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The use of phosphate buffered saline for the recovery of cells and spermatozoa from swabs

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The use of phosphate buffered saline for the recovery of cells and spermatozoa from swabs

### Abstract

In the forensic science laboratory, the recovery of spermatozoa from vaginal swabs, or vaginal cells from penile swabs, can help determine if sexual intercourse may have taken place. There are several methods used to recover spermatozoa and cells from the swabs before visualisation on a microscope slide and most of these methods use water. Phosphate buffered saline (PBS) is a non-toxic solution used in many biological laboratories. Unlike water, PBS prevents cells rupturing or shrivelling up due to osmosis. This study demonstrates that PBS can be used for the extraction of spermatozoa and cells from swabs and that PBS does not affect subsequent DNA profiling.

## Introduction

Rape cases are the most common type of sexual assault received by the Lothian and Borders Police Forensic Science Laboratory. In the majority of cases, vaginal swabs will be examined for the presence of spermatozoa in order to determine whether vaginal intercourse has taken place. However, penile swabs can also be examined for the presence of vaginal epithelial cells, which can also be transferred in this case. Subsequent DNA profiling of these cells can then be carried out to determine the possible donor of these cells.

The current method for the visualisation of spermatozoa or vaginal cells from swabs, utilised by the laboratory, involves the use of water, agitation and centrifugation to remove the cells from the swab. The pelleted cells can then be visualised on slides using high power microscopy. This method was developed historically, primarily for the extraction of spermatozoa from swabs. However, in cases where there are no spermatozoa found on the vaginal swabs, it is imperative to examine the penile swabs for the presence of vaginal epithelial

cells. The number of epithelial cells recovered from swabs is usually very low. This could be due to a number of factors; the transfer of vaginal cells to the penis may be low, the penis may have been washed since the incident removing many of the transferred cells, cells may be lost due to contact with clothing or the method used to recover cells from the swabs may disrupt the cells. It is well known that when the water concentration around cells is unbalanced, cells burst or shrivel up due to osmosis. If cells burst during the swab extraction process, DNA could be released into the supernatant, which is not routinely submitted for DNA profiling. In cases where there are very few cells to begin with, it is therefore very important to preserve these cells, thereby retaining the DNA for subsequent profiling.

PBS is a salt solution containing sodium chloride, sodium phosphate and potassium phosphate, which balances the salt concentration around cells, preventing osmosis. PBS and other buffered solutions are routinely used in biological laboratories for washing cells, for immunohistochemistry and for other cellular techniques [1-3].

This study set out to determine if PBS could replace water in the extraction of spermatozoa and cells from swabs by comparing the appearance and number of intact cells and to demonstrate that PBS has no detrimental effect on subsequent DNA profiles obtained using SGM plus.

## Experimental Method

A summary of the experimental method used in this study can be found in appendix 1.

## Samples

Vaginal swabs were obtained from three female volunteers from within the laboratory. Duplicate swabs (Sterilin, UK) were collected from each of the volunteers over three days. Semen samples were donated from three different male volunteers within the laboratory. The semen samples were diluted 1 in 100 with water or PBS and three sets of duplicate swabs were prepared from each diluted sample. Permission was obtained from one of the female volunteers to publish the DNA profile of the swabs extracted in water and PBS.

## Swab extraction and preparation of slides

Swabs were removed and placed into dolphin tubes with spin insert (Corning, MA). PBS (Sigma Aldrich, UK, 200 $\mu$ l) or distilled water (200 $\mu$ l) was added to each tube and the swabs were agitated with a cuvette stirring rod (Kartell, USA). A further 200 $\mu$ l of PBS or water was added to each tube and pummelled, as before. The tubes were then centrifuged at 20,000g for 60 seconds. This process was then repeated by adding the supernatant back through the swabs. The supernatant was removed and tested for the presence of acid phosphatase (AP) using the AP test and for blood using the Kastle-Meyer (KM) test [4-5]. For vaginal cells, the pellet was resuspended in 5 $\mu$ l of PBS or water and a 1 in 10 dilution was made. For semen samples, the pellet was resuspended in 10 $\mu$ l of PBS or water. The optimum dilution of cells and semen for counting was determined prior to this experiment, data not shown.

Each sample (5 $\mu$ l) was spotted onto a clean slide. Samples were dried and fixed by flaming. The slides were stained with Haematoxylin and Eosin (H&E) (VWR, UK) and mounted using XAM (VWR, UK). The number of spermatozoa or intact cells was counted by eye using a high power microscope (Leica, UK, magnification x500) and a graticule (16mm, Agar Scientific, UK). For all slides, all fields were counted.

