UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
DEPARTAMENTO DE CLÍNICA VETERINÁRIA

PREVALÊNCIA DE PORTADORES DA MUTAÇÃO ASSOCIADA À DEFICIÊNCIA DA ENZIMA RAMIFICADORA DE GLICOGÊNIO (GBED) EM CAVALOS DA RAÇA QUARTO DE MILHA.

CÉSAR ERINEUDO TAVARES DE ARAÚJO

Botucatu-SP
Abril/2015

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Dissertação apresentada junto ao Programa de Pós-Graduação em Medicina Veterinária para obtenção do título de mestre.

Orientador: Prof. Dr. Alexandre Secorun Borges.
Co-Orientador: Prof. Dr. José Paes de Oliveira Filho.

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LISTA DE TABELAS

CAPÍTULO II

LISTA DE FIGURAS
CAPÍTULO I

LISTA DE FIGURAS
CAPÍTULO II

LISTA DE ANEXOS

SUMÁRIO

ARAÚJO, C.E.T. **Prevalência de portadores da mutação associada à deficiência da enzima ramificadora de glicogênio (GBED) em cavalos da raça quarto de milha**. Botucatu, 2015. 61p. Dissertação (Mestrado) – Faculdade de Medicina Veterinária e Zootecnia, Campus Botucatu, Universidade Estadual Paulista.

RESUMO

A Deficiência da Enzima Ramificadora de Glicogênio (*Glycogen Branching Enzyme Deficiency* [GBED] em equinos é uma doença hereditária recessiva fatal, caracterizada principalmente por abortos, natimortos e nascimento de potros fracos. A GBED é causada por uma mutação no gene GBE1. Não existem dados acerca da existência de animais com esta mutação no Brasil. O objetivo deste estudo foi verificar a prevalência de animais portadores do alelo mutante da GBED em cavalos da raça Quarto de Milha utilizados em cinco modalidades esportivas equestres no Brasil. Amostras de sangue e pêlo foram obtidas de 740 animais. Após purificação do DNA, foram realizados as reações de PCR, sequenciamento direto automatizado e análise das sequências. Dos 740 animais testados 59 foram considerados heterozigotos para a mutação responsável pela GBED representando uma frequência de 7,97% na população estudada. As prevalências de heterozigotos foram maiores nas linhagens de apartação (20%) e rédeas (10%), seguidos por tambor/baliza (5%) e conformação (3%), não foram encontrados heterozigotos para a modalidade de corrida. Os resultados demostram que a mutação está presente no rebanho brasileiro de cavalos Quarto de milha, e sugere que a doença (homozigotos recessivos) pode estar presente de forma silenciosa. Portanto a GBED deve ser considerada no diagnóstico diferencial nos casos de abortos e morte neonatal em cavalos da raça Quarto de milha no Brasil, e medidas de prevenção da transmissão da mutação devem ser estabelecidas.

Palavras Chave: Aborto; Glicogênio; Potro; Doença muscular; Doença hereditária.

ARAÚJO, C.E.T. **Prevalence of mutation carriers associated with glycogen branching enzyme deficiency (GBED) in quarter horses.** Botucatu, 2015. 61p. Dissertação (Mestrado) – Faculdade de Medicina Veterinária e Zootecnia, Campus Botucatu, Universidade Estadual Paulista.

ABSTRACT

The deficiency of glycogen branching enzyme [GBED] in horses is a fatal recessive hereditary disease, mainly characterized by abortions, stillbirths and birth of weak foals. The GBED is caused by a mutation in the gene GBE1. The aim of this study was to determine the prevalence of mutation carriers causing GBED in a population of Quarter horse animals used in five equestrian sports practiced in Brazil. Samples of blood and were obtained from 740 animals. After DNA purification, PCR reactions, automated direct sequencing and sequence analysis were performed. Of the 740 animals tested 59 were considered heterozygous for the mutation responsible for GBED representing a prevalence of 7.97% in the population studied. The prevalences of heterozygotes were higher in cutting (20%) and reining (10%) subgroups, followed by barrel racing (5%) and halter (3%), were not found heterozygous for the racing subgroup. The results demonstrate that the mutation is present in the Quarter horse Brazilian herd, and suggests that the disease (homozygous recessive) may be present without being noticed. So the GBED should be considered in the differential diagnosis in cases of abortion and stillbirths in Brazilian Quarter horses and strategies should be developed to prevent transmission of the mutation.

Keywords: Abortion; Glycogen; Foal; Muscle disease; Hereditary disease.

CAPÍTULO 1

INTRODUÇÃO

1. INTRODUÇÃO

O efetivo Brasileiro de equinos é de 5,5 milhões de equinos (IBGE, 2010). Estimativas apresentadas em 2006 indicam que o complexo mercado da equideocultura brasileira movimenta valor econômico superior a R$ 7,5 bilhões anuais e 3,2 milhões de empregos diretos e indiretos, relacionados ao cavalo no Brasil (Lima *et al.*, 2006).

Dentre as diversas raças de cavalos que existem no mundo, a Quarto de milha ocupa lugar de destaque, principalmente, devido a sua versatilidade, a qual viabilizou seu uso em diversas modalidades esportivas (Harris e Swinney, 2011).

A raça QM foi a primeira desenvolvida na América, surgindo nos EUA a partir do século XVII. Sua origem decorre de animais reprodutores trazidos da Arábia, Turquia e Inglaterra à América do Norte pelos colonizadores europeus. O resultando destes cruzamentos foram cavalos compactos, fortes, velozes e muito versáteis, usados para atividades que vão desde a lida com o gado até a disputa de corridas que tinham em média um quarto de milha (402m), originando assim, o nome da raça. A implantação da raça no Brasil começou em 1955, com animais importados dos Estados Unidos que despertaram notoriedade suficiente para gerar a difusão da raça no país (Abqm, 2014a; Aqha, 2014).

Para o ano de 2013 a população mundial de QM era da grandeza de 2,93 milhões (Aqha, 2013). No Brasil o número de QM registrados na Associação Brasileira de Criadores de Cavalo Quarto de Milha (ABQM) é de mais de 424 mil animais, com valor estimado de 827,3 milhões de dólares dividido entre 79,7 mil criadores, proprietários e associados Toda essa atividade gera mais de 318 mil empregos diretos no Brasil (Abqm, 2014a). Nos eventos oficiais da ABQM são distribuídos mais de 4 milhões em prêmios anualmente. A raça também movimenta o mercado de leilões, apurando mais de 195 milhões com a comercialização de mais de 4,5 mil animais em 115 leilões (Abqm, 2014c).

Atualmente raça QM possui três linhagens principais: conformação, velocidade e trabalho. Destas linhagens derivam animais para as mais diversas modalidades esportivas como: conformação, corrida, tambor/baliza, rédeas, apartação, etc. Estas modalidades esportivas são muito praticadas no Brasil, com inúmeras competições durante o ano (Abqm, 2014b).

Diversas práticas vêm sido empregadas na criação de QM visando principalmente o melhoramento na raça no âmbito de desempenho atlético, a seguir: rigoroso controle

do livro de registros (studbook), a seleção de cruzamentos baseados no desempenho atlético dos animais, uso repetido de garanhões populares, a endogamia e a aplicação de biotécnicas reprodutivas. Essas técnicas aceleram o progresso genético, mas também estão associadas com o aumento da doenças hereditárias (Bettley *et al.*, 2012).

