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Research Article

Forensic discrimination of vaginal epithelia by DNA methylation analysis through pyrosequencing

The accurate identification of body fluids from crime scenes can aid in the discrimination between criminal and innocent intent. This research aimed to determine if the levels of DNA methylation in the locus PFN3A could be used to discriminate vaginal epithelia from other body fluids. In this work we bisulfite-modified and amplified DNA samples from blood, saliva, semen, and vaginal epithelia using primers for PFN3A. Through pyrosequencing we were able to show that vaginal epithelia present distinct methylation levels when compared to other body fluids. Mixtures of different body fluids present methylation values that correlate with single-source body fluid samples and the primers for PFN3A are specific for primates. This report successfully demonstrated that the analysis of methylation in the PFN3A locus can be used for vaginal epithelia discrimination in forensic samples.

Keywords:
DNA methylation / Epigenetics / Forensic science / Pyrosequencing / Vaginal epithelia
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1 Introduction

In forensic casework, determining the presence of vaginal secretions can aid in the distinction between innocent and sexual contact. The current method used in forensic laboratories to presumptively identify vaginal fluids depends on successful histological staining of the glycogen-rich vaginal cells by the Schiff reagent. However, the method's sensitivity depends on the reproductive age and menstrual cycle of the female and it easily gives false positives by unspecific staining of male buccal and urogenital cells [1]. More sensitive and reliable methods are those that rely on the quantification of certain RNAs as a product of cell-specific gene expression. Gene expression can occur at the transcription level which is quantifiable by the levels of messenger RNA (mRNA) present in a specific type of cells [2]; or at the post-transcription level that occurs through micro RNA (miRNA) presence in specific tissues [3]. The most successful reported methods that are able to quantify mRNA or miRNA include capillary electrophoresis [4–6], high-resolution melt analysis [7], and quantitative RT-PCR [8]. All three methods show promising results since they are automatable, easy to implement in a forensic laboratory, and sensitive. However, the use of RNA requires all consumables, water, and reagents to be RNase free and a postextraction treatment with DNaseI [4, 6, 8] to digest any residual DNA. Accidental release of such enzyme may cause degradation of casework samples in forensic laboratories. Regarding mRNA analysis, the quantification of tissue-specific mRNA transcripts for body fluid identification relies on the normalization with mRNA levels of housekeeping genes such as GAPDH [1, 9]. Methods currently available for RNA quantification do not discriminate between human and bacterial RNA, which is commonly present in most body fluids [1, 7]. Lack of a precise quantification of RNA in the sample can sometimes lead to lack of results [7].

Due to the aforementioned difficulties, we propose the analysis of different DNA methylation levels as a way to discriminate body fluids. DNA methylation is one of the epigenetic mechanisms for gene regulation in the human body. Levels of DNA methylation in certain loci are thought

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Abbreviations: **CG**, dinucleotide cytosine-guanine on the same strand of DNA; **IRB**, Institutional Review Board; **PFN3**, gene name. Profilin 3; **RNA**, Ribonucleic Acid; **tDMRs**, tissue-specific differentially methylated regions

Colour Online: See the article online to view Figs. 1–7 in colour.

to control gene expression by silencing or activating specific genes. The presence of a methyl group on the 5′ carbon of a cytosine belonging to the dinucleotide CG (CpG) is believed to prevent the binding of the transcription machinery in the promoter region of genes [10]. Some loci in the genome called tissue-specific differentially methylated regions (tDMRs) can therefore be used for body fluid discrimination [11–13] since they present different levels of DNA methylation depending on the cell studied [14, 15].

To determine the pattern of DNA methylation in a locus, the most commonly used methods include the bisulfite modification of genomic DNA that chemically converts the unmethylated cytosines to uracils but does not react with methylated cytosines. During the polymerase chain reaction (PCR) uracils are copied as thymines and the resultant amplicons can then be sequenced to determine the presence of a cytosine or a thymine at each specific CpG. One of the most commonly used methods for DNA methylation analysis is pyrosequencing because it allows individual quantification of CpGs and provides a percent methylation value displayed on a pyrogram created by the instrument software [16].

To successfully use pyrosequencing in forensic laboratories, more loci capable of discriminating several body fluids, including vaginal epithelia need to be identified. One study that aimed to uncover more loci was the work of Lee *et al*. (2010) where samples of blood, saliva, semen, menstrual blood, and vaginal epithelia were collected from volunteers. After extraction, the DNA from each body fluid was pooled, Sanger sequencing was performed and the results were compared to in silico converted sequence in order to obtain a comparison of methylation levels. The methylation map revealed that the tDMR named PFN3 presented an overall different DNA methylation level for vaginal fluid when compared to other body fluids [17].

