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Implementation of rhAmp SNP genotyping for the detection of the B3GALNT2 gene mutation associated with congenital hydrocephalus in Friesian horses in southern Sonora

Implementación del genotipado rhAmp SNP para la detección de la mutación en el gen B3GALNT2 asociada a hidrocefalia congénita en caballos Friesian del sur de Sonora

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ABSTRACT

Congenital hydrocephalus is a recessive genetic abnormal condition affecting Friesian horses, so the identification of healthy carrier animals is crucial to avoid inheriting such condition. Therefore, the objective of the present study was the detection of the B3GALNT2 gene mutation related to the presence of hydrocephalus, through the rhAmp SNP technique to identify healthy carrier horses. The study was performed in 4 locations in southern Sonora, Mexico. Blood samples were collected from 51 Friesian horses using EDTA tubes, which were centrifuged until the leuko-platelet layer was obtained. Subsequently, the TACO™ DNA extraction kit was used to obtain the DNA, which was processed by the rhAmp SNP assay to detect the mutation in the B3GALNT2 gene. A total of 7 (13.72 %) Friesian horses were found to be heterozygous carriers (C/T). In conclusion, the presence of the mutant allele was confirmed by the rhAmp SNP assay, which was proposed as an effective molecular technique for the detection of horses as carriers of genetic abnormal conditions.

Keywords: Hydrocephalus; heritability; rhAmp SNP; recessive; heterozygous.

RESUMEN

La hidrocefalia congénita es una condición genética recesiva anormal que afecta a los caballos Friesian, por lo que la identificación de animales portadores sanos es crucial para evitar heredar dicha condición. Por ello, el objetivo del presente estudio fue la detección de la mutación en el gen B3GALNT2 relacionada con la presencia de hidrocefalia mediante la técnica de rhAmp SNP para identificar caballos portadores sanos. El estudio se realizó en 4 localidades del sur de Sonora, México. Se tomaron muestras de sangre de 51 caballos Friesian utilizando tubos con EDTA, los cuales fueron centrifugados hasta obtener la capa leuco-plaquetaria. Posteriormente, se utilizó el kit de extracción de ADN TACO™ para obtener el ADN, el cual fue procesado mediante el ensayo rhAmp SNP para detectar la mutación en el gen B3GALNT2. Un total de 7 (13.72 %) caballos Friesian resultaron ser portadores heterocigotos (C/T). En conclusión, se confirmó la presencia del alelo mutante mediante el ensayo rhAmp SNP, el cual es propuesto como una técnica molecular eficaz para la detección de caballos portadores de anomalías genéticas.

Palabras clave: hidrocefalia; heredabilidad; rhAmp SNP; recesiva; heterocigoto.

INTRODUCTION

The Friesian horse breed, originated in the Netherlands, was registered in 1879 and currently has a large active breeding population. History shows that this population was smaller, causing an accumulation of inbreeding and a decrease in genetic diversity, resulting in a high incidence of genetic defects that exceed the 1% inbreeding rate indicated by the FAO. All equine breeds have a different genetic variety, but that with the least genetic diversity is the Friesian breed, which is characterized by having a small population with an uneven genetic contribution of ancestry (Ducro *et al*., 2015).

In recent years, the horse industry in Mexico has shown annual growth rates of 10 % with more than 150 horses breeding establishments, making Mexico the second country with the largest number of horses in the world, despite their minor genetic divergence. In 2000 arrived the first Friesian called "Theet van de Pluum" and currently it has been estimated that there are around 7000 horses registered in the Mexican Friesian Horse Association (Asociación Mexicana del Caballo Frisón, 2020).

One of the most important hereditary pathologies observed in horses is the congenital hydrocephalus, a birth defect that results in a significant and harmful increase in the volume of the cranial cavity due to the accumulation of cerebrospinal fluid in the brain's ventricles (Ojala and Ala-Huikku, 1992). Such disease is associated with a single nucleotide substitution from cytosine to thymine (XM_001491545 c.1423C > T) in the b-1,3-N-acetylgalactosaminyltransferase 2 (B3GALNT2) gene, and it has been reported in several breeds such as Quarter Horse, American Miniature Horse, Friesian Horse, Standarbred, Hanoverian Warmblood and Thoroughbred (Foreman *et al*., 1983; Ojala and Ala-Huikku, 1992; Ferris *et al*., 2011; Oey *et al*., 2011; Sipma *et al*., 2013).

