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

PREVALÊNCIA DAS MUTAÇÕES GENÉTICAS CAUSADORAS DA TROMBASTENIA DE GLANZMANN EM EQUINOS NO BRASIL

RAÍSSA OLIVEIRA LEITE

Botucatu - SP
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Orientador: Prof. Ass. Dr. José Paes de Oliveira Filho

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SUMÁRIO

LEITE, R. O. **Prevalência das mutações genéticas causadoras da Trombastenia de Glanzmann em equinos no Brasil**. Botucatu – SP, 2019. 43p. Dissertação (Mestrado) – Universidade Estadual Paulista, Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu.

RESUMO

A Trombastenia de Glanzmann (TG) é uma doença hereditária autossômica recessiva caracterizada por alterações na agregação plaquetária, culminando em sinais clínicos como hemorragias e epistaxe. Duas mutações (c.122G>C e g.1456_1466del) no gene *Integrin subunit alpha2β* (*ITGA2B*) foram descritas como responsáveis pela ocorrência da TG em equinos de diversas raças, dentre elas: Quarto de Milha, Puro Sangue Inglês, Standardbred, Oldenburg e Passo Fino. Este gene codifica a subunidade αIIb da integrina αIIbβ3, que é receptora de fibrinogênio nas plaquetas. Embora a TG tenha sido diagnosticada nos EUA, Canadá, Japão e Austrália, estudos de prevalência em equinos no Brasil e no mundo são inexistentes. Objetivou-se determinar a prevalência destas mutações em equinos no Brasil. Utilizou-se 1.053 amostras, oriundas do banco de DNA do LBMCV, de equinos Quarto de Milha (n=679) e Warmblood (n=374) clinicamente saudáveis. Segmentos do DNA foram amplificados por PCR e sequenciados. O genótipo de cada animal foi analisado e comparado à sequência de nucleotídeos do gene *ITGA2B* depositada no GenBank™. As mutações responsáveis pela TG não estavam presentes na população estudada. Em outras palavras, todos os animais testados apresentaram genótipo *wild type*. Sendo assim, nas condições em que este estudo foi realizado, pode-se inferir que, apesar de não ser possível afirmar que não existam cavalos carreadores de alelos mutados para a doença no Brasil, a TG parece ser uma doença extremamente rara na população de cavalos Quarto de Milha e Warmblood no Brasil.

Palavras-chave: epistaxe, doença genética, hemorragia, receptor de fibrinogênio.

LEITE, R. O**.** Prevalence of the mutations responsible for Glanzmann Thrombasthenia in horses in Brazil. Botucatu – SP, 2019. 43p. Dissertação (Mestrado) – Universidade Estadual Paulista, Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu.

ABSTRACT

Glanzmann thrombasthenia (GT) is an autosomal recessive inherited disorder characterized by changes in platelet aggregation, leading to hemorrhage and epistaxis. Two different mutations (c.122G>C and g.1456_1466del) in the Integrin subunit alpha2β gene (*ITGA2B*) have been identified in different breeds, i.e., Quarter Horse, Thoroughbred, Standarbred, Oldenburg and Paso Fino. ITGA2B codifies the αIIb subunit of the αIIbβ3 integrin, also termed platelet fibrinogen receptor. Horses with GT have been diagnosed in USA, Canada, Japan and Australia. However, there are no studies on prevalence of GT in equines in the world. The aim of this study was to evaluate the prevalence of the mutations responsible for GT in horses in Brazil. A total of 1,053 DNA samples of clinically healthy Quarter Horse (n = 679) and Warmblood horses (n = 374) were used. DNA fragments were amplified by PCR and sequenced. Subsequently, the genotype of each animal was analyzed and compared to the nucleotide sequence of the ITGA2B gene found on GenBank™. The mutations involved with GT in horses were not prevalent in the cohort of horses assessed. In other words, all animals tested were wild type. Therefore, under the conditions in which this study was carried out, it can be inferred that, although it is not possible to state the absence of mutated allelles in Brazilian horses, GT seems to be extremely rare in the population of Quarter Horse and Warmblood in Brazil.

Key words: epistaxis, genetic disease, hemorrhage, fibrinogen receptor.

Capítulo I

1. Introdução

O Brasil possui o quarto maior rebanho mundial de equinos (FAO, 2013) o que corresponde a um efetivo de 5.577.539 animais (IBGE, 2016) que movimenta anualmente cerca de 16 bilhões de reais e gera mais de três milhões de empregos diretos e indiretos (MAPA, 2016).

Diversas raças compõem o plantel brasileiro de equinos, dentre as de maior popularidade encontram-se os Quarto de Milha (QM), o Warmblood (WB) e Puro Sangue Inglês (PSI) (MAPA, 2016).