To determine if this tDMR can be used to discriminate vaginal epithelia from crime scenes, we performed pyrosequencing on a subregion of PFN3–PFN3A. Contrary to the method used by Lee *et al*. (2010), each sample from each body fluid pyrosequenced originated from a single individual and no pooling of DNA was performed. The region selected for this study–PFN3A–includes only the CpGs that in the work of Lee *et al*. (2010) showed the highest methylation difference when compared to other body fluids. The aim of this work was to explore the potential of PFN3A to become a biomarker for vaginal fluid discrimination in forensic laboratories using pyrosequencing.

2 Materials and methods

Blood, buccal, vaginal swabs, and semen samples were collected from volunteers according to IRB-13-0555 from Florida International University and Pontifícia Universidade Católica do Rio Grande do Sul (CONEP #723.619/ CEP #845.747). Swabs were air-dried and DNA extraction was performed using the EZ1® DNA Investigator kit (Qiagen, CA, USA) and the BioRobot® EZ1 automated purification workstation (Qiagen, CA, USA) according to the manufacturer's specifications. Quantification was performed using the PicoGreen® method [18] (Life Technologies, CA, USA). Fifty nanograms of DNA were bisulfite modified using the EpiTect® Fast DNA Bisulfite Kit (Qiagen, CA, USA) according to manufacturer's instructions and using 20 μL of eluting buffer as a final volume for bisulfite-modified DNA. For the sensitivity studies, six vaginal epithelia samples were serial diluted to obtain 10, 5, and 1 ng of input genomic DNA for bisulfite conversion. For the mixture studies, DNA samples of blood, vaginal epithelia, and semen were quantified and mixed in different ratios of each two body fluids in order to have 100 ng total of genomic DNA input to the bisulfite modification reaction. The ratios and mixtures used were 75% blood to 25% vaginal epithelia or semen, 50% blood to 50% vaginal eptihelia or semen, 25% blood to 75% vaginal epithelia or semen and 75% vaginal epithelia to 25% semen, 50% vaginal epithelia to 50% semen, 25% vaginal epithelia to 75% semen. For the species-sensitivity studies, DNA from cat, dog, chicken, cow, mouse, bacterial pool (*Escherichia coli, Staphylococcus aureus, Enterococcus faecali,* and *Pseudomonas aeruginosa*), horse, gorilla, orangutan, and chimpanzee were collected and processed as described above. DNA from one sample of human vaginal epithelia was used as positive control.

Table 1. Sequence of PFN3A primers used in this study

Primers	Sequence
Forward	5′-GTG TAT AGT TTT GTT GAG GAT GTT TT-3′
Reverse[a)]	5′-ACA AAC ACA CCT TCC TAC AA–3′
Sequencing	5′-GTT TTG TTG AGG ATG TTT TT–3′

a) Biotinylated primer.

DNA amplification reactions were performed using the PyroMark® PCR kit (Qiagen, CA, USA) by adding 2 μL of bisulfite-modified DNA to each reaction and following manufacturer's instructions. Primers for PFN3A were designed using the PyroMark® Assay Design software version 2.0 (Qiagen, CA, USA) and are displayed in Table 1. The PCR products and a low molecular weight ladder (New England Biolabs, MA, USA) were run in 2% agarose gels, stained with ethidium bromide to confirm the presence and size of amplicons.

Pyrosequencing reactions were performed using a PyroMark® Q24 pyrosequencer (Qiagen) using 10 μL of PCR product and the protocol established by the manufacturer https://www.qiagen.com/pt/resources/download.aspx?id=59f0275d-e60f-4517-b786-b0e0ca13952e&lang=en [19]. The percent methylation for each individual CpG was automatically calculated by the PyroMark® Q24 software version 2.0.6 (Qiagen).

Statistical analysis was performed using SPSS version 22. The percent methylation was compared between body fluids for each CpG performing a one-way ANOVA and a Welch ANOVA followed by Tukey-HSD posthoc test. For the sensitivity studies, a dependent *t*-test was performed to compare the percent methylation of each CpG from each dilution of vaginal epithelial cells to the 50 ng group. A *p*-value

< 0.05 represents that the difference observed is significant for all statistical tests performed.