Congenital hydrocephalus is an uncommon genetic defect that in the last 30 years has presented an increase in

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reports in Friesian horses, compared to other breeds, due to their low genetic diversity (Ayala-Valdovinos *et al*., 2017). Currently, an effective treatment to cure equine hereditary hydrocephalus is not available; then, it is necessary to continue working on the evaluation of the inheritance of hydrocephalus through different genetic tests, to avoid the reproduction of animals affected with this mutation (Finno, 2020).

Since hydrocephalus in Friesian horses is an autosomal recessive hereditary disease, the method of choice to identify and prevent this disease is molecular testing, which allows the detection of animals heterozygous for the mutation in the B3GALNT2 gene associated with hydrocephalus (Ayala-Valdovinos *et al*., 2017). Results of this technique enable the identification of unaffected carrier animals, facilitating their exclusion from reproductive programs, and thus, reducing the incidence of hydrocephalus in the population (Ducro *et al*., 2015). The rhAmp SNP assay, an enzymatic system with the best allele specificity, uses RNasa H2 to eliminates or reduces the presence of dimers, and generates a higher fluorescence than other genotyping techniques resulting in better clustering and separation of genotypes. Also, the use of universal reporter markers makes possible the multiplex genotyping of SNPs, achieving the simultaneous detection of two SNPs in a single PCR reaction, which reduces the cost of the assay and increases the yield (Beltz *et al*., 2018).

Therefore, the objective of the present study was the implementation of rhAmp SNP genotyping assay as a novel molecular technique, to detect the presence of alleles in the B3GALNT2 gene associated with congenital hydrocephalus in Friesian horses from southern Sonora, México.

MATERIALS AND METHODS

Ethical statement

All animal procedures performed in this study were revised and approved by the "Bioethics Committee from the Agronomic and Veterinary Sciences Department", at the Instituto Tecnológico de Sonora (ITSON) located in Ciudad Obregón, Sonora, Mexico (Protocol code 2022_003).

Animal population

The present study included a total of 51 Friesian horses (29 males and 22 females), 3 to 6 years old, and optimal body condition, randomly selected from 4 different farms. All the horses had a phenotype without hydrocephalus, since animals showing this disease die at birth. The collaborative farms were open equine stables located in Huatabampo, Navojoa, Cajeme and Hermosillo, which are municipalities in the southern of Sonora, Mexico, and geographically located between 32°29' and 26°14' North latitude and between 108°26' and 105°02' West longitude of the Greenwich Meridian.

Blood collection and DNA extraction

A blood sample (4 mL) was collected from each horse by jugular vein puncture using BD Vacutainer® tubes with EDTA K2, which were identified and transported to the ITSON Veterinary Laboratory. Blood samples were centrifuged for 40 min at 30,000 rpm to separate the leuko-platelet layer, which was transferred into 1.5 mL tubes, and homogenized by agitation and inversion. Afterward, the DNA extractions were carried out by the taco™ Nucleic Acid Automatic Extraction System (GeneReach Biotechnology Corp.) using the taco™ DNA/RNA Extraction Kit according to the manufacturer's instructions. Both quality and quantity of DNA per sample were obtained with UV spectrophotometry (BioSpect-Nano, Shimadzu®). DNA integrity was verified by electrophoresis on a 1 % agarose gel stained with ethidium bromide using 90 V for 45 min. The gel was visualized in the system with ultraviolet light and recorded by BioDoc-It Imaging SystemTM (Analytik Jena US LLC, Upland, CA, USA). DNA extractions were placed in sterile vials, stored at -20 °C, and subsequently processed for molecular analyses with the rhAmp SNP assay.

