UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
CAMPUS DE BOTUCATU

FREQUÊNCIA ALÉLICA DO POLIMORFISMO DE NUCLEOTÍDEO ÚNICO c.2032G>A NO GENE *PLOD1,* RESPONSÁVEL PELA SÍNDROME DA FRAGILIDADE CUTÂNEA EQUINA

NATALIA MORAES DIAS

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Orientador: Prof. Dr. Alexandre Secorun Borges

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RESUMO

A síndrome da fragilidade cutânea equina, conhecida como *warmblood fragile foal syndrome* (WFFS), é uma enfermidade do tecido conjuntivo de caráter autossômico recessivo e é caracterizada clinicamente por lesões cutâneas e mucosa da cavidade oral, articulações hiperextensíveis e abortamento. Tais características são incompatíveis com o desenvolvimento dos animais afetados. O polimorfismo de nucleotídeo único (SNP) c.2032G>A no gene *PLOD1* (*procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1*) em homozigose é responsável pela enfermidade em equinos de raças com aptidão para esportes equestres olímpicos. Existe apenas um caso publicado de um potro afetado com a confirmação da mutação, na Suíça, e apenas dois estudos de prevalência do SNP causador da doença, na Alemanha e nos Estados Unidos. Casos descritos com sinais clínicos compatíveis com a enfermidade e descritos antes da mutação ser descoberta estão presentes na literatura. Atualmente as associações de equinos na Europa tem dado grande atenção para esta enfermidade em equinos *warmblood*. A escassez de estudos e de informações sobre a existência da enfermidade no Brasil e no mundo leva-nos a acreditar que a mesma possa ser subdiagnosticada em nosso e nos outros países. O objetivo desse estudo foi estimar a frequência alélica do SNP c.2032G>A no gene *PLOD1* em equinos da raça BH no Brasil, utilizando a reação em cadeia de polimerase e o sequenciamento direto da região do SNP como metodologia diagnóstica. Foram coletadas amostras de sangue e de bulbo piloso de 374 animais para extração de DNA, posterior amplificação da região desejada e, por fim, sequenciamento gênico. Nesse estudo, encontrou-se frequência alélica de 5,48%, inferindo que é necessária maior atenção por parte dos criadores para prevenir a propagação da doença nos animais.

Palavras chave: abortamento; colágeno; tecido conectivo; mutação; gene *PLOD1*.

DIAS, N. M. **Allele frequency of the single nucleotide polymorphism c.2032G>A in the *PLOD1* gene, responsible for warmblood fragile foal syndrome**. Botucatu – SP, 2018. 59p. Dissertação (Mestrado) – Universidade Estadual Paulista, Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu.

ABSTRACT

Warmblood fragile foal syndrome (WFFS), is a connective tissue disease that presents an autosomal recessive character and is clinically characterized by cutaneous lesions, lesions in the gums and mucosa of the oral cavity, hyperextensible joints and abortions. Such characteristics are incompatible with the development of the affected animals. The single nucleotide polymorphism (SNP) c.2032G> A in the *PLOD1* gene (*procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1*) in homozygosis is responsible for the disease. There is only one report, describing an affected foal and the mutation in Switzerland and there are two studies of prevalence of the causative SNP of the disease (one in Germany and the other in the United States). Cases report presenting clinical signs compatible with the disease and described before the mutation was discovered are present in the literature. Currently the equine associations in Europe are giving great attention to this disease in warmblood horses. The scarcity of studies and information about the existence of the disease in Brazil and in the world leads us to believe that it can be underdiagnosed in our and in other countries. The objective of this study was to estimate the allelic frequency of c.2032G>A SNP in the *PLOD1* gene in Brazilian Sport Horses in Brazil, using the polymerase chain reaction and the direct sequencing of the SNP region as a diagnostic tool. Blood and hair bulb samples were collected from 374 animals for DNA extraction, subsequent amplification of the desired region and, finally, Sanger sequencing. In this study, the allelic frequency was 5.48%, what infers that more attention is needed from the breeders and, also, control measures to prevent the spread of the disease in Brazilian animals must be adopted.

Keywords: abortion; collagen; connective tissue; mutation; *PLOD1* gene.

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Capítulo I

1. Introdução

A síndrome da fragilidade cutânea equina (*warmblood fragile foal syndrome* - WFFS) é uma enfermidade genética, de herança autossômica recessiva. Assemelha-se com as manifestações clínicas da síndrome de Ehlers-Danlos (EDS) tipo VI em humanos, conhecida como tipo cifoscoliótico (FUGIMOTO et al., 1997).

