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

FREQUÊNCIA ALÉLICA DA MUTAÇÃO *APAF*1 EM BOVINOS DA RAÇA HOLANDESA (*HOLSTEIN-FRIESIAN*) NO BRASIL

LUKAS GARRIDO ALBERTINO

BOTUCATU – SÃO PAULO
JANEIRO DE 2021

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Dissertação apresentada junto ao programa de Pós Graduação em Medicina Veterinária para obtenção do título de Mestre.

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

Co-Orientadora: Prof.ª Dra. Thais Helena Constantino Patelli

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JANEIRO DE 2021

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ROSEMEIRE APARECIDA VICENTE-CRB 8/5651

Albertino, Lukas Garrido.
Frequência alélica da mutação APAF1 em bovinos da raça Holandesa (Holstein-friesian) no Brasil / Lukas Garrido Albertino. - Botucatu, 2021

Dissertação (mestrado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Faculdade de Medicina Veterinária e Zootecnia
Orientador: José Paes de Oliveira-Filho
Coorientador: Thais Helena Constantino Patelli
Capes: 50501062

1. Bos taurus - Doenças. 2. Desenvolvimento embrionário e fetal. 3. Genética animal. 4. Mutação.

Palavras-chave: Bos taurus; Doença genética; Perda embrionária e fetal.

Nome do Autor: Lukas Garrido Albertino

Título: FREQUÊNCIA ALÉLICA DA MUTAÇÃO *APAF*1 EM BOVINOS DA RAÇA HOLANDESA (*HOLSTEIN-FRIESIAN*) NO BRASIL

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Data da defesa: 21 de Janeiro de 2021

AGRADECIMENTOS

Aos meus Pais, Vagner e Rita, por todo o amor, carinho, paciência e confiança em mim depositados, e por todas as broncas, que não foram poucas, conselhos e ensinamentos que moldaram a pessoa quem sou. Obrigado por serem quem são e por se sacrificarem tanto para que seu filho pudesse realizar seu sonho.

Ao meu irmão, Matheus, por ter sido meu companheiro, ouvinte e ombro amigo ao longo de todos esses anos.

Aos meus avós paternos, Zélia e Nilton, e avó materna, Marcília, e a todos os familiares, sejam de sangue ou aqueles que considero como sendo, por me apoiarem e me incentivarem.

Ao meu orientador, Prof.º Dr. José Paes de Oliveira-Filho, pelo apoio, paciência, confiança e ensinamentos, que ocorrem desde o estágio, e pela oportunidade na realização deste projeto.

A Prof.ª Thais Helena C. Patelli, Co-orientadora deste projeto, pela ajuda na coleta das amostras e pelas considerações neste trabalho.

Aos docentes da Clínica de Grandes Animais, Prof.º Simone Biagio Chiacchio, Prof.º Rogério Martins Amorim e Prof.º Alexandre Secorun Borges, e também a Prof.ª Thais Gomes Rocha, pelo apoio, paciência, confiança e ensinamentos durante a Residência.

Aos colegas de Residência e amigos, Luiza S. Zakia, Raphael Teixeira, Gustavo G. Macedo, Beatriz C. Kamura, Lucas V. de Oliveira, Cristiana R. Bromberger, Karina Oliveira, Guilherme M. Fávaro, Neri Hellen V. da Cunha e João Pedro M. de Oliveira por toda a ajuda com os pacientes, por fazerem parte do meu crescimento profissional e pessoal, por me permitir fazer parte do crescimento de vocês e por todos os momentos vividos juntos.

Aos funcionários da Clínica de Grandes Animais e da FMVZ, Marco Antônio, César Leme e Lucas Rocha, pela ajuda com os pacientes e rotina da Clínica.

Aos colegas Pós Graduandos e amigos da salinha, Ana Luisa H. de Albuquerque, Pedro N. Bernardino, Raíssa O. Leite, Roberta M. Basso, Larissa Andrade, Anelize Trecenti e Danilo Andrade, por toda a ajuda neste e demais projetos, conversas e momentos compartilhados.

Às alunas de iniciação científica, companheiras de laboratório e amigas, Julia F. Ferreira e Amanda Manara, pela ajuda com este projeto e demais projetos e com a rotina do LBMCV.

Aos amigos, Rebeca Dourado, Vitor Castro, Renata Cordeiro, Albert Vinicius, Caio Fernando, Samantha Braune e Leticia Lemes, por todo o apoio, ajuda, conversas e conselhos.

A todos os docentes, funcionários, Médicos Veterinários, colegas de estágio, colegas que Residência, colegas de Pós Graduação, proprietários, as demais pessoas e aos animais que contribuiram de maneira direta ou indireta para este projeto e para que eu chegasse aonde estou.

Ao meu avô materno, Antônio (*in memoriam*), minha bisavó paterna, Maria (*in memoriam*), e tia-avô, Joana (*in memoriam*). Gostaria que estivessem aqui para ver seu neto, bisneto e “preferido” realizando este sonho.

Aos meus companheiros de quatro patas, Luke e Titã, por terem me ensinado tanto, por terem me mostrado que estava, e ainda estou, seguindo o caminho certo e por todos os momentos que vivemos juntos.

À CAPES, pela concessão da bolsa de estudo, sem a qual a realização deste projeto seria muito mais difícil.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento Pessoal de Nível Superior – Brasil (CAPES) – código de financiamento 001.

