ASCUS</td>
<td>CIN 1</td>
<td>CIN 1 fragments and dyskeratosis</td>
</tr>
<tr>
<td>7</td>
<td>ASCUS</td>
<td>CIN 1</td>
<td>CIN 1 fragments and metaplasia</td>
</tr>
<tr>
<td>8</td>
<td>ASCUS</td>
<td>Atypia</td>
<td>Dyskeratosis and metaplasia</td>
</tr>
<tr>
<td>9</td>
<td>ASCUS</td>
<td>Warty atypia</td>
<td>Koilocytosis</td>
</tr>
<tr>
<td>10</td>
<td>ASCUS</td>
<td>Negative</td>
<td>Metaplasia</td>
</tr>
<tr>
<td>11</td>
<td>HSIL</td>
<td>CIN 3</td>
<td>CIN 3 fragments and dyskeratosis</td>
</tr>
<tr>
<td>12</td>
<td>HSIL</td>
<td>CIN 3</td>
<td>CIN 3 fragments and koilocytosis</td>
</tr>
<tr>
<td>13</td>
<td>HSIL</td>
<td>CIN 3</td>
<td>CIN 3 fragments and koilocytosis</td>
</tr>
<tr>
<td>14</td>
<td>HSIL</td>
<td>CIN 3</td>
<td>CIN 3 fragments and dyskeratosis</td>
</tr>
</tbody>
</table>
suspended in BoonFix, PCR is possible on archival samples. In the study presented in this paper, the HPV PCR was done in Leiden one year after sampling in Tanzania.

In our Leiden screening practice we have encountered many carcinoma cases in which the ThinPrep cytology contains thick cell groups with blurry nuclei. Overall, this represents approximately 1% of all slides. Often, these slides also contain single atypical cells which raise the suspicion of significant changes in the cervical epithelium, but these blurred minibiopsies are not fit for an unequivocal diagnosis [18]. We observed that in the Cellient 4 μ thin paraffin sections carcinomatous tissue fragments can be studied in detail. Moreover, the architecture of the cytohistology is highlighted by the p16 immunostaining.

It is our experience that the brushes (Cervex-Brush Combi) used for cervical sampling, collect remarkable large carcinomatous tissue fragments. Nevertheless, care should be taken to dislodge all these valuable tissues from the brushes by placing the vials with the brushes in a shaker. Note that the Hologic protocol dictates the removal of the brush from the vial: if the clinician has not shaken all the cellular material from the brush, it ends up, with the brush, in the waste basket. The vials brought to Leiden from Tanzania contained the brushes, accordingly all sampled carcinomatous fragments of case 11, 12, 13, and 14 were rescued.

As far as the HPV genotypes are concerned, there were only three cases with one HPV genotype, 11 contained multiple HPV genotypes. Four scrapes had as many as three HPV genotypes. In the four CIN 3 cases, there was only one with HPV16.

In conclusion, in this pilot study of HIV-positive HPV-positive Tanzanian women, the Cellient system resulted in high quality histology with perfect p16 images.

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