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DNA extraction methods of canine sperm cells to sexing by quantitative PCR

Métodos de extração de DNA de espermatozóides caninos para sexagem por PCR quantitativo

Métodos de extracción de DNA de espermatozoides caninos para sexado por PCR cuantitativa

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Abstract
Despite similarity to other cell types, the sperm has a compacted chromatin which makes DNA extraction more difficult. Based on this, this study compared different sperm concentrations and three DNA extraction techniques to use in qPCR. Four protocols were tested: a non-commercial using phenol:chloroform (M1) alone or associated with a preparatory kit (Differex-System-Kit-M2), and two commercial with mini-columns extraction: M3 (Illustra-Blood-Genomic-Prep-Mini-Spin) and M4 (Wizard®-Genomic-DNA-Purification). Four sperm concentrations were used (1, 10, 30 and 50x106) from ejaculates of 3 dogs. After extraction, the DNA was used for qPCR with primers directed against X and Y chromosomes. M1 and M2 protocols recovered high DNA concentration independently of initial sperm concentration, with a satisfactory ratio of 260/280 absorbances (mean 1.80 and 2.10, M1 and M2 respectively). In contrast, other methods recovered low DNA concentration and with poor quality in M3 (mean 1.60 in M3 and 1.85 in M4). The DNA extraction of canine sperm cells using protocols with phenol:chloroform (M1 and M2) was efficient. The M1 protocol notably allowed the quantification of X and Y chromosomes in qPCR using low number of sperm cells (1x106). This study provides the first comparison of DNA extraction techniques of canine sperm and it is concluded that independent of sperm concentration used, including use of only 1x106 sperm, DNA extraction from these cells using phenol:chloroform has satisfactory results regarding the quantity and quality of the extracted sample, and enabled quantitation of target gene sequences in qPCR, allowing subsequent use in sperm sexing and other biotechnologies.
Keywords: Chromosome; Dog; Phenol-chloroform; Semen; Sexing sperm.

Resumo
Apesar da semelhança com outros células, o espermatozoide possui cromatina compacta, dificultando a extração do DNA. Com base nisso, este estudo comparou diferentes concentrações espermáticas e três técnicas de extração de DNA para uso em PCRquantitativo. Quatro protocolos foram testados: um não-comercial utilizando fenol:clorofórmio (M1) sozinho ou associado a um kit preparatório (Differex-System-Kit-M2), e dois comerciais com mini colunas de extração – M3 (Illustra-Blood-Genomic-Prep-Mini-Spin) e M4 (Wizard®-Genomic-DNA-Purification). Quatro concentrações espermáticas foram utilizadas (1, 10, 30 and 50x106 células) do ejaculado de 3 cães. Após a extração, o DNA foi utilizado para qPCR com primers direcionados contra os cromossomos X e Y. Os protocolos M1 e M2 recuperaram alta concentração de DNA independente da concentração espermática inicial, com proporção satisfatória em absorbâncias de 260/280 (média 1,80 e 2,10, M1 e M2 respectivamente). Em contrapartida, os outros métodos recuperaram baixa concentração de DNA com baixa qualidade em M3 (média 1,60 em M3 e 1,85 em M4) independente da concentração espermática. A extração do DNA de células espermáticas caninas utilizando protocolos com fenol:clorofórmio (M1 e M2) foi eficiente. O protocolo M1 notavelmente permitiu a quantificação dos cromossomos X e Y no qPCR utilizando baixo número de células espermáticas (1x106). Este estudo fornece a

primeira comparação de técnicas de extração de DNA de espermatozoide canino. Pode-se concluir que independente da concentração de espermatozoides utilizada, a extração do DNA de espermatozoides utilizando fenol:clorofórmio é satisfatória e possibilita a quantificação da sequência de genes alvo em qPCR, permitindo subsequente utilização na sexagem espermática e em outras biotecnologias.
Palavras-chave: Cromossomo; Cão; Fenol-clorofórmio; Sêmen; Sexagem espermática.