Os animais da raça Quarto de Milha têm se destacado em função de suas qualidades fenotípicas, tais como sua estrutura morfológica, velocidade, docilidade e, principalmente, sua versatilidade para o trabalho e para as mais diversas modalidades esportivas (ABQM, 2018). Com mais de 500 mil animais registrados junto à Associação Brasileira de Criadores de Cavalo Quarto de Milha (ABQM), o QM representa aproximadamente 10% de toda a população nacional de cavalos.

A raça foi desenvolvida a partir do século XV nos Estados Unidos, resultando do cruzamento de animais provenientes da Arábia, Turquia e Inglaterra visando à obtenção de exemplares compactos, com musculatura bem desenvolvida e que fossem capazes de percorrer curtas distâncias em uma velocidade incomparável com as demais raças (ABQM, 2019).

Por outro lado, cavalos Brasileiro de Hipismo (BH) são selecionados a partir do cruzamento de diversas raças principalmente as pertencentes ao grupo warmblood (WB), com grande influência dos Puro Sangue Inglês (PSI), visando boa estrutura, conformação e aptidão para os esportes equestres olímpicos (Dias et al., 2000). Além disso, são animais que atendem às exigências e necessidades das Polícias Militares, apresentando o melhor padrão racial para executar o policiamento montado em vários Estados do país (ABCCH, 2018).

De acordo com a Associação Brasileira de Criadores de Cavalo de Hipismo (ABCCH, 2018), existem em torno de 23 mil animais registrados no país, distribuídos por diversos Estados, concentrando o maior plantel no Estado de São Paulo.

No mercado de equinos, se inserem animais de alto valor zootécnico e destacados geneticamente, o que foi possibilitado principalmente pelos avanços na genética (Coelho e Oliveira, 2008). Contudo, diversas práticas utilizadas por criadores de cavalos, como utilização de *studbook* fechado, endogamia, reprodução seletiva e uso repetido de padreadores populares contribuem significativamente pra o aumento dos distúrbios hereditários (Bettley et al., 2012). Portanto, conhecer a genealogia dos animais é imprescindível para o melhoramento genético e também para a identificação de alterações genéticas que possam causar características indesejáveis, dentre elas as doenças hereditárias (Coelho e Oliveira, 2008).

Dentre as enfermidades genéticas descritas em equinos, a Trombastenia de Glanzmann (TG) é uma doença hereditária de caráter autossômico recessivo que causa alterações na agregação plaquetária, manifestando-se clinicamente com graus variados de hemorragias e/ou epistaxe (Livesey et al., 2005; Sebastiano et al., 2010). Embora a epistaxe seja um sinal clínico comumente observado em animais atletas, estudos de prevalência de doenças que cursam com este sinal clínico que incluíssem análises moleculares ainda não foram realizados no Brasil.

2. Revisão de literatura

2.1. Doenças genéticas nos equinos

O aumento das enfermidades de caráter hereditário em equinos está associado a técnicas de criação adotadas por criadores tais como cruzamento consanguíneo - ou inbreeding - e uso repetido de determinados padreadores com genótipo desconhecido, o que pode aumentar a probabilidade de ocorrer homozigose de alelos mutados e doenças autossômicas recessivas (Bettley et al., 2012). Além disso, com o desenvolvimento dos recursos de mapeamento genético, foram possíveis abordagens científicas que permitiram acelerar a taxa de descoberta de mutações causadoras de doenças em equinos (Finno et al., 2009).

Os testes genéticos possibilitam o diagnóstico de doenças específicas e permitem aos proprietários e criadores tomarem melhores decisões quanto às escolhas dentro da criação de uma raça (Tryon et al., 2009).

Bettley e colaboradores (2012) verificaram que mais de 20% das raças de equinos no mundo apresentam doenças com predisposição genética, sendo os animais da raça PSI e QM os mais acometidos. Destas doenças, as autossômicas recessivas são as mais comuns.

Mesmo com o número significativo de doenças genéticas afetando os equinos e com o aumento gradativo de pesquisas abrangendo este assunto, ainda há escassez de informações para grande parte dos distúrbios potencialmente hereditários na espécie (Bettley et al., 2012).

De 235 alterações genéticas descritas em equinos (OMIA, 2019), aproximadamente 40 possuem dados de prevalência (Bettley et al., 2012). Estudos de prevalência e padrão de herança tem papel fundamental na determinação do impacto de uma doença em uma criação (Tryon et al., 2009).

No Brasil, estudos de prevalência de doenças de caráter hereditário em equinos já foram realizados, dentre eles: Imunodeficiência severa combinada (Teixeira et al., 2001), Astenia dérmica regional equina (HERDA) (Badial et al., 2014), Paralisia periódica hipercalêmica (HYPP) (Delfiol et al., 2015), Miopatia por acúmulo de polissacarídeo (PSSM), Hipertermia maligna (Delfiol et al., 2018), Deficiência da Enzima Ramificadora de Glicogênio (Araújo et al., 2018)

e Síndrome da fragilidade cutânea equina (Dias et al., 2019). Muitos desses estudos demonstraram resultados similares aos encontrados em outros países, como os Estados Unidos (Tryon et al., 2009; McCue et al., 2006). Isto pode estar relacionado ao fato de que as linhagens dos principais equinos do Brasil são provenientes das linhagens americanas.

