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Investigation of optimal procedures for storage and use of plasma samples suitable for gene doping tests

Teruaki TOZAKI[1#*], Aoi OHNUMA[1#], Mio KIKUCHI[1], Taichiro ISHIGE[1], Hironaga KAKOI[1], Kei-ichi HIROTA[1], Yuji TAKAHASHI[2] and Shun-ichi NAGATA[1]

[1]Genetic Analysis Department, Laboratory of Racing Chemistry, Tochigi 320-0851, Japan
[2]Equine Research Institute, Japan Racing Association, Tochigi 329-0412, Japan

Gene doping, which is prohibited in horseracing and equestrian sports, can be performed by introducing exogenous genes, known as transgenes, into the bodies of postnatal animals. To detect exogenous genes, a method utilizing quantitative polymerase chain reaction (qPCR) with a hydrolysis probe was developed to test whole blood and plasma samples, thereby protecting the fairness of competition and the rights of stakeholders in horseracing and equestrian sports. Therefore, we aimed to develop sample storage methods suitable for A and B samples in gene doping tests using blood. For sample A, sufficient qPCR detection was demonstrated after refrigeration for 1 to 2 weeks post collection. For sample B, the following procedures were confirmed to be suitable for storage: 1) centrifugation after sample receipt, 2) frozen storage, 3) natural thawing at room temperature, and 4) centrifugation without mixing blood cell components. Our results indicated that long-term cryopreservation yielded good plasma components from frozen blood samples even though it destroyed blood cells, indicating its applicability to the gene doping test using sample B, which can be stored for later use. Sample storage procedures are as important as detection methods in doping tests. Therefore, the series of procedures that we evaluated in this study will contribute to the efficient performance of gene doping tests through qPCR using blood samples.

Key words: *gene doping, sample splitting, Thoroughbred, transgene*

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The International Federation of Horseracing Authorities (IFHA) and the Fédération Equestre Internationale (FEI) prohibit gene doping in horseracing and equestrian sports, respectively, in their rules [11]. The abuse and/or misuse of gene therapy is a form of gene doping in which exogenous genes are introduced into the bodies of postnatal animals [4, 7, 18]. Transgenes are sequences of mRNA structures without introns that are cloned into plasmid vectors or viral vectors [16, 18]. These structures have enabled the development of methods that utilize quantitative polymerase chain reactions (qPCRs), such as the real-time PCR and digital PCR, with hydrolysis probes designed at exon/exon junctions for transgene detection [1, 2, 5, 13, 14, 16]. Additionally, vector sequences are targeted to detect exogenous genes cloned into a vector [6].

While conventional doping tests use urine samples, gene doping tests are performed on plasma separated from whole blood [1, 2, 5, 14, 16]. Ethylenediaminetetraacetic acid (EDTA) blood collection tubes are generally recommended for sample collection in gene doping tests because heparin is a PCR inhibitor [15]; however, the long-term storage of blood samples in EDTA tubes—in a refrigerator or at room temperature—renders them prone to hemolysis. Hemolysis is the rupture of red blood cells and subsequent release of the host's genome into plasma; the excess genomic DNA may affect exogeneous gene detection in qPCR experiments.

In gene doping tests, whole blood should be centrifuged immediately after collection, ideally before hemolysis, to separate the plasma. Then, the plasma should be stored under frozen conditions for gene doping tests, because transgenes (e.g., naked plasmid) in frozen plasma can be

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*Corresponding author. e-mail: ttozaki@lrc.or.jp
#These authors contributed equally.

stored for a long time [14, 16]. However, gene doping tests in horseracing may be carried out as in-competition testing or out-of-competition testing using blood collected at racetracks or horseracing facilities (e.g., training centers and stables), and the collected samples are then sent to a laboratory. Unless the laboratory is adjacent to the racetrack, it takes several days for shipped samples to reach the laboratory. Therefore, validating the storage and shipping procedures of the samples during this period is necessary.

In general, the system of A/B split samples has been introduced to protect the fairness of competition and the rights of stakeholders in horseracing and equestrian sports, as well as human sports [9]. Sample A is used for conventional testing, while sample B is used by most racing/equestrian authorities for confirmation in another laboratory based on the request of a stakeholder, such as in the case of sample A testing positive. This system may also be necessary in gene doping tests to ensure fairness, hence the need for long-term storage of sample B.