É bem entendido que, um cavalo quando conquista expressiva notoriedade a partir do sucesso em uma das disciplinas competitivas, é selecionado para produzir um número muito maior de descendentes em relação ao um cavalo normal. Nesta linha de pensamento além das características desejáveis, pode também ser amplificada a frequência de alelos mutantes relacionados à doença hereditária, aumentando sua incidência, se o cavalo selecionado para a reprodução for portador de uma mutação que cause uma doença (Tryon *et al.*, 2009).

A partir da publicação do genoma do cavalo, o número de pesquisas acerca de doenças hereditárias que os acometem aumentou consideravelmente, possibilitando a identificação da base genética destas doenças (Brosnahan *et al.*, 2010). Não se sabe acerca da incidência destas doenças no rebanho Quarto de milha brasileiro, provavelmente estão sendo transmitidas para gerações sucessivas e causando perdas financeiras para os criadores de cavalos e sofrimento para os animais acometidos e seus proprietários.

Uma revisão sistemática conduzida por Bettley (2012) possibilitou a conclusão de que 102 doenças potencialmente hereditárias (que possuem um componente hereditário) existem no cavalo doméstico (*Equus caballus*) nas mais diversas raças.

Contudo somente existem oito doenças hereditárias que são causadas por mutações genéticas conhecidas (Finno *et al.*, 2009). Destas, cinco existem na raça QM: a Paralisia Periódica Hipercalêmica (HYPP)(Rudolph *et al.*, 1992), Deficiência da Enzima Ramificadora do Glicogênio (GBED)(Ward *et al.*, 2004), Astenia Cutânea (HERDA)(Tryon *et al.*, 2007), Miopatia por Acúmulo de Polissacarídeo 1 (PSSM1)(Mccue *et al.*, 2008) e a Hipertermia Maligna (HM)(Aleman *et al.*, 2009). Destas doenças somente o HYPP possui teste comercial de diagnóstico molecular disponível no Brasil. Pesquisas desenvolvidas no país já padronizaram o teste molecular para HERDA(Badial *et al.*), HYPP(Battazza *et al.*, 2014) e PSSM(Souza *et al.*, 2014). As demais doenças ainda não possuem o diagnóstico molecular confirmatório padronizado no país.

A GBED é uma doença hereditária autossômica monogênica recessiva fatal (Ward *et al.*, 2004), causada por uma mutação pontual de substituição (c.102C>A) no gene

(GBE1) que codifica a Enzima Ramificadora de Glicogênio (GBE). A GBED ocasiona abortos, natimortos e nascimentos de potros fracos, que morrem nas primeiras horas de vida (Valberg *et al.*, 2001; Wagner *et al.*, 2006).

Em 1999, três casos de animais quarto de milha (um feto abortado, um natimorto e um potro que nasceu fraco e veio a óbito) foram avaliados sob aspectos clínicos e histopatológicos, caracterizando uma miopatia por acúmulo de polissacarídeo no músculo esquelético e cardíaco e também em outros tecidos corpóreos como cérebro, medula espinhal e fígado, nesta momento esta doença foi denominada amilopectinose (Render *et al.*, 1999)

Dois anos depois, um estudo descreveu sete animais da raça quarto de milha com variedades clínicas desde natimortos até animais que tiveram óbito por falência cardíaca, Neste estudo o diagnóstico de GBED foi estabelecido com base na análise de pedigree, atividade enzimáticas, histopatologia e ensaios imunoenzimáticos (Valberg *et al.*, 2001).

O aborto e natimorto equino constituem a maior causa de mortalidade equina, causando prejuízos econômicos para o agronegócio do cavalo(Hong *et al.*, 1993; Laugier *et al.*, 2011). A frequência de abortos nesta espécie varia entre 8% e 19% (Bain, 1969; Whitwell, 1980; Acland, 1993). Entretanto pesquisas realizadas no Reino Unido, França e Estados Unidos constataram que entre 7,8% a 25,1% dos casos de aborto a etilogia não é identificada (Hong *et al.*, 1993; Smith *et al.*, 2003; Laugier *et al.*, 2011); no Brasil essa proporção é de 47,2% a 54% (Moreira *et al.*, 1998; Marcolongo-Pereira *et al.*, 2012). Nos Estado Unidos estima-se que entre 1,3% e 3,8% dos casos de aborto em cavalos QM são causados pela GBED (Wagner *et al.*, 2006).

O diagnóstico da GBED é realizado com a identificação da mutação por meio de testes genéticos em amostras de DNA que podem ser extraídas de sangue e/ou pelo do animal afetado. O diagnóstico molecular confirmatório padronizado para a GBED não está comercialmente disponível no Brasil. Não se sabe se existem animais portadores da mutação no país, bem como a frequência destes. Entretanto é provável que a mutação esteja presente no rebanho Brasileiro, visto que a base genética destes animais é derivada dos animais pertencentes ao rebanho dos Estados Unidos.

O conhecimento da frequência de um alelo do gene da doença em uma população e o modo de hereditariedade é crítico para determinar o impacto de uma doença em uma determinada raça (Tryon *et al.*, 2009). O objetivo desta dissertação foi verificar a prevalência de cavalos Quarto de milha portadores da GBED no Brasil.

REVISÃO DE LITERATURA

2. REVISÃO DE LITERATURA

2.1 GLICOGÊNIO

O Glicogênio é um homopolissacarídeo altamente ramificado (Figura 1) composto de resíduos de D-glicose (Figura 2) unidos por ligações glicosídicas α-1,4 (Figura 3), formando longas cadeias que apresentam ramificações por meio de ligações glicosídicas α-1,6 (Figura 4) a cada dez resíduos (aproximadamente) de D-glicose (Roach, 2002; Berg *et al.*, 2003). Cerca de 8% das ligações são α-1,6 o restante são ligações α-1,4(Melendez Hevia *et al.*, 1993).

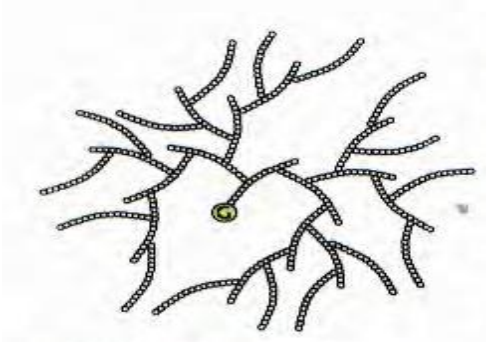

Figura 1. Desenho esquemático da região central de uma molécula de glicogênio evidenciando grande número de ramificações
Fonte: Berg (2008).

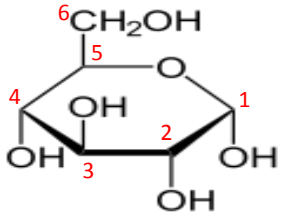

Figura 2. Desenho esquemático de uma molécula de D-Glicose com seus carbonos numerados.
Fonte: Adaptado de Berg (2008).

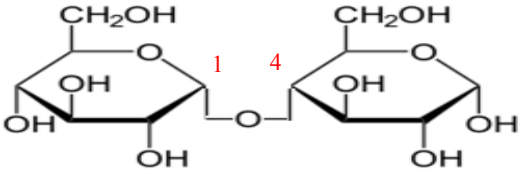

Figura 3. Desenho esquemático de uma ligação glicosídica α -1,4 entre duas moléculas de D-Glicose
Fomte: Adaptado de Berg (2008).