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ABCBRH – Associação Brasileira de Criadores de Bovinos da Raça Holandesa

ADP – Difosfato de adenosina

*APAF*1 – *Apoptotic protease activating factor*-1

APCBRH – Associação Paranaense de Criadores de Bovinos da Raça Holandesa

ARMS-PCR – *Amplification refractory mutation system* PCR

AS-PCR – *Allelic specific* PCR

ATP – Trifosfato de adenosina

*BTA*5 – Cromossomo 5

cm – Centímetros

CVM – Complexo de malformação vertebral

DUMPS – Deficiência da sintase de monofosfato de uridina

EUA – Estados Unidos da América

FANCI – Fanconi anemia complementation group I

HH1 – Haplótipo 1

HH3 – Haplótipo 3

HH4 – Haplótipo 4

HH5 – Haplótipo 5

HH6 – Haplótipo 6

HH7 – Haplótipo 7

HOLSTEIN USA – Holstein Association USA

kg – Quilos

PCR – Reação em cadeia da polimerase

*SLC*35*A*3 – *Solute carrier family* 35, *member A*3

*SNP*s – Polimorfismos de nucleotídeo único

SSCP-PCR – *Single stranded conformation* PCR

UMPS – *Uridine monophosphate synthetase*

SUMÁRIO

ALBERTINO, L. G. **FREQUÊNCIA ALÉLICA DA MUTAÇÃO *APAF*1 EM BOVINOS DA RAÇA HOLANDESA (*HOLSTEIN-FRIESIAN*) NO BRASIL**. Botucatu, 2020. X p. Dissertação (Mestrado) – Faculdade de Medicina Veterinária e Zootecnia, campus de Botucatu, Universidade Estadual Paulista.

RESUMO

A mutação *APAF*1 é uma enfermidade autossômica recessiva, associada ao haplótipo 1 (HH1) e caracterizada pela substituição de uma citosina (C) por uma timina (T) na posição *p.Q*579*X* (c.1741C>T) do cromossomo 5 (*BTA*5). A mutação está associada com a raça Holandesa e causa perda fetal e embrionária, durante o 60º e 200º dia de gestação, e redução nas taxas de concepção. Estudos demonstram uma prevalência de 1.48% a 65%, entretanto, não se há estudos e conhecimento sobre ocorrência da mutação no Brasil. O objetivo deste estudo é verificar a prevalência da mutação *APAF*1 em bovinos holandeses no Brasil. Um total de 248 amostras de DNA (210 vacas e 38 touros) foram utilizadas neste estudos, obtidas de quatro propriedades de leite e três Centrais de Reprodução dos estados do Paraná e São Paulo. As amostras foram submetidas às técnicas de PCR e *ARMS*-PCR, analisadas por eletroforese em gel de agarose, purificadas e submetidas ao sequenciamento direto de Sanger, para confirmação do genótipo e comparação e possível validação do método e primers desenvolvidos para a técnica de *ARMS*-PCR. Todos os animais analisados neste estudos foram classificados como não carreadores da mutação, ou seja, homozigotos para o alelo C (*wild-type*) tanto na técnica de *ARMS*-PCR quanto no sequenciamento Sanger. Concluímos que a mutação associada ao gene *APAF*1 não teve prevalência na população brasileira de Holandeses sob a qual este estudo foi realizado e que aparenta ser extremante rara.

Palavras chave: doença genética, perda embrionária e fetal, *Bos taurus*

ALBERTINO, L. G. **ALLELIC FREQUENCY OF *APAF*1 MUTATION IN HOLSTEIN CATTLE (HOLSTEIN-FRIESIAN) IN BRAZIL**. Botucatu, 2020. X p. Dissertação (Mestrado) – Faculdade de Medicina Veterinária e Zootecnia, campus de Botucatu, Universidade Estadual Paulista.

ABSTRACT

The *APAF*1 mutation is an autosomal recessive inherited disease, associated with the haplotype 1 (HH1) and characterized by a substitution of a cytosine (C) for a thymine (T) in *p.Q*579*X* (c.1741C>T) position of chromosome 5 (*BTA5*). The mutation is associated with the Holstein cattle breed and causes fetal and embryonic loss, during the 60th and 200th day of gestation, and reduced conception rate. Studies demonstrated prevalence from 1.48% to 65%, however, there are no studies and knowledge about the mutation in Brazil. This study aimed to verify the prevalence of the *APAF*1 mutation in Brazilian Holstein cattle. A total of 248 Holstein DNA samples (210 cows and 38 bulls) were used in this study, obtained from four dairy farms and three animal reproduction centers from Paraná and São Paulo states. DNA samples were submitted to PCR and *ARMS*-PCR techniques, analyzed via agarose gel electrophoresis, purified, and subjected to Sanger direct sequencing, for confirmation of the genotype and comparison and possible validation of the method and primers developed for *ARMS*-PCR. All animals assessed in this study were identified as non-carriers or, allelic C homozygous (wild-type) for *APAF*1 mutation, by *ARMS*-PCR technique and Sanger direct sequencing. We concluded that the mutation associated with the *APAF*1 gene was not prevalent in the Brazilian Holstein population under which this study was carried out and it appears to be extremely rare.