Em humanos, a EDS tipo VI é rara, possui uma prevalência de 1:100.000 nascidos vivos e frequência de portadores do alelo mutado em 1:150 pessoas (YEOWELL et al., 2000; GIUNTA et al., 2005). Manifestações clínicas de EDS foram relatadas em uma variedade de animais domésticos, sendo estes: gatos (FREEMAN et al., 1987), cães (HEGREBERG et al., 1969), ratos (SINKE et al., 1997), bovinos (WITZIG et al., 1984), ovinos (HELLE et al., 1972) e equinos (SOLOMONS, 1984).

Em humanos, inúmeras mutações ocorrem no gene *PLOD1* (*procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1*), sendo frequentes a duplicação no éxon 10 e no éxon 16. Já em equinos, a WFFS é ocasionada por um SNP *missense* no gene *PLOD1*, localizado no éxon 19 do cromossomo 2, no qual a substituição pontual de uma guanina por uma adenina leva à alteração da estrutura da enzima lisil hidroxilase 1 e, consequentemente, ao seu mau funcionamento (YEOWELL et al., 2000). Caracteriza-se pelos sinais clínicos de lesões em mucosas e pele, hiperextensão das articulações, orelhas caídas, hidropisia, enfisema subcutâneo e abortamento.

Em estudo realizado na Suíça, Monthoux et al. (2015), citam que, para padronizar o teste genético da enfermidade, Winand, em 2011, utilizou 124 animais e obteve uma prevalência de heterozigotos de 11,11%. Ainda, um estudo com 500 animais realizado por Gunreben na Alemanha, em 2013, encontrou prevalência de heterozigotos de 9,5% (Monthoux et al., 2015).

Segundo a Associação Brasileira de Criadores de Cavalo de Hipismo (ABCCH), existem 22.914 animais registrados no Brasil até dezembro de 2017. A ABCCH tem como objetivo promover o desenvolvimento da criação do Cavalo Brasileiro de Hipismo, conhecido como BH. Os criadores buscam no cruzamento entre raças formadoras, um animal de boa estrutura e conformação, com grande aptidão para os esportes equestres olímpicos.

Partindo de animais rigorosamente selecionados já existentes no país, os fundadores da ABCCH definiram como raças formadoras nacionais e estrangeiras, aquelas que comprovadamente eram reconhecidas como altamente dotadas para os esportes equestres. Segundo Dias et al. (2000), vinte raças foram utilizadas na formação do BH, sendo as principais: o próprio BH (22,5%), animais sem genealogia conhecida (21,9%), Puro Sangue Inglês (PSI) (15,0%), Hanoveriana (8,1%), Westfalen (5,2%), Holsteiner (4,8%) e Trakehner (4,1%). Até o presente momento não existe no Brasil nenhum estudo de prevalência da enfermidade e consequentemente nenhum estudo que demonstre a(s) linhagem(s) com maior prevalência.

2. Revisão de literatura

2.1 Panorama da equideocultura brasileira

Atualmente o Brasil possui cerca de 5.510.601 equinos, ocupando a quarta posição no mundo em cabeças de animais, segundo dados da Organização das Nações Unidas para a Alimentação e a Agricultura (*Food and Agriculture Organization* – FAO) de 2013, se mantendo estável na última década (IBGE, 2012) (Tabela 1).

O agronegócio equino no Brasil movimenta cerca de R$ 7,5 bilhões e gera cerca de 3,2 milhões de empregos diretos e indiretos. Em 2015 o PIB da equideocultura chegou à marca histórica de 16 bilhões de reais, apresentando um crescimento bruto de 11,3% ao ano. Segundo economistas, nenhum setor formal da economia brasileira obteve um crescimento econômico como o setor de equinos (IBGE, 2012).

TABELA 1 - Classificação mundial da população de equinos.

País	Número de cabeças	Classificação mundial
Estados Unidos	10.350.000	1º
México	6.356.000	2º
China	6.337.380	3º
Brasil	5.510.601	4º

Fonte: FAO, 2012.

Segundo dados do Ministério da Agricultura e Abastecimento (MAPA) (LIMA et al., 2006), como atividade organizada, o esporte ganhou evidência a partir do início do século XIX, com a inclusão da equitação, em 1810, entre as disciplinas da Academia Real Militar (Rio de Janeiro) e as provas de corrida. No início do século seguinte, surgem os primeiros clubes hípicos como, por exemplo, o Club Esportivo de Equitação (hoje, Centro Hípico do Exército), no Rio de Janeiro, e a Sociedade Hípica Paulista, em São Paulo, ambos em 1911. O hipismo foi introduzido nos Jogos Olímpicos em 1900 (Paris) e a primeira participação brasileira ocorreu nos Jogos de 1948 (Londres). Sete anos antes, em 19 de dezembro de 1941, surgiu a Confederação Brasileira de Hipismo (CBH), órgão máximo do esporte nacional, sediada no Rio de Janeiro (RJ).