Some gene doping tests use plasma components [5, 16] that require appropriate long-term storage. In addition to the development and validation of detection methods, the handling of the test samples in doping tests is crucial. In this study, we investigated the storage stability of blood used to obtain plasma samples for gene doping tests and examined the preferred specimen storage method. We also investigated the effect of blood storage on gene doping tests.

Materials and Methods

Ethical considerations and animals

This study was approved by the Animal Care Committee of the Equine Research Institute (ERI), Japan Racing Association (JRA; Shimotsuke, Tochigi, Japan; approval number 22-12). In this study, six Thoroughbred horses (horse 1, 6-year-old gelding weighing 495 kg; horse 2, 5-year-old male weighing 570 kg; horse 3, 4-year-old female weighing 484 kg; horse 4, 5-year-old male weighing 566 kg; horse 5, 3-year-old male weighing 492 kg; horse 6, 7-year-old female weighing 510 kg) were used, and all the experiments using the horses were performed in facilities of the ERI, JRA.

Design of exogenous genes

In our previous study, Control_A and Control_C were designed as models of exogenous genes or positive control substances in gene doping studies. Based on a similar concept, two plasmid DNAs, namely Control_B_SET1 and Control_B_SET2, were designed as exogenous gene models in this study. These plasmids contained the sequences between forward and reverse primers for pre-amplification designed for 12 targeted transgenes, including the erythropoietin (*EPO*) gene in our previous study [14], and their sequences were tandemly connected. These sequences were artificially synthesized and cloned into pUCFa (r-Amp$^+$, ColE1_ori$^+$, Fasmac, Atsugi, Kanagawa, Japan). Large-scale (15 mg=5 mg × 3 horses) sequences of Control_B_SET1 and Control_B_SET2 were prepared by GenScript (Nanjing, Jiangsu, China).

Administration of exogenous genes

Care and handling of the six horses were in compliance with animal ethics and welfare guidelines. In our experiments, no mRNA was transcribed from the administered plasmid, and the plasmids were excreted in urine. Therefore, there were no welfare concerns for the horses. Control_B_SET1 (5 mg) was intramuscularly injected into three Thoroughbred horses (horses 1, 2, and 3), and Control_B_SET2 (5 mg) was intramuscularly injected into the other three Thoroughbred horses (horses 4, 5, and 6). Blood samples were collected in BD Vacutainer® spray-coated K2EDTA tubes (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) at 15 min, 1 hr, 3 hr, 6 hr, 9 hr, 12 hr, 1 day, 2 days, 3 days, 4 days, 1 week, 2 weeks, and 3 weeks after administration.

Sample storage and DNA extraction

Blood was collected in three tubes and processed in three different ways. The first tube was centrifuged within 24 hr of sampling and stored at −40°C until use. The second tube was refrigerated at 4°C for 1 week after sampling and was then centrifuged and stored at −40°C until use. The third tube was refrigerated at 4°C for 2 weeks after sampling and then centrifuged and stored at −40°C until use. Frozen tubes were thawed by letting them stand at room temperature (15–25°C) and then centrifuged to separate the hemolyzed blood cells and plasma components. Plasma was collected from thawed tubes.

DNA was extracted from 1.5 m*l* plasma using a Custom NEXTprep cfDNA Auto Kit (1.5 m*l*; PerkinElmer, Waltham, MA, USA) with a Chemagic 360 instrument (PerkinElmer). The extract was dissolved in Milli-Q water (Merck Millipore, Burlington, MA, USA) to a final volume of 50 μl.

Design of the primers and probe

To quantify the genomic DNA in plasma, primers (forward, 5′-GCCCCTCTTTTTCCACATTTT-3′; reverse, 5′-TCTTGCTGGTCCAGTGGATCT-3′) and a probe (5′-TGCTCGCTGTTC TCAT-3′) were designed on the same exon of the myostatin (*MSTN*) gene in the horse genome. The probe was labelled with VIC as a TaqMan–MGB probe. The primers and probe were designed in one of our previous studies [17].