3 Results

We first aimed to determine if the DNA methylation levels in the PFN3A locus are different for vaginal epithelia when compared to blood, saliva, and semen. To address this question, we performed pyrosequencing of 8–12 DNA samples from each body fluid. These samples were treated using bisulfite modification and analyzed using pyrosequencing. The percent methylation values for each CpG from each body fluid was recorded and the arithmetic average and standard deviations calculated (Fig. 1). Statistical analysis was performed, and only the CpGs that had a $p < 0.05$ between vaginal epithelia and all other body fluids were reported. Our analysis showed that nine out of ten CpGs have an intermediate level of mean percent methylation for vaginal epithelia that is significantly different when compared to other body fluids. CpG9 presented a *p*-value greater than 0.05, indicating that methylation levels between blood and vaginal epithelia are more similar. As this particular CpG will not be useful to discriminate vaginal epithelia, its percent methylation value will not be displayed in any subsequent results.

Forensic evidence often contains low amounts of DNA that can hinder the ability to obtain reliable values for percent of methylation. In order to determine the sensitivity of our method, six DNA samples of vaginal epithelia were quantified and diluted to obtain inputs of 10, 5, and 1 ng to the bisulfite conversion reaction, followed by PCR amplification and pyrosequencing. The mean percent methylation of the diluted samples was then compared with those of samples where 50 ng of genomic DNA were used as input to bisulfite conversion. The results obtained (Fig. 2) show that for the 5 ng group only one CpG (CpG6) showed significant differences ($p < 0.05$) in the levels of methylation when compared to the 50 ng group. When the 1 ng group was compared to the 50 ng group, our results indicated that CpGs 2 and 3 also showed statistically significant ($p < 0.05$) differences in methylation levels. Moreover, all the pyrograms generated for the 1 ng group of samples produced warnings due to low peak height (as example, Supporting Data, Fig. 1). Such warnings are the result of a minimal peak height threshold set by the manufacturer (20 relative light units). In the future, this threshold may be lowered and validated, however since that is beyond the scope of this study, we consider the default threshold established by the manufacturer as a quality parameter of our data.

To further establish the minimal amount of genomic DNA that can be used to obtain reliable distinction of vaginal epithelia using pyrosequencing and the primers for PFN3A, we also compared the methylation percent values of the vaginal epithelial samples where 5 and 1 ng of genomic DNA were bisulfite-modified with that of DNA from blood, saliva, and semen where 50 ng was used for bisulfite conversion. The analysis of variance showed that for the 5 ng group all CpGs, including CpG6 present significant differences ($p < 0.05$) when compared to other body fluids (Fig. 3) and can therefore be used to distinguish vaginal epithelia. Contrary to this, for the 1 ng group, only three out of nine CpGs presented significant differences ($p < 0.05$) between vaginal epithelia and other body fluids. Due to all statistical analysis and low peak-height observed for the 1 ng group, we suggest a minimum of 5 ng of genomic DNA to be used for this method.

Another issue in forensic samples is that mixtures of body fluids are sometimes present in crime scenes samples. This work aimed to identify vaginal epithelia in samples collected from specimens that are part of cases of suspected sexual assault. For that reason we decided to ascertain how the methylation levels in the PFN3A locus change when different ratios of blood, vaginal epithelia, and semen are present in one sample. Three randomly selected DNA samples, one from each body fluid, were quantified for DNA methylation levels in the PFN3A locus and compared to the average percent methylation for each body fluid observed in Fig. 1. The same randomly selected DNA samples were mixed with each other in ratios of 75:25, 50:50, and 25:75 and their methylation levels assessed. Figure 4 shows that samples containing mixtures of blood and vaginal epithelia have methylation values intermediate to those of samples containing only blood or vaginal epithelia. Moreover there is a decrease in methylation percent as the ratio of blood to vaginal epithelia decreases. A similar correlation was also observed for the samples containing blood and semen (Fig. 5) as well as samples where vaginal epithelia and semen are mixed (Fig. 6).

To determine whether this method could also be hindered by the presence of non-human DNA, we tested DNA from cat, dog, chicken, cow, mouse, bacterial pool (*Escherichia coli, Staphylococcus aureus, Enterococcus faecali,* and *Pseudomonas aeruginosa*), horse, gorilla, orangutan, and chimpanzee. One human DNA sample was used as a positive control. Even though in the agarose gel samples from cat, cow, orangutan, and gorilla showed faint bands and only the human and chimpanzee showed bright bands (data not shown), all PCR products were pyrosequenced to confirm the presence/absence of peaks on the pyrograms. We believe that the presence of bands in the agarose gel in the nonprimate samples are the result of nonspecific amplification since 45 PCR cycles are necessary to amplify bisulfite-modified DNA. If that was the case, when pyrosequencing was performed, the sequence should not correspond to the expected sequence and the software should display red warnings.

