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TECHNICAL NOTE

CRIMINALISTICS

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Efficacy of Several Candidate Protein Biomarkers in the Differentiation of Vaginal from Buccal Epithelial Cells*

ABSTRACT: Currently, there is no accurate method to differentiate vaginal epithelial cells from buccal epithelial cells in biological samples typically encountered in forensic casework. This study tested the expression of a selection of candidate proteins in buccal and vaginal epithelial cells. We investigated six candidate biomarkers, such as loricrin, vimentin, stratifin, cytokeratin 4, cytokeratin 13, small proline-rich protein 2, and involucrin, using Western blot analysis on whole protein extracts and immunohistochemistry (IHC) on intact cells in an attempt to identify cell-specific markers that would differentiate these cells by microscopy. Involucrin, loricrin, and stratifin showed differential expression during Western blot analysis and were carried through to IHC. Although proteins unique to vaginal epithelial cells and buccal epithelial cells were not identified from among the proteins tested, the increased expression levels of two proteins, loricrin and stratifin in vaginal cells, when compared to buccal cells, do provide encouraging results in the search for epithelial cell-specific markers.

KEYWORDS: forensic science, immunohistochemistry, Western blot analysis, epithelial cells, vaginal, buccal, biomarkers

The identification of the types of cells from which DNA originates can be of great importance in a forensic investigation. When parts of the body come in contact with an item, cells from the superficial layers of the epithelia; which can be skin, vaginal, or buccal in origin, may be transferred. DNA profiles from epithelial cells left behind at a crime scene can assist in the identification of the people involved, but there can be different evidential implications depending on whether the cells originate from skin, vagina, or the mouth. Epithelial cells from skin can be identified by microscopy owing to the prevalence of nonnucleated cells and their highly keratinized state (1). However, buccal and vaginal epithelial cells are morphologically indistinguishable when examined microscopically using standard histological staining methods.

Currently, there is no test available to determine whether epithelial cells are vaginal or buccal in origin. The Lugol's staining method is no longer regarded as specific for vaginal epithelia as subsequent studies have demonstrated that glycogen-containing epithelial cells can also be present in oral mucosa and male urethral mucosa (2–4). Previous studies have also investigated the differential expression of protein and carbohydrate markers but no unique marker has yet been identified (5–7). One recent study found that the application of a modified Danes stain showed different staining patterns for skin, buccal, and vaginal cells (8). However, there are likely to be limitations to the application of this method on forensic samples because of the difficulty of interpreting staining results from mixed cell samples. The results are encouraging, as they do provide a method of distinguishing vaginal cells from buccal and skin cells in unmixed samples. Furthermore, the staining patterns produced suggest that there are differences in the protein makeup of the various cell types.

Recently developed mRNA profiling methods can now assist in the identification of body fluids, for example vaginal fluid (through the use of markers for vaginal-specific bacteria) and saliva (via the expression of statherin and histatin) (9–11). However, mRNA profiling may not specifically associate a DNA profile with a particular set of cells as would be possible with a cell-specific marker that allowed the collection of a cell type by laser microdissection for DNA analysis.

This study investigates a selection of candidate biomarkers chosen from the literature with the potential to specifically label either terminally differentiated vaginal or buccal cells. The candidate biomarkers selected were first tested on whole protein extracts from buccal and vaginal cells of female participants by Western blot analysis using antibodies specific to the candidate protein. Any biomarker that was expressed in a significantly higher concentration in the whole protein content of either buccal or vaginal cells after Western blot analysis was then tested using immunohistochemistry (IHC). IHC enables *in situ* testing for protein biomarkers in cell samples and has been used previously in a forensic investigation to identify a specific cell type (12). IHC is compatible with DNA extraction and profiling methods used in routine forensic casework (13) so cells labeled by IHC can be specifically identified and separated from a cell mixture for downstream DNA analysis.

Methods and Materials

Collection of Cell Samples

Sterile rayon tip swabs (Biolab, Auckland, New Zealand) were used to collect superficial epithelial cell samples. Female

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volunteers, between 20 and 50 years of age, each provided self-collected samples swabbed from the inner cheeks and upper palate of the mouth and the internal wall of the vagina. Swabs were dried in a laminar flow cabinet and stored at room temperature for up to 1 week in paper envelopes until they were required for further study. Skin samples to be used as positive controls were self-collected by volunteers from patches of skin peeling after unintentional sunburn. Loose, peeling skin was collected using tweezers and stored in microfuge tubes at −20°C. All samples were collected according to guidelines approved by the University of Auckland Human Subjects Ethics Committee (reference #2004/217).