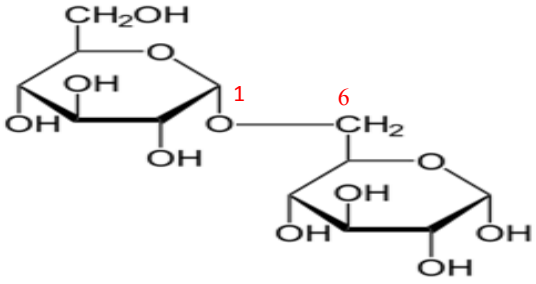

Figura 4. Desenho esquemático de uma ligação glicosídica α -1,6 entre duas moléculas de D-Glicose
Fonte: Adaptado de Berg (2008).

O glicogênio é a principal forma de armazenamento prontamente mobilizado de glicose que ocorre em todo o reino animal(Calder, 1991). A sua importância se dá por três principais aspectos: primeiro, sua degradação controlada mantém o nível de glicemia entre as refeições dentro da normalidade; segundo, a glicose provinda da

metabolização do glicogênio é prontamente mobilizada caracterizando-se como uma boa fonte energética para a atividade física repentina; terceiro a manutenção dos níveis de glicemia através do glicogênio é de suma importância para alguns tipos celulares como as hemácias, o tecido nervoso e a retina para os quais a glicose é o principal substrato energético (Lund-Andersen, 1979; Berg *et al.*, 2003).

O glicogênio não tem um peso molecular fixo, porque é constantemente modificado de acordo com as exigências metabólicas privilegiando a síntese ou degradação da molécula (Bonen *et al.*, 1985; Hutber e Bonen, 1989; Azevedo *et al.*, 1998). É encontrado no citosol sob a forma de grânulos com diâmetro de 10 a 40 nm, existe na maioria dos tecidos do mamífero, contudo os principais locais de armazenamento são fígado (10% do peso) e o músculo esquelético (2% do peso) que apesar de ter uma menor concentração de glicogênio em relação ao fígado, contém quantidade absoluta maior, devido à maior massa absoluta de tecido muscular (Calder, 1991; Berg *et al.*, 2003; Lehninger *et al.*, 2005). A função do glicogênio hepático é manter os níveis glicêmicos dentro da normalidade, ao passo que a função do glicogênio muscular é servir de fonte de energia para a atividade do próprio musculo (Calder, 1991).

O glicogênio hepático existe sob a forma de partículas de peso molecular de 107 Da, equivalente a cerca de 55000 unidades de glicose (Goldsmith *et al.*, 1982). Tem uma estrutura otimizada para maximizar a glicose total armazenada no menor volume possível e possibilitar o armazenamento de glicose respeitando uma pressão osmótica compatível com a vida(Lehninger *et al.*, 2005).

A estrutura altamente ramificada do glicogénio também favorece a velocidade da liberação de "combustível", devido à maximização de unidades de glicose na periferia da molécula que podem ser liberadas prontamente quando necessário (Melendez Hevia *et al.*, 1993).

2.2 SÍNTESE DO GLICOGÊNIO

A síntese do glicogênio ocorre na maioria dos tecidos, contudo principalmente no fígado e músculo esquelético (Lehninger *et al.*, 2005). O glicogênio é sintetizado a partir de uma forma ativada de glicose, a uridina difosfato glicose (UDP-glicose). A partir da disponibilidade de UDP-glicose, resumidamente ocorre:

1- A Glicogenina, uma proteína que realiza autoglicolisação (atribui resíduos de glicose para si) age pré-formando uma cadeia de até oito resíduos de glicose (extraídos da UDP-glicose) que funciona como um molde inicial da síntese de glicogênio (Berg *et al.*, 2003; Lomako *et al.*, 2004; Lehninger *et al.*, 2005).
2- A enzima Glicogênio Sintase liga-se a Glicogenina, e a partir de UDP-glicose catalisa a adição de novos resíduos de glicose à extremidade não redutora da molécula de glicogênio em formação, por meio da formação de ligações glicosídicas α-1,4. (Berg *et al.*, 2003; Lehninger *et al.*, 2005).
3- Uma outra enzima é necessária para formar as ramificações do glicogênio, a Enzima Ramificadora (glicosil-4,6-transferase) que atua catalisando a formação de ligações glicosídicas α-1,6 entre os resíduos de glicose conferindo a molécula de glicogênio sua característica ramificada (Berg *et al.*, 2003; Lehninger *et al.*, 2005).

Portanto as ramificações além de aumentarem a solubilidade do glicogênio, criam um grande número de terminais, que são locais de ação das enzimas da síntese (glicogênio sintase) e degradação (glicogênio fosforilase). Desse modo a ação da Enzima Ramificadora que gera as ramificações aumenta a velocidade de síntese e degradação do glicogênio (Berg *et al.*, 2003).

2.3 DOENÇAS DE DEPÓSITO DE GLICOGÊNIO E GBED

As doenças de depósito de glicogênio (*Glycogen storage diseases*-GSD) ou Glicogenoses compreendem um grupo de doenças metabólicas hereditárias causadas por anormalidades das enzimas que regulam a síntese ou degradação do glicogênio (Mahler, 1969; Roach, 2002; Berg *et al.*, 2003). Existem em humanos 12 tipos de anormalidades em etapas conhecidas do metabolismo da glicose e glicogênio musculares (glicogenoses I a XII), contudo muito mais do que 12 etapas enzimáticas estão envolvidas no metabolismo, portanto é provável que novas "GSDs" sejam descobertas (John Vissing *et al.*, 2010). A classificação de cada GSD decorre do tipo de alteração enzimática ou proteica envolvida, o órgão acometido e os sinais clínicos apresentados (Moses, 1990).

Um dos tipos de GSD é a GBED, em humanos é chamada de Glicogenose tipo IV (GSD IV), Amilopectinose, Doença do Poliglucosano e Doença de Andersen (Andersen, 1956; John Vissing *et al.*, 2010) e ocorre devido a mutações no gene

(GBE1), acarretando deficiência da enzima de ramificação de glicogênio (GBE, glicosil-4,6-transferase) (Nolte *et al.*, 2008).

A apresentação clínica da GBED varia tanto entre espécies como também dentro de uma espécie (Render *et al.*, 1999). Descrita pela primeira vez em humanos (Andersen, 1956) apresentando três variações clínicas com envolvimento neuromuscular: congênita, juvenil e adulta (Bruno *et al.*, 2007). Esta heterogeneidade clínica está relacionada a diferentes mutações identificadas ao longo do gene GBE1 nos diferentes tipos de fenótipos (Bao *et al.*, 1996). Em animais a GBED foi descrita pela primeira vez em uma família de gatos (Fyfe *et al.*, 1992) com variações clínicas incluindo: natimorto, morte pós-parto imediata, aparecimento juvenil de miopatia e neuropatia progressiva letal.

2.4 GBED EM CAVALOS

Em cavalos é uma doença hereditária autossômica monogênica recessiva fatal (Ward *et al.*, 2004) descrita pela primeira vez em três animais QM (um feto abortado, um potro natimorto e um potro com um mês de vida) (Render *et al.*, 1999) com avaliações histológica e ultra-estrutural caracterizando-a como doença de depósito de glicogênio tipo IV.