The results showed that only the samples from human and chimpanzee present a pyrogram without any warnings recorded by the software (Fig. 7). For the samples of cat, cow, gorilla, and orangutan the pyrogram showed peaks that do not correspond to the expected DNA sequence (Fig. 7C), which means that the DNA sequenced was not specific for the PFN3A locus. All other samples, including that of DNA from bacterial pool (Fig. 7D), provided negative results with no peaks present in the pyrograms. The absence of peaks is expected since no bands were observed in the agarose gel for these samples.

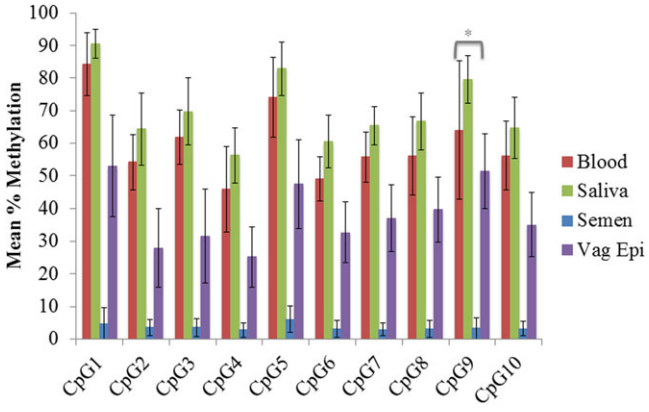

Figure 1. Graph showing mean percent of methylation for samples of blood ($n = 8$), saliva ($n = 11$), semen ($n = 12$), and vaginal epithelia ($n = 10$) with 50 ng input to bisulfite. * CpG where the difference in methylation levels is not statistically significant ($p < 0.05$) between vaginal epithelia and blood.

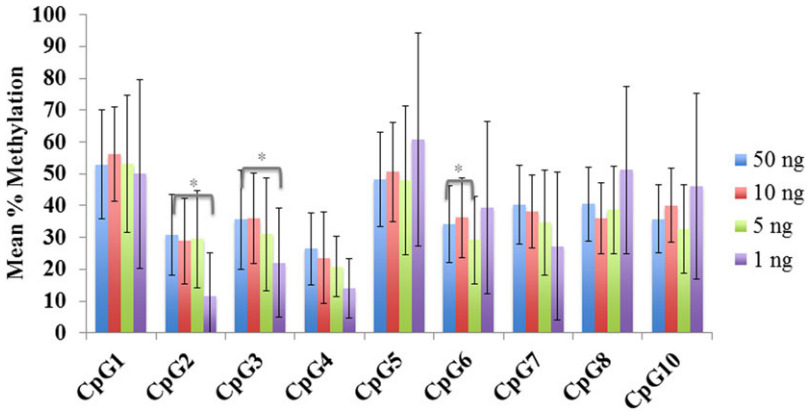

Figure 2. Graph showing mean percent of methylation for vaginal epithelia samples ($n = 6$) with different input of DNA. *CpGs and nanograms of DNA that present methylation levels with statistically significant differences ($p < 0.05$) as determined by a dependent *t*-test performed between groups.

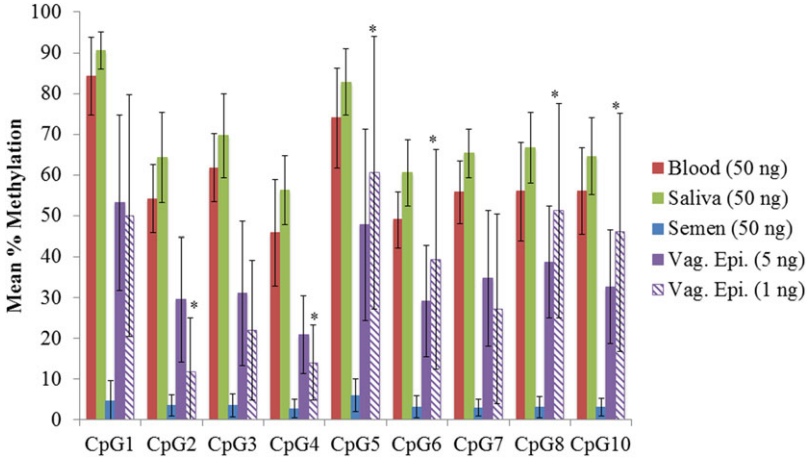

Figure 3. Graph showing mean percent of methylation for vaginal epithelia samples ($n = 6$) with input of 5 and 1 ng of genomic DNA to bisulfite conversion and blood ($n = 8$), saliva ($n = 11$), and semen ($n = 12$) with 50 ng of genomic DNA bisulfite modified. *CpGs where the difference in methylation percent between the 1 ng samples and the 50 ng ones is not statistically significant ($p < 0.05$), meaning that vaginal epithelia cannot be distinguished from other body fluids on these CpGs.