Keywords: genetic disease, embryonic and fetal loss, *Bos taurus*

CAPÍTULO I

1. Introdução

Dentre as metas nos programas de seleção genética, a fertilidade e a produção leiteira são consideradas como um dos principais objetivos. O endocruzamento, ou *inbreeding*, tem como principal objetivo a seleção de animais com características desejáveis, como fertilidade, longevidade produtiva e maior produção de leite, entretanto tem como consequências o aparecimento de mutações letais e subletais, e diminuição da efetividade reprodutiva em um animal. No gado holandês, a mutação associada ao gene *APAF*1 (*Apoptotic protease activating factor-1*) é responsável por causar morte embrionária e abortos espontâneos, entre o 60º e 200º dia de gestação, e baixa taxa de fertilidade. Considerada como uma mutação autossômica recessiva, onde há a substituição de uma citosina (C) por uma timina (T) na posição *p.Q*579*X* (c.1741C>T) do gene *APAF*1 do cromossomo 5 (*BTA*5). Estima-se que cerca de 500 mil abortos ocorreram mundialmente decorrentes desta mutações, o que corresponde a um prejuízo estimado de 420 milhões de dólares para a indústria leiteira. A mutação foi associada por pesquisadores ao touro Pawnee Farm Arlinda Chief, considerado como um dos mais importantes reprodutores da história do gado holandês, deixando mais de 2,5 milhões de descendentes, o que corresponde a 14% do genoma mundial dos Holandeses. De acordo com estudos realizados na França, Estados Unidos (EUA), Rússia, Japão e Polônia, a prevalência da mutação varia entre 1.48% a 65%. No Brasil, não se há estudos ou conhecimento sobre a mutação em nosso rebanho, sendo este o primeiro trabalho a avaliar a prevalência da mutação em nossos animais.

2. Revisão de literatura

2.1. História do gado holandês no mundo e no Brasil

A origem do gado holandês (*Holstein-friesian*) é pouco conhecida por pesquisadores (ABCBRH). De acordo com autores, existem relatos da origem desses animais datados de dois mil anos atrás, onde acredita-se que a raça teve origem nas mãos das tribos frísias e batavas (PRESCOTT et al., 1930; LUSH, HOBERT e WILLHAM, 1936) na região que hoje é conhecida como Holanda.

Nos Estados Unidos (EUA), há relatos da importação de animais originários da Holanda antes da Guerra da Independência Americana, entretanto, não ocorreu o registro desses animais e de seus descendentes. O primeiro relato com subsequente registro dos animais ocorreu em 1852 (LUSH, HOBERT e WILLHAM, 1936). Em 1872, publicou-se o primeiro *herd-book* (tradução livre para “livro da raça”) nos EUA, onde foi sugerido o nome de “Holstein-friesian” aos animais (ABCBRH; LUSH, HOBERT e WILLHAM, 1936). Atualmente, a raça é apenas conhecida por “Holstein” (ABCBRH).

De acordo com a Associação Brasileira de Criadores de Bovinos da Raça Holandesa (ABCBRH), que cita Paulino Cavalcanti (1935), acredita-se que o gado holandês foi trazido para o nosso país entre os anos de 1530 e 1535. O *herd-book* brasileiro foi fundado em 1935 com os reprodutores “Colombo St. Maria” e “Campineira”. Até 2018, o número total de animais registrados no *herd-book* era de 772.608 (APCBRH). No Brasil, a maioria dos produtores encontram-se nos estados de São Paulo, Paraná e Minas Gerais (ABCBRH).

2.2. Características da raça

A raça Holandesa é facilmente reconhecida por sua pelagem característica, com animais de coloração preta e branca ou vermelha e branca (HOLSTEIN USA) (Figura 1), por sua alta performance produtiva (HOLSTEIN USA; SIEKLICKI et al., 2020), decorrente de seleção genética dos animais

(WALSH, WILLIAMS e EVANS, 2011; RODRIGUEZ-RAMILLO et al., 2015) e por sua disponibilidade de material genético em todos os continentes (SIEKLICKI et al., 2020).

Figura 1. Representação do padrão moderno esperado para um animal fêmea (esquerda) e macho (direita) da raça Holandesa.

Fonte: HOLSTEIN USA.

Os holandeses são animais grandes, sendo considerados como a maior raça leiteira do mundo (McGUFFEY e SHIRLEY, 2011), com fêmeas adultas pesando em torno de 680 quilos (kg) e com 1,50 centímetros (cm) de cernelha (HOLSTEIN USA).

É esperado que as fêmeas tenham sua primeira cria entre o 24º e 26º meses de idade, com uma gestação de aproximadamente nove meses. Machos podem se reproduzir a partir do 13º mês de vida, quando devem estar pesando cerca de 363 kg (HOLSTEIN USA).

Nos EUA, a média de produção de leite anual é de 11.339 kg/vaca adulta em três ordenhas/dia com uma vida produtiva de quatro anos (HOLSTEIN USA). No Brasil, a média de produção, no ano de 2019, foi de 8.900 kg/vaca adulta em duas ordenhas/dia e 11.326 kg/vaca adulta em três ordenhas/dia em lactações de 305 dias (APCBRH).

2.3. Doenças genéticas nos holandeses

A produção de leite depende resumidamente de dois fatores, uma gestação bem sucedida e uma boa taxa de parição durante a vida útil de uma vaca leiteira (ADAMS et al., 2016). A relação entre fertilidade e produção leiteira fez da performance reprodutiva uma importante meta nos programas de seleção genética (WALSH et al., 2011; ADAMS et al., 2016).