For specifically detecting the *EPO* transgenes, primers

were designed on different exons, and probes were designed on the exon/exon junctions. These primers and probes were used in one of our previous studies [14]. The probes were labelled with FAM as a TaqMan–MGB probe. To quantify the transgene cloned into the plasmid vector, three sets of primers and probes, Amp_3, ColE1_3, and pUCintergenic_3, were newly designed on the plasmid vector sequences. Amp_3, ColE1_3, and pUCintergenic_3 were labelled as Hex, Cy5, and Cy5.5, respectively. Information on gene doping control was not included in this study.

Digital PCR detection

Digital PCR was performed using a T100 thermal cycler and QX200 droplet reader (Bio-Rad, Hercules, CA, USA) for single-plex PCR to count copy numbers of the genome in plasma, and a QX ONE Droplet Digital PCR system (Bio-Rad) was used for multiplex PCR to quantify plasmid DNA, according to the manufacturer's instructions. No-template controls (NTCs) were prepared for quality control.

To quantify the Control_B_SET1 plasmid in the collected plasma, multiplex digital PCRs using EPO_SET1 (FAM), Amp_3 (HEX), ColE1_3 (CY5), and pUCintergenic_3 (CY5.5) were designed for horses 1, 2, and 3. To quantify the Control_B_SET2 plasmid in the collected plasma, multiplex digital PCRs using EPO_SET2 (FAM), Amp_3 (HEX), ColE1_3 (CY5), and pUCintergenic_3 (CY5.5) were designed for horses 4, 5, and 6. The detection value was the mean of the four markers.

Results

Procedures of blood sample storage and their conditions

Blood samples collected in EDTA collection tubes were centrifuged, and the tubes were frozen at −40°C (Fig. 1, samples 1–3). The samples were allowed to thaw at room temperature (18−25°C) for approximately 50 min before using them for gene doping detection (Fig. 1, samples 4–6). In the naturally thawed state, protruding blood cell components were observed between the blood cells and plasma components; therefore, the thawed blood collection tubes were centrifuged (Fig. 1, samples 7–9). A small amount of hemolysis in the blood cells and plasma was visually confirmed in samples 4–9.

Copy numbers of genomic DNA in the collected plasma

Genomic DNA in the plasma was quantified by digital PCR using primers and probes designed in the same exons of the *MSTN*. DNA extracts (each 78=13 time points × 6 horses; 3 weeks of non-refrigerated storage, 1 week of refrigerated storage, and 2 weeks of refrigerated storage) from all the plasma collected from the six animals were used for quantifying genomic DNA in the plasma.

As shown in Figure 2, 3.6 copies/*µl* (standard deviation (SD): 2.43), 13.6 copies/*µl* (SD: 13.7), and 35.1 copies/*µl* (SD: 54.0) were observed in 50 *µl* of elution extracted from 1.5 m*l* of plasma after non-refrigerated storage, 1 week of

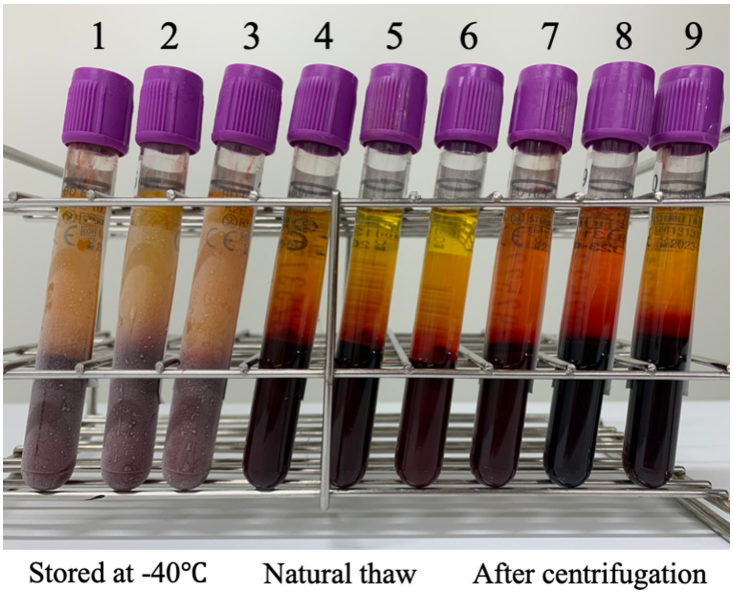

Fig. 1. Sample processing procedure using samples that had been refrigerated for 1 week. Samples 1–3: stored under frozen conditions (−40°C). Samples 4–6: naturally thawed at room temperature for 40 min. Samples 7–9: centrifuged to separate plasma and hemolyzed blood cells. A small amount of hemolysis was observed in all tubes because samples refrigerated for 1 week were used.