Atualmente possui 19 federações filiadas, além da Comissão de Desportos do Exército. A CBH é filiada ao Comitê Olímpico Brasileiro (COB) e à Federação Equestre Internacional (FEI) (LIMA et al., 2006).

Segundo o estudo realizado pela Confederação da Agricultura e Pecuária do Brasil - CNA, em 2004, estima-se que cerca de 50 mil atletas praticam esportes hípicos, nas suas diversas modalidades. O esporte hípico no Brasil abrange diversas modalidades, como: rodeio, conformação, enduro, hipismo paraolímpico, hipismo rural, adestramento, salto, concurso completo de equitação (CCE), volteio, cavalgada, vaquejada, polo, equitação de trabalho, corrida, *horseball* e arquearia a cavalo (MAPA, 2016). O Brasil conta com cerca de 200 clubes e escolas de equitação onde estes esportes são praticados. Além disso, centenas de eventos são promovidos por associações de criadores e empresas de turismo equestre. A população equina no Brasil encontra-se em expansão, assim como as modalidades atléticas, sendo importantes geradores e fontes de renda (MAPA, 2016).

2.2 Formação da raça Brasileiro de Hipismo no Brasil

Diversas raças de equinos têm sido utilizadas na formação da raça Brasileira de Hipismo, como pode ser observado na Tabela 2. As principais raças de garanhões foram: Puro Sangue Inglês (20,9%), Hanoveriana (16,1%), Westfalen (10,5%), Holsteiner (9,6%) e Trakehner (8,2%), além de machos da própria raça BH com contribuição de 8,7%. As principais raças de fêmeas foram: éguas base (42,8%), que são éguas nacionais com ou sem genealogia conhecida, Puro Sangue Inglês (10,8%) e éguas da própria raça BH (36,2%) (DIAS et al., 2000).

Ainda, no trabalho realizado por Dias et al. (2000), os autores evidenciam a grande influência do Puro Sangue Inglês na formação das raças de equinos atletas, como ocorre em outras raças de equinos utilizadas para a prática de esportes hípicos do mundo. Segundo Moureaux et al. (1995), a raça Sela Francesa, possui aproximadamente 52% de PSI e a raça Hanoveriana, aproximadamente 15%. Também tiveram forte influência do Puro Sangue Inglês as raças Holsteiner, Trakehner, Oldenburger e Zangersheide (DIAS et al., 2000).

TABELA 2 – Frequência (%) das raças de garanhões e éguas utilizados na formação da raça Brasileira de Hipismo.

Raça	Garanhão	Égua
Anglo-Árabe	2,7	-
Animais base	1,0	42,8
Brasileira de Hipismo	8,7	36,2
Budjorney	1,5	-
Hanoveriana	16,1	-
Holsteiner	9,6	-
Oldenburger	3,6	-
Orloff	1,2	-
Puro Sangue Inglês	20,9	10,8
Rheinland	1,0	-
Sela Argentina	2,0	1,9
Sela Belga	2,5	-
Sela Francesa	5,3	-
Sela Holandesa	2,1	-
Trakehner	8,2	-
Westfalen	10,5	-
Outras	1,5	5,6
Sem pais conhecidos	1,5	2,8

Fonte: DIAS et al. (2000).

Identifica-se ainda na Tabela 2, a grande diferença entre a utilização de garanhões (8,7%) e éguas (36%) da raça Brasileira de Hipismo como reprodutores. O pequeno percentual de garanhões pode ser explicado pela dificuldade de aprovação de garanhões da raça pela Associação de Criadores do Cavalo de Hipismo e pelo uso cada dia mais frequente da inseminação artificial, graças à crescente facilidade de importação de sêmen de garanhões das raças formadoras (ABCCH). A maior porcentagem de éguas BH deve-se, certamente, ao desenvolvimento da raça com boa seleção das éguas e ao alto custo de importação de fêmeas de raças europeias (DIAS et al., 2000 e ABCCH).

Dias et al (2000) acreditam que mesmo com 22 anos de seleção controlada pelo Registro Genealógico, a raça Brasileira de Hipismo encontra-se no início de sua formação. No período de julho de 1977 a setembro de 1998 as raças que tiveram maior influência na formação do cavalo BH foram Puro Sangue Inglês, Hanoveriana, Westfalen, Holsteiner e Trakehner. O grande número de raças utilizadas na formação do Brasileiro de Hipismo contribuiu para um coeficiente de endogamia próximo de zero. A grande variabilidade genética do cavalo BH é uma característica que possibilita a implantação de futuros programas de melhoramento genético da raça (WRIGHT, 1992).