Epithelial Cell Protein Extraction

Epithelial cells were recovered from the swabs using a method that promoted loss of protein from the nonepithelial cell sources present in the body fluid but minimal loss of epithelial cell protein. Three to five swabs for each sample were soaked in 4 mL of 1× phosphate-buffered saline (PBS) and incubated at room temperature for 1 h on an orbital shaker at 60 rpm. Swab heads were squeezed with tweezers to maximize cell recovery, and then 4 mL of a 3:1 solution of Ficoll/PBS was underlaid beneath the resuspended cells. Cells were centrifuged through the Ficoll/PBS for 10 min at $400 \times g$, the supernatant discarded, and the cells resuspended in 3 mL of 1× PBS and centrifuged again to wash. This wash step was repeated. Depending on the amount of cells recovered, the final cell pellet was incubated with 50–200 μL of protein lysis buffer (8 M Urea, 3 M Thiourea, 2% amidosulfobetaine-14 [ASB-14], 1% dithiothreitol) for an hour at room temperature to extract the proteins. Any remaining debris was separated from the extraction by centrifugation for 5 min at $15{,}000 \times g$. An aliquot of the protein extract was quantified using Bradford's reagent (14), and the remaining whole protein extracts were stored at −80°C until such time as they were required for testing by Western blot analysis.

Western Blot Analysis

Whole protein extracts (5 μg total protein) from vaginal and buccal cell samples were separated by electrophoresis on 4–12% acrylamide 2-(*N*-morpholino) ethanesulfonic acid (MES) gels with MES running buffer (Invitrogen, Auckland, NZ), and then transferred to polyvinylidene fluoride (PVDF) membranes (Invitrogen) by Western blotting. Primary antibodies (from rabbit or mouse) specific to the protein of interest were used to detect the presence of the biomarker on the membrane. Anti-rabbit and anti-mouse Novex Western Breeze™ Chemiluminescent kits from Invitrogen were used to develop these membranes. These kits employ secondary antibodies conjugated to alkaline phosphatase that bind to any primary antibodies made within a particular species that are present on the PVDF membrane. The CPD-Star® chemiluminescent substrate used in the kit releases light when it reacts with the alkaline phosphatase enzyme conjugated to the secondary antibody. Biomarkers of interest were detected on the membrane using commercially available primary antibodies against the particular proteins; loricrin—AV41738 (Sigma-Aldrich, Auckland, NZ), involucrin—I1908 (Sigma-Aldrich), cytokeratin 4 (CK 4)—NCL-CK4 (Vision Biosciences, Melbourne, Australia), cytokeratin 13 (CK 13)—NCL-CK13 (Vision Biosciences), small proline-rich protein 2 (SPRR2)—ALX-210-901-R100 (Sapphire Biosciences, Hamilton, NZ), stratifin—WH0002810M1 (Sigma-Aldrich), and vimentin—AV48226 (Sigma-Aldrich) in combination with the solutions from the Western Breeze™ kit. A pan-cytokeratin polyclonal antibody raised to detect cytokeratins found within all epithelial tissues (18-0059; Invitrogen) was used as the positive control.

Chemiluminescent signals from the Western blots were detected by exposure to BioMax XAR X-ray film (Kodak, Auckland, NZ). The films were scanned to tiff files, and the relative levels of protein present were determined using the ImageJ analysis program (15). For each individual tested, the pixel intensity of each band was measured, and the background intensity was subtracted from each reading. The relative expression level of the biomarker was then normalized against the sample (buccal or vaginal) that provided the lowest pixel intensity in the band.