2.2. Doenças de hemostasia de caráter hereditário

Mutações genéticas acometendo os componentes celulares do sistema hematopoiético, tais como hemácias, células de defesa e plaquetas, acarretam manifestações clínicas como anemia, infecção e hemorragias (Bauer et al., 2009).

As desordens plaquetárias podem ter origem intrínseca ou extrínseca, sendo as alterações intrínsecas caracterizadas por defeitos na anatomia das plaquetas, levando a disfunções das mesmas. Por outro lado, as alterações extrínsecas envolvem glicoproteínas de membrana, grânulos, proteínas de transdução de sinais ou proteínas estruturais culminando na redução ou disfunção na adesão e agregação plaquetária, entretanto são clinicamente indistinguíveis (Boudreaux et al., 2007).

Alterações hemostáticas de caráter hereditário são menos frequentes que as adquiridas, entretanto doenças genéticas que levam a essas alterações vêm sendo descritas em equinos (Brooks, 2008). Tais doenças resultam de mutações em genes codificantes de proteínas hemostáticas específicas, que levam ao prejuízo em sua síntese, ou a produção de proteínas afuncionais (Patrushev, 2002). Essas mutações são perpetuadas dentro de uma população quando portadores em heterozigose (clinicamente normais) são mantidos em programas de reprodução (Brooks, 2008).

O diagnóstico de distúrbios plaquetários é complexo devido às limitações de conhecimento a respeito dos mesmos, aliado ao fato de existirem várias doenças descritas, das quais a ocorrência muitas vezes é rara (Harrison et al., 2011). Em função da complexidade dos fatores que comprometem a hemostasia faz-se necessária uma abordagem adequada para proceder o diagnóstico (Brooks, 2008). Quando o quadro clínico e resultados laboratoriais sugerirem comprometimento plaquetário de caráter hereditário, torna-se

importante avaliar a participação destas enfermidades e/ou descartar outras alterações de coagulação (Harrison et al., 2011).

Um estudo realizado por Boudreaux e colaboradores (2015) avaliou a função de genes codificantes de duas proteínas (CD39 e CD39L1) com papel na regulação da hemostasia em cavalos com e sem clínica de alterações hemorrágicas, além de testarem animais que apresentavam Hemorragia Pulmonar Induzida por Exercício. Dentre os equinos avaliados, três haviam sido diagnosticados previamente com TG e destes, dois também apresentaram mutação no gene codificador de CD39 (um homozigoto e um heterozigoto) o que sugere que pode haver mais de um fator genético acometendo animais em condições patológicas de hemorragia.

Dentre as doenças hereditárias que causam hemorragias já descritas em equinos podemos citar: deficiência de fator de von Willebrand (vWD), hemofilia A e Trombastenia de Glannzman (TG).

2.2.1. Trombastenia de Glanzmann

A Trombastenia de Glanzmann (TG) é uma doença hereditária de caráter autossômico recessivo. Portanto, machos e fêmeas são igualmente acometidos, os pais dos afetados são clinicamente normais e existe uma correlação de consanguinidade (Boudreaux & Lipsocomb, 2001).

A TG foi descrita pela primeira vez pelo pediatra suíço Dr. Eduard Glanzmann em 1918, entretanto sua caracterização molecular somente foi possível a partir de 1990 (Bray e Shuman, 1990).

Esta doença ocorre devido às alterações quantitativas ou qualitativas no complexo da glicoproteína IIb-IIIa, também conhecida como integrina αIIbβ3 ou receptor de fibrinogênio (Brooks et al, 2008).

A integrina αIIbβ3 é encontrada principalmente na superfície das plaquetas e possui um papel essencial na agregação plaquetária por meio da resposta aos agonistas desta agregação (Sanz et al., 2011) que, por sua vez, provoca uma alteração na conformação do receptor, proporcionando habilidade do mesmo em interagir com o fibrinogênio, promovendo a agregação das plaquetas (Patrushev, 2002).

Duas subunidades distintas compõem o receptor de fibrinogênio: a subunidade αIIb e a β3, codificadas pelos genes *integrin subunit alpha 2β* (*ITGA2B*) e *integrin subunit β3* (*ITGB3*), respectivamente, sendo ambas necessárias para o funcionamento correto do receptor (Boudreaux & Lipsocomb, 2001).

A epistaxe é a manifestação clínica mais frequente, entretanto hemorragias de mucosas, gastrointestinais e formação de petéquias podem ocorrer sendo a anemia a complicação mais comum (Boudreaux & Lipsocomb, 2001).

