Epigenetic markers for identification of body fluids

Deborah Silva, Joana Antunes, Kuppareddi Balamurugan, Clarice Alho, Bruce McCord
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Identification of body fluids

Body fluids

• Important evidence recovered from crime scenes.

• DNA evidence identify a suspect or victim or exonerate an innocent person.

• Determine type and origin of a biological material reconstruct crime scenes.
Difficulties in identifying a body fluid: similarities to another fluid or substance hard to identify a spot in a scene with the naked eye

When the identity of a stain seems to be obvious

**Absolute confirmation** is necessary for the use of the evidence in court

Possible **occurrence of mixtures**: a stain may contain multiple bodily fluids from more than one donor.
Physical tests are used to **identify** a fluid or **confirm** the absence of one.

**Ideal test to detect body: specific to human, sensitive and robust.**

- **Presumptive** and **confirmatory** tests: developed for identification of bodily fluids.

  - **Presumptive tests:** screening tests, but tend to have **limited specificity.**
  
  - **Confirmatory tests:** absolute identification of tissues.
• Identification of a particular body fluid and its composition is very informative for the investigation.

• **Type of analysis is critical.**

The destructive nature of a screening test may be important when only a small amount of material is available.

• Many common techniques have been used for decades.

  Presumptive tests to identify heme in blood, acid phosphatase in semen, and amylase in saliva.
Disadvantages of using current identification tests:

• Destruction of the sample.

• Detection of a particular body fluid
  ▫ The investigator has to decide which test to perform based on the fluid that is most likely to be present.

• They are based on enzymatic or immunological assays.
  ▫ Dependent on the stability of the target molecules.
  ▫ Require the presence of the intact protein and may not work in environmentally degraded or damaged samples.
  ▫ Low specificity: cross-reaction with other molecular species or tissues.
Disadvantages of using current identification tests:

• Most commercial kits do not provide quantitative results.
  ▫ Indicates only the presence or absence of the tested material sample.

• Unable to quantify the contributions from multiple sources tissues.

• There is no statistical confidence associated with any outcome, positive or negative.

• There is more sample consumption to perform the tests.
• New methods developed → detection of specific mRNA.

Problems: RNA is **less stable** than DNA and requires **additional consumption of samples**.

• DNA ← ideal source for body fluid identification.

DNA provides quantitative results, it is more stable than RNA and no additional sample processing is needed.

Suggested procedure → Epigenetics
Epigenetics is the study of changes in gene expression unrelated to DNA base pairing.

- It includes the study of:
  - how patterns of expression are passed to offspring
  - how changes in gene expression occurs during differentiation of a cell type
  - how environmental factors can change the way genes are expressed
• Epigenetic changes:
  ▫ Play an important role in cellular differentiation process
  ▫ It is transmitted to the subsequent generation through mitosis and meiosis
  ▫ They are more frequent than Genetic changes
  ▫ Changes in chromatin and DNA are stable over many cell divisions
  ▫ Do not involve changes in the DNA sequence of the organism
Identical twins differ in their behavior and physiology.

- They may differ in susceptibility to infectious and degenerative diseases.

- Why is this? What are the reasons? Is the genotype not the same?

Epigenetic differences arise during the lifetime of monozygotic twins (Fraga et al. 2005)
Mechanisms of epigenetic changes

DNA methylation is an epigenetic modification involved in transcriptional regulation.

- Methyl residues bind to certain areas of the DNA chain.
• Typical mammalian genome:
  ▫ approximately 3 billion base pairs
  ▫ 40% are base pairs G:C

• CpG dinucleotides distribution is uneven, forming **CpG islands**.
  
  ➡️ Most of these islands are located near transcription start sites.

  ➡️ Methylation can occur at position C5 of cytosine in some CpG dinucleotides.

Source: http://www.ks.uiuc.edu/Research/methylation
• **5-methylcytosine** correlates with **cytosine** in the same way that **thymine** correlates with **uracil**.

Methylation is important in cell differentiation.

Genomic loci are differentially methylated between tissues.