O endocruzamento, ou *inbreeding*, é definido como o cruzamento entre indivíduos geneticamente relacionados (RODRIGUEZ-RAMILLO et al., 2015; ADAMS et al., 2016; SIEKLICKI et al., 2020) e irá ocorrer em qualquer rebanho ou população, ao menos que medidas específicas seja utilizadas para evitá-lo (THOMPSON, EVERETT e HAMMERSCHMIDT, 2000). O *inbreeding* tem como principal objetivo a seleção de animais com características desejáveis, como fertilidade, longevidade produtiva e maior produção de leite (WHITE, VINSON e PEARSON, 1981; PRYCE et al., 2014), entretanto tem como consequências o aparecimento de mutações letais e subletais e diminuição da efetividade reprodutiva de um animal (RODRIGUEZ-RAMILLO et al., 2015; ADAMS et al., 2016; SIEKLICKI et al., 2020). As mutações letais causam grandes perdas econômicas, pois nenhum produto careando essas mutações irá sobreviver para reprodução (HOENING e SIMIANER, 2006; ADAMS et al., 2016).

Dentre as principais mutações relacionadas ao gado holandês, podemos destacar a deficiência da sintase de monofosfato de uridina (*DUMPS*) (ROBINSON et al., 1983), complexo de malformação vertebral (*CVM*) (AGERHOLM et al., 2001), síndrome *brachyspina* bovina (AGERHOLM, McEVOY e ARNBJERG, 2006) e os haplótipos associados à raça, incluindo o HH1 associado ao gene *APAF*1, responsáveis por causar morte embrionária e baixa taxa de fertilidade.

2.3.1. Deficiência da sintase de monosfosfato de uridina

Enfermidade caracterizada pela não conversão do orotato, também conhecido como ácido orótico ou vitamina B13, em monofostato de uridina, um

importante componente dos ácidos nucleicos (ROBINSON et al., 1983; SHANKS e ROBINSON, 1989). Causada por uma mutação autossômica recessiva (ROBINSON et al., 1983), onde há a substituição de uma citosina (C) por uma timina (T) no gene *UMPS* (*Uridine monophosphate synthetase*) no cromossomo 1 (SCHWENGER, SCHÖBER e SIMON, 1993). De acordo com a literatura, a prevalência da *DUMPS* em estudos é de 0.01% a 1.4% (POLI et al., 1986; SHANKS, BRAGG e ROBINSON, 1987; SUN et al., 2011; VANRADEN et al., 2011).

De acordo com Shanks e Robinson (1989) e Shanks e colaboradores (1992), animais homozigotos para a mutação sofrem morte embrionária por volta do 35º ao 40º dia de gestação. Animais heterozigotos são clinicamente saudáveis e com desenvolvimento normal, apresentando somente altos teores de oratato no sangue, urina e leite durante o período de lactação (ROBINSON et al., 1983).

2.3.2. Complexo de malformação vertebral

O *CVM* está associado à substituição de uma guanina (G) por uma timina (T) no gene *SLC*35*A*3 (*Solute carrier family* 35, *member A*3) do cromosso 3. É considerada uma mutação autossômica recessiva (THOMSON et al., 2006), com prevalência em trabalhos de 1.37% a 16.62% (BERGLUND, PERSON e STÅLHAMMAR, 2004; MEYDAN, YILDIZ e AGERHOLM, 2010; SUN et al., 2011; WANG et al., 2012; RUŚĆ et al., 2013; COLE et al., 2016) e foi descrita nas raças Holandesa (AGERHOLM et al., 2001) e e Montbeliarde (BOURNEUF et al., 2017).

Os animais portadores da mutação podem ser abortados a qualquer momento da gestação, nascerem prematuros, natimortos ou, em raros casos, ainda vivos (JOHNSON et al., 2003), e apresentam alterações como défice de crescimento e ganho de peso, encurtamento das vértebas cervicais e torácicas, contração bilateral simétrica das articulações metacarpofalangeanas e metatarsofalageanas, artrogripose, hemivértebras, escoliose, sinostose, dismorfia craniofacial e má formação cardíaca (AGERHOLM et al., 2001;

DUNCAN et al., 2001; NAGAHATA et al., 2002; AGERHOLM et al., 2004; THOMSON et al., 2006)

2.3.3. Síndrome brachyspina bovina

A síndrome *brachyspina* bovina é uma mutação autossômica recessiva rara (TESTONI et al., 2008; AGERHOLM et al., 2010; CHARLIER et al., 2012) associada à deleção dos exons 25 a 27 do gene *FANCI* (*Fanconi anemia complementation group I*) no cromossomo 21 (CHARLIER et al., 2012). A prevalência da mutação em trabalhos varia entre 2.76% até 7.5% (CHARLIER et al., 2012; FANG et al., 2013; FRITZ et al., 2013; SAHANA et al., 2013; RUŚĆ e KAMIŃSKI, 2015; COLE, NULL e VANRADEN, 2016).

Os animais acometidos podem sofrer morte embrionária no início da gestação ou natimortos (AGERHOLM, McEVOY e ARNBJERG, 2006; CHARLIER et al., 2012) com alterações corporais como défice de crescimento e ganho de peso, encurtamento de todas as vértebras, alongamento dos membros, bragnatismo inferior, mal posicionamento das orelhas e má formação e posicionamento de órgãos, como fígado, rim, coração e gônodas (AGERHOLM, McEVOY e ARNBJERG, 2006; ALGERHOLM e PEPERKAMP, 2007; TESTONI et al., 2008; AGERHOLM et al., 2010; CHARLIER et al., 2012).