Immunohistochemistry

Epithelial cells from buccal and vaginal swabs were recovered as described previously for protein extraction, but the final cell pellet was resuspended in 100–200 μL of PBS (depending on the size of the pellet). A 10 μL aliquot of the cell suspension was spread onto a clean glass slide and dried at 37°C for 10 min. Skin slides were prepared by applying small pieces of skin peel (*c.* 2 mm^2) to clean glass slides. The slides were fixed by immersion for 1 min in a 50:50 solution of methanol/acetone and then dried in a laminar flow cabinet. Antigen retrieval was performed using a Digest-All proteinase kit (Invitrogen) following the manufacturer's instructions. The pepsin, trypsin, and ficin solutions from the Digest-All kit were tested with each of the primary antibodies being tested. Horseradish peroxidase-diaminobenzidine (HRP-DAB) IHC was carried out as described previously (7) using the EnVision™+ System-HRP (DAB) kit (DAKO, Global Science, Auckland, NZ). Slides were counterstained with Mayer's hematoxylin for 1 min before dehydration and sealing with permanent mountant and cover slips. Stained cells were visualized using a Leica DM1000 LED light microscope (Leica Microsystems, Wetzlar, Germany) and recorded using the Leica LAS EZ system.

Results and Discussion

Selected Biomarkers

The biomarkers selected in this study were loricrin, vimentin, stratifin, CK 4, CK 13, SPRR2, and involucrin. These proteins were selected as they have been reported to be specifically involved in the differentiation of stratified squamous epithelial layers. The cytokeratins are a large family of major cytoplasmic proteins found in human epithelia and are expressed in a pairwise manner to form heterodimeric intermediate filaments (16,17). CK 4 has been suggested as a biomarker for buccal cells (18), and CK 13 is its heterodimeric partner. Loricrin (19), involucrin (20), and vimentin (21) are also intermediate filaments found in stratified epithelia and are used as biomarkers for epithelial carcinomas of various types. Stratifin is a 14-3-3 protein that binds to receptors and is found predominantly in squamous epithelia in humans (22).

SPRR2 is also involved in differentiation of squamous epithelia as well as being linked to inflammation and defense (23). IHC on tissue biopsy samples has shown that the protein is expressed in the superficial layers of the buccal mucosa, but not the superficial layers of the skin or hard palate (20), and so it may be a candidate for differentiation of buccal cells.

Western Blot Analysis

Examination of the relative protein expression of the biomarker candidates by Western blot analysis on whole protein extracts from

superficial vaginal and buccal cell samples suggests that CK 13, stratifin, and loricrin are present in larger amounts in vaginal cells than in buccal cells (Fig. 1). The mean levels of expression of both SPRR2 and involucrin are higher in buccal than in vaginal cells, but a large amount of variation is observed between individuals with these two markers.

One-factor analysis of variance (ANOVA) was carried out in EXCEL™ to determine whether there was any statistical significance to the differences observed in the amounts of the protein candidates detected in the extracts of the two cell types. The analysis showed that there was no significant difference in the expression levels of the pan-cytokeratin ($F = 1.75$, p-value = 0.22, d.f. = 9), SPRR2 ($F = 1.49$, p-value = 0.27, d.f. = 7), involucrin ($F = 1.09$, p-value = 0.34, d.f. = 7), vimentin ($F = 1.932$, p-value = 0.213, d.f. = 7), or CK 4 ($F = 0.36$, p-value = 0.57, d.f. = 7) proteins. The ANOVA statistic gave indications of some difference in the expression of CK 13 between vaginal and buccal cells ($F = 4.99$, p-value = 0.056, d.f. = 9) with the protein expressed at a slightly higher level in vaginal cells and confirmed a significant increase in the expression levels of stratifin ($F = 46.32$, p-value = 1.37E-04, d.f. = 9) and loricrin ($F = 159.21$, p-value = 1.52 E-05, d.f. = 9) in vaginal cells compared to buccal cells.

After Western blot analysis, a subset of the candidate biomarkers was selected for further investigation using IHC. Loricrin and stratifin were selected as the Western blot analysis indicated these proteins were significantly more abundant in the vaginal cell samples tested. As the increase in expression of the CK 13 protein in vaginal cells was not shown to be statistically significant, this biomarker was not carried forward for testing at the IHC stage. Although none of the biomarkers tested at the Western blot analysis stage showed significantly more protein in buccal cells compared to vaginal cells, the large variation in the expression of involucrin within the buccal cells of the participants suggested that this protein might also benefit from further testing.

