A proof of principal study on the use of direct PCR of semen and spermatozoa and development of a differential isolation protocol for use in cases of alleged sexual assault

Shanan S. Tobe[1,2*], Yuvaneswari C. Swaran[3,4], Lynn Dennany[3], Ursula Sibbing[5], Kristina Schulze Johann[5], Lindsey Welch[3] and Marielle Vennemann[5]

1. Department of Chemistry and Physics, Arcadia University, Glenside, Pennsylvania, USA
2. School of Biological Sciences, Flinders University, Adelaide, Australia
3. Centre for Forensic Science, WestCHEM, Technology and Innovation Centre, University of Strathclyde, Glasgow, UK
4. Current address: Forensic Division, Department of Chemistry Malaysia, Jalan Sultan, Malaysia
5. Institute of Legal Medicine, University of Münster, Germany

* Corresponding author:
450 S. Easton Rd.
Glenside, PA 19038
USA
tobes@arcadia.edu

Abstract

Sexual assault samples are some of the most common samples encountered in forensic analysis. These samples can require a significant time investment due to differential extraction processes. We report on the first record of successful direct amplification of semen for STR analysis. Neat seminal fluid, dilutions ranging from 1:5 to 1:160 and GEDNAP samples were successfully amplified using a direct method. A mild differential isolation technique to enrich spermatozoa was developed and successfully implemented to separate and directly amplify a mixture of semen and female epithelial cells. Aliquots of samples subjected to the differential isolation protocol were stained with Haemotoxylin and Eosin for sperm scoring. Samples stained after PCR showed a complete lack of intact spermatozoa demonstrating that the cells are lysed during the PCR process. This paper demonstrates the potential to incorporate direct PCR in cases of sexual assault to more rapidly obtain results and achieve a higher sensitivity. (150)

Keywords

Direct PCR; Sexual Assault; Semen; Seminal Fluid; Differential Isolation; spermatozoa

1. Introduction

Amplification without prior DNA extraction, known as direct PCR, has gained increased interest in forensic science due to the reduced time to DNA profile and increased sensitivity of the technique. Although its application to forensic science is relatively recent, the technique itself has been used since the 1990s [1-3].

Benefits of direct PCR include reduced time and expenses compared to standard DNA extraction prior to amplification. In direct PCR, manual processing of the sample as well as the amount of buffers and consumables needed are limited to a minimum which helps to avoid the introduction of contamination [4, 5]. Furthermore, DNA extraction results in significant loss of DNA from the samples due in part to multiple tube changes and also due to sometimes low DNA recovery rates [6]. This is especially concerning in samples originally containing very low amounts of DNA.

Direct PCR has already been tested successfully for various sample types, including touch DNA (epithelial cells and cell free DNA) [4]; blood [1, 2, 7-10]; buccal swabs/cells [8-10]; hair [2, 11]; fingernails [12]; tissues [3], and plant material [13]. Other highly significant forensic sample types are those relating to sexual assault allegations, specifically semen and spermatozoa; however, to our knowledge, investigation for the use of direct PCR in these types of crimes has not yet been undertaken. Direct PCR has been used to identify pathogens in semen (e.g. [14, 15]), but never for the identification of the semen donor, as required in forensic investigation. Sexual assault allegations can make up a large proportion of casework samples and can also be more time intensive due to specific aspects such as differential extraction [16] and, microscopy and sperm scoring [17].

Presumptive identification of suspected semen is well documented and mainly consists of the use of Acid Phosphatase (AP) [18, 19] and Prostate Specific Antigen (P30/PSA) [20, 21], however these presumptive tests are known to cross react with a variety of substances [22-26]. Direct observation of spermatozoa is therefore needed in some regions as conclusive proof of ejaculation so microscopy and sperm scoring may still be required, however direct PCR would still offer major benefits including: minimal amount of sample destruction; faster analysis; greater sensitivity; decreased cost, and; reduced risk of contamination. Semen can be found on intimate body swabs (often as a mixture), on skin, clothing and bedding items, all of which are routinely submitted for forensic examination [27, 28]. Dependant on if the semen is neat and expected as a single source, as it may be with bedding and clothes, or a mixture is expected, as with intimate swabs, will determine the downstream analysis method. Mixed samples from intimate swabs are processed using a two-step differential extraction [16] to separate the sperm fraction from the epithelial fraction.

As the samples are not purified through an extraction process, direct PCR incorporates all elements of a sample in to the analysis. This includes the total DNA of a sample, but also cellular components and other potential inhibitors, which can have an adverse effect on the PCR process. Semen has been demonstrated to have between 15 and 200 million spermatozoa per mL for males with normal sperm counts [29] and up to 5 million other non-spermatozoa cells per mL, mainly constituting leucocytes and round cells (debris and immature sperm) [30], but which will still contain the donors DNA. Heme, a component of blood, has been shown to act as a PCR inhibitor by blocking the active site of the polymerase [31, 32]. Other cellular or tissue based inhibitory substances that can be commonly encountered in forensic cases include: polysaccharides and humic acid, found in soils and plants [33-35]; urea, found in urine [36], and calcium, found in bones [37]. Other inhibitory

substances such as indigo dye, found in blue jeans, have also been demonstrated to pose problems with the amplification process [38]. The overall inhibitory effect of different substances can be reduced based on the particular polymerase used in the PCR [39, 40], but to date various sample types have been successfully typed using direct PCR without problem.