2.3.4. Haplótipos

Atualmente, na raça Holandesa, existem 17 haplótipos responsáveis por causar morte embrionária e baixa taxa de fertilidade (GHANEM e NISHIBORI, 2018). Destes, somente seis haplótipos, HH1 (ADAMS et al., 2016), HH3 (VANRADEN et al., 2011; McCLURE et al., 2014), HH4 (FRITZ et al., 2013), HH5 (VANRADEN et al., 2011; SCHŰTZ et al., 2016), HH6 (FRITZ et al., 2018) e HH7 (FRITZ et al., 2013; HOZÉ et al., 2020), descritos na tabela 1, foram elucidados a nível molecular.

Tabela 1. Haplótipos descritos em nível molecular na raça Holandesa.

Haplótipo	Gene	Cr.[1]	Variante	Prev.[2]	Referência
HH1	*APAF*1	5	c.1741C>T	2%	Adams et al., 2016
HH3	*SMC*2	8	c.3404T>C	2.95%	Cole et al., 2016
HH4	*GART*	1	c.869ª>C	0.37%	Cole et al., 2016
HH5	*TFB*1*M*	9	Deleção 138kpb	2.22%	Cole et al., 2016
HH6	*SDE*2	16	g.29773628ª>G	1.3%	Fritz et al., 2018
HH7	*CENPU*	27	Deleção 4pb	0.8%	Hozé et al., 2020

1. Cromossomo; 2. Prevalência da mutação.

Conforme citado por Hoening e Simianer (2006) e por Adams e colaboradores (2016), as mutações letais são responsáveis por causar grandes perdas econômicas. A mutação *APAF*1 é a única mutação associada aos holandeses na qual pesquisadores estimaram o impacto econômico gerado pela mesma, afetando a produção de leite e fertilidade dos animais, e sua importância na raça (Adams et al., 2016).

2.4. *APAF*1

A proteína *APAF*1 é uma importante molécula chave no processo intrínseco, ou mitocondrial, da apoptose celular (MÜLLER et al., 2005; REBOULD, WOHLGEMUTH e ESCHENBURG, 2011; SHAKERI, KHEIROLLAHI e DAVOODI, 2017) e no desenvolvimento do sistema nervoso central durante a embriogênese (YOSHIDA et al., 1998). Tal processo inicia-se com a ligação entre o citrocomo C e a proteína, havendo a troca de um ADP (difosfato de adenosina) por um ATP (trifosfato de adenosina), fazendo com que *APAF*1 assuma uma estrutura heptamétrica quartenária e recrute moléculas inativas da procaspase-9, tal complexo é conhecido como apoptossoma, uma plataforma de ativação da procaspase-9 (ZHOU et al., 2015; SHAKERI, KHEIROLLAHI e DAVOODI, 2017). A procaspase-9 inicia sua

função, seguida da ativação da procaspase-3 ou procaspase-7 (REBOULD, WOHLGEMUTH e ESCHENBURG, 2011), levando ao processo de apoptose celular.

Müller e colaboradores (2005), utilizando ratos como modelo experimental, descobriram que a expressão da proteína inicia-se no sétimo ao nono dias de gestação, com sua maior expressão por volta do 12º dia. A inibição, ou deficiência, da *APAF*1 tem como consequências letalidade embrionária tardia nesta espécie, com fetos apresentando má formações craniofaciais, como crescimento exacerbado do encéfalo e massas na região da cabeça, desenvolvimento inadequado do sistema auditivo interno e a presença de membranas interdigitais (YOSHIDA et al., 1998; MÜLLER et al., 2005).

Em bovinos da raça Holandesa, a mutação está associada a morte embrionária e abortos espontâneos, entre o 60º e 200º dia de gestação, e baixa taxa de fertilidade (ADAMS et al., 2016). O primeiro estudo realizado sobre a mutação foi feito por VanRaden e colaboradores (2011), entretanto a mesma só foi completamente descrita em nível molecular, e rastreada até um descendente em comum e possível responsável pela disseminação da mutação, por Adams e colaboradores no ano de 2016.

É considera como uma mutação autossômica recessiva, caracterizada pela substituição de uma citosina (C) por uma timina (T) na posição *p.Q*579*X* (c.1741C>T) no gene *APAF*1 do cromossomo 5 (*BTA*5). Essa substituição leva à formação de um *stop codon*, ou códon de terminação, truncando 670 aminoácidos (53.7%) de 1.248 aminoácidos que compõem a proteína (ADAMS et al., 2016).

Como citado anteriormente, a mutação foi rastreada por pesquisadores até um descente em comum, o touro Pawnee Farm Arlinda Chief, ou apenas “Chief”. Chief é considerado como um dos mais importantes reprodutores na história do gado holandês, deixando mais de 2,5 milhões de descendentes, o que corresponde a 14% do genoma mundial dos holandeses. De acordo com os pesquisadores, é se estimado que Chief trouxe um lucro de 300 bilhões de dólares para a indústria leiteira, entretanto, cerca de 500 mil abortos ocorreram mundialmente decorrentes da mutação, o que corresponde a um prejuízo de 420 milhões de dólares (ADAMS et al., 2016).

Além de Chief, o genoma de três de seus filhos (Walkway Chief Mark, Milu Betty Ivanhoe Chief e SWD Valiant), que também foram touros importantes na reprodução do gado holandês, foram utilizados para validar a mutação e o estudo realizado. Dois deles, “Mark” e “Ivanhoe Chief”, foram identificados como carreadores da mutação (heterozigotos), enquanto que “Valiant” foi classificado como não carreador, ou seja, homozigoto para o alelo C (*wild-type*) (ADAMS et al., 2016).