Os padrões de hemorragia são consistentes com os achados laboratoriais, que se caracterizam por um número normal de plaquetas, porém com ausência de agregação plaquetária frente aos agonistas, culminando em prejuízo severo à retração do coágulo (Boudreaux & Lipsocomb, 2001).

Em humanos, apesar de rara, a TG apresenta uma frequência relativamente alta em populações em que a consanguinidade é comum (Sebastiano et al, 2010) podendo apresentar frequência similar à outras doenças de hemostasia como como hemofilia e deficiência de fator de von Willebrand (George et al., 1990). Este fato caracteriza uma distribuição geográfica desigual da doença em humanos (George et al., 1990). Muitas mutações afetando os genes *ITGA2B* e *ITGB3* têm sido relatadas, incluindo mutações sem sentido (*nonsense*), deleções inserções e inversões (George et al., 1990). Existe um banco de dados disponível online onde são depositados informações pertinentes as causas da TG em humanos, disponível em: https://glanzmann.mcw.edu/.

Apesar de o fenótipo da TG estar bem definido na espécie humana, existe uma diferença considerável no grau de severidade entre os pacientes afetados, com casos variando desde pequenos sangramentos de mucosa a hemorragias severas e possivelmente fatais (Nurden et al., 2011).

O tratamento mais comum é a transfusão de plaquetas, embora em casos em que haja isoanticorpos induzidos por transfusão plaquetária prévia, recomenda-se o uso de fator VII recombinante ativado (Nurden et al., 2011).

Em medicina veterinária, o diagnóstico clínico da doença também já foi reportado na espécie canina (Catafalmo et al., 1986; Boudreaux et al., 1996;

Haysom et al., 2016) com a primeira descrição molecular ocorrendo em 2000 em um animal da raça Cão de Montanha dos Pirineus (Lipscomb et al., 2000).

Os cães e os equinos são modelos experimentais da doença para estudos em humanos, uma vez que o fenótipo apresentado por esses animais é similar ao que ocorre na espécie humana (Bauer et al., 2009).

2.2.1.1. Trombastenia de Glanzmann em equinos

Na espécie equina, casos de animais com sinais clínicos característicos de TG têm sido relatados em diversas raças, dentre elas: PSI (Livesey et al., 2005; Fry et al., 2005), QM (Christopherson et al., 2007), Oldenburg (Macieira et al., 2007) e Passo Peruano (Sanz et al., 2011).

Em 1987, Miura e colaboradores relataram o caso de um potro PSI de seis meses de idade cujo histórico era compatível com trombastenia e os sinais clínicos incluíam formação de hematomas e hemorragias de mucosas. Pouco depois, ocorreu o primeiro relato de diagnóstico clínico de TG em um macho, castrado, da raça American Trotter, de quatro anos de idade que apresentava episódios de epistaxe há mais de três anos (Sutherland et al., 1989). Livesey e colaboradores (2005) descreveram a ocorrência de TG em dois equinos, uma égua da raça QM e um macho castrado meio sangue PSI, cujo histórico incluía epistaxe crônica e intermitente.

Christopherson et al. (2006) caracterizaram a sequência de DNA codificante (cDNA) dos genes *ITGA2B* e *ITGB3*, em cavalos normais e compararam com a sequência em humanos, cães e dois equinos afetados por TG. Os genes *ITGA2B* e *ITGB3* codificam, respectivamente, as subunidades αIIb e β3 do receptor de fibrinogênio e, nesse estudo, foi descrita uma mutação por substituição de guanina por citosina (C<u>G</u>G por C<u>C</u>G) no códon 41 do gene *ITGA2B,* resultando numa substituição de prolina por arginina em uma região altamente conservada da proteína. Neste mesmo estudo, dos dois animais clinicamente afetados genotipados, um apresentou genótipo homozigoto e o outro heterozigoto para a mutação de substituição. Portanto, concluiu-se que este último poderia ser um heterozigoto composto, o que foi confirmado posteriormente quando outra mutação causadora da TG na espécie equina foi descrita, e este mesmo animal apresentou genótipo heterezigoto para esta

nova mutação. Fato semelhante acontece em humanos acometidos por TG, em que diversos casos de mutações envolvendo o gene *ITGA2B* são de heterozigotos compostos (Christopherson et al., 2006).

Esta nova mutação consistia em uma deleção de 10 pares de base incluindo os três últimos pares do éxon 11 e os sete primeiros do íntron 11 do gene *ITGA2B* (Christopherson et al., 2007).

Essas mutações culminam em alterações da expressão do receptor αIIβ3 na superfície plaquetária, o que consequentemente ocasiona ausência de retração do coágulo e falha na ligação do fibrinogênio (Norris et al., 2007).

Clinicamente, a TG pode se manifestar como hemorragias cutâneas, de mucosas e gastrointestinais, sendo a epistaxe o sinal clínico mais frequente (George et al.,1990). Entretanto, diferentemente de outros distúrbios hemostáticos, o número de plaquetas e dosagem de fator de von Willebrand são compatíveis com os valores padrões normais (Boudreaux e Lipscomb, 2000).