Resumen
Apesar de la similitud con otras células, el esperma tiene una cromatina compacta, lo que dificulta la extracción de DNA. En base a esto, este estudio comparó diferentes concentraciones de esperma y tres técnicas de extracción de DNA para su uso en la PCR cuantitativa (reacción en cadena de la polimerasa). Se probaron cuatro protocolos: no comercial usando fenol:cloroformo (M1) solo o asociado con un kit de preparación (Differex-System-Kit- M2) y dos protocolos comerciales con mini columnas de extracción-M3 (Illustra-Blood-Genomic-Prep-Mini-Spin) y M4 (Wizard®-Genomic-DNA-Purification). Se utilizaron cuatro concentraciones de esperma (1, 10, 30 y 50x106 células) del eyaculado de 3 perros. Después de la extracción, el DNA se utilizó para qPCR con primers dirigidos contra los cromosomas X e Y. Los protocolos M1 y M2 recuperaron una alta concentración de DNA independientemente de la concentración inicial de espermatozoides, con una relación satisfactoria en absorbancias de 260/280 (media 1,80 y 2,10, M1 y M2 respectivamente). Mientras que los otros métodos recuperaron baja concentración de DNA con baja calidad en M3 (media 1,60 en M3 y 1,85 en M4) independientemente de la concentración de espermatozoides. El DNA de espermatozoides caninos utilizando protocolos de fenol:cloroformo (M1 y M2) fue eficiente. El protocolo M1 permitió en particular la cuantificación de los cromosomas X e Y en qPCR utilizando un bajo número de espermatozoides (1x106). Según nuestro conocimiento, este estudio proporciona la primera comparación de las técnicas de extracción de DNA de esperma canino. Teniendo en cuenta los resultados obtenidos, se puede concluir que independientemente de la concentración de espermatozoides utilizada, la extracción de DNA de espermatozoides utilizando fenol:cloroformo tiene resultado satisfactorio de la muestra extraída y permite la cuantificación de la secuencia de genes diana en qPCR, lo que permitió su uso posterior en el sexado de espermatozoides y otras biotecnologías.
Palabras clave: Cromosoma; Perro; Fenol-cloroformo; Semen; Sexado de esperma.

1. Introduction

PCR allows DNA replication in vitro and amplification of specific sequences with high sensitivity and specificity (Bueno, 2004; Mothé et al., 2018a). However, appropriate quality and quantity of DNA are necessary for efficient PCR use. DNA extraction is therefore the first step in molecular biology research field (Coelho et al., 2004).

Several methods of DNA extraction are described in the literature however there are still problems such as contamination with foreign DNA, enzyme inhibitors and DNA fragility (Coelho et al., 2004). For these reasons an efficient extraction method, free of inhibitors and which maintains the integrity of genetic material, is essential to the correct application of PCR and other molecular techniques. One of the conventional methods for extraction of other cell types uses phenol and chloroform that promote protein denaturation, based on the hydrophobic properties of these macromolecules and their affinity with organic solvents (Oliveira et al., 2007).

Other conventional protocols for obtaining DNA are based on commercial kits, as in case of criminal investigation for sexual abuse, in which sperm cells are mixed with epithelial cells (Tsukada et al., 2006) besides others kits that use somatic cells (Lee et al., 2003; Namba et al., 2003; Abu-amero et al., 2013; Edwards et al., 2011; Kamani et al., 2013).

However, most of routinely methods used for isolating DNA from somatic cells have been ineffective for sperm cells. It is recognized that sperm DNA is six times more condensed than a mitotic chromosome DNA (Pógany et al., 1981). In somatic cells, DNA is composed of histones, but in spermatozoa the histones are gradually replaced during spermatogenesis by transition proteins and later by low molecular weight proteins (protamines), making the nucleus extremely condensed (Marushige and Marushige, 1975). Due to this strong chromatin condensation, the nucleus of the spermatozoa is smaller than that of somatic cells (Eddy, 2006), making it difficult to extract DNA from these cells by existing methods. Usually, for DNA extraction from other cells such as blood samples, a volume of 200 µL to 1 mL or a total number of cells greater than or equal to 1x108 cells are used (GE manual, 2007). However, unlike other animal species such as bovine (Scott et al., 2016; Scott et al., 2018), dog's ejaculate volume and sperm concentration are low, requiring technique improvement that allows DNA

extraction from smaller volumes and subsequent qPCR.

Thus, the objective of the present study was to evaluate different methods of extracting sperm DNA from dogs to standardize the most appropriate technique for this type of sample and to enable its use in qPCR to determine the percentage of X and Y chromosomes in sexed samples. In addition to standardizing the qPCR technique for analysis of the X and Y chromosomes in the canine species.