In this study, we investigated the potential for direct amplification of spermatozoa and seminal fluid in order to determine the donor. We subjected stains containing seminal fluid (pure and mixtures) to the technique of direct amplification and investigated a differential isolation method prior to direct amplification.

2. Materials and Methods

All samples were collected and obtained in accordance with ethical requirements as set out by the University of Strathclyde.

2.1 Semen

Semen was obtained from a fertility clinic and anonymous donors. All samples contained normal sperm counts between 15 and 200 million spermatozoa/mL [29], which equates to between 15,000 and 200,000 spermatozoa/µL and between 48.7 and 649.3 ng/µL DNA.

2.2 Samples in cases of sexual assault

Semen dilutions (1:5, 1:10, 1:20, 1:40, 1:80 and 1:160) were spotted on sterile cotton, air dried in a safety cabinet and subjected to direct PCR. Dilutions were performed in sterile distilled water to a final volume of 1 mL. Two repetitions at each dilution were performed. Four stains from previous proficiency tests of the German DNA profiling (GEDNAP) group were subjected to direct PCR: stain D of GEDNAP 38 (semen on toilet paper), stain 4 of GEDNAP 43 (blood-semen-mixture on tissue), stain 2 of GEDNAP 45 (semen on condom, stain was transferred to a swab), and stain 2 of GEDNAP 46 (semen on toilet paper). GEDNAP stains were each processed twice to ensure consistency. Neat semen (10 µL) and blood (approximately 10 µL) were deposited on to the various substrates and allowed to air dry in a safety cabinet to avoid contamination. This was done in duplicate for each fluid and each substrate.

2.3 Differential lysis

Body fluid mixtures often encountered in sexual assault allegations were prepared to test a short differential enrichment protocol. Mixtures of semen and saliva (1:10), to a final volume of 100 µL, were prepared and kept in liquid form and also spotted onto sterile cotton. Saliva was donated by a female volunteer to more closely simulate expected male/female mixtures. Several short fibres, for dried stains, and 5 µL, for liquid samples, were submerged in 18 µL Swab solution (Promega). Proteinase K (2 µL, 20mg/mL) was added and samples were incubated for 15, 30 and 60 min at 70°C. After centrifugation 2µL of the supernatant was transferred for direct PCR while the pellet was washed twice with 100 µL swab solution and then transferred for direct PCR.

Five µL of the resuspended sperm fraction (SF) and non-sperm fraction (NSF) were slide mounted and stained using Haemotoxylin and Eosin (HE). Slides were assessed for spermatozoa using a Leica DM 2500 Microscope (Wetzlar, Germany) with magnification of 500x (ocular 10x, objective 50x).

2.4 Direct PCR

Samples deposited on fabric, including toilet paper and tissue, were collected for direct PCR by taking two to three fibres, approximately 2-3mm in length, from the substrate and adding directly to the PCR reagents. NSF samples were added at 8 µL to the PCR and the total pellet from the SF was added, minus the sample removed for sperm scoring.

All samples were amplified without extraction using PowerPlex ESX 16 and 17 Kits (Promega Corp.). All samples were processed according to manufacturer's specifications; reaction volumes were reduced to 12.5 µL for samples processed with ESX 17. Samples treated with the differential enrichment protocol additionally had 1 x Amp Solution (Promega) added to the mastermix. For solid substrate samples (fibres, tissue and toilet paper) amplification-grade water was added up to the final volume required by the PCR kit.

Amplification took place in a 2720 Thermal Cycler (Life Technologies) and all batches were processed with both a negative and positive controls. All PCR products were visualised on a 3130 Genetic Analyser (Life Technologies, UK). Data were analysed using Genemapper ID v.3.2.1 software (Life Technologies, UK) with a threshold of 50 rfu used as the limit of detection, and 150 rfu as the limit for a homozygote. Local and global balance were calculated according to [41].

2.5 Lysis of spermatozoa

To determine if the spermatozoa were being lysed during the PCR process, samples were centrifuged post-PCR and 10 µL was removed from the bottom of the PCR tube (where a pellet would have formed) and then slide mounted, HE stained and examined as previously described.

3. Results and Discussion

3.1 Lysis of spermatozoa

Samples HE stained post-PCR showed a complete lack of intact spermatozoa (Figure 1). This is the first instance that the lysis of spermatozoa during PCR has been demonstrated and supports the use of direct PCR in cases of sexual assault for SF samples. Complete lysis of the spermatozoa indicates that direct PCR can be used for sexual assault samples and that despite the protein coat, samples can be fully analysed without the risk of missing potentially vital genetic material, important with low level samples. In this way case samples that may previously have yielded insufficient levels of DNA, due to minimal levels of DNA combined with low extraction efficiency, can now be analysed with the total DNA compliment offering resolution of difficult, low level, case samples.