De acordo com estudos realizados na França (FRITZ et al., 2013), EUA (ADAMS et al., 2012; ADAMS et. al., 2016), Rússia (ROMANENKOVA et al., 2016; ROMANENKOVA et al., 2017; KHATIB, MAZURR e PROKHORTCHOUK, 2020), Japão (GHANEM et al., 2018) e Polônia (KAMIŃSKI, 2020), a prevalência da mutação varia de 1.48% a 65%.

No estudo realizado por Fritz e colaboradores (2013), foram analisados 47.878 animais, sendo 9.388 touros e 38.072 vacas, obtidos do banco de dados genômicos da França. Os autores encontraram a prevalência de 2.6% (1.244 animais) nos animais estudados.

Adams e colaboradores (2012), analisaram 758 animais e encontraram uma prevalência de 65% (497 animais) da mutação. Já no ano de 2016, utilizando o banco de dados genômicos de holandeses nos EUA, os mesmos autores analisaram 246.773 animais e encontraram uma prevalência de 2% (5.299 animais). Esta redução no número de carreadores e na frequência da mutação, ocorreu devido a implantação de métodos para reprodução destes animais, visando eliminar este haplótipo na população (Adams et al., 2016).

Na Rússia, Romanenkova e colaboradores (2016), analisaram 863 animais, sendo 593 touros e 270 vacas, e os autores encontraram uma prevalência de 6.5% (56 animais) na população estudada. Em 2017, Romanenkova e colaboradores, analisaram 1.268 animais, sendo 638 touros e 630 vacas, e a prevalência encontrada foi de 1.89% (24 animais). Khatib e colaboradores (2020), analisaram 1.521 animais, sendo 1.114 touros e 407 vacas, e os autores encontraram uma prevalência de 1.48% (23 animais).

Ghanem e colaboradores (2018), analisaram 240 vacas, originárias de 12 propriedades de leite em Hiroshima, e 15 fetos mumificados da Universidade Rakuno Gakuen (Hokkaido, Japão), e encontraram uma prevalência de 2.9% (7 animais) nas vacas e 33.3% (5 animais) nos fetos, o

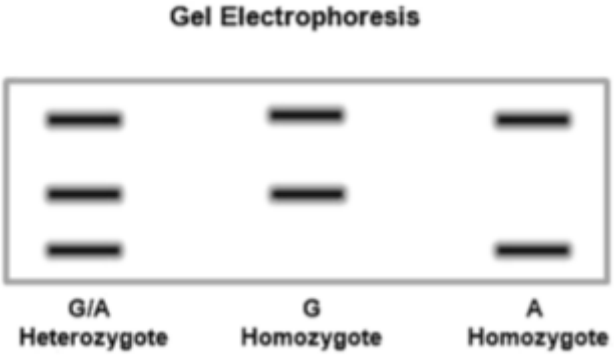
que gerou uma prevalência de 4.7% (12 animais) na população. Este estudo relata o primeiro e único caso de um feto mumificado, cruza entre uma vaca Holstein e um touro Wagyu, heterozigoto para a mutação, sugerindo que a mesma possa ocorrer em animais de geração F1.

O diagnóstico da mutação é feito através da reação em cadeia da polimerase (PCR) e análise genômica dos animais através do sequenciamento genético. Além da técnica comum de PCR, técnicas como *single stranded conformation* PCR (*SSCP*-PCR) (KAMIŃSKI, 2020), *allelic specific* PCR (*AS*-PCR) (GHANEM et al., 2018) e *amplification refractory mutation system* (*ARMS*-PCR) (KUMAR et al., 2020) foram utilizadas por autores como método de diagnóstico.

A técnica de *ARMS*-PCR é um método simples e de baixo custo para determinar *SNP*s (polimorfismos de nucleotídeo único) sem a necessidade de sequenciamento genético do animal (MEDRANO e OLIVEIRA, 2014). É considerada uma técnica melhor quando comparada a *AS*-PCR, em razão a sua alta especificidade na amplificação de produtos em uma única reação. Vem sendo utilizada em mapeamentos de diversas mutações genéticas e na detecção do vírus causador da hepatite B em seres humanos (KUMAR et al., 2020).

Essa técnica consiste na utilização de dois pares de *primers* em uma mesma reação de PCR, os *primers outer* são não alelo específicos e são responsáveis por amplificar a região onde se encontra o *SNP*, enquanto os *primers inner* são alelo específicos e irão produzir fragmentos relacionados a estes (Figura 2) (MEDRANO e OLIVEIRA, 2014). Kumar e colaboradores (2020), recentemente validaram a técnica e os *primers* desenvolvidos para a detecção do haplótipo HH1 em bovinos da raça Holandesa.

Figura 2. Representação esquemática de um gel de eletroforese com reação de *ARMS*-PCR.



Fonte: MEDRANO e OLIVEIRA, 2014

A genética da raça Holandesa no Brasil é constituida da importação de sêmen, embriões e animais provenientes dos EUA, Europa e Canadá (COSTA et al., 2000). Em 2017, de acordo com autores, o Brasil importou 2,4 milhões de doses de sêmen provenientes de diversos países (SIEKLICKI et al., 2020). No Brasil, não se há estudos ou conhecimento sobre a prevalência da mutação em nosso rebanho, sendo este considerado como o primeiro trabalho a avaliar a prevalência da mutação em nossos animais.

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CAPÍTULO II

Trabalho científico

O trabalho a seguir for redigido de acordo com as normas da revista *The Veterinary Journal* (<https://www.journals.elsevier.com/the-veterinary-journal>).