Não existem estudos sobre a prevalência da TG em equinos no mundo, e não se sabe sobre a presença destas mutações na população brasileira de equinos. Tendo em vista que o cruzamento consanguíneo é uma prática comum em equinos (Norris et al., 2006), pode haver diferenças entre a frequência da ocorrência da TG em determinadas populações da espécie. Ademais, conhecer a frequência alélica de uma doença em uma população de animais possibilita estabelecer o impacto e relevância da mesma para a espécie, especialmente para raças específicas (Tryon et al., 2009).

Sendo assim, estudos de prevalência em outros países podem contribuir para definir a importância da doença para a espécie, uma vez que hemorragias são sinais clínicos comuns em equinos cujas causas genéticas permanecem não muito bem elucidadas (Norris et al., 2007).

2.2.2. Deficiência do fator de von Willebrand (vWD)

Caracterizada por alterações na estrutura, função ou concentração do fator de von Willebrand (vWF), o qual atua na mediação da adesão plaquetária ao endotélio vascular lesionado, ativação celular e na transferência do fator VIII (Patrushev, 2002).

Em humanos, diversas mutações causadoras da doença têm sido descritas incluindo mutações *missense* e *nonsense,* microdeleções e inserções (Patrushev, 2002).

Existem três classificações principais descritas para humanos, com diferentes padrões de herança, que podem ser extrapoladas para os animais: a tipo 1 consiste em uma diminuição quantitativa de vWF, com padrão de herança autossômico dominante; a tipo 2 é caracterizada pelo comprometimento funcional, sendo portanto uma alteração qualitativa, resultando em alterações na adesão plaquetária dependente do vWF ; a tipo 3 apresenta caráter autossômico recessivo, marcada pela ausência ou diminuição severa da quantidade de vWF. Em equinos, as alterações do tipo 1 e 2 já foram identificadas (Rathgeber et al., 2001; Brooks et al., 1991).

A doença já foi descrita em equinos das raças QM (Brooks et al., 1991; Laan et al., 2005), Árabe (Brooks, 2008) e PSI (Rathgeber et al., 2001). Os sinais clínicos mais frequentes são hemorragias de mucosa, aumento do tempo de sangramento pós traumas, hematomas e hemoartrose (Laan et al., 2005; Brooks, 2008).

2.2.3. Hemofilia A

A hemofilia A é uma doença hereditária recessiva ligada ao cromossomo X, portanto machos são clinicamente afetados e fêmeas são portadoras em heterozigose (Norton et al., 2016).

Em humanos, diversas mutações têm sido descritas no gene codificador do fator VIII, o que afeta sua estrutura e função (Oldenburg et al, 2004). Em equinos, Norton e colaboradores (2016) identificaram uma possível mutação no íntron 1 do gene codificador do fator VIII com provável herança materna, entretanto as causas moleculares permanecem desconhecidas. Apesar disso, a doença tem sido diagnosticada clinicamente e reportada na literatura nesta espécie (Mills e Bolton, 1983; Littlewood et al., 1991).

Potros hemofílicos tendem a vir a óbito ou serem eutanasiados com poucos dias de vida devido à sangramento umbilical exacerbado. O prognóstico para equinos comparativamente a outras espécies é desfavorável, devido à ocorrência de hemoartrose recorrente (Brooks, 2008).