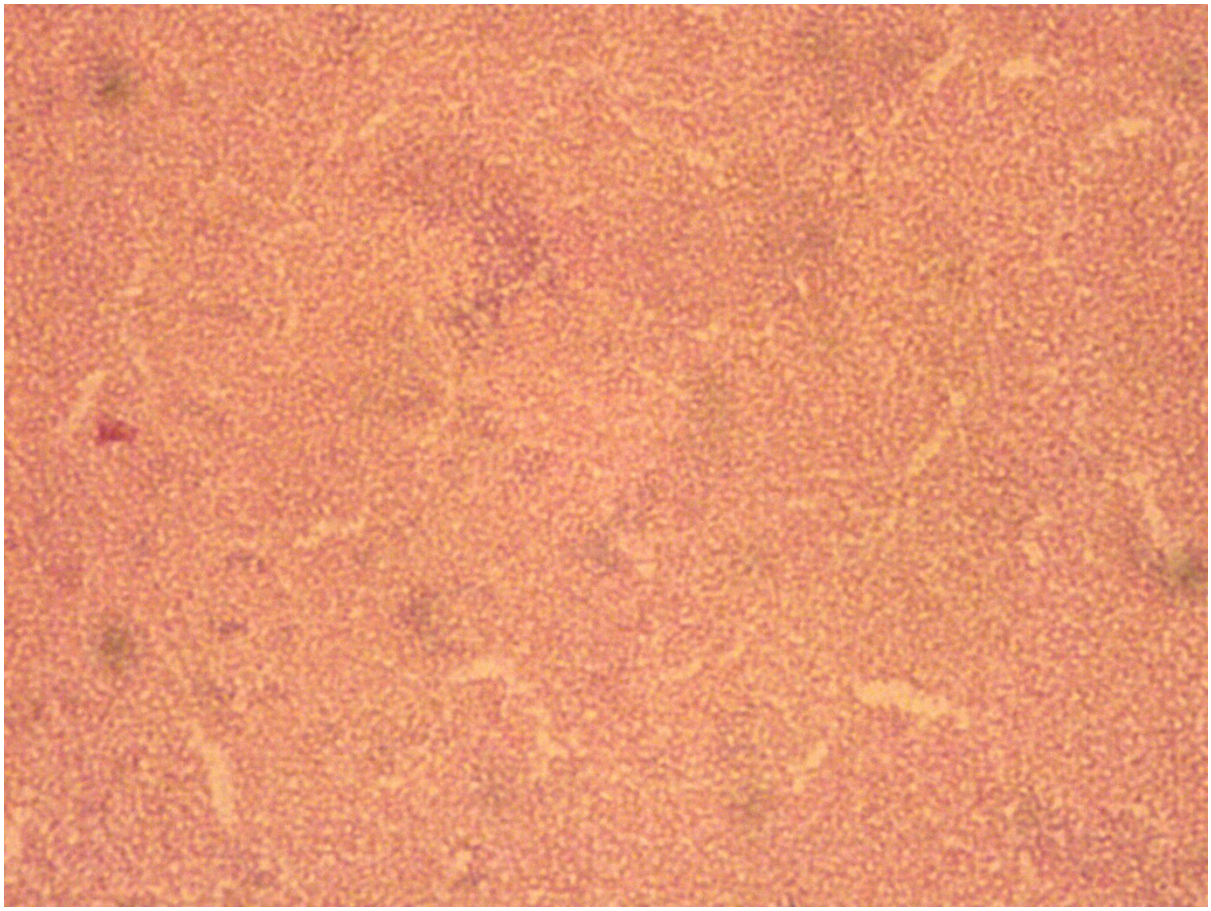


Figure 1: Post-PCR sample (1:1), centrifuged and pellet area mounted and HE stained; magnified at 500x demonstrating the lack of intact spermatozoa.

3.2 Dilution of semen and GEDNAP samples

The dilution series of seminal fluid produced full profiles at all dilutions to 1:80 demonstrating the potential for direct PCR in cases of sexual assault. Global balance for full profiles ranged from 0.78 (one 1:40 dilution) to 0.91 (one 1:10 dilution) with an average of 0.84. Local balance showed a minimum range from 0.4 (SE33 in one 1:20 dilution) to 0.72 (D19 in one 1:10 dilution) and an average of 0.6. One sample at 1:80 produced a full profile, while the other sample showed one locus (D19) and one allele (THO1) dropping out. Both samples at 1:160 showed higher levels of dropout, with one sample containing 50 % of the expected profile and the other 47 %. Peak heights reduced as the dilution increased (Figure 2). Stains demonstrating a weak presumptive positive, or which are known to be diluted as in the case of washing, may therefore benefit from the addition of more fibres or concentration via a differential isolation protocol as described below.