2.3 Síndrome da fragilidade cutânea equina

A síndrome de Ehlers-Danlos em humanos é causada por várias mutações em diferentes genes com diferentes modos de hereditariedade, foi categorizada em diferentes subtipos, devido aos sinais clínicos evidenciados (DE PAEPE & MALFAIT, 2012). Em vários casos de EDS, em diferentes espécies, mutações foram detectadas em genes que codificam enzimas envolvidas no metabolismo de colágeno ou outros componentes da matriz extracelular dérmica (BYERS et.al, 2012).

A classificação da EDS em humanos tem sido modificada ao longo do tempo (BURROWS, 1999). Recentemente, esta síndrome foi classificada em 12 doenças diferentes, baseada nas características clínicas, no padrão de herança e na alteração molecular e/ou bioquímica envolvida (DE PAEPE & MALFAIT, 2012).

Segundo Beighton et al. (1998), em humanos, a EDS tipo VI é caracterizada no nascimento por hipotonia muscular grave, muitas vezes requerendo tratamento neuromuscular invasivo; cifoscoliose, que é progressiva e grave; hipermobilidade e luxações articulares e hiperelasticidade cutânea grave. Além disso, ocorre fragilidade da pele com cicatrizes anormais; osteopenia sem tendência a fraturas; micro córnea; esclera azulada e ocasionalmente ruptura de artérias e globo ocular (AL-HUSSAIN et al., 2004).

O diagnóstico da síndrome de Ehlers-Danlos tipo VI em humanos se dá pela análise da atividade da enzima lisil hidroxilase em fibroblastos de pele

cultivada e/ou diretamente por análise de mutações no gene *PLOD1*, no qual mais de 20 mutações diferentes já foram descritas (GIUNTA et al., 2005; WALKER et al., 2005; BYERS et. al. 2012).

Segundo Walker et al. (2005), diferentes mutações foram relatadas no gene *PLOD1* humano e são associadas à síndrome clínica de Ehlers-Danlos tipo VI, sendo a duplicação do éxon 10 e do éxon 16 a mutação mais comum (25%). Ainda, dez variantes patogênicas humanas tiveram mutações pontuais em diferentes regiões do éxon 10 e do éxon 16 de *PLOD1*; que demonstraram associação a uma diminuição da atividade enzimática em pacientes com EDS tipo VI (WALKER et al., 2005).

O gene *PLOD1* é responsável por codificar uma importante enzima modificadora pós-tradução na biossíntese de colágeno, que hidroxila lisinas específicas em colágeno (YEOWELL et al., 2000). Esta enzima hidroxila os resíduos de lisil nas sequências - Xaa-Lys-Gli - do colágeno, que servem como locais de ligação para unidades de hidratos de carbono (galactose ou glucosil-galactose) e desempenham um papel essencial na formação de ligações cruzadas de colágeno intra e intermolecular. Assim, a deficiência de lisil hidroxilase resulta em sub-hidroxilação de resíduos de lisil e sub-glicosilação de resíduos de hidroxilisil em colágenos e, consequentemente, na formação de ligações cruzadas prejudicadas com consequente instabilidade mecânica dos tecidos afetados, como observado em indivíduos acometidos com EDS VIA (GIUNTA et. al., 2005).

Essas hidroxilisinas atuam como precursoras de reticulação, que são responsáveis pela resistência à tração, estabilidade mecânica das fibrilas de colágeno e estão envolvidas na formação de fibras (YEOWELL et al., 2000; WALKER et al., 2005; DE PAEPE & MALFAIT, 2012). De acordo com o estudo realizado por Takaluoma et al. (2007), observou-se que ratos com mutações no gene *PLOD1* são flácidos, têm anormalidades na locomoção e cerca de 15% deles morrem por causa da ruptura aórtica. As células do músculo liso apresentaram alterações degenerativas e as fibrilas de colágeno da aorta e da pele apresentaram morfologia anormal.

Na medicina veterinária, a caracterização de EDS baseado somente nos achados clínicos é bastante difícil, uma vez que a mesma é similar a mais de um subtipo de EDS. A doença genética caracterizada por fragilidade do tecido

conjuntivo que acomete a pele dos animais domésticos tem sido referida como dermatosparaxia (HELLE et. al., 1972; WITZIG et. al.,1984), EDS, astenia cutânea e hiperelastose cutânea (MINOR, 1980; WHITE, et al., 2004).

A Astenia Dérmica Regional (HERDA), é uma doença autossômica recessiva, descrita em cavalos Quarto de Milhas e raças relacionadas. É causada por um polimorfismo de nucleotídeo único (SNP) *missense* (c.115G>A) no gene da Ciclofilina B (*PPIB*), na qual os animais afetados apresentam clinicamente pele frouxa, fina, hiperextensível e frágil que lacera facilmente ao menor trauma, formando extensas cicatrizes seromas, hematomas e ulcerações que desenvolvem inicialmente no dorso do animal (TRYON, *et. al*., 2007).