2.4.1 Etiologia

A caracterização do defeito molecular responsável pela GBED em equinos foi realizada pela primeira vez em 2004 (Ward *et al.*, 2004). Neste estudo, os pesquisadores verificaram que esta doença ocorre devido a uma mutação pontual que consiste na substituição de uma citosina por um adenina na posição 102 da região codificante, implicando na troca de uma Tirosina (TAC) por um sinal de parada (TAA) prematuro no códon 34 do exón 1 do gene GBE1, caracterizando uma mutação "sem sentido" (*nonsense mutation*). O gene GBE1 equino, é composto por 15 éxons, que juntos (cDNA) apresentam 2100 bases que em uma transcrição normal codificam 699 aminácidos, na situação de transcrição de uma sequência com a mutação este processo é interrompido prematuramente no códon 34, resultado numa proteína truncada e sem atividade funcional, sendo assim, potros homozigotos recessivos não são capazes de sintetizar uma proteínca GBE funcional (Ward *et al.*, 2004).

2.4.2 Epidemiologia

Estudos de pedigree de animais portadores da mutação apontam dois garanhões o Zantanon e o seu filho King P234 como disseminadores da mutação (Valberg, 2006). O garanhão King P234 foi um importante cavalo que viveu entre 1932 e 1958, produziu muitos descendentes e influenciou bastante a raça QM nos primeiros anos de existência da *American Quarter Horse Association* (Aqha, 2014). Devido à sua importância ele foi incluído no "hall da fama" desta associação em 1989. Nos Estados Unidos, portadores da mutação são observados de forma mais frequente em certas linhagens de cavalos QM, utilizados em competições esportivas de apartação (Tryon *et al.*, 2009).

No Brasil não se sabe se existem animais acometidos (homozigotos recessivos) ou portadores (heterozigotos), contudo estudos realizados nos Estados Unidos em 2006 verificaram um percentual de portadores da mutação causadora da doença de 8,3% 11% (Wagner *et al.*, 2006; Tryon *et al.*, 2009), valores maiores foram encontrados nos subgrupos das modalidades de *Western Pleasure (26,3%)* e Apartação (13,6%) (Tryon *et al.*, 2009). Outro trabalho atribui que entre 1,3% e 3,8% dos casos de aborto dos equinos QM são causados pela GBED (Wagner *et al.*, 2006).

2.4.3 Fisiopatologia

A enzima GBE é importante para glicogênese, por catalisar a formação de pontos de ramificação. Embora não existam danos relacionados as enzimas de degradação do glicogênio, que são as responsáveis pelo catabolismo, os tecidos de potros acometidos por GBED são incapazes de sintetizar o glicogênio ramificado normalmente (Valberg *et al.*, 2001) e o armazenam com estrutura anormal, menos ramificada comprometendo a velocidade de síntese e degradação da molécula de glicogênio e desta forma a manutenção da homeostase da glicose, predispondo a que ocorra o acúmulo desse polissacarídeo nos tecidos (Valberg *et al.*, 2001; Sponseller *et al.*, 2003; Wagner *et al.*, 2006).

O glicogênio serve como um substrato de energia primária para a maioria dos tecidos corporais, sendo essencial para o crescimento e desenvolvimento do feto e para homeostase da glicose nos recém nascidos. Na ausência de glicogênio a musculatura cardíaca, esquelética e o encéfalo são incapazes de manter a atividade adequada levando

o animal à morte (Valberg *et al.*, 2001; Wagner *et al.*, 2006; Brosnahan *et al.*, 2010). Ademais o glicogênio anormal acumula-se nas fibras de Purkinje que somado ao possível aporte insuficiente de glicose ao miocárdio, justifica a característica dos potros acometidos apresentam arritmia e morte súbita quando exercitados/estimulados (Valberg *et al.*, 2001).

Um aspecto interessante é que heterozigotos portadores da mutação GBE1 demonstram cerca de 50% da atividade normal da GBE (Valberg *et al.*, 2001), no entanto isto ainda não demonstrou exercer efeitos adversos sobre a saúde destes animais.

2.4.4 Sinais clínicos

Os sinais clínicos podem estar relacionados com o tecido que estiver com maior limitação do fornecimento de glicose disponível naquele momento. A falta de glicose no músculo cardíaco pode levar à disfunções cardíacas, crises de hipoglicemia podem ocorrer quando houver depleção de glicogênio no fígado, e fraqueza muscular pode ser mais aparente em potros com a depleção de glicogênio muscular significativa (Valberg *et al.*, 2001).

De modo geral, em equinos, a doença é caracterizada por abortos, natimortos e nascimentos de potros fracos, estes animais que nascem vivos podem apresentar deformidades flexurais, convulsões, dificuldade ou incapacidade de permanecer em estação, ausência do reflexo de sucção, sinais de insuficiência cardíaca e respiratória e morte súbita. Geralmente os animais morrem nas primeiras horas de vida, mas podem sobreviver por até 18 semanas quando submetidos a tratamento intensivo em unidades especializadas(Valberg *et al.*, 2001; Wagner *et al.*, 2006).

2.4.5 Alterações histopatológicas

No exame histopatológico, os equinos afetados podem apresentar na musculatura, material cristalino eosinofílico ou glóbulos biofílicos na coloração de hematoxilina e eosina (Valberg *et al.*, 2001; Ward *et al.*, 2004). A coloração especial de ácido periódico-Schiff (PAS) pode demonstrar depósitos globulares anormais de polissacarídeos e inclusões cristalinas (Valberg *et al.*, 2001). Inclusões globulares são

evidenciadas nas células de Purkinje do miocárdio e também podem ser encontradas nos miocardiócitos, tecido neural e de forma inconsistente, no fígado (Render *et al.*, 1999).

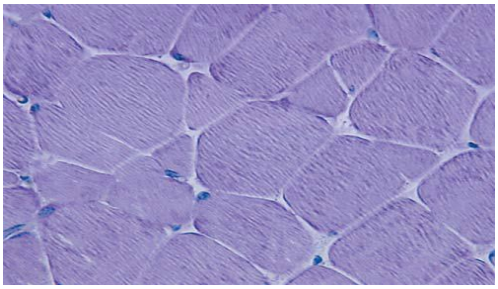

Figura 5. Corte histológico fixado em formalina e feita coloração PAS, de secção transversal de músculo glúteo médio de um potro sadio. Notar a distribuição uniforme da cor violeta relacionado ao glicogênio.
Fonte: Sponseler (2003)

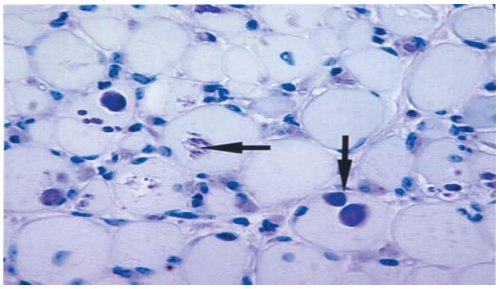

Figura 6. Corte histológico fixado em formalina e feita coloração PAS, de secção transversal de músculo glúteo médio de um potro afetado por GBED. Notar a ausência de distribuição uniforme da cor violeta relacionado ao glicogênio e a presença de glóbulos positivos para PAS (seta vertical) e inclusões cristalinas (seta horizontal)
Fonte: Sponseler (2003)

2.4.6 Diagnóstico

Não é confiável diagnosticar a GBED utilizando a análise de pedigree, porque a maioria dos cavalos Quarto de milha é descendente dos garanhões King P234 e Zantanon (Valberg, 2006).