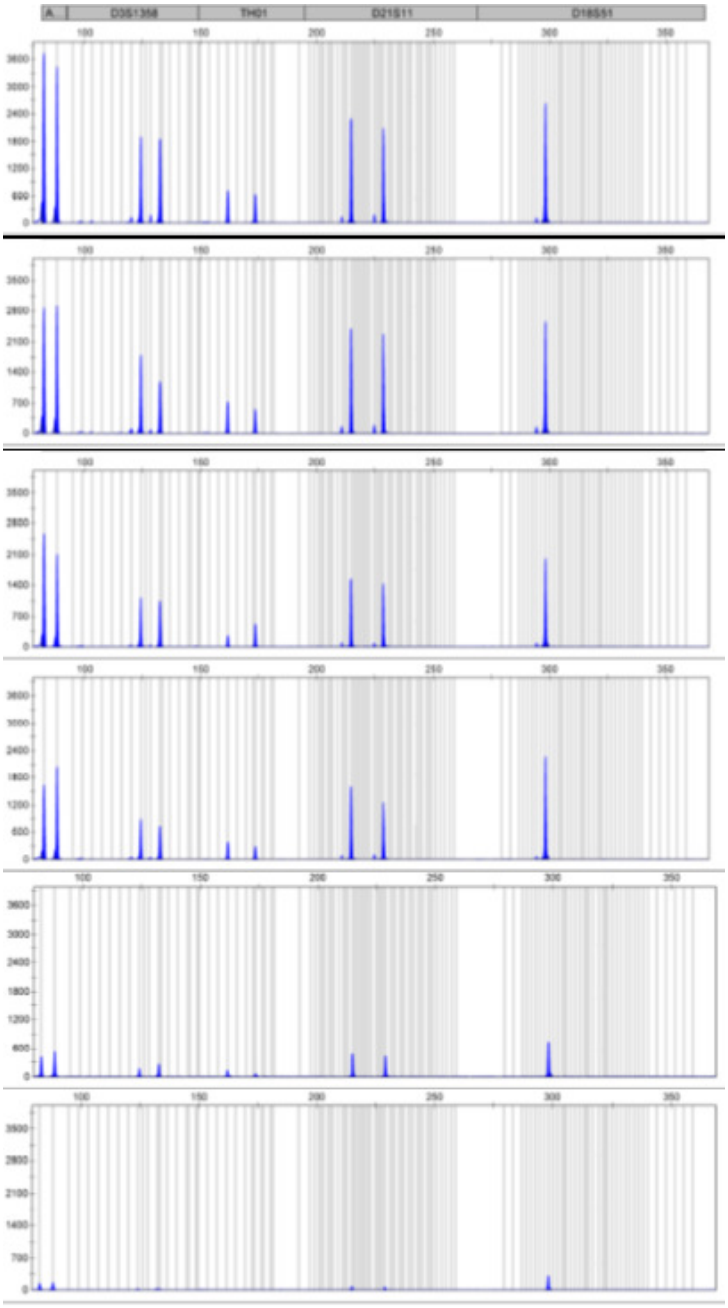
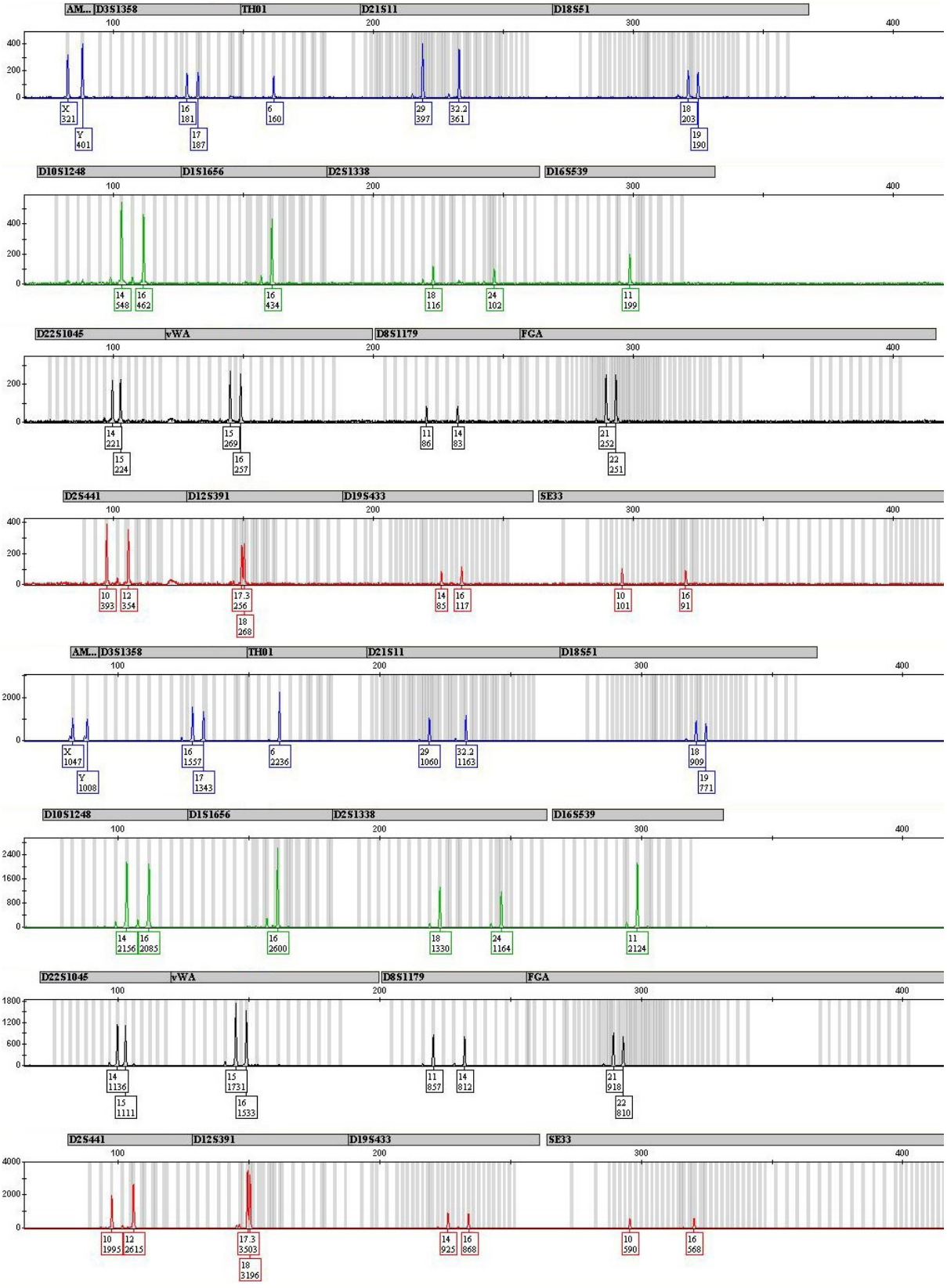