Different methylation patterns between tissues and cells can provide the basis of an assay for body fluid identification.
Note differences occurring between sperm, keratinocytes (skin cells), and lymphocytes (white blood cells) (Eckhardt et al. 2006).
Measure Methylation

Step 1

- Extraction of samples

BioRobot® EZ1

EZ1® DNA Investigator kit
Step 2

- Conversion of unmethylated cytosines to uracils
- Methylated cytosine does not react

EpiTect® Fast DNA Bisulfite Kit
Allele 1 (methylated)  
---ACTCCACGG---TCCATCGCT---  
---TGAGGTGCC---AGGTAGCGA---  

Bisulfite treatment  
Alkylation  
Spontaneous denaturation  

---A\text{UT\text{U\text{U\text{A\text{U\text{G\text{G---T\text{U\text{U\text{A\text{T\text{C\text{G\text{T---}}}}}}}}}}}}}}  
---A\text{UT\text{U\text{U\text{A\text{U\text{G\text{G---T\text{U\text{U\text{A\text{T\text{G\text{U---}}}}}}}}}}}}}}  

---TGAGGTG\text{U\text{U---AGGTAGCGA---}}  
---TGAGGTG\text{U\text{U---AGGTAGUGA---}}  

Non-methylation-specific PCR  
Methylation-specific PCR  

Differentiation of bisulfite-generated polymorphisms  

Step 3

- Amplify target DNA methylation sites
- Specific amplification of bisulfite converted DNA
Step 4

Pyrosequencing

- DNA sequencing based on the ‘sequencing by synthesis’
- Detection of pyrophosphate release on nucleotide incorporation

PyroMark® Q24 Pyrosequencer
Data analysis

- Marker
  - Methylation % from pyrograms
  - Average
  - Results listed for each cell type.

- One-way ANOVA test: compare across cell types.

- \( P < 0.05 \).
Published epigenetic markers for identification of body fluids

Research Article

The determination of tissue-specific DNA methylation patterns in forensic biofluids using bisulfite modification and pyrosequencing

The goal of this study is to explore the application of epigenetic markers in the identification of biofluids that are commonly found at the crime scene. A series of genetic loci were examined in order to define epigenetic markers that display differential methylation patterns between blood, saliva, semen, and epithelial tissue. Among the different loci tested, we have identified a panel of markers, C20orf117, ZC3H12D, BCAS4, and FGF7, that can be used in the determination of these four tissue types. Since methylation modifications occur at cytosine bases that are immediately followed by guanine bases (CpG sites), methylation levels were measured at CpG sites spanning each marker. Up to 11 samples of each tissue type were collected and subjected to bisulfite modification to convert unmethylated CpG-associated cytosine bases to thymine bases. The bisulfite
<table>
<thead>
<tr>
<th>Markers</th>
<th>PCR and sequencing primers</th>
<th>Identification of body fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>C20orf117</td>
<td>Not informed*</td>
<td>Blood</td>
</tr>
<tr>
<td>ZC3H12D</td>
<td>Not informed*</td>
<td>Semen</td>
</tr>
</tbody>
</table>
| BCAS4   | Forward PCR– AGTGGGTGAGGTTGTGAAATGT  
Reverso PCR– CCATCCTACTAAAACATCTAATT  
Forward Sequencing Primer– AGTTTTTTGGTGAAAGTTTAT  
Forward PCR– GGTTTATATGGTATTATTTGGGTGGT  
Reverse PCR– ATTATATACTCCTCAAACACACAC  
Forward sequencing primer– TATATACTCCTCAAACAC | Saliva                       |
| FGF7    |                             | Semen                       |
C20orf117_hypermethylated in blood

![Mean % Methylation Graph](image)

- **CpG site**
  - CpG 1
  - CpG 2
  - CpG 3
  - CpG 4
  - CpG 5
  - CpG 6
  - CpG 7