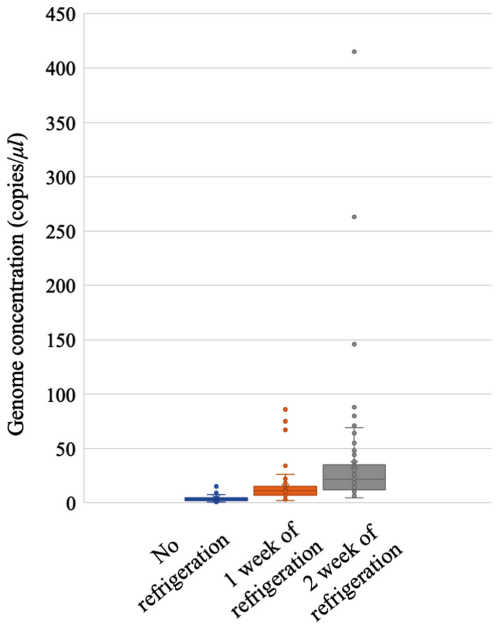

Fig. 2. Genome copy numbers in 1.0 m*l* of plasma after non-refrigerated storage, 1 week of refrigerated storage, and 2 weeks of refrigerated storage.

refrigerated storage, and 2 weeks of refrigerated storage, respectively. Long-term refrigerated storage increased the copy number of genomic DNA in the plasma. Refrigerated storage caused hemolysis, which was thought to increase the copy number of genomic DNA in the plasma. The plasma turned reddish black in color and was suspended because of the extended storage period.

Detecting exogenous genes using digital PCR

Plasma without refrigerated storage was used in this experiment. The distributions of plasmids detected in the plasma were different among all horses (Fig. 3). The detection value up to 1 day after administration was over 100 copies/μl, which provided clear detection values in digital PCR. The detection values at 2 days after administration in horses 1, 2, and 3 were 3.1, 5.3, and 3.9 copies/μl, respectively, whereas the detection values at 2 days after administration in horses 4, 5, and 6 were 27.7, 32.6, and 11.2 copies/μl, respectively. In horse 1, 13.9 and 10.6 copies/μl were observed at 4 days and 3 weeks after administration, respectively, but in others, it was <10.0 copies/μl.