Figure 2: Blue dye channel of ESX17 showing direct PCR result from dilutions of 1:5, 1:10, 1:20, 1:40, 1:80 and 1:160 (top to bottom).

All GEDNAP-stains produced low-level, but full and reproducible profiles (Figure 3) containing the same alleles as when analysed using a traditional differential extraction technique. Total peak height and average peak height were 7,570 and 222.65 ± 123.68 for the direct PCR sample, and 52,829 and 1553.79 ± 464.67 for the traditionally extracted sample. The traditionally extracted and amplified sample showed a local allelic balance between 0.79 and 1, with a global balance of 0.928, whereas the direct PCR sample showed a local allelic balance between 0.73 and 1, with a global balance of 0.925. While the average peak height for the direct PCR sample (Figure 3 top) is 14.3 % the height of the traditionally extracted sample (Figure 3 bottom), it is still complete and could be fully called using the Genemapper ID v.3.2.1 software. The local and global balances are similar, with only a 0.32 % difference between the global balances, indicating that the direct PCR approach amplified

efficiently and as would be expected from a traditionally extracted sample. Considering that only two 2 mm fibres were sampled for direct PCR relative to a sample of approximately 2 mm^2 section for the traditional extraction sample, the direct amplification of seminal fluid and stains is a less destructive process, leaving valuable evidence behind for subsequent analysis or re-evaluation. GENAP samples represent simulated case samples that are used for proficiency testing relating to presumptive testing and STR analysis. The ability to amplify these samples using direct PCR therefore demonstrates the robustness and applicability of direct amplification of semen to forensic casework.



180

181

182 **Figure 3: Top - Full profile obtained by direct PCR from 2 fibres (2mm each) of stain 2 of GEDNAP 46 (semen on toilet**
183 **paper). Bottom – Full profile obtained by traditional differential extraction of 2 of GEDNAP 46.**

3.4 Differential isolation of spermatozoa in male/female mixtures

The ability to distinguish between male and female fractions of a mixed sample in cases of sexual assault is of primary importance to forensic investigators. This has been customarily accomplished using a differential extraction protocol [16] or through other techniques such as laser microdissection of spermatozoa or epithelial cells [42]. These techniques can add several hours to the analysis of sexual assault evidence and contribute to backlogs in the processing of sexual assault casework. A more rapid method for differentiating cells from the complainer (female) and accused (male) in allegations of sexual assault would therefore offer obvious benefits in reduction of time for analysis and, in conjunction with a direct amplification approach, greater sensitivity.

The mild differential isolation technique used to enrich spermatozoa and remove the female fraction was successful as demonstrated by the HE stained samples (Figure 4). Most non-sperm cellular components were digested after 15 minutes (Figure 4b) and after 60 minutes of incubation the non-sperm cells were completely digested (Figure 4d). Our results indicated that the optimal incubation time was 60 minutes (data not shown). This incubation time showed the best separation between the male and female STR profiles, with the female profile component reducing in intensity as the incubation time increased.

Profiles resulting from the differentially isolated samples show high levels of amplification with good balance across all loci. Global balance ranged from 0.78 to 0.87 for liquid mixtures and 0.78 to 0.9 for dried mixtures on cotton.

While the differential isolation method adds a small amount of time to the direct amplification process, it resulted in strongly enhanced male profiles. Even though the female fraction was still present at low levels after enrichment, the male profile was clearly distinguishable (Figure 5). The female fraction, derived from the supernatant, similarly showed a mixture with the female fraction accounting for the major contributor (Figure 5).

We conclude that even in samples with a massive excess of female cells, direct PCR can produce male profiles with surprisingly strong peak heights if combined with the described differential isolation protocol. Using the whole cell pellet directly in the PCR means that little or no genetic material is lost during the DNA extraction processes. This ability to use the whole sample makes direct amplification a powerful technique for forensic analysis.

