Microsatellite analysis of cryopreserved stallion semen stored on FTA® paper

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ABSTRACT

The aim of this study was to establish and validate a method to permit microsatellite analysis of DNA profiles obtained from frozen–thawed stallion sperm cells. This would provide reliable and accurate verification of the identification of a semen donor. Ejaculates from 5 pony stallions were collected, processed and frozen in 0.5 mℓ plastic straws. Aliquots of 100 μℓ of the frozen-thawed semen thus obtained were either placed directly, or diluted (1:10; 1:100; and 1:1000) and placed on slides of FTA® paper. Similarly, blood samples obtained from each of the stallions were placed onto slides of FTA® paper. A punch was removed from each sample after drying. Each sample was mixed with FTA® purification reagent, Dithiothreitol and Proteinase K before incubation and processing. All samples were processed with a set of 13 microsatellite markers. Further analysis permitted a comparison of the DNA profiles of the frozen–thawed semen and the blood samples. A full profile of markers was obtained from the 1:10 and 1:100 dilutions of the frozen-thawed semen samples as well as from the blood samples. The DNA profiles from the frozen-thawed semen and blood samples obtained from the stallions matched in all cases.

Key words: cryopreserved semen, DNA microsatellites, stallion.

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INTRODUCTION

The use of cryopreserved or frozen semen for artificial insemination (AI) is a common practice in many species, including the horse. The ability to match the cryopreserved semen sample to the donor or sire has become increasingly important. This would allow reliable and highly accurate verification of the identification of a specific semen donor by comparison with the microsatellite analysis of the DNA profile of a blood sample obtained from the same stallion. The potential applications include the certification of cryopreserved semen, genetic screening of cryopreserved semen intended for commercial AI programmes, and heritability studies involving genetic traits transferred *via* the insemination of frozen–thawed stallion semen.

Blood samples have been routinely stored on FTA® paper since the beginning of 2002 by the Veterinary Genetics Laboratory at Onderstepoort. DNA profiles obtained from these samples are used for parentage testing in various animal species. FTA® paper provides a means of storing biological samples for DNA analysis at room temperature for several years. Cells applied to the paper are lysed and the DNA is trapped within the fibres. Fungal and bacterial growth is inhibited and the DNA is protected from ultraviolet light and oxidative damage within the paper matrix. Cell debris and proteins are removed and polymerase chain reaction (PCR) is then performed directly on the immobilised DNA within the paper.

A request to the Laboratory for donor verification in the certification of cryopreserved stallion ejaculates led to an investigation into a reliable method for routine storage of cryopreserved semen on FTA® paper for DNA analysis. Sperm cell membranes, however, contain thiol-rich proteins and are resistant to cell lysis. This was evident following the routine handling of earlier ejaculates of raw and frozen–thawed semen samples placed on FTA® paper. A method of disrupting the cell membranes and allowing the DNA to bind to the paper for further processing was required.

MATERIALS AND METHODS

Ejaculates were collected using an artificial vagina from each of 5 pony stallions. The gel-free semen obtained from each ejaculate was processed before being aspirated at a concentration of 500 million sperm per mℓ into 0.5 mℓ plastic French straws (Taurus, Irene, SA). The straws had been indelibly marked with an inscription that included the name of the stallion as well as the date of collection and processing. The straws were then frozen using minor modifications to an established method[3] and were transferred for further storage in liquid nitrogen for approximately 12 months.

A straw from each stallion was identified and was transported in liquid nitrogen to the Veterinary Genetics Laboratory. The straws were thawed in a water bath at 35 °C for 30 seconds. The frozen–thawed semen content of each straw was transferred to a 1.5 mℓ Eppendorf tube. A 100 μℓ aliquot of the undiluted frozen–thawed semen was placed on a slide of FTA® paper (Whatman Bioscience, Cambridge, UK). Subsequently, a series of 3 dilutions (1:10, 1:100 and 1:1000) of the frozen–thawed semen were made with a semen-processing diluent (1.50 g glucose; 25.95 g sodium citrate dihydrate; 3.7 g disodium EDTA; and 1.20 g sodium bicarbonate in 1 ℓ deionised water, at approximately 296.9 mOsm/kg and a pH = 6.9). Aliquots of 100 μℓ of each dilution were placed on separate slides of FTA® paper and allowed to dry overnight.