O colágeno é uma proteína fundamental do organismo, sendo o principal componente da matriz extracelular (KOIDE & NAGATA, 2005). Segundo Minor (1980), grande parte dos processos patológicos levam a alterações na síntese ou na degradação do colágeno. Ainda, diferentes isótopos de colágeno compreendem aproximadamente um terço da proteína corpórea total dos organismos vertebrados. Essas proteínas desempenham funções estruturais e de manutenção da integridade tecidual (BYERS, 2000; MYLLYHARJU & KIVIRIKKO, 2001), proporcionando aos tecidos força de tensão, flexibilidade e extensibilidade (MINOR, 1980).

Em equinos, a EDS foi descrita em raças como: raças de tração, raças com aptidão para os esportes equestres olímpicos, Árabes, Quarto de Milhas, bem como PSI; em neonatos, equinos jovens e adultos. Duas enfermidades genéticas que causam fenótipos de EDS em cavalos foram descritas: astenia dérmica regional hereditária equina (HERDA) que afeta equinos da raça Quarto de Milha (TRYON, et al., 2005) e a síndrome da fragilidade cutânea equina (WFFS), afetando equinos *warmblood* (MONTHOUX et al., 2015).

A síndrome da fragilidade cutânea equina é uma doença genética de caráter autossômico recessivo, que acomete o tecido conjuntivo e é caracterizada por pele hiperextensível e anormalmente frágil, bem como hiperextensibilidade das articulações (MONTHOUX et al., 2015). Descrita pela primeira vez na Suíça, a WFFS é causada por um polimorfismo de nucleotídeo único (SNP) (c.2032G>A, p.Gly678Arg) no éxon 19 do gene *PLOD1*, presente no cromossomo 2. O gene *PLOD1* em equinos possui 20 éxons, possuindo

26523 pares de bases. Em 2015, um potro com sinais clínicos semelhantes a essa síndrome, foi diagnosticado com WFFS, com teste genético confirmatório (MONTHOUX et al., 2015). Monthoux et al. (2015), descreveram um potro com sinais clínicos semelhantes a HERDA, porém, contrariando todos os padrões dessa enfermidade (Tabela 3). O potro recém-nascido foi submetido à eutanásia logo após o nascimento devido ao seu mau prognóstico.

Ainda, acredita-se que a WFFS pode apresentar sinais clínicos reprodutivos, pois à medida que as membranas fetais encontram sua origem no embrião, o tecido conjuntivo dessas membranas pode ser afetado, levando a sua ruptura prematura e causando as complicações na gestação (BARABAS, 1966).

TABELA 3 – Comparativo das lesões cutâneas de WFFS e HERDA em equinos

Doença	WFFS	HERDA
Nome	Síndrome da fragilidade cutânea equina	Astenia dérmica regional hereditária equina
Raça	*Warmblood*	Quarto de Milha (QM)
Gene	*PLOD1* (procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1)	*PPIB* (ciclofilina B)
Sinais clínicos	• Lesões da pele/mucosa; • Hiperextensão de articulações; • Orelhas flexíveis; • Hidropisia; • Enfisema subcutâneo; • Hematomas; • Parto prematuro;	• Pele frágil e hiperextensível; • Lacerações; • Seromas; • Hematomas; • Ulcerações; • Cicatrizes;
Localização das lesões	Cabeça, pescoço, tórax, membros, abdômen, cernelha, mucosas orais, cartilagem articular.	Tendões, ligamentos, grandes vasos, córnea.
Idade de aparecimentos dos sinais clínicos	Mais comum em neonatos.	Principalmente em cavalos mais velhos, mas também é possível em neonatos.
Herança genética	Autossômica recessiva	Autossômica recessiva
Teste genético disponível	Sim	Sim
Prevalência da mutação	Até 11%	Até 9,2% em QM
Estudos de prevalência	Winand N (2012)	Ishikawa et al. (2012) Até 28% em linhagens de apartação Tryon et al. (2009) Até 5,8% em QM Badial et al. (2014)

Fonte: modificado Monthoux et al. (2015).

No exame necroscópico o potro apresentava múltiplas lacerações cutâneas nos membros anteriores e na face. O abdômen estava aberto na linha média (aproximadamente 30 cm de comprimento) com exposição do intestino delgado. A pele era extremamente frágil e fina (1 a 2 mm), friável e ligeiramente presa ao tecido subcutâneo subjacente; quando comparada a pele do potro afetado com a pele de um potro normal (membro anterior), observava-se nitidamente a diferença de espessura da pele afetada (MONTHOUX et al., 2015).