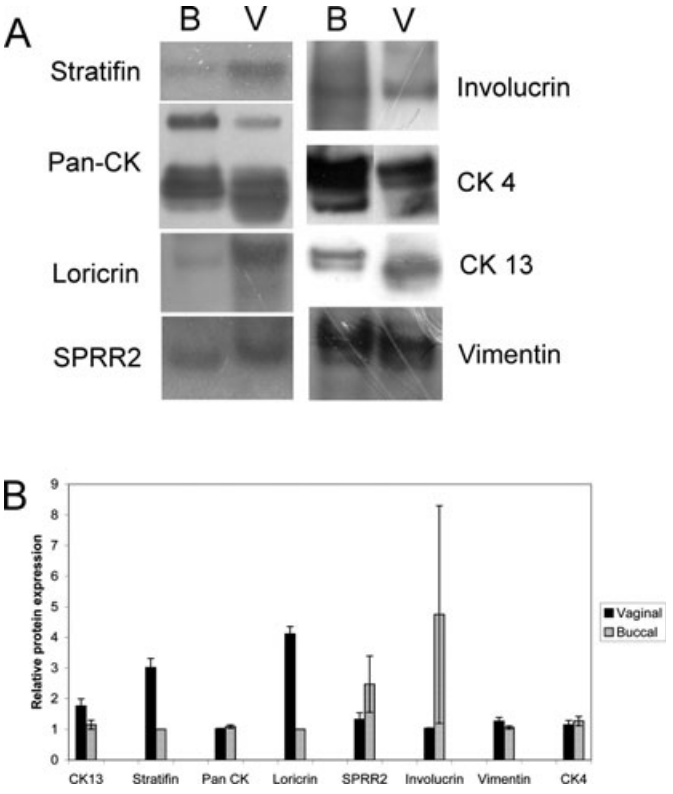

FIG. 1—*Western blot analysis to determine the protein expression levels of biomarker candidates. Representative images from scanned films are shown (A) and the relative expression level of the proteins in vaginal (V) and buccal (B) cells was calculated from pixel intensities provided by ImageJ analysis. Data presented in (B) are mean relative protein expression levels. Black bars indicate vaginal expression, and gray bars indicate buccal expression of the proteins, with error bars representing the standard error of the mean, $n \geq 4$.*

Immunohistochemistry

HRP-DAB IHC was carried out on glass slides containing smears of superficial cells of either buccal or vaginal origin from volunteers, as well as the skin tissue used as a positive control. Antigen retrieval was carried out on all of the slides examined including negative controls to improve binding of the antibodies to the proteins of interest. Antigen retrieval using pepsin, ficin, and trypsin was tested in combination with all of the primary antibodies (data not shown), and the combinations that provided the best staining results were carried out for the experiments with samples from multiple volunteers. Ficin antigen retrieval was used on samples with antibodies against involucrin. Trypsin antigen retrieval was used in combination with primary antibodies against loricrin, stratifin, and pan-cytokeratin, and the negative controls were carried out with both ficin and trypsin.

HRP-DAB immunochemistry produces a brown precipitate at the site of antibody binding to the relevant protein of interest. Both buccal and vaginal cells stained quite strongly with the antibody against involucrin (Fig. 2*C,D*). Although the pattern of staining seemed to differ slightly, with vaginal cells staining more darkly around the cell periphery, this is unlikely to be sufficient for use as a test in a forensic setting. Loricrin was detected at significantly higher levels in vaginal cells by Western blot analysis; however, during immunohistochemical detection, the staining pattern indicated that this overall increase is caused by strong staining in only a proportion of the vaginal cells (Fig. 2*F,G*). Stratifin was detected in a stronger and more evenly expressed manner in vaginal than in buccal cells (Fig. 2*I,J*), but given that it is also reported as being prevalent in skin cells (24) and stained the skin epithelial peel quite strongly (Fig. 2*K*), this may preclude its use in a forensic context.

It is important to note that the skin epithelial peel is a different sample type to the cell smears, consisting of multiple layers of highly keratinized, enucleated cells, as well occasional nucleated cells from the lower levels of the stratum corneum that have not been as fully differentiated. These nucleated cells can be seen to be stained in the skin peel when loricrin (Fig. *2F*) and involucrin (Fig. *2E*) were used as the biomarkers, while the fully differentiated, enucleated cells that would be typically collected from a forensic situation did not stain.