2. Methodology

The study is qualitative study (Pereira et al., 2018) and was conducted according to the ethical principles recommended by Brazilian College of Animal Experimentation (COBEA) and was approved by Commission ethics on Animal Use (CEUA), Faculdade de Medicina Veterinária e Zootecnia da Universidade Estadual Paulista, under protocol number 146/2014.

All reagents used were of high purity and purchased from GE Healthcare (Uppsala, Sweden), Sigma-Aldrich (São Paulo, Brazil), Merck SA (São Paulo, Brazil) or Bio-Rad Laboratories Brazil Ltda (São Paulo, Brazil) or they are cited among the text.

Three healthy crossbred dogs were used with 2 to 5 years-old from Faculdade de Medicina Veterinária e Zootecnia – UNESP (Botucatu, SP, Brazil). All were clinically and reproductively examined before the experiment. Animals were conditioned to semen collection by digital manipulation of the penis (Seager and Platz, 1977) in absence of a female, being collected 4 ejaculates from each dog.

Semen volume, motility, vigor, concentration and morphology were evaluated. Only samples containing >70% morphologically normal and live spermatozoa (motility and vigor) were used. After analysis, the samples were divided into four aliquots and submitted to different treatments. Then, every aliquot was separated according to sperm concentrations at 1, 10, 30 and 50x106 cells.

Three protocols were tested for technique standardization including one described by Taylor (2005) with modifications, alone (M1) or associated with a preparatory method for somatic cells separation (M2 - Differex System Kit, Promega Biotechnology Brazil, Ltda, Brazil); and two commercial protocols using extraction mini-columns - M3 (Illustra Blood Genomic Prep mini Spin Kit, GE HealthCare, UK) and M4 (Wizard® Genomic DNA Purification Kit, Promega Biotechnology in Brazil Ltda, Brazil).

Prior to extraction, cells were washed twice with PBS (pH 7.2) by centrifugation at 2.000 xg for 10 minutes to remove seminal plasma. After washing, the supernatant was discarded and lysis buffer (10 mM TRIS, 10 mM EDTA, 10 Mm NaCl, 2% mercaptoethanol, 0.5% Sodium dodecyl sulfate, pH 8.0 adjusted with 1M NaOH) was added to M1. In M2, M3 and M4 protocols the buffers were supplied by kit. Next, the samples were frozen at -80°C overnight and then thawed and sonicated in ice bath using a 3.0 mm probe, in 20% amplitude for 30 seconds repeated for 10 times at intervals of 1 minute between series. After this, the protocols M3 and M4 followed manufacturer's instructions. For M1 and M2 protocols the samples were heated to 50°C in a water bath for 50 minutes. Thus, 5 µL of 20 mg/mL K proteinase were added, and the samples were gently mixed and incubated for 14 to 18 hours at 50°C in a water bath. After incubation, 250 µL phenol:chloroform:isoamyl alcohol (25: 24: 1) were added.

Samples were homogenized by inverting the tube and centrifuged at 15.000 xg for 10 minutes. The lower density phase (upper tube) containing DNA was removed and transferred to a new tube. The process was performed again. One mL of cold absolute ethanol was added to the samples in a new tube and gently mixed. The tubes were centrifuged at 15.000 xg for 1 minute, ethanol was removed (supernatant) and the tubes remained open to dryness. The DNA was eluted in 25µL buffer (10mM TRIS, 1mM EDTA, pH 7.4) and stored at -20ºC.

Optical density was performed by a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific Inc, USA) to evaluate the DNA concentration. The DNA purity was evaluated by data obtained of absorbances ratio at 260 and 280 wavelengths.

Subsequently, the extracted DNA was submited to qPCR using primers directed against Y chromosome (AF_107021.1) or X chromosome (NM_001003323.2). The GoTaq® qPCR Mastermix kit (Promega, Madison, WI, USA) and the 7500 Fast Real Time PCR equipment (Life Technologies™, São Paulo, SP, Brazil) were used for qPCR. The standard curve of qPCR was prepared with a pool of samples submitted to 10-fold serial dilutions in distilled water, starting at 10-2 and ending at 10-6. The concentration of each DNA sample was calculated based on the standard curve by 7500 Software 2.0.6 program (Life Technologies™, São Paulo, SP, Brazil).

The results of DNA concentration were presented as mean ± standard error and analyzed by ANOVA and multiple comparisons by the Student-Newman-Keuls test for different protocols and sperm concentrations using the SigmaPlot software for Windows, v.11.0, 2008 Systat Software Inc. The results of 260/280 relative absorbance were presented by descriptive statistics.