Probe and synthetic positive design

To identify the single nucleotide variations with the cytosine to thymine (C/T) change position at the nucleotide 75,907,505 in the chromosome 1 *Equus caballus* (NC_009144.3) covering B3GALNT2 gene, the sequence was downloaded from the Ensembl database (https://www.ensembl.org/index.html) and then analyzed using the SnapGene Viewer program (https://www.snapgene.com).

Based on the previous information, two sequences of 250 bp were generated and the variation (C/T) was placed in the middle of the amplicon to be used as synthesized controls. Subsequently, from the already selected sequences, the primers and probes for mutation detections were designed using the Integrated DNA Technologies software (i.e., rhAmp SNP genotyping section), in which the fluorophore FAM was assigned for the reference allele and the fluorophore VIC for the alternative allele.

Standardization of the rhAmp SNP technique

To develop the rhAmp SNP reaction, gBlocks were used as synthetic controls, with the normal equine sequence and a synthetic positive with the mutant allele. These components were activated in 50 µL nuclease-free water and diluted from a concentration of 101 to 103 ng/µL, following the protocol proposed by Integrated DNA Technologies (https://www.idtdna.com). The volumes of reagents used for the rhAmp SNP reaction were 2.65 µL of combined master mix and reporter mix, 0.25 µL of rhAmp SNP assay (20X), 0.10 µL of nuclease-free water, and 2 µL of DNA at a concentration of 10 ng/µL.

Reactions were then carried out with different concentration gradients of controls until triplicates were obtained to select the most suitable concentration for the rhAmp SNP technique. This technique was performed on the Bio-Rad CFX96TM real-time thermal cycler (Bio-Rad Lab Inc., Hercules CA, USA) using FAM and VIC fluorophores, with an amplification program of 1 initial cycle of enzyme activation at 95 °C for 10 min, followed by 39 cycles of denaturation extension at 95 °C for 10 sec, 60 °C for 30 sec and 68 °C for 20 sec.

Genotyping to detect B3GALNT2 gene mutation

Genotyping using rhAmp technique was performed using 5 ng of DNA mixed with rhAmp Genotyping Mix, composed by rhAmp Genotyping Master Mix (catalogue number: 1076017), rhAmp Reporter Mix (catalogue number: 1076028) and custom rhAmp SNP assays (Integrated DNA Technology, Coralville, Iowa, USA). All of these procedures were performed according to the manufacturer's recommended protocols.

Results of the allele discrimination process were interpreted as follows: Homozygous allele 1 (C/C) lies along the horizontal axis (high FAM and low VIC); homozygous allele 2 (T/T) is expected to be parallel with the vertical axis (high VIC and low FAM signal). Finally, heterozygote alleles (C/T) are expected to cluster along the diagonal, centrally in the graphic (nearly equal FAM and VIC signals).

Statistical analysis

The sample size was determined by a simple completely random sampling using the formula described by Schaeffer *et al.* (1996), and limit on the estimation error of less than 5 % ($P < 0.05$). The frequency of carrier animals was determined as the proportion of heterozygous animals with respect to the total number of sampled animals.

RESULTS AND DISCUSSION

Probes design for rhAmp SNP assay and synthetic control

The B3GALNT2 gene sequence was analyzed to detect the position of the mutation and select the 250 bp amplicon. Subsequently, we obtained the region and the specific base pairs of the C/T change, which were required to design the probes and the primers. The rhAmp SNP genotyping tool from the Integrated DNA Technology (IDT) software was used for this purpose (Table 1). Such genotyping tool is a fully integrated genotyping solution including a predesigned assay and synthetic control templates.

Currently, the use of a synthetic positive is essential for the detection of microorganisms and genetic mutations that cause a pathology. The rhAmp SNP genotyping technology allowed detecting the alleles more quickly and easily due to RNasa H2. This methodological procedure generates reliable results that make possible to differentiate carrier from non-carrier individuals of a certain class of alleles, helping in the diagnosis of the congenital hydrocephalus (Cárdenas, 2018).