One of the technological gaps in forensic biology is the lack of a reliable method to differentiate epithelial cell types. This is particularly important when the cell type the DNA originates from will have considerable impact on the relevance of the biological evidence in a case. This study has focused on biomarkers potentially specific to the cell types themselves rather than to a body fluid, as use of biomarkers secreted into a fluid could, in some instances, result in the misassignment of a DNA profile to a cellular source when analyzing mixtures of cells, or simply mean that the profile could not be assigned to a cellular source. Laser microdissection is becoming increasingly used in forensic laboratories, as it enables separation of cell mixtures that can be difficult to interpret by traditional DNA analysis methods. One of the main advantages in using biomarkers specific to particular epithelial cell types is that they not only allow identification of the cell type during microscopy but

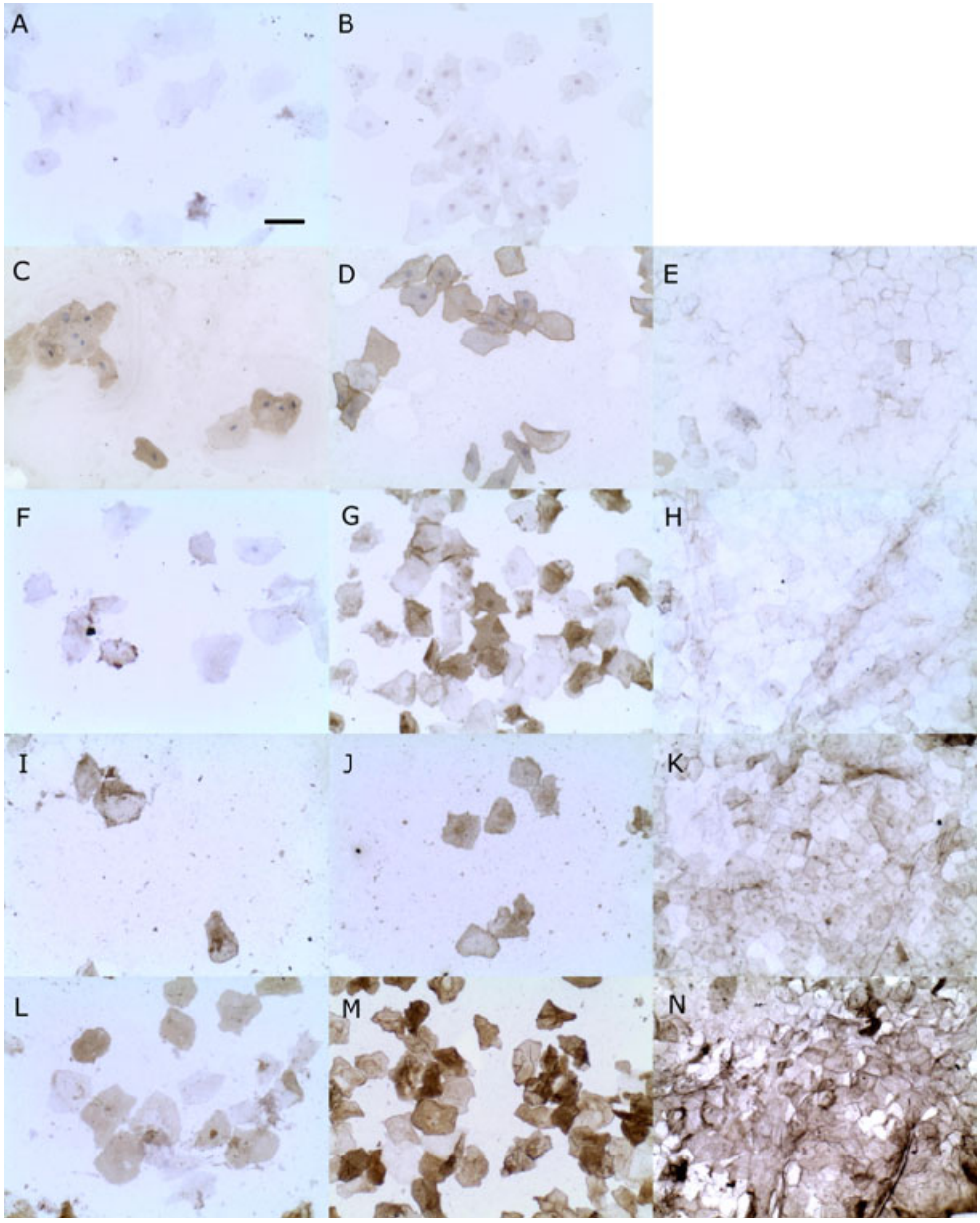

FIG. 2—*Representative images from immunohistochemical detection of candidate biomarkers in surface buccal (A, C, F, I, L) and vaginal (B, D, G, J, M) epithelial cells on glass slides, with skin peels used as positive controls (E, H, K, N). Bright field microscopy images produced using Leica LAS EZ system. Primary antibodies against involucrin (C–E), loricrin (F–H), stratifin (I–K), and pan-cytokeratin (L–N) were used in combination with the DAKO EnVision™+ detection kit. A and B are negative controls. Brown staining from the DAB precipitate indicates the presence of the candidate biomarker within the cell. The scale bar in A represents 50 μm and is the same for all images.*

also allow target cells to be collected by laser microdissection for subsequent DNA analysis.