- **Samples**
  - Blood
  - Saliva
  - Sperm
ZC3H12D_hypomethylated in sperm

Mean % Methylation

CpG Site

Blood  Saliva  Sperm

CpG 1  CpG 2  CpG 3  CpG 4  CpG 5
FGF7_hypermethylated in sperm

Mean % Methylation

CpG Site

Blood  Saliva  Sperm
Bcas4_hypermethylated in saliva

Mean % Methylation

Blood  Saliva  Sperm

CpG Site

CpG 1  CpG 2  CpG 3  CpG 4  CpG 5  CpG 6  CpG 7

CpG 1  CpG 2  CpG 3  CpG 4  CpG 5  CpG 6  CpG 7
To ensure the efficiency of these epigenetic markers, developmental validation studies need to be performed to determine the conditions and limitations of this new tool for forensic analysis.
“The ability to detect genetic information from non-targeted species should be determined. The detection of genetic information from non-targeted species does not necessarily invalidate the use of the assay, but may help define the limits of the assay.”
<table>
<thead>
<tr>
<th></th>
<th>BCAS4</th>
<th>ZC3H12D</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Positive control</td>
<td>Positive control</td>
</tr>
<tr>
<td>2</td>
<td>Negative control</td>
<td>Negative control</td>
</tr>
<tr>
<td>3</td>
<td>Positive control</td>
<td>Positive control</td>
</tr>
<tr>
<td>4</td>
<td>Negative control</td>
<td>Negative control</td>
</tr>
</tbody>
</table>

1- Dog saliva
2- Cat saliva
3- Cat blood
4- Dog blood
5- Chicken
6- Cow
7- Mice
8- Bacteria
9- Horse
PC- Human blood
Sensitivity studies

“The ability to obtain reliable results from a range of DNA quantities should be evaluated.”
• DNA input of **500 ng** → good results

• Purpose: **lower the DNA yield** while keeping the same quality in results.

• A saliva sample was processed five separate times.
  
  ▫ The concentration of DNA subjected to bisulfite modification was varied.
- Marker: ZC3H12D
- Sample: saliva

DNA input 385 ng
DNA input 100 ng
DNA input 50 ng
DNA input 10 ng
DNA input 5 ng
Stability studies

“The ability to obtain results from DNA recovered from biological samples deposited on various substrates and subjected to various environmental and chemical insults should be evaluated.”
Inhibition study:

- Final inhibitors concentrations: hematin (0.08 mM) and humic acid (0.24 mg/mL).
- Marker: ZC3H12D
- Sample: saliva

- Published data
- Humic acid added before bisulfite conversion
- Hematin added before bisulfite conversion
• Bisulfite modification kit: bisulfite conversion and cleanup of DNA for methylation analysis.

  Inhibitors are washed away in the washing steps for the bisulfite modification.
- Humic acid added after bisulfite conversion
• Hematin added after bisulfite conversion
• Negative control
Degradation study:

• Samples were heated at 95°C for 10, 15, 20, and 25 min.
- Marker: ZC3H12D
- Sample: saliva
- Marker: FGF7
- Sample: saliva

Published data
- 10 min
- 15 min
- 20 min
- 25 min

CpG2 | CpG3 | CpG4 | CpG5 | CpG6 | CpG7 | CpG8
Aged samples:

- Blood in FTA for 9 yrs has same DNA methylation.
ZC3H12D

Mean % Methylation

CpG sites

FGF7

Mean % Methylation

CpG sites

Regular samples
Blood in FTA paper
• 20-year old samples
Mixture studies

“The ability to obtain reliable results from mixed-source samples should be determined. These studies will assist the laboratory to establish guidelines for mixture interpretation, which may include determination of the number of contributors to the mixture, determination of the major and minor contributor profiles, and contributor ratios or proportions.”
- Marker: ZC3H12D
- Samples: blood and sperm
Conclusion/overview

- Epigenetic markers have a promising application in forensic casework.

- The ability to identify the biological sample using DNA would be a powerful tool.

- Body fluids recovered from crime scenes can provide crucial information.

- Results obtained so far indicate a good perspective for the overall validation of the tested markers.

Forensic markers for the identification of cell type will provide the community with new and improved methods to interpret the crime scene.
Acknowledgements

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Thank you