DNA extraction

The DNA evaluated by spectrophotometry showed an average OD 260/280 of 1.95 and a concentration of 184.75 ng/µL. The purity of the nucleic acids was based on the optical density (OD) indicator, obtaining values from 1.8 to 1.9 in the 260/280 ratio. According to the National DNA Bank, a 260/280 ratio is very stable, and values between 1.8 and 2.0 are required to consider that the DNA is optimal for the genotyping assay. Therefore, the purity of the samples processed in the current study were optimal for performing the rhAmp SNP technique.

According to Cornejo *et al*. (2014), the magnetic bead kit (TACO™ DNA kit) used for the nucleic acid extraction procedures adsorbs negatively charged DNA onto a selective matrix. The DNA is held together during the removal of cellular remnant, whereas secondary metabolites are removed by lysis and washing solutions to release the molecule from the matrix. This method decreases the extraction time by reducing steps in the procedure and guarantees a high extraction purity, then DNA recovery and contaminant removal processes are very efficient.

To confirm that the samples analyzed had a high molecular weight and to verify that they can be conserved in the long term, an agarose gel electrophoresis was performed and bands with intensity were obtained after reading (Figure 1). The results of the electrophoresis agreed with the description made by Cornejo *et al*. (2014). They explained that when the DNA is intact, a well-defined band should be observed close to the well where the sample is placed, on the other hand, when a band presents sweeping or the lane is luminous, it is indicative of a degraded or fragmented DNA product.

Table 1. Sequences of primers used to perform the rhAmp SNP technique.
Tabla 1. Secuencias de los iniciadores usados para realizar la técnica de rhAmp SNP.

Primer	Sequence 5' 3'
Specific primer 1	GAATGTTGTCTTCCCCGT
Specific primer 2	AATGTTGTCTTCCCCGC
Reverse primer	CGCTTACTATTAGACTCACATTCACG

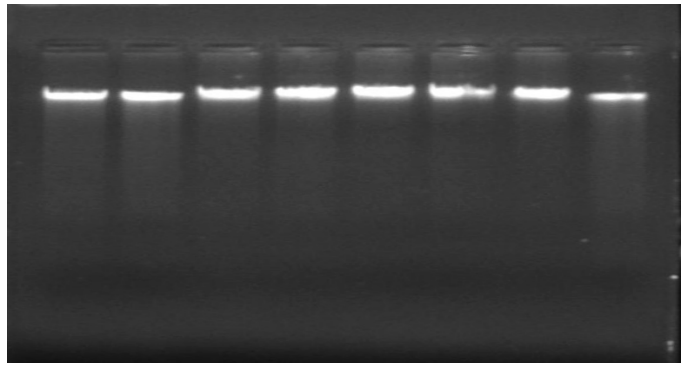

Figure 1. DNA extraction on 1.5 % agarose gel stained with ethidium bromide. Lanes 1 to 8 contain DNA products from blood samples of Friesian horses.
Figura 1. Extracción de ADN en gel de agarosa al 1.5 % teñido con bromuro de etidio. Los carriles del 1 al 8 contienen los productos de ADN a partir de muestras sanguíneas de caballos Friesian.

Standardization of rhAmp SNP technique

The standardization of the rhAmp SNP technique was performed with the gBlocks, which were designed to visualize the allelic discrimination plot. The gBlocks gene fragments are double-stranded, sequence-verified genomic blocks up to 500 base pairs. At the end of the process, the ideal concentration of the gBlocks for the reaction was 10^5 and the final volume per sample was 5 µL. The results after standardization showed both controls and synthetic positive samples (Figure 2).