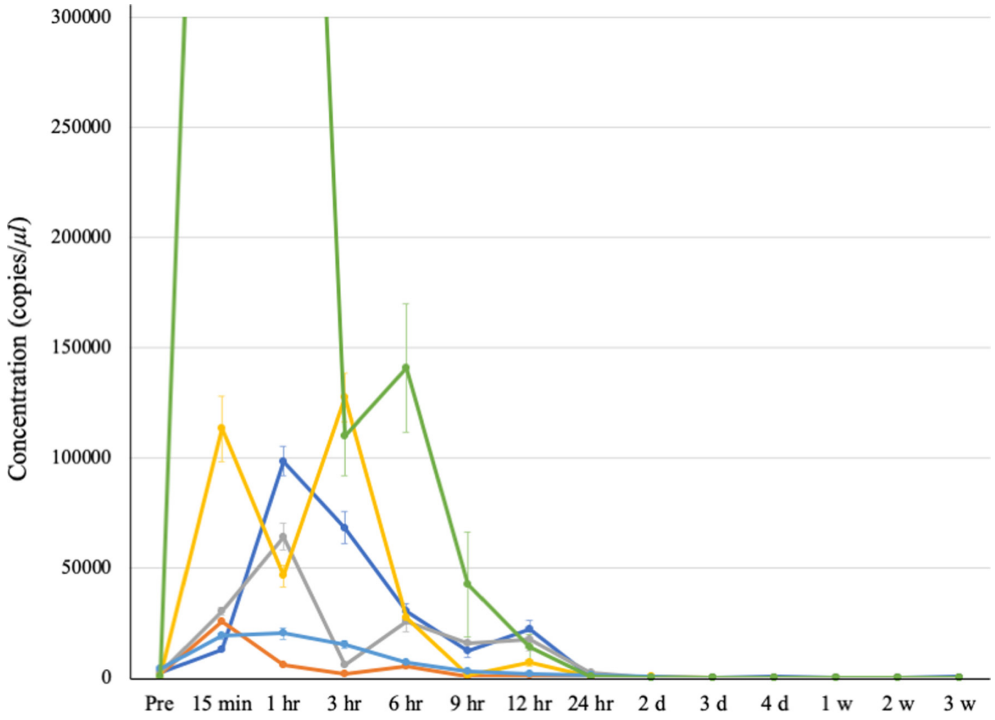

Fig. 3. Copy numbers of the plasmid vectors detected in 1.5 m*l* of plasma following plasmid administration to six horses. Blue, horse 1 (Control_B_SET1); orange, horse 2 (Control_B_SET1); gray, horse 3 (Control_B_SET1); yellow, horse 4 (Control_B_SET2); sky blue, horse 5 (Control_B_SET2); and green, horse 6 (Control_B_SET2). The copy numbers at the 15 min and 1 hr time points for horse 6 were 827,361 and 779,471 copies/μl, respectively, and only this horse showed higher values than the other horses.

Effect of refrigerated storage on gene doping detection

To evaluate the effect of refrigerated storage, blood at 1 and 2 days after administration, which were the cutoffs for certainty detection in this study, were used. These samples were stored at −40°C after non-refrigerated storage, 1 week of refrigerated storage, and 2 weeks of refrigerated storage. Subsequently, all blood samples were naturally thawed at room temperature and centrifuged. DNA was extracted from 1.5 m*l* of plasma, and the plasmid detection values were compared.

The administered plasmids in the samples with non-refrigerated storage, 1 week of refrigerated storage, and 2 weeks of refrigerated storage were detected. The detection ratios of plasmids at 1 week of refrigerated storage and 2 weeks of refrigerated storage to non-refrigerated storage were 30–199% (Table 1). A tendency for the plasmid vectors to degrade was observed; however, they were confirmed to be detected without any issues even in the samples stored in a refrigerator for 1 to 2 weeks.

Discussion

In this study, we investigated the optimal procedures for storage and use of A and B samples suitable for gene doping tests on plasma. In conventional doping tests for low-molecular weight compounds, authorities take urine samples, split them into samples A and B, and send sample A to a testing laboratory. If sample A is positive, sample B is sent to another lab (or the same lab) for confirmation. Therefore, sample A requires short-term storage (1 to 2 weeks), and sample B requires long-term storage (several months). If urine is used for the tests, the A and B samples can be frozen safely, without any quality concerns. However, for gene doping tests using blood, especially plasma, the storage method, in addition to maintenance of the sample quality, is of great importance.

For gene doping testing in horses, sample collection occurs at the racetrack or training center, where it may be stored for several days before being transported to the testing laboratory. Therefore, we investigated the effects of refrigerated storage on blood samples. After refrigerated storage for 1 and 2 weeks, samples showed increased elution of the host genome into plasma compared with those after non-refrigerated storage. Although the detected plasmid copy numbers varied between non-refrigerated storage and 1 or 2 weeks of refrigerated storage, the detection term (2 days) was the same among them. Ideally, centrifugation should be performed immediately after sample collection to separate plasma components and use them for DNA extraction. However, it takes several days for the blood collected at a horseracing location to be transported to a testing laboratory and used. Refrigerated storage for approximately 1–2 weeks may have a negligible effect on qPCR-based gene doping tests.