Short Communication

Allelic frequency of *APAF*1 mutation in Holstein cattle in Brazil

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Abstract

*APAF*1 is an autosomal recessive inherited mutation, associated with haplotype 1 (HH1) and characterized by a substitution of a cytosine for a thymine (c.1741C>T) in chromosome 5. The mutation is associated with the Holstein cattle breed and causes fetal and embryonic loss, during the 60th and 200th day of gestation, and reduced conception rate. Studies about the mutation were performed in the USA, France, Russia, Japan, and Poland, where the frequency of the mutation varies from 1.48% to 65%. However, there are no studies and knowledge about the mutation in Brazil. This study aimed to verify the prevalence of the *APAF*1 mutation in Brazilian Holstein cattle. A total of 248 DNA samples of clinically healthy animals (n=210 cows and n=38 bulls) 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 *APAF*1 gene found on GenBank. There were no carriers in the analyzed samples, that is, all animals tested were wild type. Therefore, under the conditions in which this study was carried out, it can be inferred that *APAF*1 seems to be extremely rare in the population of Holstein cattle in Brazil, although it is not possible to affirm that no animals are carrying mutated alleles in Brazil.

Keywords: *Bos taurus*; Embryonic and fetal loss; Genetic disease; Prevalence study

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Each Table should be typed on a separate page, numbered (1, 2 etc.) and a brief title given directly above each table. Footnotes to tables should be indicated by a, b etc. and typed at the bottom of the relevant table. Information in tables should not be duplicated in figures and vice versa.

References

Citation in text
Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Citation of a reference as 'in press' implies that the item has been accepted for publication. Review articles should be cited only to support generally acknowledged principles, and at the discretion of the Handling Editor. Conference proceedings and textbook references are only acceptable where other peer-reviewed sources do not exist, and only at the Handling Editor's discretion. Statements citing such sources should make it clear that the citation has not been peer-reviewed.

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If a website is used to reference any document in a manuscript, it should be set out as a footnote on the page where it first appears; it should not appear in the References at the end of the manuscript. If an individual web reference is cited more than once in the manuscript, the footnote number used is as for the first time the website was cited. The footnote number should be inserted manually in the text where the website is cited for the second or subsequent time. At the foot of the page, provide the link as follows: '[1]See: Basic Local Alignment Search Tool. http://www.ncbi.nlm.nih.gov/blast.(accessed 19 April 2018).' It is the Authors' responsibility to check that all URLs are active and live at proof stage and if not then the text must be amended accordingly.

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they should be placed first chronologically and then alphabetically, e.g. (Philbey et al., 2003; Cassidy and Mills, 2005; Litster, 2010). If two or more references by the same Author(s) published in the same year are cited, they should be distinguished from each other by placing a, b, etc. after the year, e.g., (Laven, 2011a, b; Laven and Smith, 2010a, b). Personal communications should be designated as '(E.A. Blomme, personal communication)'.

Papers that are in press may be cited using the year of acceptance where the digital object identifier (doi number) has been allocated. This can be updated to the year of print publication at the proof stage if the cited paper has been published. In the Reference list, quote the doi number where details of the journal volume and page numbers are yet not known.

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References should be set out as follows:

Journal reference - Yang, Y., Dahly-Vernon, A.J., Blomme, E.A.G., Lai-Zhang, J., Kempf, D.J., Marsh, K.C., Harrington, Y.A., Nye, S.H., Evans, D.L., Roman, R.J. et al., 2010. Liver transcriptomic changes associated with ritonavir-induced hyperlipidemia in sensitive and resistant strains of rats. The Veterinary Journal 185, 75-82.

Book reference - Cunningham, J.C., Klein, B.G., 2007. Endocrinology. In: Textbook of Veterinary Physiology, Fourth Edn. Saunders Elsevier, St. Louis, MO, USA, pp. 439-448.

Proceedings - Elbers, A.R., Mintiens, K., Staubach, C., Gerbier, G., Meiswinkel, R., Hendrinckx, G., Backx, A., Conraths, F.J., Meroc, E., Ducheyne, E., et al., 2007. Bluetongue virus serotype 8 epidemic in North-western Europe in 2006: Preliminary findings. Proceedings of the Annual Meeting of the Society for Veterinary Epidemiology and Preventive Medicine, Dipoli, Finland, 28th-30th March 2007 pp. 231-245.

Theses - Duz, M. 2009. Assessment of a methodology for determination of H^2O^2 concentration and pH in exhaled breath condensate in horses with and

without lower airway inflammation. Thesis, Master of Veterinary Medicine, University of Glasgow, United Kingdom.

Web addresses - FAOSTAT, 2008. Food and Agricultural Organization Statistical Database: Live Animals. http://faostat.fao.org (accessed 15 July 2010).

Supplementary material

Supplementary material such as applications, images and sound clips, can be published with your article to enhance it. Submitted supplementary items are published exactly as they are received (Excel or PowerPoint files will appear as such online). Please submit your material together with the article and supply a concise, descriptive caption for each supplementary file. If you wish to make changes to supplementary material during any stage of the process, please make sure to provide an updated file. Do not annotate any corrections on a previous version. Please switch off the 'Track Changes' option in Microsoft Office files as these will appear in the published version.

Where Supplementary data are provided (see further information below), use the following wording in the main text after the Acknowledgements:

Appendix: Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/...'

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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.stard-statement.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.reflect-statement.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).

ANEXOS

ANEXO I

Figura 1. Fotodocumentação de gel de agarose 1,5% com reação de PCR de 18 amostras de animais testados para o gene *APAF*1. Seta branca: ladder de 100pb; seta amarela: produto com 239pb.