## AP and KM tests

A few drops of the supernatant from each sample were placed onto separate filter papers and tested for the presence of AP using the AP test or blood using the KM test [4-5]. A positive AP test was determined by the presence of a purple colour after 2 mins.

## DNA analysis

### Extraction of DNA

Cells and spermatozoa were separated using the preferential lysis method as previously described [6].

#### (i) DNA extraction from spermatozoa

DNA was then extracted from the spermatozoa fractions using QIAamp Mini DNA extraction kit (Qiagen, UK). The spermatozoa pellet was resuspended in 500  $\mu$ l of pre – warmed QIAamp ATL buffer (Qiagen, UK) and 50  $\mu$ l of QIAamp Proteinase K (10mg/ml) (Qiagen, UK). Samples were mixed gently, incubated at 56°C for 45 mins and centrifuged at 20,000g for 3 mins. The supernatant was removed and discarded. The pellet was resuspended in 500  $\mu$ l of pre – warmed QIAamp ATL buffer (Qiagen,UK) and centrifuged again at 20, 000g for a further 3 mins. Again the supernatant was removed and discarded. The final pellet

was resuspended in 180  $\mu$ l of pre – warmed QIAamp ATL buffer (Qiagen, UK), mixed and centrifuged briefly, before incubating at 85°C for 10 – 15 mins. QIAamp Proteinase K (10mg/ml, 20 $\mu$ l) (Qiagen,UK) and Dithiothreitol (DTT, 1M, 20  $\mu$ l) were added to each sample, mixed, and centrifuged briefly. Samples were incubated at 56°C for 1 hr with occasional mixing. Samples were centrifuged briefly, and 180  $\mu$ l of pre – warmed QIAamp AL buffer (Qiagen, UK) added to each sample and mixed. Ethanol (100%), equal to half the current sample volume was added to each sample, mixed and centrifuged briefly.

(ii) DNA extraction from vaginal epithelial cells

DNA was then extracted from the cellular fractions using QIAamp Mini DNA extraction kit (Qiagen, UK). QIAamp Proteinase K (10mg/ml, 20 $\mu$ l) (Qiagen, UK) was added to each sample. Samples were then mixed gently before being incubated at 56°C for 45 mins. Samples were centrifuged briefly and pre – warmed QIAamp AL buffer (180  $\mu$ l) (Qiagen, UK) added to each sample, mixed and incubated at 56°C for 10 – 15 min. Ethanol (100%), equal to half the current sample volume was added to each sample, mixed and centrifuged briefly.

Both semen and vaginal samples were then transferred to a labelled QIAamp column (Qiagen, UK) placed in a 2 ml collection tube (Qiagen, UK) and spun at 6,800g for 1 min at RT. The used collection tubes were discarded and the

columns placed in a fresh 2 ml collection tubes. Columns were washed with 500  $\mu$ l of QIAamp AW1 Buffer (Qiagen, UK) and centrifuged at 6,800g for 1 min. The used collection tubes were discarded and the columns placed in fresh 2 ml collection tubes. Columns were washed again with QIAamp AW2 buffer (500  $\mu$ l) (Qiagen,UK) centrifuged at 20,000g for 3 mins. The used collection tubes were discarded and the columns transferred to a 1.5 ml labelled safelock tube (Eppendorf, UK). Samples were eluted from the columns with pre – warmed, sterile distilled water (50 – 100  $\mu$ l) and incubated for 5 mins at RT.

A final elution volume (ranging from 50 – 100  $\mu$ l) of pre –warmed sterile distilled water (Millipore, UK) was added to each column, incubated for 5 – 10 mins at RT and centrifuged at 6,000g for 1 min. The columns were discarded and DNA extracts stored at –20°C.

### DNA quantitation

The quantity of DNA was determined using the quantiblot or pico green systems. The choice of method depended on the method used routinely in the laboratory at that time.

DNA samples were quantitated using the QuantiBlot™ Human DNA Quantitation kit (PE Applied Biosystems, UK) with the chemiluminescent detection method (ECL reagent, Amersham, UK) according to the manufacturer's recommendation [7].

Picogreen<sup>®</sup> quantification was performed using Fluoroskan Ascent apparatus and associated software, by comparison of known standards of DNA [8].