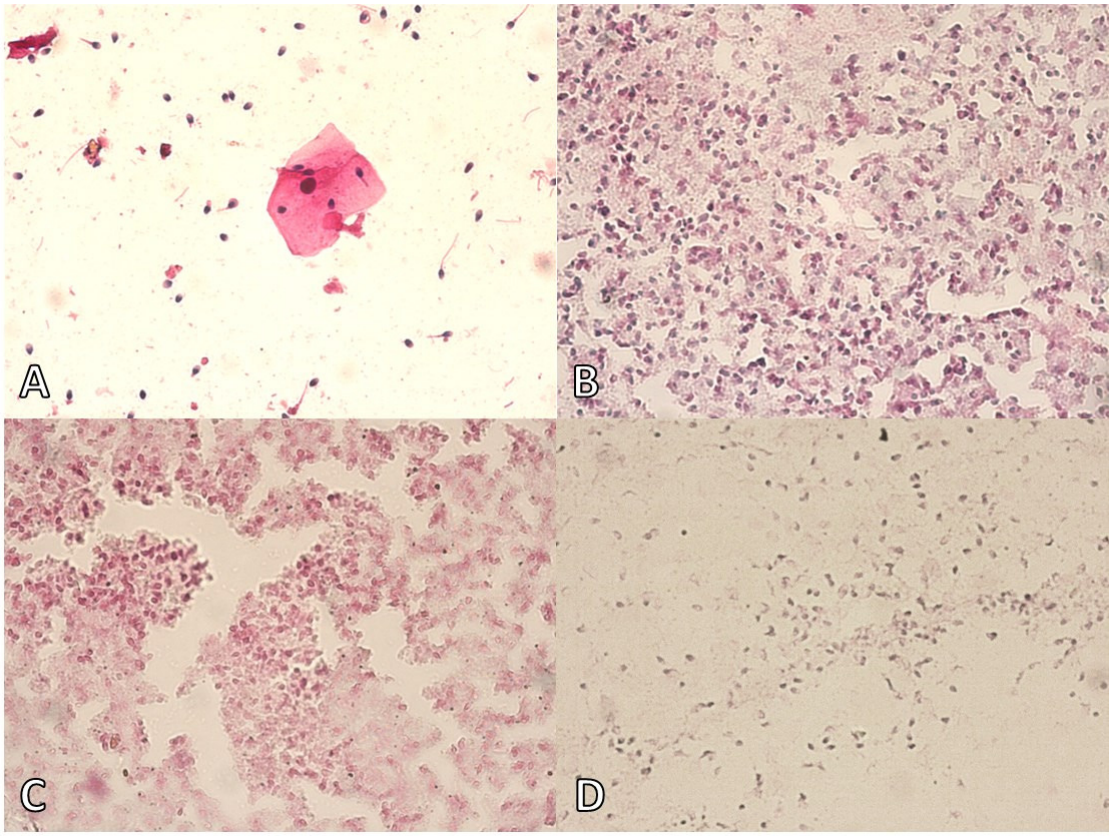


Figure 4: Differentially isolated samples stained using HE at various incubation times, magnified 500x. a) no incubation; b) 15 min incubation; c) 30 min incubation, and; d) 60 min incubation.

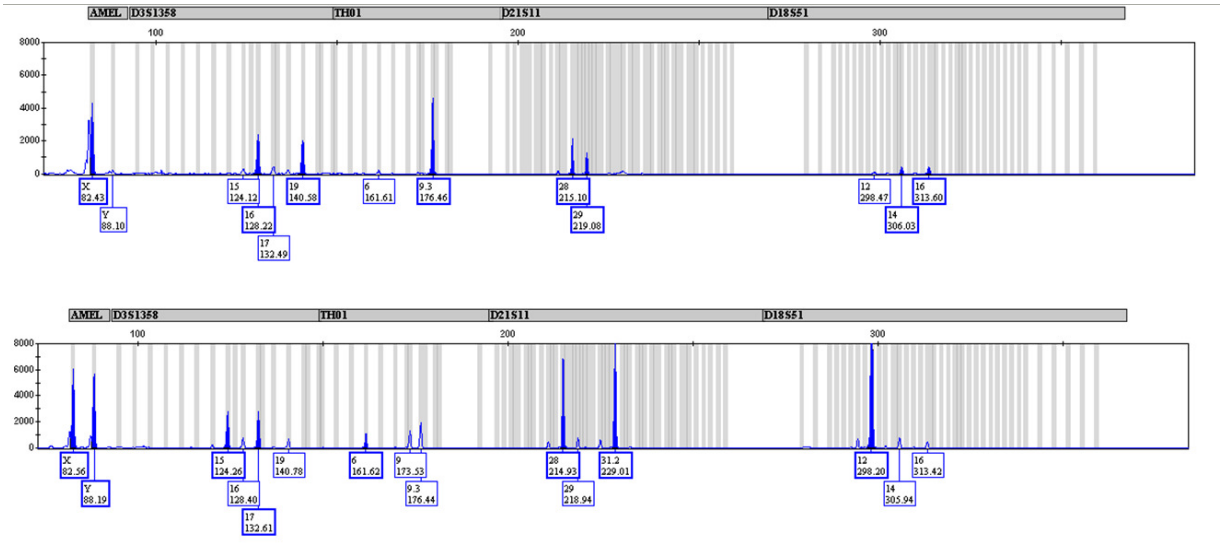


Figure 5: Direct PCR after differential enrichment of a semen-saliva mixture (1:10). The blue channel of the ESX amplification is shown exemplarily, containing the markers amelogenin, D3S1358, TH01, D21S11, and D18S51. The male DNA profile obtained from the cell pellet after differential lysis (top) reveals surprisingly high peaks, while the female fraction in the supernatant (bottom) is also clearly distinguishable from the male fraction.