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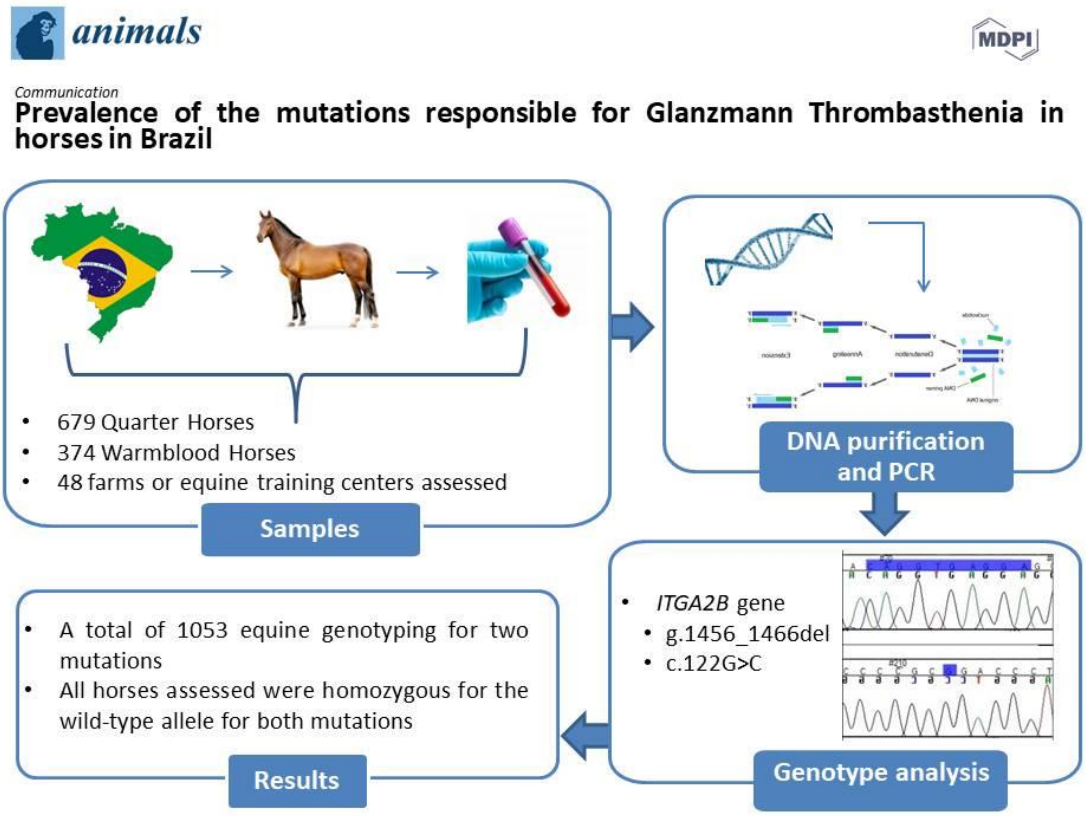
Capítulo II

O trabalho a seguir foi redigido em conformidade com as normas da revista "*Animals*".



Communication

Prevalence of the mutations responsible for Glanzmann Thrombasthenia in horses in Brazil

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Simple Summary: Hemodynamic hereditary disorders occurs in different species due to mutations in genes coding specific hemostatic proteins, leading to alterations in their synthesis, or to the production of non-functional proteins which leads to impairment of hemostasis. Some of these disorders have been described in equines, i.e.: von Willebrand factor deficiency (vWD), hemophilia A and Glannzman's thrombasthenia (GT). GT is an inherited disease characterized

by hemorrhages and has been described in different species, including horses of varied breeds, such as: Thoroughbred, Standardbred, Oldenburg, Paso Fino and Quarter Horse. Although hemorrhages are clinical signs frequently found in the equine internal medicine, its etiology is often not well defined. The aim of this study was to evaluate the prevalence of the mutations for Glanzmann Thrombasthenia in horses in Brazil.

Abstract: Glanzmann thrombasthenia (GT) is an autosomal recessive inherited disorder characterized by changes in platelet aggregation, leading to hemorrhage and epistaxis. Two different mutations (c.122G>C and g.1456_1466del) in the Integrin subunit alpha2β gene (ITGA2B) has been identified in different breeds, i.e., Quarter Horse, Thoroughbred, Oldenburg and Paso Fino. ITGA2B codifies the αIIb subunit of the αIIbβ3 integrin, also termed platelet fibrinogen receptor. Horses with GT have been diagnosed in USA, Canada, Japan and Australia. However, there are no studies on prevalence of GT in equines in the world. The aim of this study is to evaluate the prevalence of the mutations responsible for GT in horses in Brazil. A total of 1053 DNA samples of clinically healthy Quarter Horse (n = 679) and Warmblood horses (n = 374) were used. DNA fragments were amplified by PCR and sequenced. The genotype of each animal was analyzed and compared to the nucleotide sequence of the ITGA2B gene found on GenBank™. GT was not prevalent in the Brazilian equine population, in other words, all animals tested were wild type. Therefore, under the conditions in which this study was carried out, it can be inferred that: although it is not possible to affirm that there are no horses carrying mutated alleles in Brazil, GT seems to be extremely rare in the population of Quarter Horse and Warmblood in Brazil.

Keywords: Epistaxis; Genetic disease; Hemorrhage; Fibrinogen receptor.

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1. Introduction

Hemodynamic hereditary disorders occur due to mutations in genes coding specific hemostatic proteins, leading to alterations in their synthesis, or to the production of non-functional proteins [1]. Some of these disorders have been described in equines, i.e.: von Willebrand factor deficiency (vWD) [2], hemophilia A [3] and Glannzman's thrombasthenia (GT) [4,5,6,7,8,9].

Glanzmann's thrombasthenia (GT) is an autosomal recessive inherited disorder that occurs due to quantitative or qualitative changes in the glycoprotein IIb-IIIa complex, also known as αIIbβ3 integrin or fibrinogen receptor, which is found on the surface of the platelets and have an essential role in platelet aggregation [10]. Glanzmann's thrombasthenia is clinically characterized by cutaneous, mucosal and gastrointestinal hemorrhages, and epistaxis is the most frequent clinical sign [6]. This disorder has been described in Thoroughbred [4,7,8,11,12,], Standardbred [11], Oldenburg [12], and Quarter Horse [8].