No exame histológico, a derme anormalmente fina (aproximadamente 30% a menos da espessura da pele normal), possuía uma quantidade reduzida de feixes de colágeno dérmico, estes eram de espessura e orientação variável e disposta com espaços anormalmente grandes entre as fibras dérmicas profundas. A epiderme e as estruturas anexas eram normais. A microscopia eletrônica foi realizada em amostras cutâneas de vários locais do corpo, revelando apenas anormalidades leves. As fibrilas de colágeno apresentavam-se razoavelmente organizadas em feixes paralelos, contudo, houve aleatoriedade leve na orientação e variabilidade das secções transversais das fibrilas de colágeno (MONTHOUX et al., 2015).

Após todos os achados clínicos, patológicos e histológicos do potro, realizou-se o teste genético da égua (mãe) para HERDA, tendo resultado negativo. Em 2011, quando a padronização do teste genético de WFFS tornou-se disponível comercialmente, testou-se o potro e obteve o genótipo homozigoto recessivo (A/A), revelando então, o primeiro animal diagnosticado com a WFFS com mutação caracterizada; uma nova enfermidade genética em equinos de raças com aptidão para os esportes equestres olímpicos na Europa, semelhantes aos BH no Brasil. Todos os parentes próximos do potro foram testados e; tanto a égua, quanto as duas meio irmãs, filhas do mesmo garanhão, revelaram ser heterozigotos (A/G) (MOUNTHOUX et al., 2015).

O não conhecimento da enfermidade, em conjunto com a não disponibilidade de um teste genético, resultou na falha de diagnóstico definitivo de vários casos de EDS (SOLOMONS, 1984; GUNSON et al., 1984). Estes cavalos apresentaram sinais como: hiperelasticidade da pele, perda da cápsula do casco, lesões no dorso, flanco e membros, formação de cicatrizes e abortamento ou perda embrionária em cruzamentos consanguíneos.

Casos em cavalos *warmblood* com sinais clínicos semelhantes à EDS tipo VI já foram relatados anteriormente nos trabalhos de Witzig et. al. (1984), Winter et. al. (2004), Rufenacht et al. (2010) e Marshall et al. (2011), porém a mutação responsável pela WFFS ainda não havia sido descoberta, resultando na falta de diagnóstico definitivo para esses animais.

Os relatos de Witzig et al. (1984) e Winter et al. (2004), descreviam potros *warmblood* recém-nascidos, que apresentaram lesões cutâneas nos membros, região abdominal, cernelha e embaixo da cauda, com hiperextensão das

articulações dos membros, hidropisia, enfisema subcutâneo e uma pele anormal. Estes foram submetidos a eutanásia nas primeiras horas de vida. A semelhança dos sinais clínicos desses dois potros com o relato de Mounthoux et. al. (2015), hoje, nos levam a hipótese de casos suspeitos de WFFS.

Marshall et al. (2011), descreveram um potro *warmblood* de 6 semanas de idade, que desenvolveu hematomas, lacerações múltiplas, cicatrizes, edema e apresentou lesões cutâneas localizadas nas regiões do flanco, do dorso e dos membros, também foi submetido a eutanásia nas primeiras horas de vida. Rufenacht et al. (2010), relataram um potro Sela Suíça, de 1 ano e 5 meses, que apresentou problemas de cicatrização de feridas e alterações dermatológicas no flanco e no dorso. Além disso, a pele da região dorsal e da região toracolombar era hiperelástica. Esse caso divergiu dos demais, tanto com relação à idade, quanto à localização das lesões, portanto, estes dois casos clínicos descritos sem associação a um genótipo específico, tornaram-se casos suspeitos para a WFFS, já que todos foram testados para HERDA e apresentaram resultados negativos.

Segundo Steelman et al. (2013), a síndrome de Ehlers-Danlos em equinos é uma coleção de fenótipos semelhantes com origem genética diferente, portanto a diferenciação entre HERDA, WFFS e outros casos de EDS possuem um diagnóstico difícil se não estiverem disponíveis e padronizados os testes genéticos. Podemos observar essa afirmativa no relato de Oliveira-Filho et. al. (2017), no qual uma égua Mangalarga-Campolina apresentava lesões de pele frágil e hiperextensível ao longo do corpo, porém negativa para as mutações HERDA e WFFS.