4 Discussion

The development of PCR-based methodologies for forensic identification of body fluids constitutes an important area of research in forensic serology. In order to accurately identify a body fluid left at a crime scene, methods developed for forensic application must have sufficient sensitivity to detect trace levels of degraded DNA as well as distinguish single-source from mixtures of body fluids, and be primate-specific. We explored a small region within the PFN3 tDMR previously described by Lee *et al.* (2010). By selecting the CpGs within the PFN3 tDMR that presented higher methylation differences between vaginal epithelia and other body fluids, we were able to quantify methylation for ten individual CpGs by designing

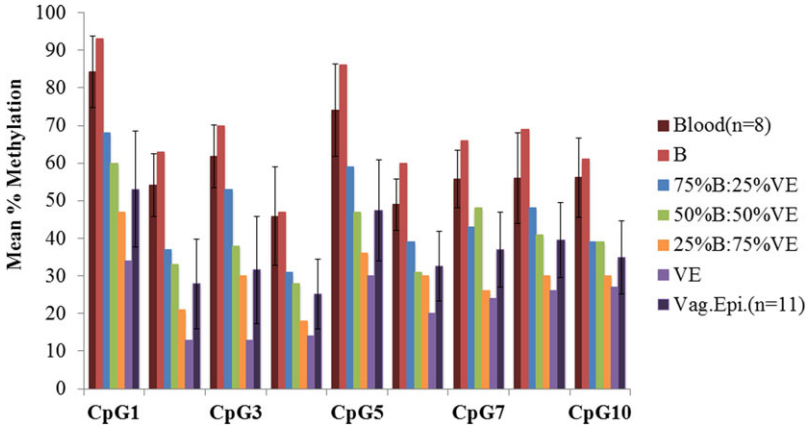

Figure 4. Mean percent of methylation for samples containing different ratios of DNA from blood (B) and vaginal epithelia (VE). With the decrease in the content of DNA from blood compared to vaginal epithelia, a decrease in the percent of methylation is also observed. The average percent methylation for the blood (n = 8) and vaginal epithelia (n = 11) samples are shown as control. The samples labeled B and VE are the DNA from blood and vaginal epithelia, respectively, used to make the mixture.

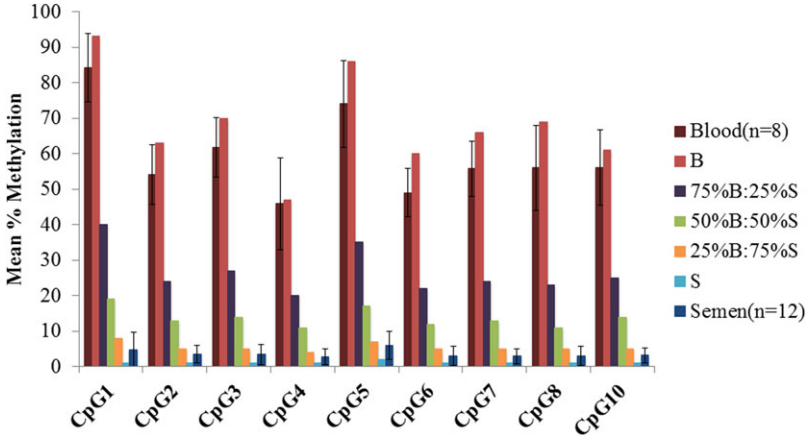

Figure 5. Mean percent of methylation for samples containing different ratios of DNA from blood (B) and semen (S). With the decrease in the content of DNA from blood compared to semen, a decrease in the percent of methylation is also observed. The average percent methylation for the blood (n = 8) and semen (n = 12) samples are shown as control. The samples labeled B and S are the DNA from blood and semen, respectively, used to make the mixture.