The various IHC staining patterns that may be observed during testing of a putative biomarker are given in Fig. 3. The staining pattern in example A shows the best-case scenario, where the protein being tested has only been detected in one cell type, in this instance vaginal epithelial cells. DNA profiling results from stained cells can, in this scenario, be attributed to vaginal epithelial cells. However, the staining pattern in example C shows no specificity between the two cell types. In example B, although the protein has been detected in both cell types, it may still be possible to select and assign resultant DNA profiles as likely originating from vaginal epithelial cells to those cells that display heavy staining. The staining pattern for buccal and vaginal cells using loricrin as a biomarker in Fig. 2(*F,G*) strongly resembles example B. IHC using loricrin as the biomarker was carried out on cells contributed by further volunteers, and although there was still an increase in staining of a proportion of the vaginal cells within the sample (Fig. 4), the staining pattern was not as clear.

Conclusions

Buccal and vaginal epithelial tissues are very similar in structure and function; both being stratified, squamous, nonkeratinizing tissues with mucous membrane activity. Because of these similarities, it is perhaps not surprising that there are also many similarities in the patterns of protein expression between the two cell types. This similarity was evident in the results of Western blot analysis testing and immunohistochemical analysis of the group of selected

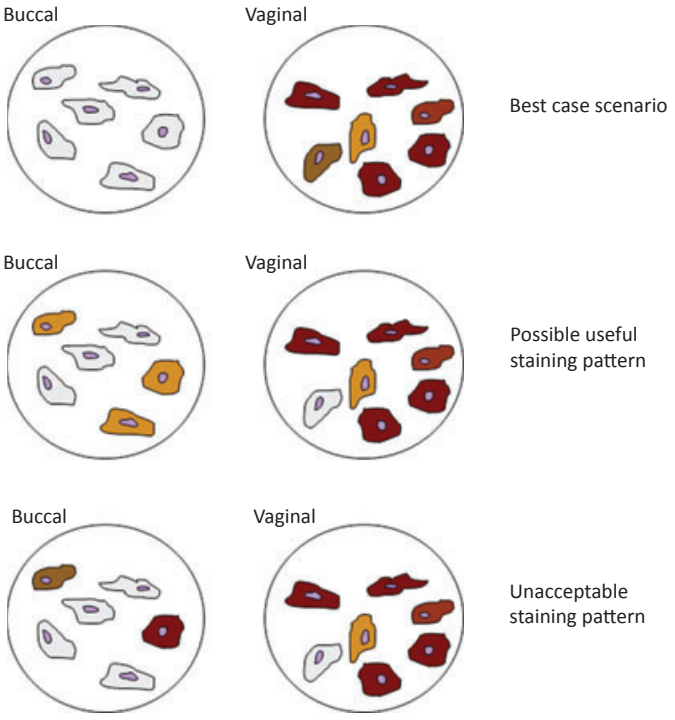

FIG. 3—*Possible staining patterns observed during validation of a vaginal immunohistochemical cell identification test. The dark shading indicates binding of the antibody and staining of the cells. In the best-case scenario (A), only the cells of interest (vaginal) stain with the test and buccal cells do not stain. An alternate staining pattern that could be acceptable (B) would be where most of the cells of interest stain strongly, some light staining might be observed in other (buccal) cell types, but only strongly staining cells are selected for DNA analysis. An unacceptable staining pattern (C) would be one where a few alternate cells (buccal) stained darkly and could not be differentiated from vaginal cells.*