An aliquot of 100 μℓ venous blood, collected from each of the 5 stallions in Vacutainer™ tubes containing EDTA, at the time of analysis was also placed on a slide of FTA® paper and allowed to dry. A 2-mm punch was removed from the stored FTA® paper containing each frozen–thawed semen sample as well as from the blood samples. Each punch was placed into a 200 μℓ, thin-walled PCR tube. An amount of 200 μℓ FTA® purification reagent, 20 μℓ 1 M Dithiothreitol (DTT) (Sigma-Aldrich, Johannesburg) and 5 μℓ Proteinase K (Roche Diagnostics, Johannesburg) (20 mg/mℓ) was added to the tube[1]. This was incubated at 56 °C for 1 hour in a GeneAmp 9700 PCR System (Applied Biosystems, Johannesburg). The punch was again washed twice with 200 μℓ FTA® purification reagent (Whatman® Bioscience, Cambridge, UK) and

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Table 1: **Number and percentage of amplified loci recognised with 13 microsatellites in venous blood and different dilutions of frozen–thawed semen from 5 pony stallions.**

Sample	Stallion 1	Stallion 2	Stallion 3	Stallion 4	Stallion 5
Blood	13 (100)	13 (100)	13 (100)	13 (100)	13 (100)
Undiluted semen	5 (38.4)	ND	ND	ND	ND
1:10 semen	13 (100)	13 (100)	13 (100)	13 (100)	13 (100)
1:100 semen	13 (100)	13 (100)	13 (100)	10 (76.9)	13 (100)
1:1000 semen	0 (0)	12 (92.3)	11 (84.6)	10 (76.9)	11 (84.6)

ND = no data.

twice with 200 $\mu\ell$ TRIS-EDTA buffer. The punches were allowed to air-dry for at least 1 hour prior to PCR.

All samples were processed with a set of 13 microsatellite markers and PCR parameters that are routinely used by the Veterinary Genetics Laboratory at Onderstepoort for the parentage testing of horses. An amount of 1 $\mu\ell$ of the PCR product was transferred to a loading plate and 10 $\mu\ell$ HiDi Formamide (Applied Biosystems, Johannesburg) and 0.25 $\mu\ell$ Genescan 400HD ROX® size standard were added to each well. Following this, the PCR fragments were sized using capillary electrophoresis on a 310 Genetic Analyser (Applied Biosystems, Johannesburg). Further analysis was performed using STRand (Board of Regents, University of California) on a personal computer. The DNA profiles of the frozen–thawed semen and the blood samples from each stallion were compared.

RESULTS

The lysis of the cryopreserved and subsequently thawed sperm cells within the FTA® paper was successfully achieved using the mixture of DTT and Proteinase K in the FTA® purification reagent and incubating at 56 °C for 1 hour.

A complete set of results was obtained of each stallion from the blood samples as well as from the 1:10 and 1:100 dilution of the frozen–thawed semen samples. A full profile of markers could not be obtained from either the undiluted frozen–thawed semen samples or the 1:1000 dilution (Table 1).

Each stallion was assigned a set of allele codes according to the internationally accepted nomenclature for each of the 13 microsatellite markers tested. The DNA profile of the blood sample of each stallion matched the profile of the frozen–thawed semen sample of the same animal in each case.

DISCUSSION

The lysis of the cryopreserved and subsequently thawed sperm cells within the FTA® paper was achieved using the mixture of DTT and Proteinase K in the FTA® purification reagent and incubating this at 56 °C for 1 hour. DNA within the cells was released and trapped within the paper allowing PCR to be successfully performed.

Semen contains 150 000 to 300 000 ng/mℓ DNA as opposed to blood that contains 20 000 to 40 000 ng/mℓ depending on the number of white blood cells within the sample[2]. These data apply specifically to the human. Frozen–thawed stallion semen is more concentrated than ejaculated raw semen due to the requirements of most cryopreservation methods. Dilution of the sample, therefore, becomes necessary to avoid overloading the PCR reaction.

This method will permit verification of the identification of a specific semen donor by comparison with the DNA profile of a blood sample obtained from the same stallion. The potential applications include the certification of cryopreserved semen and both the genetic screening of commercial frozen stallion semen and heritability studies involving genetic traits transferred *via* artificial insemination of frozen–thawed stallion semen.

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