### PCR amplification

Samples were amplified using the AMPFISTR<sup>®</sup> SGM Plus<sup>™</sup> PCR amplification Kit (P/N 4307133, PE Applied Biosystems, UK) containing locus specific 5 – FAM, JOE and NED – labelled and unlabelled primers in buffer, that amplify the following ten STR loci [9]: D3S1358 (D3), HUMVWF31 (VWA), D16S539 (D16), D2S1338 (D2), D8S1179 (D8), D21S11 (D21), D18S51 (D18), D19S433 (D19), HUMTH01 (TH01), and HUMFIBRA (FGA) and the sex determinant marker, Amelogenin.

PCR master mix was prepared containing AmpFISTR<sup>®</sup> PCR reaction mix (MgCl<sub>2</sub>, dATP, dGTP, dCTP, and dTTP, Bovine Serum Albumin (BSA), and 0.05% sodium azide in buffer and salt), AmpFISTR<sup>®</sup> SGM Plus<sup>™</sup> Primer set, and AmpliTaq Gold<sup>®</sup> DNA polymerase (5U/μl), then 15 μl aliquoted into 0.2 ml thin walled PCR tubes (Treffclear, Anachem, UK).

Each DNA sample (10μl) was amplified in a total reaction volume of 25 μl.

Samples were amplified in 0.2 ml thin – walled PCR tubes without mineral oil on 9700 thermal cycler (PE Applied Biosystems, UK) following standard PCR conditions as recommended by the manufacturer (95°C for 11 min, 28 cycles of

94°C for 1 min, 59°C for 1 min and 72°C for 1 min, 60°C extension for 45 min, hold at 25°C).

A positive PCR control (AMPFISTR® control DNA 007 (0.1 ng/μl)) (See Appendix DNA type) and a negative PCR control (Sterile distilled water (Alpha Q System, Millipore, UK) added in place of DNA) were included in each PCR run.

### Electrophoresis

Amplified PCR products were run on a 5% polyacrylamide denaturing gel (molar concentration 18g Urea (Amresco, UK), 0.1% Amberlite (AG 501 – X 8, Biorad), 5% Longranger acrylamide (50% stock solution) (Cambrex, UK), 1X TBE buffer (Amresco, UK), 30ml deionised water (BDH)) and run for 2.5 hours using 1 X TBE buffer (89mM Tris, 89mM borate, 2 mM EDTA (Amresco, UK)) using an automated DNA sequencer (ABI Prism 377 DNA sequencer, PE Applied Biosystems, UK).

Amplified PCR product (1μl) was added to an equal volume of formamide – loading buffer suspension (FLS; 100 μl Formamide (Sigma Aldrich, UK), 20μl loading buffer (blue dextran, 50mg/ml; EDTA, 25mM)) and mixed with GeneScan – 500 ROX internal size standard labelled with the dye ROX (Applied Biosystems, UK). GeneScan – 500 ROX was used as an internal lane

standard, designed to size DNA fragment ranging from 35 bp to 500 bp. It provided 16 single – stranded DNA fragments of 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490, 500, a single peak of each observed for each fragment, allowing high precision sizing of DNA fragments.

The samples were then heat denatured on a 9700 thermal cycler (PE Applied Biosystems, UK) for 2 minutes at 95°C and snap cooled in a cooling block (Anachem, UK) before being loaded onto a 36-lane gel.

AmpFLSTR® SGM Plus™ Allelic ladder, containing amplified alleles for all loci, was included with the AMPFISTR® SGM Plus™ kit (PE Applied Biosystem, UK) and was run in two lanes (in one odd and one even).

Following electrophoresis, the data was collected using ABI Prism Collection software (v 2.1, PE Applied Biosystems, UK).

### Analysis and Interpretation

AMPFLSTR SGM Plus DNA profiles were obtained as previously described [9] using GeneScan® software v 3.1.2 (PE Applied Biosystems, UK) and Genotyper® v 2.5 (PE Applied Biosystems, UK).

### Statistics

Using a paired t-test, the differences in the number of cells recovered between water and PBS were not significant ( $t=-1.717$ ,  $df=17$ ,  $p=0.1041$ ) when no other factors were taken into account. However, the power of the test was only 0.37 at 95% confidence. It is estimated that approximately 85 pairs would have to be used to raise the power to beyond 0.95.

Two way analysis of variance revealed no evidence for any significant effect for type of cell, or occasion, or the interaction between cell type and occasion on the numbers of cells recovered using PBS or water.