4. Conclusion

We have demonstrated that even though spermatozoa are enclosed in a firm cell wall that they can be analysed by direct PCR, without the need of labour and time intensive extraction methods. While the DNA in the sample may in part be derived from the non-spermatozoa cells of the semen donor, our results from the post-PCR HE stained samples demonstrate that the spermatozoa are being lysed during the PCR process and are directly contributing to the resultant profile.

Simulated case samples and GEDNAP proficiency testing samples were all successfully typed offering significant improvements in time and sensitivity in the analysis of sexual assault evidence. For the first time the successful direct amplification of semen and spermatozoa on various substrates and in simulated sexual assault samples has been achieved.

Without the need for extraction, up to 5 hours (if using differential extraction) can be saved and the chance of success is increased. Standard extraction techniques can lose greater than 70 % of the genetic material, therefore direct PCR of semen can allow amplification of samples which may have previously failed to yield results. The use of standard equipment and reagents means that this technique can be implemented rapidly into most forensic laboratories with simple validation testing. The successful use of direct PCR with semen samples will allow rapid and more sensitive analyses in sexual assault casework samples.

Acknowledgments

YCS was funded through the Government of Malaysia. We would also like to thank Lisa Dierig and Marianne Schürenkamp for excellent technical assistance and Prof. Peter Schmidt (Münster) for help with microscopic images.

References

1. Mercier B, Gaucher C, Feugeas O, Mazurier C (1990) Direct PCR from whole blood, without DNA extraction. Nucleic acids research 18: 5908.
2. Ohhara M, Kurosu Y, Esumi M (1994) Direct PCR of whole blood and hair shafts by microwave treatment. Biotechniques 17: 726, 8-, 8.
3. Panaccio M, Georgesz M, Hollywell C, Lew A (1993) Direct PCR from solid tissues without DNA extraction. Nucleic acids research 21: 4656-.
4. Linacre A, Pekarek V, Swaran YC, Tobe SS (2010) Generation of DNA profiles from fabrics without DNA extraction. Forensic science international: genetics 4: 137-41.
5. Vallone PM, Hill CR, Butler JM (2008) Demonstration of rapid multiplex PCR amplification involving 16 genetic loci. Forensic Science International: Genetics 3: 42-5.
6. Butts ELR, Kline MC, Almeida JL, Vallone PM. (2010) DNA extraction efficiency: is it what you thought? Mid-Atlantic Association of Forensic Scientists State College, PA
7. Aki K, Kawazoe K, Izumi A, Tada T, Minakuchi K, Hosoi E (2014) Direct determination of ABO blood group genotypes from whole blood using PCR-amplification of specific alleles method. American Journal of BioScience 2: 49-55.
8. Park SJ, Kim JY, Yang YG, Lee SH (2008) Direct STR Amplification from Whole Blood and Blood-or Saliva-Spotted FTA® without DNA Purification*. Journal of forensic sciences 53: 335-41.
9. Wang DY, Chang C-W, Oldroyd NJ, Hennessy LK (2009) Direct amplification of STRs from blood or buccal cell samples. Forensic Science International: Genetics Supplement Series 2: 113-4.
10. Vandewoestyne M, Van Hoofstat D, Franssen A, Van Nieuwerburgh F, Deforce D (2013) Presence and potential of cell free DNA in different types of forensic samples. Forensic Science International: Genetics 7: 316-20.