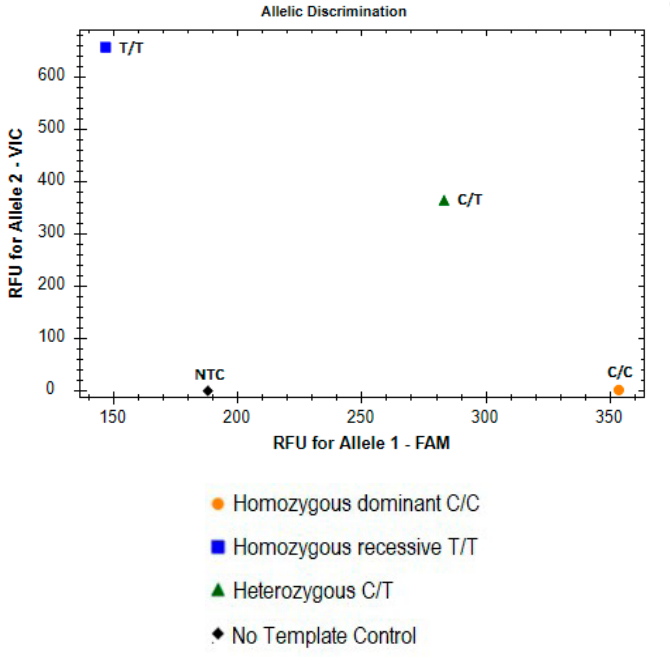

Figure 2. Graph of standardization of rhAmp SNP genotyping directed to amplicons with synthetic alleles, with reference allele C and alternative allele T. Post-PCR read data were obtained and the normalized reporter signal was measured in Relative Fluorescence Units (RFU) for allele 1 (FAM) and allele 2 (Yakima Yellow alternative of VIC) along the X-Y axes respectively. The allelic discrimination showed three distinct genotype groups, including homozygous reference allele C/C (yellow), heterozygous C/T (green) and homozygous alternative allele T/T (blue).

Figure 2. Gráfica de estandarización de genotipado rhAmp SNP dirigido a amplicones con alelos sintéticos, con alelo de referencia C y alelo alternativo T. Se obtuvieron datos de lectura post-PCR y se midió la señal reportera normalizada en Unidades Relativas de Fluorescencia (RFU) para el alelo 1 (FAM) y el alelo 2 (Yakima Yellow alternativo de VIC) a lo largo de los ejes X-Y respectivamente. La discriminación alélica mostró tres grupos de genotipos distintos, incluyendo homocigoto alelo de referencia C/C (amarillo), heterocigoto C/T (verde) y homocigoto alelo alternativo T/T (azul).

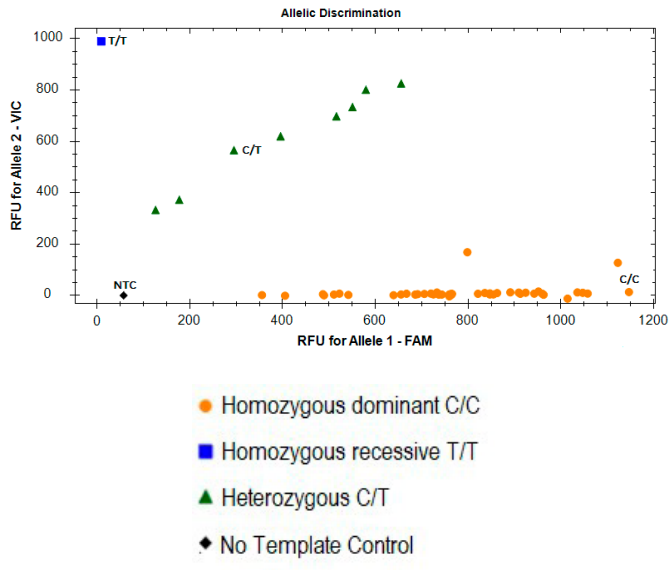

Figure 3. Graph of allelic discrimination results of rhAmp SNP genotyping with the reference allele amplicon C and alternative allele T (XM_001491545) using horse biological samples. Post-PCR read data showed quantification of signals to identify RFU along the X-Y axes respectively for allele 1 (FAM) and allele 2 (Yakima Yellow alternative of VIC). This assay used 51 DNA samples extracted from Friesian horses.

Figure 3. Gráfica de resultados de discriminación alélica de genotipado rhAmp SNP con el amplicón del alelo de referencia C y alelo alternativo T (XM_001491545) utilizando muestras biológicas de caballo. Los datos de lectura post-PCR mostraron la cuantificación de señales para identificar las RFU a lo largo de los ejes X-Y respectivamente para alelo 1 (FAM) y el alelo 2 (Yakima Yellow alternativo de VIC). En este ensayo se utilizaron 51 muestras de ADN extraídas a partir de caballos Friesian.