## Results

### Effect of PBS on H&E staining

There was no visible difference in H&E staining of spermatozoa or vaginal cells extracted using PBS or water, see figure 1.

No samples were KM positive. All semen samples were AP positive (between 10 and 50 s) and there was no difference between those extracted with PBS or water. Of the vaginal samples, 4 out of the 18 were AP positive at 2 mins. However, there was no difference between those extracted with PBS or water.

#### Effect of PBS on the recovery of vaginal cells and spermatozoa

Statistical analysis of the results showed that there was no significant difference in the number of intact cells or spermatozoa extracted from swabs using PBS or water, see figure 2.

#### DNA profiling

Full SGM plus profiles were obtained from all samples, see figure 3. There was no difference in the DNA profiles obtained from samples extracted using water or PBS.

#### Discussion

Cells and spermatozoa are routinely stained with H & E in the laboratory. This method stains the nuclei of cells purple and the cytoplasm pink. The heads of

spermatozoa have a characteristic two-tone appearance, with the base of the head staining purple and the upper portion staining a very pale purple. Spermatozoa tails stain pink. PBS was not found to alter the staining of cells or spermatozoa in any way. Other tests carried out routinely on swabs from sexual assaults are the KM test for blood and the AP test for the presence of seminal fluid. The results of these tests were comparable when the swabs were extracted with PBS or water. As expected, all semen samples were AP positive. Of the vaginal swabs, 22% were AP positive within 2 mins. This reaction was much slower than that seen when the semen swabs were tested. Acid phosphatase is present in vaginal secretions, although the level is lower than in semen. The presence of large numbers of bacteria and/or yeast can also result in a positive AP test [5]. Several of the swabs analysed in this experiment did have high numbers of bacteria, however, no yeast was found (data not shown).

The aim of this study was to show that PBS could be used in place of water for the recovery of cells and spermatozoa from swabs received in sexual assault cases. This study showed that there was no significant difference in the number of intact cells or spermatozoa recovered from vaginal swabs or swabs of semen samples when extracted using PBS or water. However, there was generally a slight increase in the number of intact cells when extracted with PBS, although not significant. The relatively high degree of variation (as shown by the standard deviation) in the numbers of cells and spermatozoa in samples from the same individual may be due to a number of reasons. The

number of cells will depend on the time of day the swab was taken and is likely to vary from day to day. Part of this problem was overcome by taking samples at the same time each day. Another reason is the method of extraction and preparation of slides. The use of centrifugation, vortexing and pipetting will all have a deleterious effect on some of the cells. However, the same method was used for all samples and therefore this should limit this as a significant contributor. Finally, variation could occur through the counting process. The counting of cells and spermatozoa was done by eye, as accurately as possible, using a graticule attached to the high power microscope. This method allowed only intact cells to be counted. Some other methods, such as cell counters would not be able to distinguish between the number of free nuclei and the number of intact cells. Interestingly, the results obtained from the same slides, counted by a second scientist were virtually the same (data not shown). It is acknowledged that this study looked at a relatively low number of samples and that the analysis of many more samples from a larger population may provide significant results.

Given that PBS is already commonly used in the wider scientific population for many laboratory procedures, further such analysis for the purpose of this study was deemed unnecessary.

The number of vaginal cells recovered from each sample in this study is relatively high and would not reflect the low numbers of cells expected to be

recovered from swabs such as penile swabs in cases such as rape. A comparison of the recovery of a few cells per sample would not provide accurate data and so was not done in this case.

DNA profiling using SGM plus is an extremely reliable and useful tool in determining the possible perpetrator/s of a particular crime. Although useful DNA profiles can be obtained from low amounts or low quality DNA, the best results are obtained from samples with sufficient good quality DNA. This study demonstrated that the recovery of vaginal cells or spermatozoa from swabs extracted using PBS or water did not affect the subsequent DNA profile. Profiling of all samples resulted in full DNA profiles from high quality DNA.