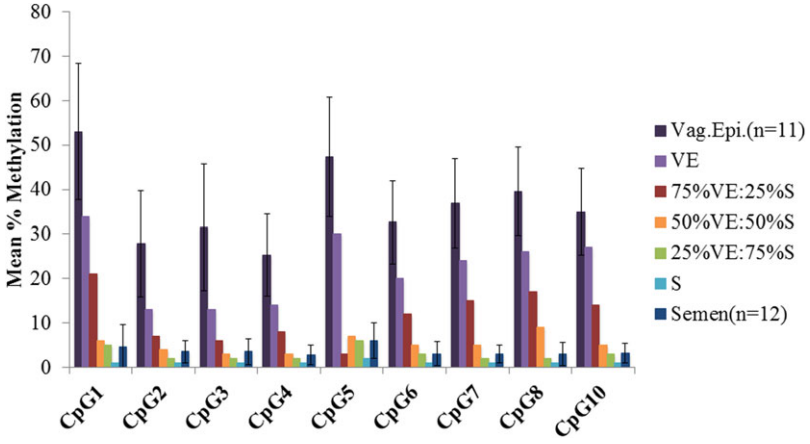

Figure 6. Mean percent of methylation for samples containing different ratios of DNA from vaginal epithelia (VE) and semen (S). With the decrease in the content of DNA from vaginal epithelia compared to semen, a decrease in the percent of methylation is also observed. The average percent of methylation for the vaginal epithelia (n = 11) and semen (n = 12) samples are shown as control. The samples labeled VE and S are the DNA from vaginal epithelia and semen, respectively, used to make the mixture.

primers specific for the PFN3A locus. Furthermore, we were able to prove that nine of those CpGs present significant differences ($p < 0.05$) in their methylation values for vaginal epithelia when compared to blood, saliva, and semen. Moreover we also showed that the average methylation percent values for vaginal epithelia range between 25 and 55% methylation for the nine CpGs sequenced. Similarly, two CpGs reported by Lee and colleagues (2015) [20] also present intermediate levels of methylation ranging from 40 to above 80% (for 11 vaginal samples analyzed by SNaPshot). Two other CpGs reported by Park *et al.* (2014) [21] present variation in methylation levels from 20 to above 80% (for 20 vaginal samples analyzed by pyrosequencing). All three reports demonstrate that vaginal epithelia present intermediate levels of methylation and not hyper- or hypomethylation. In our results we establish that PFN3A can be amplified and pyrosequenced using only 5 ng of genomic DNA without any indication of PCR bias. We do note that several CpGs should be analyzed