According to Aragón-López *et al*. (2021), an increase in the use of gBlocks has been reported recently in different investigations, which has been used to achieve standardization in less time. Therefore, the use of these fragments for standardization in rhAmp SNP is recommended for an efficient detection and real comparison, in case of not having a homozygous recessive individual.

rhAmp SNP genotyping

The rhAmp SNP genotyping was performed through the allelic discrimination technique (Figure 3) and using Bio-Rad CFX96TM software to identify the presence of the B3GALNT2 gene mutation. In the rhAmp SNP assay, the biallelic discrimination process is achieved by the competitive binding of two allele-specific forward primers, one labelled with fluorophore Yakima yellow dye (YY) alternative to fluorophore Victory (VIC), and the other with fluorescein amidite (FAM) (Giglioti *et al*., 2020). This technology uses a unique two-enzyme system coupled with RNA-DNA hybrid primers to interrogate target SNPs.

According to standardization of rhAmp SNP technique, the 51 horses were genotyped using the allele C as reference amplicon and the allele T as positive control, and a mixture of both to form the heterozygous control. The genotyping results revealed the presence of 7 heterozygous horses (1 female and 6 males) carrying the mutant allele (C/T) and 44 homozygous dominant horses (21 females and 23 males) with normal genotypes (C/C). Then, the estimated frequency of the mutant allele was 13.72 % (7/51x100). In an autosomal recessive condition, the heterozygote horses have one normal allele and one mutated allele; they do not show the disease because the normal allele is sufficient to produce the functional protein codified by the gene B3GALNT2. However, in some cases, there could be subtle variations in the function of such protein but not sufficient to manifest the disease.

The incidence found in this study was higher than the 9.6 % reported by Ayala-Valdovinos *et al*. (2017) and lower than the 17.3 % reported by Ducro *et al*. (2015). Kolb and Klein (2019) reported the first case of congenital hydrocephalus associated with a nonsense mutation of the B3GALNT2 gene in Belgian draft horses. The authors emphasized the importance of genotyping testing to identify carriers' animals in different horse breeds as potential tool to study congenital diseases.

Additional research involving a larger population size of Friesian horses is expected to find a higher frequency of

heterozygous carrier individuals in the state of Sonora. The detection of individuals carrying the B3GALNT2 gene mutation is a relevant indicator, considering that the frequency of the mutant allele is higher than 1 % in the population under study. Because of this, in this population the mutant allele will likely be inherited most frequently causing the consequent appearance of homozygous recessive animals, which will manifest the condition of hydrocephalus. According to Mendel's laws, the mating of two carrier animals has a 25 % chance of producing a diseased offspring. This would have serious economic repercussions for producers in the state of Sonora.

The rhAmp SNP technique use RNase H2, that eliminates or significantly reduces the presence of dimers, which complemented with the use of universal indicators, make possible the multiplex genotyping of SNPs, achieving simultaneous detection of two SNPs in a single PCR reaction, reducing the cost of the assay and increasing throughput (Beltz *et al*., 2018; Broccanello *et al*., 2018).

According to the results obtained in the current study, the rhAmp SNP technique demonstrated efficacy and high specificity, the reaction time was shorter compared to other reported techniques, and the results of all samples were obtained in a single graph. Additionally, this technique reduced the use of reagents by containing in a single mixture (Mix), which is necessary to perform the reaction. It suggests that the rhAmp SNP technique showed certain advantages over other molecular techniques that position it as one of the techniques of choice for the genotyping of mutant genes.

CONCLUSIONS

The rhAmp SNP technique demonstrated its efficacy and sensitivity through the successful identification of the mutation in the B3GALNT2 gene in Friesian horses in Sonora. The results obtained demonstrated the relevance of this molecular assay for the diagnosis of genetic mutations, which allowed differentiating carrier animals from non-carriers. This is of crucial importance in the case of congenital hydrocephalus because finding animals carrying the mutation mean that there is a risk of the birth of offspring with hydrocephalus, which will generate great economic losses for the horse breeders.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest associated with the current study.

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