3. Results

A M1 and M2 protocols recovered greater DNA concentration (P <0.001) after extraction in any of sperm concentrations used, with a satisfactory absorbance ratio 260/280 (1.80 ± 0.037, range from 1.78 to 1.83; and 2.10 ± 0.02, range from 2.05 to 2.10, respectively). In contrast M3 and M4 methods recovered a low DNA concentration (P <0.001) with inferior quality in M3 (260/280 ratio: 1.60 ± 0.10, range from 1.26 to 1.93, and 1.85 ± 0.18, range between 1.36 to 2.59, respectively), regardless sperm concentration. The amount of extracted DNA from samples was higher only in sperm concentration of 50x106 compared to 1x106 (P = 0.042). The other concentrations did not differ (Table 1).

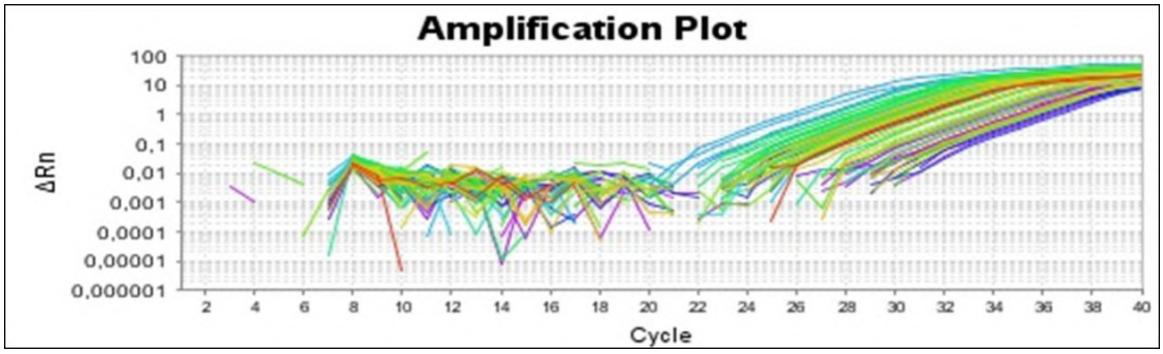
Table 1 - Mean ± standard error of canine sperm DNA concentrations (ng/µL) extracted by four different protocols

Sperm Concentration	Protocol – DNA concentration (ng/µL)			
	M1	M2	M3	M4
1x 10^6	896.5 ±368.2	3,085.0 ± 809.7	4.0 ±0.8	2.2 ± 0.5
10 x 10^6	1.364.2 ± 718.8	4,191.6 ± 406.2	3.2 ± 0.1	2.8 ± 1.1
30 x 10^6	2.614.8 ± 235.8	3,790.7 ± 802.0	3.3 ± 0.4	2.9 ± 1.1
50 x 10^6	2.842.4 ± 208.1	4,194.5 ± 976.0	5.0 ± 0.7	3.1 ± 1.5
Overall mean	1.929.5 ±308.7[a]	3,815.4 ± 358.4[b]	3.9 ± 0.3[c]	2.8 ± 0.5[c]

Different letters in the same line indicate statistical difference ($P \leq 0.05$). Source: Personal archive.

When the samples were subjected to qPCR the amplification was observed in M1 samples allowing quantitation of cells containing X and Y chromosomes, with correlation of log concentration (R2) of 0.995, slope -3.337 and 99.387% efficiency. The Cts (cycle threshold value) in these samples varied between 20 and 30 (Figure 1). In other protocols the amplification was observed in rare samples, but with high Cts (above 32), which was not considered for data analysis.

Figure 1 - qPCR amplification graphif DNA sperm samples extracted by chloroform:phenol (M1) method.



Source: Personal archive.

4. Discussion

The DNA in its original double-strand form is stable, resistant and remains unchanged in various environmental conditions. As a result, DNA extraction from a wide variety of cells has been elucidated. However, although it is also a cell, the extraction of DNA from sperm has failed, especially when compared to the isolation of DNA from somatic cells whose techniques are already well established. In this regard further studies must be conducted to enhance and/or develop other methods that allows the obtention of samples with hight quality containing nucleic acid of sperm (Almeida et al., 2009).