Two different mutations in the Integrin subunit alpha2β gene (*ITGA2B*) has been reported as responsible for GT in horses: c.122G>C, identified in Quarter Horse and Thoroughbred [5], and Oldenburg [9]; and g.1456_1466del, identified in Quarter Horse [6] and Paso Fino [10]. Studies of prevalence of theses alleles mutated have not been performed in the world, therefore, the aim of this study was to evaluate the prevalence of the mutations responsible for Glanzmann Thrombasthenia in horses in Brazil.

2. Materials and Methods

This study was approved in 21 January 2019 by the Institutional Animal Care and Use Committee (262/2011-CEUA-UNESP) and samples were collected under a strict confidentiality agreement to ensure the anonymity of establishments, owners and animals. Since the allele frequencies of the two mutation is unknown, we used the anticipated frequency of

heterozygotes of 50% [13], a population of Quarter Horse (QM) (500,000) and Warmblood (WB) horses (14,000) registered in Brazil, 5% margin of error, and 95% confidence interval to calculate the sample size (OpenEpi software).

A total of 1,053 equine DNA samples were used in this study. These samples were obtained from a genetic material database belonging to the Laboratory of Molecular Biology of the Veterinary Clinic from São Paulo State University (Unesp), School of Veterinary Medicine and Animal Science Botucatu/Brazil. This genetic database is composed by DNA samples (stored at -80ºC) which were extracted from whole blood samples obtained from adult horses (males and females), duly registered in the breeds associations (Brazilian Association of Quarter Horses Breeders and Brazilian Sport Horse Association). The samples of the QH were collected from 41 different farms and the WB samples from 7 different farms or equine training centers.

Genotype analysis were performed using specific primers previously describe for detection of the *ITGA2B* g.1456_1466del mutation [6] and for detection of the *ITGA2B* c.122G>C mutation [9]. Polymerase chain reactions were performed in a total volume of 25 μL, which contained 2.5 μL of template DNA, 0.3 μM each forward and reverse primer, 12.5 μL of GoTaq® Green PCR Master Mix (Promega), and 8.5 μL of nuclease-free water q.s.p. In addition, a no-template control reaction was performed to check for the possible presence of contamination in the PCR preparations. The amplification conditions were as follows: initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 30 s, 64°C for 30 s, and 72°C for 1 min, and final extension at 72°C for 5 minutes. Amplicons (241 bp for the deletion mutation and 359 bp for the substitution mutation) were analyzed via 1.5% agarose gel electrophoresis, purified, and subjected to Sanger direct sequencing. The obtained sequences and the electropherograms were analyzed using Sequencher 5.1 (Gene Code Corporation). The results of the genotype analysis were descriptively exposed.

3. Results and Discussion

A total of 1053 equine DNA samples were analyzed, i.e., 679 QH (483 females and 196 males) and 374 WB horses (203 females and 171 males). All horses assessed in this study were identified as homozygous for the wild-type allele for both mutations.

The present study is the first report of prevalence of the mutations (c.122G>C and g.1456_1466del) responsible for the Glanzmann Thrombasthenia in horses. Although this disease has not been described in Brazil, studies in other countries may contribute to define the importance of GT for this specie, since hemorrhages are common clinical signs in horses and its genetic causes remain poorly elucidated [14]. In addition, the knowledge about the allelic frequency of a disease in a population enables to establish its impact and relevance to the species, especially for specific breeds [15].

Other studies of prevalence of different diseases with hereditary pattern that affect horses had been performed in Brazil: Hereditary equine regional dermal asthenia [16], Hyperkalemic periodic paralysis [17], Type 1 polysaccharide storage myopathy mutation [17], Malignant hyperthermia [18], Glycogen branching enzyme deficiency [19] and Warmblood fragile foal syndrome [20]. Many of these studies demonstrated similar to results found in other countries such as USA [15,21]. This may be related to the fact that lineages of the main horses of Brazil are often closely correlated with American herd and it is suggested that the same situation for GT may be found in other countries, given the fact that United States is the largest exporter of breeds like QHs and Brazil is the fifth larger.

Not least, the prevalence of GT in humans is higher in populations where inbreeding is commonly practiced and may have frequency similar to other hemostatic diseases, such as hemophilia and von Willebrand factor deficiency [22]. Considering that inbreeding is a common practice in horses [23] there may be differences between the frequency of occurrence of GT in certain populations of this specie.

5. Conclusions

In summary, the mutations responsible for GT was not prevalent in the Brazilian equine population and under the conditions in which this study was carried out, it can be inferred that although it is not possible to affirm that GT do not occurs in horses in Brazil, this disease seems to be extremely rare in the population of QH and WB.