Recentemente, a Associação de criadores de cavalos *warmblood* da América do Norte (*Royal Dutch Warmblood Association of North America* – KWPN-NA) publicou uma nota sobre a importância da WFFS, principalmente o impacto que a mesma pode causar economicamente, além da perda afetiva ocasionada aos criadores. Ainda, a KWPN-NA ressaltou a importância da realização dos testes genéticos para identificar os animais heterozigotos e ajudar a esclarecer essa nova enfermidade. Um importante garanhão, Sternlicht (por Soliman de Hus x Rascalino x Wesley), da fazenda Hilltop foi testado, sendo o mesmo portador do alelo mutado para WFFS, porém, nenhum filho desse garanhão até o momento apresentou sinais semelhantes à WFFS

(KWPN-NA, 2018). Nenhum estudo de prevalência da WFFS em equinos *warmblood* e raças relacionadas foi descrito na literatura brasileira, justificando a importância deste trabalho por verificarmos os animais afetados no Brasil e assim, os criadores diminuírem suas perdas econômicas, reduzindo a progressão da enfermidade e consequentemente seus prejuízos.

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Capítulo II

O trabalho a seguir foi redigido em conformidade com as normas da revista "The Veterinary Journal"

Short Communication

Frequency of PLOD1 c.2032G>A SNP associated with Warmblood Fragile Foal Syndrome in Warmblood horses

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Abstract

Warmblood Fragile Foal Syndrome (WFFS) is an autosomal recessive genetic disorder caused by a mutation in the procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 (*PLOD1*) gene, associated with collagen biosynthesis. WFFS causes skin lesions and malformations of the skin in neonatal foals and abortion. The objective of this study was to investigate the allelic frequency of the SNP c.2032G>A in the *PLOD1* gene in DNA warmblood samples from Brazil. Of the 374 Warmblood horses tested, 10.96% were identified as heterozygous for WFFS polymorphism. This study reinforces that it is important that control measures should be adopted to prevent an increase in the incidence of WFFS in Warmblood worldwide.

Keywords: abortion; collagen; connective tissue; mutation; *PLOD*1 gene.

Ehlers-Danlos syndrome (EDS) in humans encompasses a group of hereditary disorders of the connective tissue, characterized by hyperextensible skin, joint hypermobility; and varying degrees of vessel and tissue fragility (Burrows, 1999; Proske et al., 2006; De Paepe and Malfait, 2012). In horses, there are two diseases with the mutations already known that resemble EDS in humans, i.e., the Hereditary Equine Regional Dermal Asthenia (HERDA) (Tryon et.al., 2007) in Quarter Horses (Solomons, 1984; White et al., 2004; Tryon et al., 2007), and the Warmblood Fragile Foal Syndrome (WFFS) in Warmblood horses (Winand, 2011, Monthoux et al., 2015).

WFFS is an autosomal recessive genetic disorder, that occurs in Warmblood horses and related breeds, characterized by lesions and malformations of the skin in neonatal foals, gum and oral cavity lesions, in addition, WFFS causes abortion and stillbirth (Monthoux et al., 2015). This disease resembles the clinical manifestations of Ehlers-Danlos syndrome (EDS) type VIA in humans (Fujimoto et al., 1997), where the deficiency of lysyl hydroxylase due to mutations in *PLOD1* (procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1) gene has already been described (Giunta et al., 2005). Similarly, WFFS in Warmblood horses is caused by the SNP c.2032G>A (p.Gly678Arg) in the equine *PLOD1* gene (Monthoux et al., 2015). The *PLOD1* gene encodes the enzyme lysyl hydroxylase, which acts on collagen biosynthesis by performing the hydroxylation of the lysine residues in the amino acid sequences (Xaa-Lys-Gly) present in the collagen molecule. Following hydroxylation, theses residues serve as binding sites for galactose or glucosyl-galactose units, which play an essential role in the formation of intra- and intermolecular collagen cross-linking (Giunta et al., 2005).

Knowing the distribution of this mutant allele in the equine population around the world is important to prevent the spread of the disease. Therefore, the objective of this study was to investigate the allelic frequency of the SNP c.2032G>A in the *PLOD1* gene in Warmblood horses from Brazil.

Our study was performed in accordance with the policies of the Institutional Animal Care and Use Committee (120/2013-CEUA) of São Paulo State University (UNESP) and the samples were collected under a strict confidentiality agreement to ensure the anonymity of establishments, owners and animals. A total of 374 (337 blood and 37 hair root) samples were collected from Warmblood horses (294, crossbred Brazilian Sport Horses; 47, Holsteiners; 12, Westfalens; eight, KEPN horses; four, Selle Francais, three, Belgian Sport Horses; two, Hanoverians, two, Argentine Warmblood Horses, one, Württemberger; and one, Oldenburg). The horses (203 females and 171 males, with ages ranging from 5 months to 28 years) were from ranches or breeding centers located in three geopolitical regions of Brazil, Southeast (296/374), Southern (41/374) and Midwest (37/374).