Potros acometidos por GBED geralmente apresentam valores hematológicos de baixa contagem de leucócitos (muitas vezes cerca de 4.000 células/μL) elevações moderadas no soro de creatina quinase (CK), aspartato transaminase (AST) e gama glutamil transferase (GGT). Exames histológicos de biópsias musculares de potros afetados podem indicar a presença de glicogênio anormal, contudo nenhum desses exames é confirmatório para a doença (Valberg *et al.*, 2001; Ward *et al.*, 2004)**.** O diagnóstico definitivo é realizado utilizando teste molecular (Valberg *et al.*, 2001). No Brasil este teste ainda não está comercialmente disponível.

2.4.7 Tratamento e prognóstico

Não há tratamento para GBED. Não existe relato de potro homozigoto para a mutação que sobrevivera mais de 18 semanas mesmo submetidos a tratamento intensivo em hospitais veterinários (Ward *et al.*, 2004). O diagnóstico precoce e eutanásia podem evitar situações desgastantes para todos os envolvidos bem como as despesas para proprietários de potros que estejam em unidades de cuidados intensivos (Valberg, 2006).

2.8 ABORTO E NATIRMORTO EQUINO

O aborto e natimorto equino constituem a maior causa de mortalidade e de prejuízos econômicos para a equideocultura (Hong *et al.*, 1993; Laugier *et al.*, 2011). A frequência de abortos nesta espécie varia entre 8% e 19% (Bain, 1969; Whitwell, 1980; Acland, 1993) e no Brasil um estudo realizado no estado do Paraná utilizando animais predominantes da raça Puro Sangue Inglês, constatou que 9,2% das gestações resultam em aborto (Moreira *et al.*, 1998). Contudo diversos trabalhos apontam que entre 7,7% a 40% a etiologia do aborto não é identificada (Acland, 1993; Hong *et al.*, 1993; Smith *et al.*, 2003; Laugier *et al.*, 2011). No Brasil a etiologia não é identificada entre 47,2% e 54% dos casos de aborto (Moreira *et al.*, 1998; Marcolongo-Pereira *et al.*, 2012). Vale salientar que segundo Wagner et al. (2006) a GBED é uma causa importante responsável por entre 1,3% e 3,8% dos casos de aborto dos equinos QM.

CAPÍTULO II

O artigo a seguir foi redigido em conformidade com as normas do periódico científico "Theriogenology".

Prevalence of mutation carriers associated with glycogen branching enzyme deficiency (GBED) in Quarter horses.

Abstract

Glycogen Branching Enzyme Deficiency [GBED] is an autosomal fatal inherited disease that affects horses, caused by a nonsense mutation (c.102C>A) in codon 34 of GBE1 gene that strongly impair the glycogen metabolism. This disease causes disorder in glycogen metabolism in the affected animals that are usually aborted or die in early life. There are no information about GBED in Brazil, however, comprehension/awareness of genotypic prevalence is important to estimate the impact of a hereditary disease in a population. The aim of this study was to determine the prevalence of GBED mutation carriers in a population of Quarter horse used in five equestrian sports practiced in Brazil. Blood samples were obtained from 740 animals. After DNA purification, PCR reactions, automated direct sequencing and sequence analysis were performed. From 740 animals tested 59 were considered heterozygous for the mutation responsible for GBED representing a prevalence of 7.97% in the studied population. The prevalence of heterozygotes were higher in cutting (20%) and reining (10%) subgroups, followed by barrel racing (5%) and halter (3%), there were no heterozygous animals in racing subgroup. The results demonstrate that the mutation exists in the Brazilian Quarter horse herd, and suggests that the homozygous recessive might be occurring without being noticed. So based on this information GBED is a disease to be considered in the differential diagnosis of cases of abortion and stillbirths in Brazilian Quarter horses and strategies should be developed to prevent transmission of the mutation.

Keywords: Abortion; Glycogen; Foal; Muscle disease; Hereditary disease.

INTRODUCTION

The American quarter horse was the first breed developed in the Americas, emerging in the United States in the seventeenth century. The insertion of this breed in Brazil began in 1955. The animals imported from the USA aroused enough notoriety to spread the breed in the country (Abqm, 2014a; Aqha, 2014). In the present days this breed is branched in three bloodlines: halter, racing and work. These strains conceived animals for many different sports modalities such as halter, racing, barrel racing, reining and cutting. These sports are very practiced in Brazil, with numerous competitions during the year (Abqm, 2014b).

The American Quarter breed is related to 18 diseases with hereditary pattern, of those five have its molecular marker elucidated: Hyperkalemic Periodic Paralysis (HYPP)(Rudolph *et al.*, 1992), Glycogen Branching Enzyme Deficiency (GBED)(Ward *et al.*, 2004), Hereditary Equine Regional Dermal Asthenia (HERDA)(Tryon *et al.*, 2007), Polysaccharide Storage Myopathy 1 (PSSM1)(Mccue *et al.*, 2008) and Malignant Hyperthermia (HM)(Aleman *et al.*, 2009).

The GBED was first described in humans (Andersen, 1956) featuring three clinical variants with neuromuscular involvement: the congenital, juvenile and adult forms (Bruno *et al.*, 2007), this clinical heterogeneity is related to different mutations identified along the GBE1 gene in different types of phenotypes (Bao *et al.*, 1996). In animals, GBED was first described in a family of cats (FYFE et al., 1992).

In horses, it is a fatal autosomal recessive monogenic disease affecting animals of the Quarter horse and Paint horse breeds, and this disease is caused by a nonsense mutation in the gene GBE1, characterized by the substitution of cytosine by adenine at position 102 of the coding region of exon 1 resulting in a premature stop codon (TAA) causing incomplete synthesis of glycogen branching enzyme (GBE, glycosyl 4,6 transferase) (Ward *et al.*, 2004).

The GBE enzyme is important for glycogenesis, catalyzing the formation of branching points. The tissues of affected foals are unable to synthesize branched glycogen normally (Valberg *et al.*, 2001) thereby impairing bioavailability and homeostasis of glucose, promoting the accumulation of polysaccharide with abnormal structure and less branched(Valberg *et al.*, 2001; Sponseller *et al.*, 2003; Wagner *et al.*, 2006). In the absence of glycogen the cardiac muscle, skeletal and brain are unable to

maintain adequate activity leading to death (Valberg *et al.*, 2001; Wagner *et al.*, 2006; Brosnahan *et al.*, 2010).

Clinically, affected foals may have flexural deformities, difficulty or inability to stand, seizures, absence of sucking reflex, signs of heart and respiratory insufficiency and sudden death, and do not survive for more than 18 weeks even under intensive medical treatment (Ward *et al.*, 2004). Due to abnormal glycogen accumulation in Purkinje fibers and possible insufficient supply of glucose to the myocardium, these foals have arrhythmias and sudden death when exercised (Valberg *et al.*, 2001). A more accurate diagnosis is performed using molecular tests that can be performed using blood and/or hair samples from affected animals (Valberg, 2006).

Under the reproductive aspect this disease is characterized by abortions, stillbirths and premature births (Ward *et al.*, 2004; Wagner *et al.*, 2006). Abortion and stillbirth are the largest cause of equine mortality, causing economic losses for the horse agribusiness (Hong *et al.*, 1993; Laugier *et al.*, 2011). The frequency of abortions in this specie is between 8% - 19% (Bain, 1969; Whitwell, 1980; Acland, 1993). But research conducted in the UK, France and the United States found that between 7,8% - 25,1% the abortion cases the etiology is not identified (Hong *et al.*, 1993; Smith *et al.*, 2003; Laugier *et al.*, 2011); in Brazil this proportion is between 47,2% - 54% (Moreira *et al.*, 1998; Marcolongo-Pereira *et al.*, 2012). In the United States is estimated that between 1.3% - 3.8% of abortion cases in Quarter horse breed are caused by GBED (Wagner *et al.*, 2006).