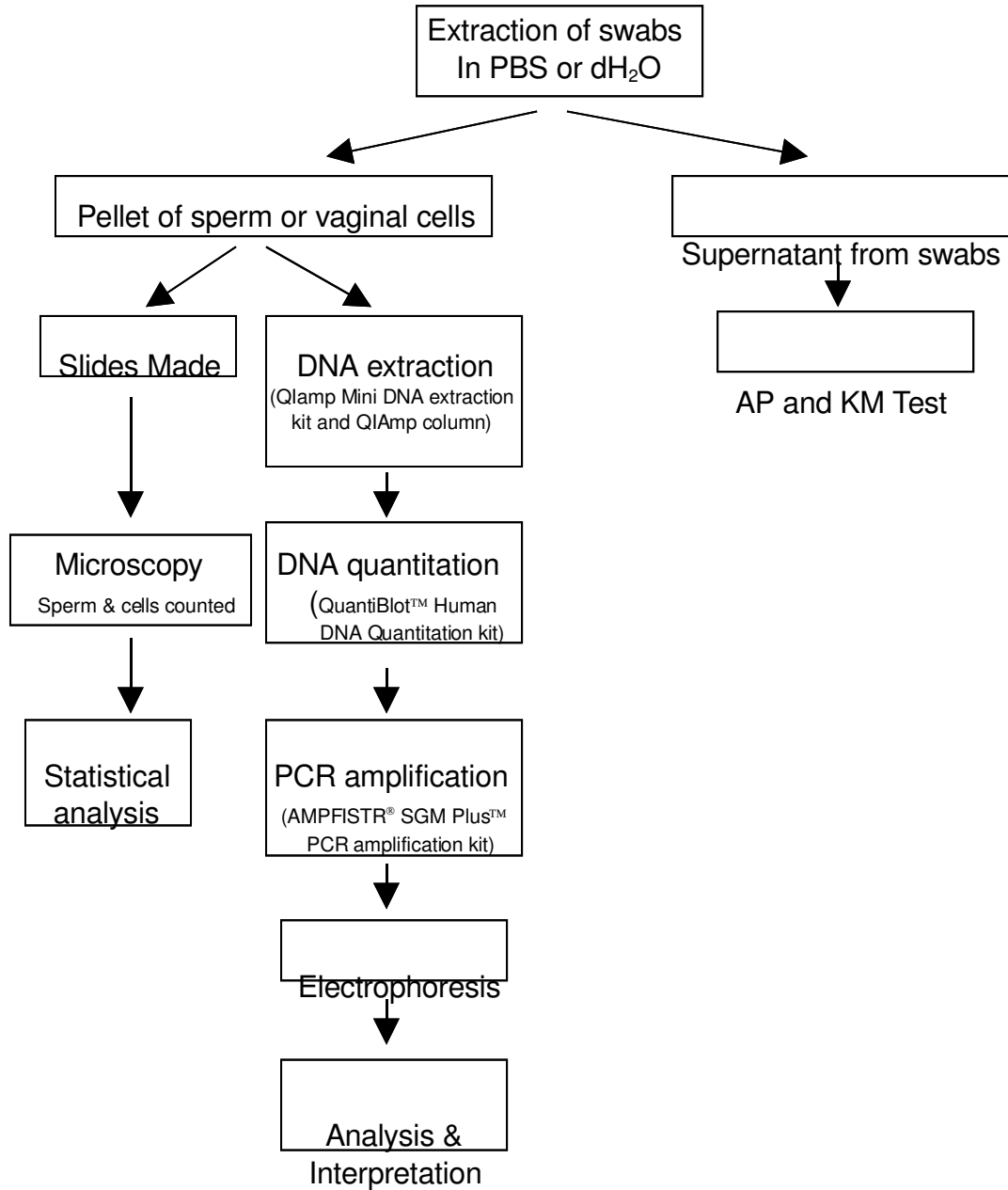
Given that PBS does not affect any of the tests or DNA profiling of vaginal cells or spermatozoa, the use of PBS has now been introduced into the swab extraction protocol in Lothian and Borders Police Forensic Science Laboratory in order to preserve, where possible, the few number of cells that may be present.

#### Acknowledgements

I would like to thank all the volunteers who provided samples for this study.

#### Appendix 1

## Summary of experimental method



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Figure 1 H&E staining of 1. Vaginal cells (nucleus stains purple and cytoplasm pink) and 2. Spermatozoa (heads stain purple/light purple and tails pink) extracted using A) PBS and B) water (Magnification x500). There is no difference in the staining of vaginal cells or spermatozoa extracted using PBS or water.

Figure 2 The number of A. intact cells and B. spermatozoa recovered from swabs. Three sets of swabs from 3 different volunteers were extracted using PBS or water and the extract (5 $\mu$ l) was spotted onto slides and counted (x500 magnification). There are no significant differences in the number of cells or spermatozoa extracted using water or PBS.

Figure 3 SGM plus profiles of vaginal cells extracted in A. PBS and B. water. Full profiles were obtained from cells extracted using PBS or water.