Genomic DNA was purified from hair root samples using an in-house method and from blood samples using the GenElute™ Genomic Blood DNA Kit (Sigma-Aldrich®) according to the manufacturer's instructions. Specific primers (forward, 5'-GTGGCTCAGATGGGAGAATG-3'; and reverse, 5'-ATTAGGGATCGACGAAGGAGA-3') were designed (online primerQuest tool - IDT®) to genotype the SNP c.2032G>A in the *PLOD1* sequence 100050902 (Winand, 2011) and specificity was evaluated *in silico* with the Basic Local Alignment Search Tool (National Center for Biotechnology Information/USA). Polymerase chain

reactions (25 μL) contained 12.5 μL of GoTaq Green PCR Master Mix (Promega), 0.3 μM of each forward and reverse primer, 2.5 μL of template DNA, and nuclease-free water up to the final volume. The amplification conditions were: initial denaturation at 95°C for 5 minutes; followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 63°C for 1 minute, and extension at 72°C for 1 minute; followed by a final extension at 72°C for 5 minutes. Amplicons (259-bp) were analysed by 1.5% agarose gel electrophoresis, purified, and subjected to direct sequencing. To sequence the DNA, 10 μL of purified PCR product, 5 μL of the reverse primer and the BigDye® Terminator Cycle Sequencing Kit were used (Life Technologies™). The sequences were determined using the ABI 3500 Genetic Analyzer (Life Technologies™). The obtained sequences and the electropherograms were analysed using Geneious® 10.0.9 software (Biomatters Ltd.). The sequences were compared with the normal *Equus caballus PLOD1* gene sequences using BLAST (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov/Blast.cgi). The allele frequencies comparison using our data X literature data (Winand, 2011, Monthoux et al., 2015) was performed using chi-squared test in the GraphPad Prism® 5.01 software (GraphPad Software).

Of the 374 Warmblood horses tested, 41 animals (10.96%; 18 females and 23 males) were identified as heterozygous for the WFFS polymorphism (N/WFFS) and 333 (89.04%) were homozygous for the wild-type allele (N/N). Warmblood horses were identified as heterozygous in 85.7% (6/7) of the rural properties assessed.

In the present study, the frequency of animals heterozygous (N/WFFS) was similar to (Winand, 2011) in United States (11.11%, 8/124) (P=0.1568) and to Gunreben study (2013) in Germany (9.5%; 47/500) (P=0.4475).

No study had previously evaluated the prevalence of WFFS SNP in Warmblood horses in Brazil and to our knowledge the clinical disease has not been reported in Brazil yet. Since all animals were apparently healthy, and no clinical signs of WFFS were observed in animals during the sampling procedures, no animal was found to be recessive homozygous for WFFS polymorphism (WFFS/WFFS). Furthermore, homozygous recessive foals are not frequently born at term; having great chances that they will be aborted at some point during gestation, since the SNP leads to changes in the synthesis of collagen and consequently the fragility of the fetal membranes (Barabas, 1966). Therefore, these are the reasons why most veterinarians in Brazil and elsewhere are unaware of this disease. Moreover, the impact that the disease has on causing abortions in Brazil is also unknown. However, the data of the present study, reinforce the hypothesis that abortion caused by WFFS are likely to occur without having the cause of abortion correctly diagnosed.

Since the WFFS allele frequency found in the present study (5.48%) was similar to that of other countries, previously described in the literature (Winand, 2011, Gunreben, 2013), control measures should be adopted to prevent an increase in the incidence of WFFS in Warmblood horses.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix

Reporting Guidelines

Reporting guidelines are available for a broad range of study designs and allow research to be critically evaluated. These guidelines have been designed by international scientific teams to promote the quality of research reporting and to ensure there is a transparent, accurate and complete account of the research. The guidelines are freely available and include the following:

1. Standards for the reporting of diagnostic accuracy studies (STARD) http://www.stardstatement.org

2. Standards for the reporting of observational studies in epidemiology (STROBE) http://www.strobe-statement.org

3. Outbreak investigation reports and intervention studies of nosocomial infection (ORION) http://www.idrn.org/orion.php

4. Consolidated standards for reporting randomised clinical trials (CONSORT) http://www.consort-statement.org

5. Systematic reviews and meta-analyses (PRISMA) http://www.prisma-statement.org

6. Randomised control trials for livestock and food safety (REFLECT) http://www.reflectstatement.org/statement

7. Enhancing the quality and transparency of health research (including good publication practice for pharmaceutical companies), economic evaluations and qualitative research (EQUATOR) http://www.equator-network.org

For further information see The Veterinary Journal (2010) 184, 249-250 (view article.