Pedigree studies of mutation carriers point to two stallions, Zantanon and his son King, the second won very expressive fame and lived between 1932 and 1958(Valberg, 2006) producing many offsprings, mainly horses with huge performance, ability and "cow sense", and substantially influencing the Quarter horse breed in the early years of the American Quarter Horse Association (Aqha, 2014; 2015) . Some of the practices employed by American Quarter Horse breeders, as artificial insemination in which a progenitor can generate numerous descendants, to improve the breed in the aspect of athletic performance, can not only accelerate genetic progress but also increase the frequency of hereditary diseases (Bettley *et al.*, 2012).

Previous studies in the United States reported a percentage of mutation carriers is between 8,3%(Wagner *et al.*, 2006) and 11% (Tryon *et al.*, 2009), highest values were found in *Western Pleasure* (26,3%) and Cutting subgroups (13,6%)(Wagner *et al.*, 2006). Genotyping tests for GBED are available in specialized laboratories in United

States, enabling the establishment of strategies to control the proliferation of the disease.

It was unknown whether there were carriers of the GBED mutation in Brazil, as well as their prevalence, this new information can promote subsequent studies, for example, the disease incidence (recessive homozygotes). However it was expected to find this mutation in Brazilian herd, since the genetic basis of these animals derived from United States herd. The aim of this study was to determine the prevalence of mutation carriers associated to GBED in a population of Quarter horse animals used in five equestrian sports practiced in Brazil.

MATERIAL AND METHODS

Animals

Seven hundred and forty pure Quarter Horses were selected, from both genders , over six months of age, from specific blood lines used in sports constituting 5 subgroups: Cutting (n=160), Halter (n=100), Race (n=160), Reining (n=160), and Barrel Racing (n=160). The convenience sampling included the participation of 42 establishments distributed throughout 16 cities belonging to São Paulo state and 1 city of Rio Grande do Sul state. Blood samples were collected without anticoagulant from all animals under strict confidentiality agreement to ensure the anonymity of establishments, animals and their owners. These samples were kept under refrigeration until that DNA extraction were performed. All procedures were approved by the Board of Ethics and Animal Experimentation of the institution (Protocol nº 262/2011 – CEUA).

DNA extraction

DNA was extracted from blood samples using a commercial kit [1] following the manufacturer's recommendations. At the end of process, the DNA was diluted in 100 uL of water-free DNase and RNase. A sample of 2 uL DNA was tested for purity (A260 / 280) and concentration using a spectrophotometer and immediately stored in a freezer at -80°C.

[1] Ilustra™ Blood GenomicPrep Mini Spin Kit (GE Healthcare)

PCR reaction

The PCR reaction was designed to amplify a 267pb fragment of GBE1 gene by using samples of genomic DNA and primers (Ward *et al.*, 2004). The standard reactions to a final volume of 25 uL, comprise: 2.0 ul of extracted DNA; 11 uL of master mix[2]; 0.4 uM of each primers (volume 1µL by reaction; under 10µM concentration); 4.0 uL of commercial buffer[3] (final concentration of 2.0X reaction) and nuclease free water q.s.p..

Thermocycler[4] was used with the following programmed cycle: 95 ° C for 5min. followed by 35 cycles of 95 ° C for 30s, 59 ° C for 1min. and 72 ° C for 1min. and final extension at 72 ° C for 7min.

Agarose gel electrophoresis

The size of the amplified products was confirmed by electrophoresis[5] system using stained[6] agarose gel 1.5% and compared to the DNA ladder[7], and then the gels were photographed[8].

Purification of the amplified products

The amplified products were purified directly from the PCR mix with a commercial kit[9], according to the manufacturer's recommendation. After purification the concentration was standardized to 10 ng / uL and submitted to the sequencing reaction.

Sequencing Reaction

The sequencing reaction was prepared to a final volume of 10 ul: 1.0 ul of the oligonucleotide sense primer used in the PCR reaction at 5 uM , 2µL (approximately 20ng) of the purified amplified product, 1.0 ul of comercial mix[10], 1.5 uL of buffer

[2] GoTaq® Green Master Mix, 2x (Promega™)
[3] PCRx Enhancer Solution® (Invitrogen)
[4] Mastercycler EP Gradient S Eppendorf®, Hamburgo, Alemanha
[5] Major Science, Saratoga, CA, USA
[6] GelRed™ (Biotium, Halward, CA, USA)
[7] LowRanger 100 pb DNA (Norgen™, BioTek Corporation, Ontario, Canada)
[8] ImageQuant®imager (GE Healthcare)
[9] PCR clean-up Gel extraction kit (Macherey-Nagel®, Duren, Germany)
[10] BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA)

solution[11] and water "nuclease-free" qsp.. After preparing the reaction all specimens were subjected to automated direct sequencing with 3500 Genetic Analyzers (AppliedBiosystems, Foster City, CA, USA). After sequencing, the quality control of the sequences and obtained electropherograms were performed using the appropriate software[12].

Analysis of the sequences

The sequences aligned against the reference genome (accession number AY505110.1) that is available in the National Center for Biotechnology Information (NCBI) database, by the use of BLAST® tool (Basic Local Alignment Search Tool) to determine the similarities and specificity of the amplified product, and prove that the target segment GBE1 horse was amplified. Were considered only the sequences that have e-value less than e-100. After confirmation, the nucleotide sequences and electropherograms were analyzed by using software[13], enabling genotypic identification by checking the position 102 of the coding region (Fig. 1).

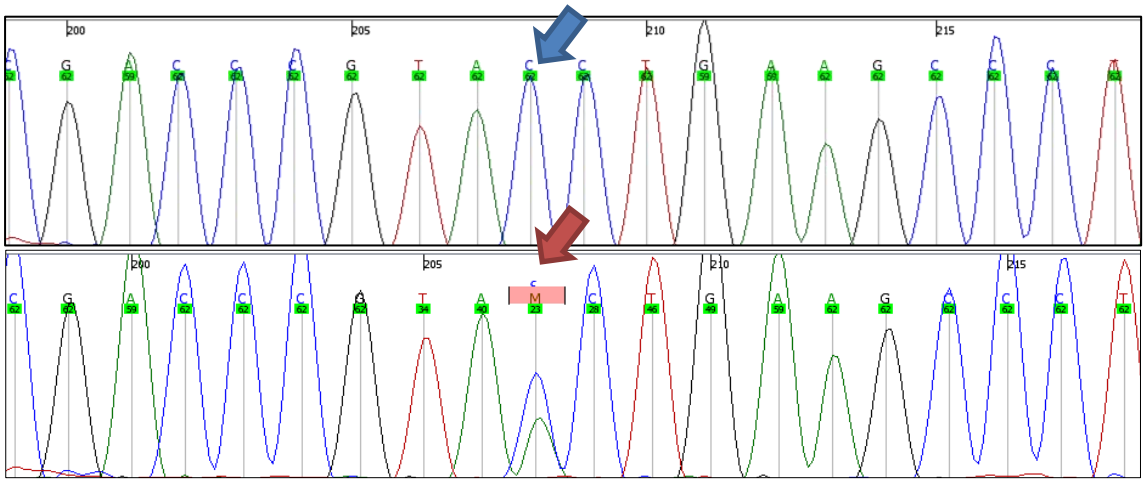

FIGURE 2- Electropherograms and GBE1gene sequences of nucleotides. Homozygous with a single peak of Cytosine (blue arrow) and a heterozygotic with a double peak of Cytosine and Adenine (red arrow).