Author Contributions:

Conceptualization, R.L., C.A., A.B. and J.O.F.; Methodology, R.L., and J.O.F.; Formal Analysis, R.L., J.F., C.A., D.D., R.T., A.B. and J.O.F.; Investigation, R.L., J.F. and J.O.F.; Writing—Original Draft Preparation, R.L., and J.O.F.; Writing—Review and Editing, R.L., J.F., C.A., D.D., R.T., AB and J.O.F.; Supervision, J.O.F.; Funding Acquisition, A.B. and J.O.F.

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Animals — Instructions for Authors

Shortcuts

- Manuscript Submission Overview
- Manuscript Preparation
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English Corrections

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Qualification for Authorship

Each author is expected to have made substantial contributions to the conception or design of the work; acquisition, analysis, or interpretation of data; the creation of new software used in the work; and/or writing or substantively revising the manuscript. In addition, all authors must have approved the submitted version (and any substantially modified version that involves the author's contribution to the study); AND agrees to be personally accountable for the author's own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even those in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature. Note that acquisition of funding, collection of data, or general supervision of the research group do not, by themselves, justify authorship.Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgements.

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1. Wager, E.; Kleinert, S. Responsible research publication: international standards for authors. A position statement developed at the 2nd World Conference on Research Integrity, Singapore, July 22-24, 2010. In Promoting Research Integrity in a Global Environment; Mayer, T., Steneck, N., eds.; Imperial College Press / World Scientific Publishing: Singapore; Chapter 50, pp. 309-16.

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Editorial Procedures and Peer-Review

Initial Checks

All submitted manuscripts received by the Editorial Office will be checked by a professional in-house *Managing Editor* to determine whether they are properly prepared and whether they follow the ethical policies of the journal, including those for human and animal experimentation. Manuscripts that do not fit the journal's ethics policy or do not meet the standards of the journal will be rejected before peer-review. Manuscripts that are not properly prepared will be returned to the authors for revision and resubmission. After these checks, the *Managing Editor* will consult the journals' *Editor-in-Chief* or *Associate Editors* to determine whether the manuscript fits the scope of the journal and whether it is scientifically sound. No judgment on the potential impact of the

work will be made at this stage. Reject decisions at this stage will be verified by the *Editor-in-Chief*.

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Once a manuscript passes the initial checks, it will be assigned to at least two independent experts for peer-review. A single-blind review is applied, where authors' identities are known to reviewers. Peer review comments are confidential and will only be disclosed with the express agreement of the reviewer.

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- *Reconsider after Major Revisions*:
  The acceptance of the manuscript would depend on the revisions. The author needs to provide a point by point response or provide a rebuttal if some of the reviewer’s comments cannot be revised. Usually, only one round of major revisions is allowed. Authors will be asked to resubmit the revised paper within a suitable time frame, and the revised version will be returned to the reviewer for further comments.

- *Reject and Encourage Resubmission*:
  If additional experiments are needed to support the conclusions, the manuscript will be rejected and the authors will be encouraged to re-submit the paper once further experiments have been conducted.
- *Reject*:
  The article has serious flaws, and/or makes no original significant contribution. No offer of resubmission to the journal is provided.

All reviewer comments should be responded to in a point-by-point fashion. Where the authors disagree with a reviewer, they must provide a clear response.

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Authors may appeal a rejection by sending an e-mail to the Editorial Office of the journal. The appeal must provide a detailed justification, including point-by-point responses to the reviewers' and/or Editor's comments. The *Managing Editor* of the journal will forward the manuscript and related information (including the identities of the referees) to the Editor-in-Chief, Associate Editor, or Editorial Board member. The academic Editor being consulted will be asked to give an advisory recommendation on the manuscript and may recommend acceptance, further peer-review, or uphold the original rejection decision. A reject decision at this stage is final and cannot be reversed.

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Production and Publication

Once accepted, the manuscript will undergo professional copy-editing, English editing, proofreading by the authors, final corrections, pagination, and, publication on the www.mdpi.com website.

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Clinical Trials Registration

Registration

Authors are strongly encouraged to pre-register clinical trials with an international clinical trials register or and to cite a reference to the registration in the Methods section. Suitable databases include clinicaltrials.gov, the EU Clinical Trials Register and those listed by the World Health Organisation International Clinical Trials Registry Platform.

CONSORT Statement

Animals requires a completed CONSORT 2010 checklist and flow diagram as a condition of submission when reporting the results of a randomized trial. Templates for these can be found here or on the CONSORT website (http://www.consort-statement.org) which also describes several CONSORT checklist extensions for different designs and types of data beyond two group parallel trials. At minimum, your article should report the content addressed by each item of the checklist. Meeting these basic reporting requirements will greatly improve the value of your trial report and may enhance its chances for eventual publication.

ANEXO 1:

Eletroferograma parcial do gene *ITGA2B* contendo os locais das mutações (em azul). (A) Sequência de 10 nucleotídeos deletadas pela mutação g.1456_1466del. (B) Nucleotídeo substituído pela mutação c.122G>C (azul).