A limitation of this study is that confirmation was only performed up to two weeks after collection. Hemolysis of blood cells increases in a time-dependent manner; therefore, the elution of additional host genomes is assumed for periods beyond two weeks. In addition, blood cells are known to lyse after exercise in racehorses. Therefore, post-race samples may be more hemolyzed than samples taken before races [3, 8]. The presence of excess genomic DNA

Table 1. Detected values of target substances in plasma using digital PCR

Samples after 24 hr	No refrigeration (A), copies/μl	Refrigeration for 1 week (B), copies/μl	Ratio (B/A), %	Refrigeration for 2 weeks (C), copies/μl	Ratio (C/A), %
1d_horse 1	283.1	473.5	167	166.5	59
1d_horse 2	105.1	69.2	66	124	118
1d_horse 3	3,116.4	1,111.8	36	3,666.4	118
1d_horse 4	486.2	969.2	199	547.6	113
1d_horse 5	1,520.8	2,277.1	150	1,716.8	113
1d_horse 6	680.6	510.5	75	385.2	57
Mean	1,032.0	901.9	116	1,101.1	96
Samples after 2 days	No refrigeration (A)	Refrigeration for 1 week (B)	Ratio (B/A)	Refrigeration for 2 weeks (C)	Ratio (C/A)
2d_horse 1	3.1	7.3	236	4.6	150
2d_horse 2	5.3	5.6	106	5.1	97
2d_horse 3	3.9	4.2	109	3.5	91
2d_horse 4	27.7	24.1	87	26.8	97
2d_horse 5	32.6	24.5	75	22.4	69
2d_horse 6	11.2	3.4	30	5.1	45
Mean	14.0	11.5	107	11.3	92

(A) Non-refrigerated storage, (B) 1 week of refrigerated storage, and (C) 2 weeks of refrigerated storage.

affects the efficiency of DNA extraction and PCR detection of target substances [15].

If a positive result is obtained from sample A, the athlete (in human sports) or trainer (in horseracing), as well as the respective authorities, may request testing using sample B. Therefore, sample B requires long-term storage before testing. To maintain the integrity of the doping test, sample B should ideally be of high quality [9]. The frozen sample storage method proposed in this study is suitable for gene doping tests using plasma. Moreover, the following procedures are suitable for the storage and use of sample B in gene doping tests: 1) centrifugation after sample receipt, 2) cryopreservation, 3) natural thawing at room temperature, and 4) centrifugation without mixing blood cell components. Although cryopreservation destroys blood cells, this procedure yields good plasma components from frozen blood samples, indicating that it is applicable to gene doping tests (transgene detection) by qPCR.

For gene doping tests using plasma, racing authorities should separate the plasma components by centrifugation or natural sedimentation after blood collection. For sample A, which undergoes testing within 2 weeks, refrigerated storage and transportation are sufficient; however, for sample B, which requires long-term storage, the proposed procedure in this study should be followed by racing authorities. Alternatively, if the same laboratory is testing both samples A and B, the laboratory should immediately centrifuge and freeze sample B for storage.

By administering 5 mg of naked plasmid to horses, detection was possible for approximately 2 days using digital PCR. These results were similar to those where 20 mg of naked plasmid was administered to one horse [16]. This suggests that naked plasmid would be detected until 2 days after administration, regardless of the dose. Additionally, small amounts of plasmids were detected in samples from 3 days to 3 weeks after administration. Therefore, highly sensitive methods, such as nested PCR with pre-amplification, may enable long-term detection. Microfluidic quantitative PCR and nested digital PCR include a pre-amplification step [12, 14]; therefore, these methods may be able to detect plasmids with higher sensitivity over a longer term than the other methods.

Since this study was conducted on naked plasmids, it cannot be applied to all gene doping methods, such as those using adenoviral vectors and plasmid vectors that are encapsulated in micellar polymers for drug delivery. For adenoviral vectors, gene doping tests have been developed using whole blood as a sample [10]. In such cases, whole blood may be frozen and stored without separation. However, in tests using plasma as a sample containing many blood cells, a large amount of the host genome is expected to be extracted; therefore, the detection sensitivity will decrease [15]. Thus, the method we have described in this study would be beneficial for gene doping tests.

In this study, the variation in distribution between individuals and the variation in detection efficiency in refrigerated storage may have been affected by the storage method proposed (collection tubes cannot be agitated after thawing). However, the procedure proposed in this study can be used as a sample storage procedure for gene doping detection, as detection by this procedure was sufficient. Thus, our study should help horseracing and equestrian sports authorities to control gene doping and should help racehorses and athletes to protect their rights.

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