The data analyzes were performed the using SAS® software and the genotype prevalence in the study population were calculated and compared the prevalence in each

[11] BigDye® Terminator v1.1 & v3.1 5X Sequencing Buffer (Applied Biosystems, Foster City, CA, USA)
[12] Sequencing Analysis 5.3.1 (Applied Biosystems, Foster City, CA, USA)
[13] Ridom® TraceEdit (Ridom GmbH, Münster, Germany)

subgroup using the statistical test Chi-square, the statistical difference was considered for $p \leq 0.05$.

Pedigree analysis

All heterozygotes had their lineage analyzed back to ten generations, consulting the Brazilian Quarter Horse Association, searching for the presence of one of the two stallions mentioned as the initial point to the dissemination of the mutation.

RESULTS

The PCR products of the all animals tested have been confirmed for the correct size (267pb) by electrophoresis (Figure 2).

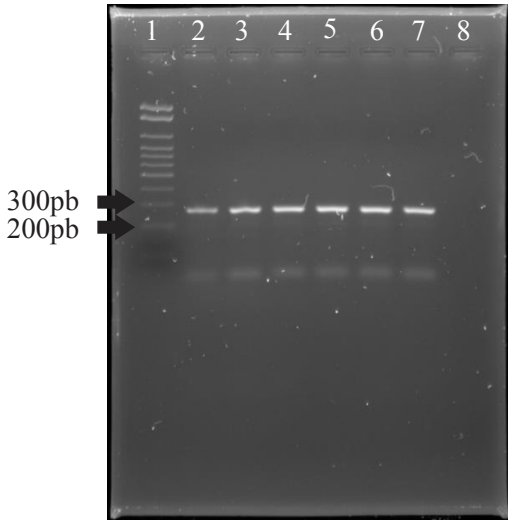

FIGURE 1- Photography of agarose gel after electrophoresis with GBED-267pb product. Well 1- LowRanger 100 pb DNA ladder, Wells 2-7 (samples), Well 8- Negative Control.

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From the 740 animals tested 59 were heterozygous for the mutation responsible for GBED representing a prevalence of 7.97% (59/740) in the studied population. Of the 59 pedigrees evaluated, all have the Zatanon or King P-234 present at least once in the mare and / or sire side, King P-234 had an average frequency 5-6 times for each pedigree.

The genotypic prevalence were different between the subgroups ($p < 0.05$ and DF = 4). The prevalence of heterozygotes was higher in cutting and reining. The descending order of genotype prevalence of heterozygotes (carriers) was 20% (32/160) in the cutting subgroup, followed by reining 10% (16/160), barrel racing 5% (8 / 160) and halter 3% (3/100). No heterozygous animals were identified in the racing subgroup (Table 1). The mutation responsible for GBED was found only in heterozygous, in animals aged from 2.5 to 29 years old and in 4 of the 5 subgroups studied. There was no statistical difference ($p < 0.05$ and DF = 1) between the rates found in both genders.

Of the 42 establishments that collaborated with the study, 20 (47.62%) had at least one heterozygous (mutation carrier). When the establishments were evaluated according to the subgroups, there were at least one heterozygous in: 53.33% of of the proprieties where there were animals of cutting (8/15); 50% (11/22) from those that had animals of reining; in 27.77% (5/18) from those that had animals of barrel racing ; and in 15.38% (2/13) from those that had animals of halter .

TABLE 1 - Distribution of herozygotes in the different subgroups studied.

Subgroup	Number of Animals	Heterozygotes for GBED	Genotypic prevalence of Heterozygotes (%)
Cutting	160	32	20
Reining	160	16	10
Barrel racing	160	8	5
Halter	100	3	3
Racing	160	0	0
Total	740	59	7,97

DISCUSSION

Considering that the total population of Quarter horses in Brazil is 424 thousand animals and that the previous data available of GBED heterozygous rate 8.3% (Wagner et al., 2006), a sample of 731 animals would be needed to estimate the prevalence of the heterozygous condition for the mutation causing GBED in Brazil, using software to calculate the sample size (Dean *et al.*, 2014) under a margin of error of 2% and 95% confidence interval. This study used a sample of 740 animals.

The mutation was not found in homozygous, since the minimum age of the animals in the study sample was 6 months and that GBED it is a fatal disease with death in up to 18 weeks of life (Ward *et al.*, 2004).

Even though is well known that the genetic basis of the Brazilian Quarter horse herd derived from United States herd (Abqm, 2014a) and that previous studies had already identified the presence of homozygous affected foals by GBED as well as heterozygous animals in the United States, it was not possible to affirm the mutation presence in the Brazilian herd. As the breed had its origin in the United States, it is likely that similar situations must find in other countries. According to data from AQHA, Brazil is the fifth largest exporter of Quarter horses, the largest exporter is the United States, which in 2014 exported 45,723 animals, all other countries together exported 2,440 animals in the same year (Aqha, 2013).

This study is the first to reveal the prevalence of heterozygous animals in Brazil for the mutation associated with GBED. The heterozygotes prevalence found was 7.97%, which is similar to those observed in previous studies 8.3% (Wagner *et al.*, 2006) and 11,0% (Tryon *et al.*, 2009) performed in United States.

A similar prevalence in both sexes corroborates with the characteristic of the GBED be an autosomal inherited disease, in this condition the mutant allele is equally likely to be transmitted by a parent to an offspring of both genders, furthermore, the mutation carriers do not develop the disease corroborating with a pattern of recessive inheritance (Nussbaum, 2008).

Different genotype prevalence was identified in the subgroups ($p < 0.05$ and $DF = 4$) and as verified by Tryon et al. (2009) no mutation carriers animals were identified in the race subgroup. In the cutting subgroup was found 20% heterozygous; for the same subgroup the rate observed in United States was 13.6%(Tryon *et al.*, 2009). This data can be related to the fact that King P-234 sired some racehorses, but is best remembered for siring horses with characteristics that are desirable in the cutting lineage. This added to the fact that King P-234 produced many offsprings and substantially influenced the Quarter horse breed (Aqha, 2015), may justify a higher prevalence in this group of mutation carrier animals as observed in this present study, as well as others. Probably any animal that belongs to this bloodline, have more chance of being a carrier of the disease, and depending on the selection of the mating a bigger number of affected foals by this disease.

A higher prevalence of heterozygotes was observed in the subgroup reinning (10%) compared to the halter subgroup (3%), but in their study Tryon et al. (2009) demonstrated a higher rate of heterozygotes in halter subgroup (5.1%) compared to subgroup reinning (3.1%). For the subgroup barrel racing was found 5% of heterozygotes in this study and 1.2% by Tryon et al. (2009).

The reproductive history of these animals was not analyzed, so it is unknown how many abortions and stillbirths were produced from mating of these heterozygotes and what diagnoses were established, this analysis along with the genotyping of these aborted or stillborn animals, would clarify how many of thosewould be homozigous for the mutation causing GBED . However, considering that in the years 2012 and 2013 were registered in the Brazilian Association of Quarter horse breeders (ABQM) over 37,000 horses, and considering the prevalence of heterozygotes in the present study, it can be projected that around 2,9 thousands heterozygotes will be registered in 2015.