Several protocols associated or not with commercial kits can be used for DNA extraction from various biological samples as tested in this study for sperm cells. Despite having obtained DNA samples using these kits the quantity and quality was lower when compared with chloroform:phenol extraction. This method has been reported successfully in other species to extract DNA sperm (Hanson and Ballantyne, 2004; Silva et al., 2014) due protein denaturation caused by this solution. In fact, this was the method with more satisfactory results in terms of DNA extraction and with appropriate quality and quantity sufficient to qPCR.

When the amplification occurred in DNA samples extracted using commercial kit (M3 and M4), high Cts was observed (above 32 cycles) in qPCR that can be explained by the unsatisfactory quantity and quality of the samples. In contrast, in samples of DNA extracted by M1 method, amplifications occurred in previous cycles (until 30 cycles), contributing to a better qPCR (Wittwer et al., 1997).

The efficiency of qPCR in the samples extracted by the chloroform:phenol (99.387%) was between 90 and 110%, at recommended limits (Lalam, 2006; Alvarez et al., 2007). Likewise, the samples quality extracted by M1 contributed for these results.

In addition, the efficiency of reaction can be measured by the R2 value (R2 = 0.995) and by the slope, and the value of -3.3% ± 10% indicates an efficiency of 100% ± 10% (Liss, 2002; Svec et al., 2015), similar to that found in our study.

In M2 protocol, developed to separate sperm cells from somatic cells in cases of sexual abuse in humans, also was used chloroform:phenol but associated with commercial kit. But, despite the superior quality of the extracted samples when compared to the M3 and M4 protocols, the amplification also occurred in rare samples and in high Cts. The PCR reaction, as occurred in M2 protocol, can occur improperly or not occur in case of incorrect handling and/or storage.

However, although possible, we do not believe in an execution error due to the number of repetitions and care of the packaging and handling, subjecting the samples to the appropriate combination of temperature and time. We assume that substances present in the commercial kit used in this protocol react adversely with those present in kits used for amplification, preventing the same, since the same method chloroform:phenol without commercial kit (M1) resulted in amplification of DNA segments without difficulty.

Also, we do not indicate the use of this method since the number of somatic cells observed in the microscope is very low (data not shown), less than one/10 fields, which would increase the analysis costs.

In M3 and M4 protocols were used commercial kits which allow the isolation of DNA by binding to the membrane of the mini-column extraction, with subsequent washing for purification and elution of the sample with a buffer. The use of kits have allowed the homogeneous and rapid extraction of DNA from various cell types (Edwards et al., 2011; Kamani et al., 2013; Abu-amero et al., 2013), however this study did not demonstrate efficacy in extracting sperm DNA. The nucleus of these cells is extremely condensate and resistant to sonication (Eddy, 2006; Lee et al., 1995; Kuretake et al., 1996), and therefore prevented the extraction by this methods that are used for other cells that do not exhibit this stable and resistent cell arrangement. We believe that phenol:chloroform method has greater capacity to destroy this structure, exposing the nucleus and enabling the DNA extraction.

Furthermore, the minimum number of cells for DNA extraction from various biological samples should be equal or greater than 100x108 (GE manual, 2007), which prevents DNA sperm extraction from some dogs once sperm concentration of this species could be less than would be needed for extraction. Despite this, most of canine breeds have sufficient number of spermatozoa in the ejaculate; however, even in these cases, the use of 100x106 cells for a single test could prevent the use of these samples in other types of analysis. With phenol:chloroform protocol the DNA extraction using only 1x106 of sperm cells was possible, enabling the extraction and use of the DNA in qPCR and other techniques (Mothé et al., 2018b).

Therefore, in the absence of a specific kit for spermatic DNA extraction the phenol:chloroform protocol shall be considered for being affordable, allow use of small sample size and especially because it is the most effective method for this purpose, despite demanding more time for implementation, be toxic and can contaminate the sample.

5. Conclusion

Based on our knowledge, this study provides the first comparison of DNA extraction techniques of canine sperm. Considering the obtained results, it is concluded that independent of sperm concentration used, including use of only 1x106 sperm, DNA extraction from these cells using phenol:chloroform has satisfactory results regarding the quantity and quality of the extracted sample, and enabled quantitation of target gene sequences in qPCR, allowing subsequent use in sperm sexing and other biotechnologies.

Conflicts of interest

We confirm that we have no financial and personal relationships with other people or organizations that could inappropriately bias or influence our work.

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