11. Ottens R, Taylor D, Abarno D, Linacre A (2013) Successful direct amplification of nuclear markers from a single hair follicle. Forensic science, medicine, and pathology 9: 238-43.
12. Tie J, Uchigasaki S (2014) Detection of short tandem repeat polymorphisms from human nails using direct polymerase chain reaction method. ELECTROPHORESIS: n/a-n/a. doi: 10.1002/elps.201400061
13. Gausterer C, Stein C, Stimpfl T (2012) Application of direct PCR in a forensic case of yew poisoning. Int J Legal Med 126: 315-9. doi: 10.1007/s00414-011-0607-0
14. Amin AS, Hamdy MER, Ibrahim AK (2001) Detection of Brucella melitensis in semen using the polymerase chain reaction assay. Veterinary Microbiology 83: 37-44. doi: http://dx.doi.org/10.1016/S0378-1135(01)00401-1
15. Van den Brule A, Hemrika D, Walboomers J et al (1993) Detection of Chlamydia trachomatis in semen of artificial insemination donors by the polymerase chain reaction. Fertility and sterility 59: 1098-104.
16. Yoshida K, Sekiguchi K, Mizuno N et al (1995) The modified method of two-step differential extraction of sperm and vaginal epithelial cell DNA from vaginal fluid mixed with semen. Forensic Science International 72: 25-33. doi: http://dx.doi.org/10.1016/0379-0738(94)01668-U
17. Tobe SS, Dennany L, Vennemann M (2015) An assessment of the subjectivity of sperm scoring. Forensic Science International 251: 83-6.
18. Sivaram S, Bami HL (1971) Identification of Seminal Stains by the Inhibition of Acid Phosphatase by L(+) Tartrate. Journal of the Forensic Science Society 11: 187-94. doi: http://dx.doi.org/10.1016/S0015-7368(71)70652-5
19. Sivaram S (1970) A modified azo-dye method for identification of seminal stains. Journal of forensic sciences 15: 120.
20. Balk SP, Ko Y-J, Bubley GJ (2003) Biology of prostate-specific antigen. Journal of Clinical Oncology 21: 383-91.
21. Hochmeister MN, Budowle B, Rudin O et al (1999) Evaluation of prostate-specific antigen (PSA) membrane test assays for the forensic identification of seminal fluid. Journal of forensic sciences 44: 1057-60.
22. Hooft PJ, van de Voorde HP (1994) Interference of body products, food and products from daily life with the modified zinc test and the acid phosphatase test. Forensic Science International 66: 187-96. doi: http://dx.doi.org/10.1016/0379-0738(94)90343-3
23. Vennemann M, Scott G, Curran L, Bittner F, Tobe S (2014) Sensitivity and specificity of presumptive tests for blood, saliva and semen. Forensic Science, Medicine, and Pathology 10: 69-75. doi: 10.1007/s12024-013-9515-6
24. Tirimanna ASL (1972) Acid phosphatases in the tea leaf. Experientia 28: 633-. doi: 10.1007/BF01944944
25. Papotti M, Paties C, Peveri V, Moscuzza L, Bussolati G (1988) Immunocytochemical detection of prostate-specific antigen (PSA) in skin adnexal and breast tissues and tumors. Basic and applied histochemistry 33: 25-9.
26. Yu H, Diamandis EP (1995) Prostate-specific antigen in milk of lactating women. Clinical chemistry 41: 54-8.
27. Allard JE, Baird A, Davidson G et al (2007) A comparison of methods used in the UK and Ireland for the extraction and detection of semen on swabs and cloth samples. Science & Justice 47: 160-7. doi: http://dx.doi.org/10.1016/j.scijus.2007.09.010
28. Nittis M (2013) Evidence Collection in Cases of Sexual Assault. Legal and Forensic Medicine: 1335-58.
29. Cooper TG, Noonan E, von Eckardstein S et al (2010) World Health Organization reference values for human semen characteristics. Human Reproduction Update 16: 231-45. doi: 10.1093/humupd/dmp048

30. Bellabarba GAD, I. Tortolero VV, Molina CZ, Bellabarba C, Velazquez E (2000) Nonsperm Cells In Human Semen And Their Relationship With Semen Parameters. Systems Biology in Reproductive Medicine 45: 131-6. doi: doi:10.1080/01485010050193896
31. Akane A, Matsubara K, Nakamura H, Takahashi S, Kimura K (1994) Identification of the heme compound copurified with deoxyribonucleic acid (DNA) from bloodstains, a major inhibitor of polymerase chain reaction (PCR) amplification. Journal of forensic sciences 39: 362-.
32. Al-Soud WA, Rådström P (2001) Purification and Characterization of PCR-Inhibitory Components in Blood Cells. Journal of Clinical Microbiology 39: 485-93. doi: 10.1128/jcm.39.2.485-493.2001
33. Tsai YL, Olson BH (1992) Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. Applied and Environmental Microbiology 58: 2292-5.
34. Watson RJ, Blackwell B (2000) Purification and characterization of a common soil component which inhibits the polymerase chain reaction. Canadian Journal of Microbiology 46: 633-42. doi: 10.1139/w00-043
35. Demeke T, Adams R (1992) The effects of plant polysaccharides and buffer additives on PCR. Biotechniques 12: 332-4.
36. Khan G, Kangro HO, Coates PJ, Heath RB (1991) Inhibitory effects of urine on the polymerase chain reaction for cytomegalovirus DNA. Journal of Clinical Pathology 44: 360-5. doi: 10.1136/jcp.44.5.360
37. Powell HA, Gooding CM, Garrett SD, Lund BM, McKee RA (1994) Proteinase inhibition of the detection of Listeria monocytogenes in milk using the polymerase chain reaction. Letters in Applied Microbiology 18: 59-61. doi: 10.1111/j.1472-765X.1994.tb00802.x
38. Shutler GG, Gagnon P, Verret G et al (1999) Removal of a PCR inhibitor and resolution of DNA STR types in mixed human-canine stains from a five year old case. Journal of forensic sciences 44: 623-6.
39. Al-Soud WA, Rådström P (1998) Capacity of nine thermostable DNA polymerases to mediate DNA amplification in the presence of PCR-inhibiting samples. Applied and environmental microbiology 64: 3748-53.
40. Hedman J, Nordgaard A, Rasmusson B, Ansell R, Rådström P (2009) Improved forensic DNA analysis through the use of alternative DNA polymerases and statistical modeling of DNA profiles. Biotechniques 47: 951-8.
41. Hedman J, Ansell R, Nordgaard A (2010) A ranking index for quality assessment of forensic DNA profiles. BMC research notes 3: 290.
42. Di Martino D, Giuffre G, Staiti N, Simone A, Le Donne M, Saravo L (2004) Single sperm cell isolation by laser microdissection. Forensic science international 146: S151-S3.