The mating of two carrier animals results in homozygous foals in 25% of the times (Tryon *et al.*, 2009), and if the principle of Hardy-Weinberg equilibrium was established, about 58 homozygous recessive are conceived and die annually affected by GBED in Brazil.

Since approximately half of the cases of abortion in Brazil the etiology is not identified (Moreira *et al.*, 1998; Marcolongo-Pereira *et al.*, 2012) and data on the prevalence of disease (GBED homozygous recessive) do not exist in this country, probably the cases of abortions caused by GBED are occurring undiagnosed, causing uncalculated loss , corroborating with the need to genotype the animals, enabling the breeders to do a proper mating selection that will not incur in the production of affected foals.

CONCLUSIONS

The mutation in the gene GBE1 responsible for GBED is present in Brazilian Quarter horses, at 47.62% of the studied proprieties and 7.97% of animals. The highest prevalence found in this study were in the cutting and reinning subgroups. The result of this study suggest that GBED should be included in the differential diagnosis of abortion cases and neonatal mortality in horses, in addition, standardized molecular testing may be used to guide the choice of mating, which is the only preventive measure.

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ANEXOS

ANEXOS

Tabela -1. Número de animais que participaram da coleta de material e cidades envolvidas.

Nº	Cidade	Nº de animais coletados
1	Avaré	142
2	Bauru	212
3	Bofete	10
4	Botucatu	73
5	Brotas	22
6	Catanduva	17
7	Cesário Lange	81
8	Garça	01
9	Ibitinga	49
10	Itapetininga	27
11	Ourinhos	10
12	Paranapanema	01
13	Pelotas	06
14	Porangaba	13
15	Quadra	55
16	Tatuí	01
17	Tupã	20
	TOTAL	740

Figura 1. Mapa do estado de São Paulo com a localização das cidades onde foram realizadas coletas

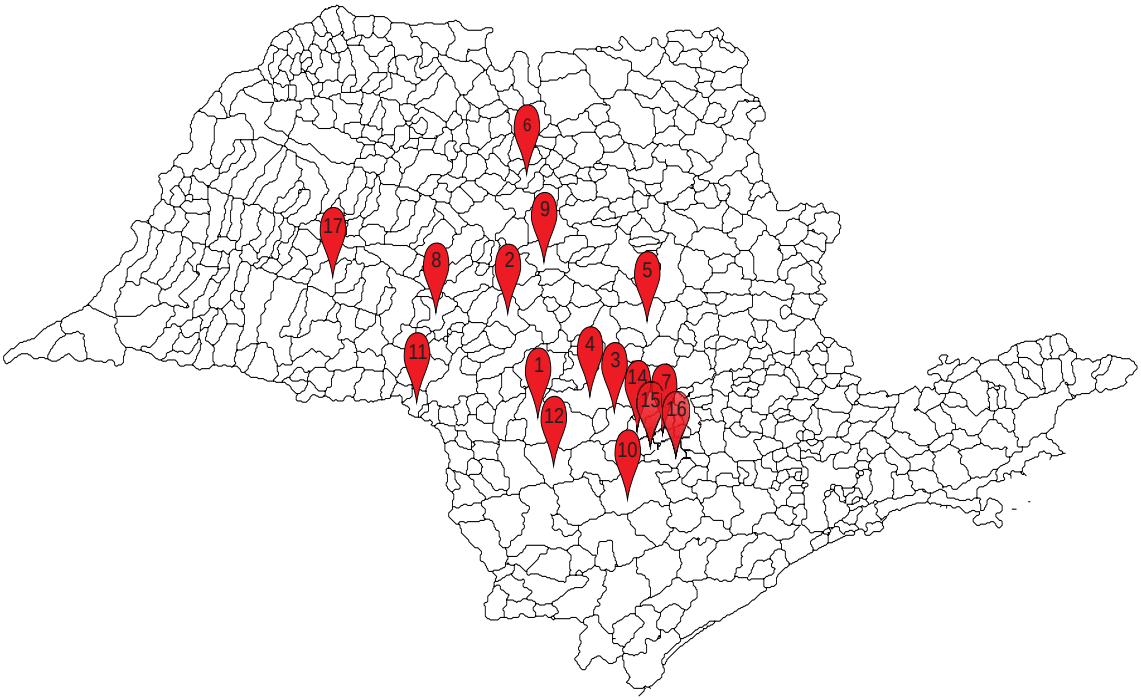
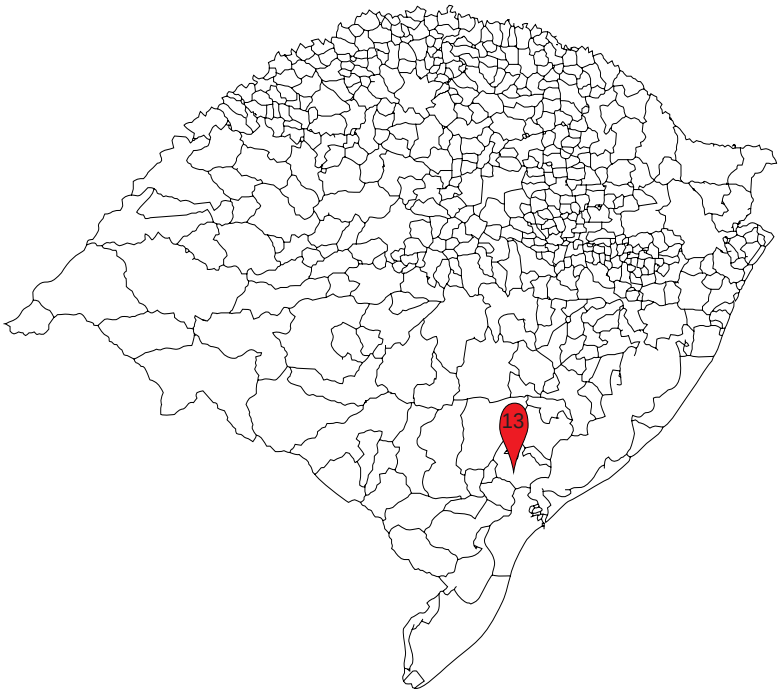


Figura 2. Mapa do estado do Rio Grande do Sul com a localização da cidade onde foi realizada coleta.



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• magnification: X 100
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• oogonium: female gamete before meiosis
• oocyte, primary: female gamete from onset of the first maturation division (meiosis) to extrusion of the first polar body
• oocyte secondary: female gamete from onset of second meiosis to extrusion of the second polar body
• ovum: female gamete from the end of both meiotic divisions until the union of the male and female pronuclei (differs from the common use of ovum as a general term for any female gamete)
• germinal vesicle: nucleus of the ovum
• zygote: a fertilized ovum, from fusion of the male and female gamete to completion of first cleavage
• embryo: a conceptus from the 2-cell stage to the stage when cell migration and differentiation are largely complete
• fetus: a conceptus after organogenesis is mostly complete (primarily increasing in size)
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kg - kilogram
L - liter
mL - milliliter
µL - microliter m - meter
min - minute mo - month
s - second
v:v - volume ratio wk - week
wt/vol - weight per volume y - year
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im - intramuscular iu - intrauterine
iv - intravenous
sc - subcutaneous po - oral
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CV - coefficient of variation df - degrees of freedom
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