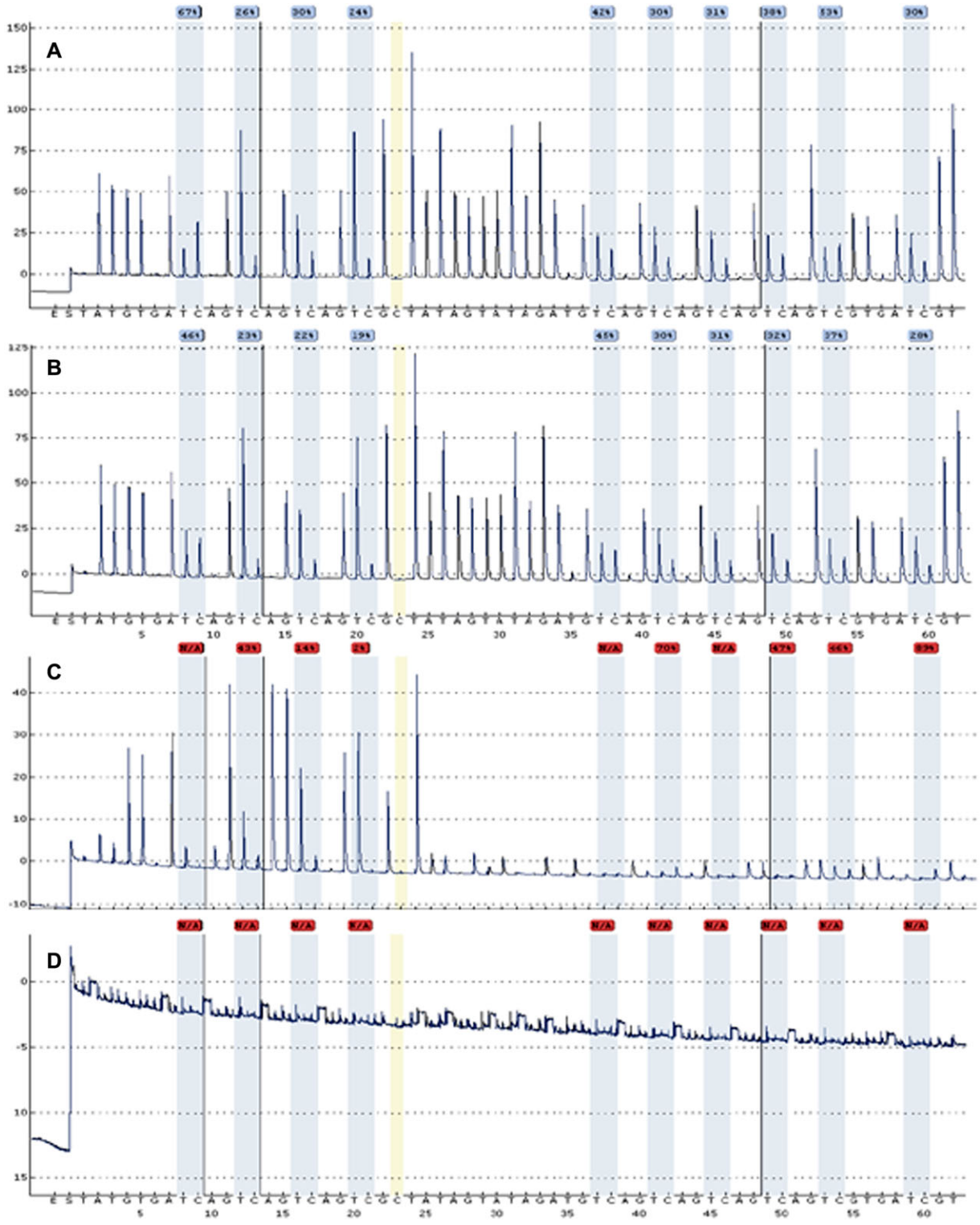

Figure 7. Pyrograms for the PFN3A locus showing DNA samples from (A) chimpanzee, (B) human, (C) cow, and (D) bacteria pool after pyrosequencing. Human and chimpanzee samples show the expected sequence and peak heights therefore the quality test imposed by the software was passed, as reflected by the blue squares showing the percent of methylation. Samples (C) and (D) show red squares either with a percent methylation value or N/A, which means that either the sequence or the peak height was not the expected by the software. The bacterial pool sample contains the following species: *Escherichia coli, Staphylococcus aureus, Enterococcus faecali,* and *Pseudomonas aeruginosa*. The pyrograms displayed for the DNA samples of dog, chicken, mice, and horse show absence of peaks similarly to this one.

in order to improve marker specificity and to overcome the variation observed between different samples from different donors. For that reason, and because PFN3A shows comparable results to those obtained by the aforementioned reports, the nine CpGs at this locus can further improve specificity in the discrimination of vaginal epithelia.

Regarding sensitivity, methods to analyze DNA methylation often require high amounts of starting material mostly due to the increase in DNA fragmentation that results from the bisulfite-modification process and the resultant loss of complementarity between the double-stranded DNA after bisulfite conversion. This process makes it difficult to design primers that produce efficient amplification of the template. For this reason, determining the sensitivity of methods that utilize DNA for body fluid identification is of paramount importance. To address this, we performed sensitivity studies in which 10, 5, and 1 ng of genomic DNA from vaginal epithelial cells were used for bisulfite conversion and subsequent analysis. We observed that when using only 1 ng of genomic DNA for bisulfite modification, the pyrograms resulted in peaks lower than the default threshold set by the software and used in this report as a data quality measure. Moreover when the percent methylation of the 1 ng group was compared to the 50 ng group of DNA from vaginal epithelial cells, only seven out of nine CpGs in the PFN3A locus presented similar methylation values. Furthermore, the variation observed for methylation values for this low level of DNA prevented vaginal epithelia forms being distinguished from other body fluids in six out of nine CpGs when comparisons are made between the 1 ng group of vaginal epithelia samples to the blood, semen, and saliva samples from the 50 ng group. Contrary to this, when a comparison was made between the 5 ng and 50 ng groups, distinction between vaginal epithelia, and other body fluids was possible in all nine CpGs. Therefore, we suggest a minimal quantity of at least 5 ng of genomic DNA as an input to bisulfite conversion prior to amplification and pyrosequencing. Future work on this project will involve a study of potential improvements to the efficiency of bisulfite conversion and amplification protocols.