biomarkers chosen in this study. Two of the biomarkers, stratifin and loricrin, showed promise at the Western blot analysis level as biomarkers that could be used to identify vaginal cells. However the stratifin biomarker did not show a pattern of staining at the IHC level that would enable its use as a marker for vaginal cells in a forensic laboratory setting. Loricrin showed promise at the immunohistochemical stage, in that at least a proportion of the vaginal cells stain in a manner that is distinct to that of buccal cells, but given the variability in the staining pattern observed between participants, it is unlikely to be suitable for use on cell mixtures in a forensic context. CK 4 has been previously suggested as a biomarker for buccal cells (18), but our data do not support this. While it may be able to reliably distinguish skin and buccal epithelial cells, our results at Western blot analysis indicate that its expression is not significantly different in vaginal and buccal cells and so it would be unable to differentiate between these two forensically important cell types. A biomarker specific to vaginal epithelial cells or buccal epithelial cells was not identified among those selected in this study. As described previously, the optimal IHC staining pattern for forensic use is one that is present in one cell type and absent in the other. Hence, we do not propose to investigate these markers further.

These results have now directed the search for vaginal and buccal epithelial cell markers using an alternative method of analysis. Proteomic studies to compare the whole protein content of vaginal and buccal epithelial cells will be undertaken in our laboratory, and regions of difference that are observed between the protein patterns on two-dimensional gels will be identified using mass spectrometry to select further candidate biomarkers. Antibodies to any proteins of interest will then be used to further test the specificity of the protein using Western blot analysis and IHC. Other factors such as possible population variability and stability of the protein biomarker over time will have to be considered. Although stability varies between different types of proteins, as is also the case with mRNA, some proteins have been shown to be stable in dried body fluid stains even while buried in soil (25) or exposed to high ambient storage temperatures (26). Identification of stable biomarkers specific to vaginal and buccal epithelial cells will be a valuable tool for the progression of forensic casework in the future.

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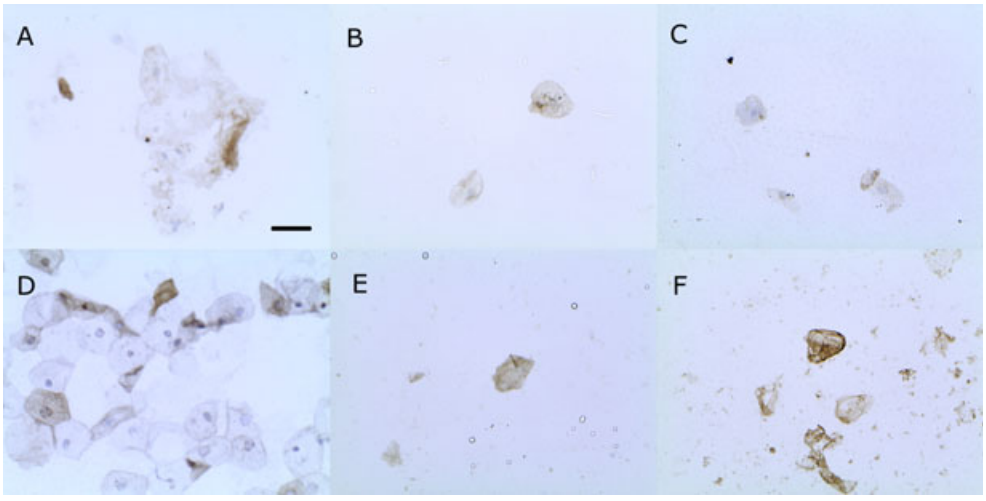

FIG. 4—*Loricin immunohistochemical staining of buccal (A–C) and vaginal (D–F) cells self-collected by three female volunteers. The cells of the first volunteer (A, D) show a similar pattern of staining of intact cells to that seen in Fig. 2, but although there is increased staining in the vaginal cells of the second (E) and third (F) volunteers, the pattern of staining is not as clear. The scale bar in A represents 50 μm and is the same for all images.*

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