For forensic purposes it is also important to estimate how much human genomic DNA is necessary to obtain reliable results so that it can be compared with current typing methodologies. The method used in this work to quantify genomic DNA (PicoGreen) is not specific for human DNA, therefore it is possible that the amounts reported here are overestimations since the samples may include nonhuman DNA especially in saliva and vaginal epithelia. Moreover, the DNA quantities reported here refer to genomic DNA added to bisulfite conversion and not amount of DNA added to PCR. Since no methods are currently available to accurately quantify bisulfite-modified DNA, we provide here an estimate with the sole purpose to clarify the significance of the reported sensitivity and to provide a rough comparison with DNA typing methods currently used in forensic laboratories. Even though the EpiTect Fast Bisulfite kit (Qiagen, Inc., CA) does not provide the user with an estimate of DNA recovery following bisulfite modification and cleanup, a study performed by Holmes and colleagues (2014) [22] determined that 73% of the DNA was recovered when using the EpiTect Fast Bisulfite kit (Qiagen). In such case, after the bisulfite modification of 5 ng of genomic DNA, we estimate that approximately 3.65 ng (73% of 5 ng) of bisulfite-modified DNA is present in 20 μL of elution buffer. That final solution will then have an estimated concentration of 0.18 ng/μL of bisulfite-modified DNA. To amplify the region PFN3A, we added 2 μL of 0.18 ng/μL to PCR, which corresponds to an estimated amount of 0.36 ng of bisulfite-modified DNA. For forensic typing methodologies, 1 ng is the optimal amount of genomic DNA to be added to PCR. Our study shows that we are able to obtain reliable quantification of methylation in the PFN3A locus using pyrosequencing for an estimated amount of 0.36 ng of bisulfite-modified DNA added to PCR.

Another characteristic of some forensic samples is that they are composed of a mixture of body fluids. We quantified the percent methylation of mixtures of three different body fluids in different ratios. Even though the levels of methylation correlate properly with the decrease or increase of one body fluid in comparison to the other, an unknown sample from a crime scene could still be mistakenly considered mixture or single source. It is our opinion that in order to properly deconvolute mixtures, more than one marker specific for each body fluid should be evaluated. Therefore, amplifying other markers like the ones described in the work of Silva *et al.* (2016) [23] may allow mixture deconvolution using pyrosequencing. However, the identification of more genome locations with better sensitivity would be useful. For example, genome locations that present either hyper- or hypomethylation, such as the case of the semen marker ZC3H12D [14, 15] show better specificity for single source samples and will aid in the identification of mixtures of body fluids.

Due to the potential for co-extraction of nonhuman DNA (mostly bacteria and/or yeast) from body fluid samples and also environmental exposure, we examined the primers for PFN3A to determine if they were able to amplify DNA from nonhuman sources. After running pyrosequencing reactions for all nonhuman DNA samples, we were able to observe three different types of results. Chimpanzee DNA provides a result similar to that of a human DNA sample which is most likely due to a similar genome sequence between the primates. The pyrograms for samples of cat, cow, gorilla, and orangutan present unspecific peaks leading the software to produce a very specific warning message declaring that the samples do not match the reference sequence. Pyrosequencing with respect to DNA methylation analysis requires the user to input the target sequence to analyze so that the software can automatically compare the peaks on the pyrogram with the expected peaks from the sequence to analyze. Comparing Fig. 7B and 7C one can see that the observed peak height for the tested sample does not correspond to the peak height of the expected sequence. This agrees with the notion that unspecific amplification may have occurred during PCR due to the large number of cycles required by this protocol. The third type of results obtained were pyrograms similar to that in Fig. 7D. These pyrograms correspond to samples where

either the amplification primers or the sequencing primers did not anneal, preventing amplification and/or sequencing.

Thus using specific primers for bisulfite-converted DNA, we can obtain amplicons that correspond to a genome region that are primate-specific. Forensic samples commonly contain DNA from other species, either due to exposure to the environment or due to the presence of several bacterial species that are part of the human microbiome. For that reason a method that uses nucleic acids for body fluid discrimination must be specific enough to tolerate the presence of nonhuman DNA or RNA. Our data showed that the locus PFN3A and the primers designed are specific for primates and do not provide pyrosequencing data for bacterial DNA. The fact that the chimpanzee DNA provided a pyrogram comparable to the human DNA is expected for primate-specific assays.

The results presented here demostrate the specificity of the PFN3A locus as a vaginal marker for forensic discrimination of body fluids. However, a full developmental validation should be performed prior to the use of this technology in forensic laboratories. This research provides the data necessary to demonstrate the potential of PFN3A methylation as a method for detection of vaginal epithelia by pyrosequencing.

4.1 Conclusion

This report demonstrates that the methylation status of the PFN3A locus can be used to successfully discriminate the presence of vaginal epithelia in extracted samples using as little as 5 ng of genomic DNA. Moreover this report also shows that methylation levels for mixtures of body fluids have intermediate values when compared to single source DNA, and that the primers designed for PFN3A are specific to humans and higher primates. The use of epigenetic methods such as this one for body fluid identification can simplify current sample analysis as serological tests can be performed directly on extracted DNA from casework samples. Overall, the results of this paper define specific levels of methylation for this locus over a range of CpG islands, making the work useful in sample identification and discrimination.

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