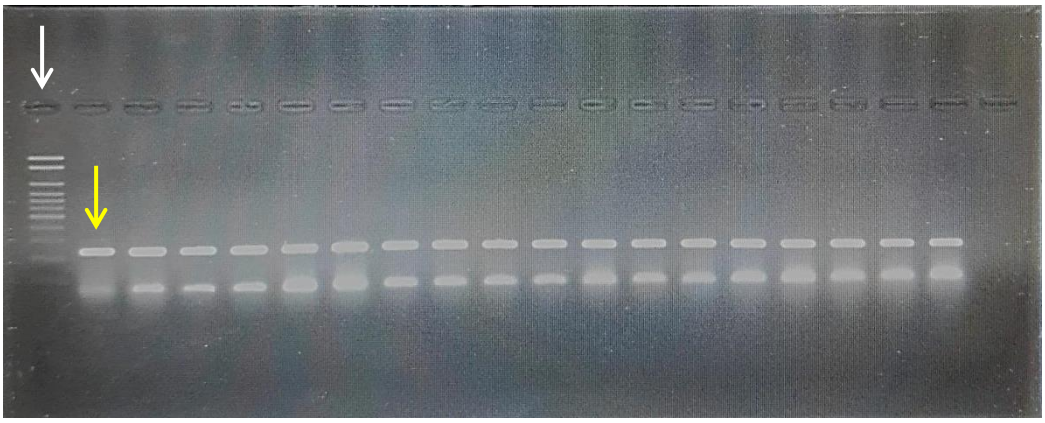


Figura 2. Fotodocumentação de gel de agarose 1,5% com reação de *ARMS*-PCR de 18 amostras de animais testados para o gene *APAF*1. Seta branca: ladder de 100pb; seta azul: produto dos *primers outer* (439pb); seta verde: produto indicando o alelo C (286pb); seta vermelha: animal em que não houve produto dos *primers outer*.

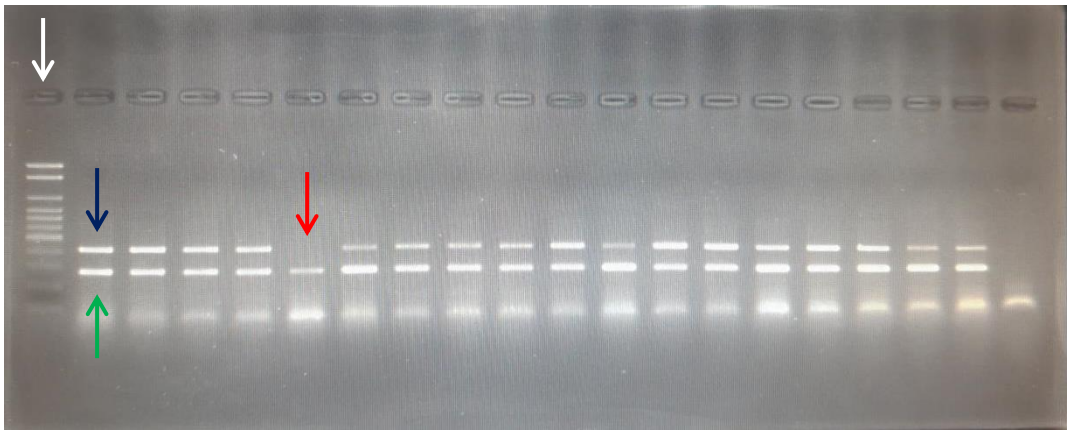


Figura 3. Eletroferograma parcial do gene *APAF*1 contendo o local da mutação (em preto). Nucleotídeo substituído na posição *p.Q*579*X* (c.1741C>T).

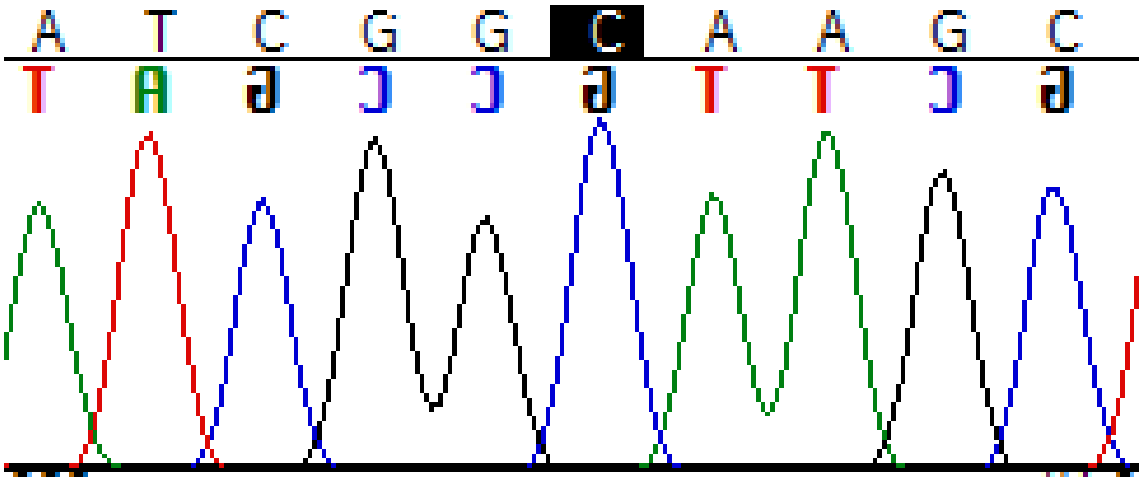
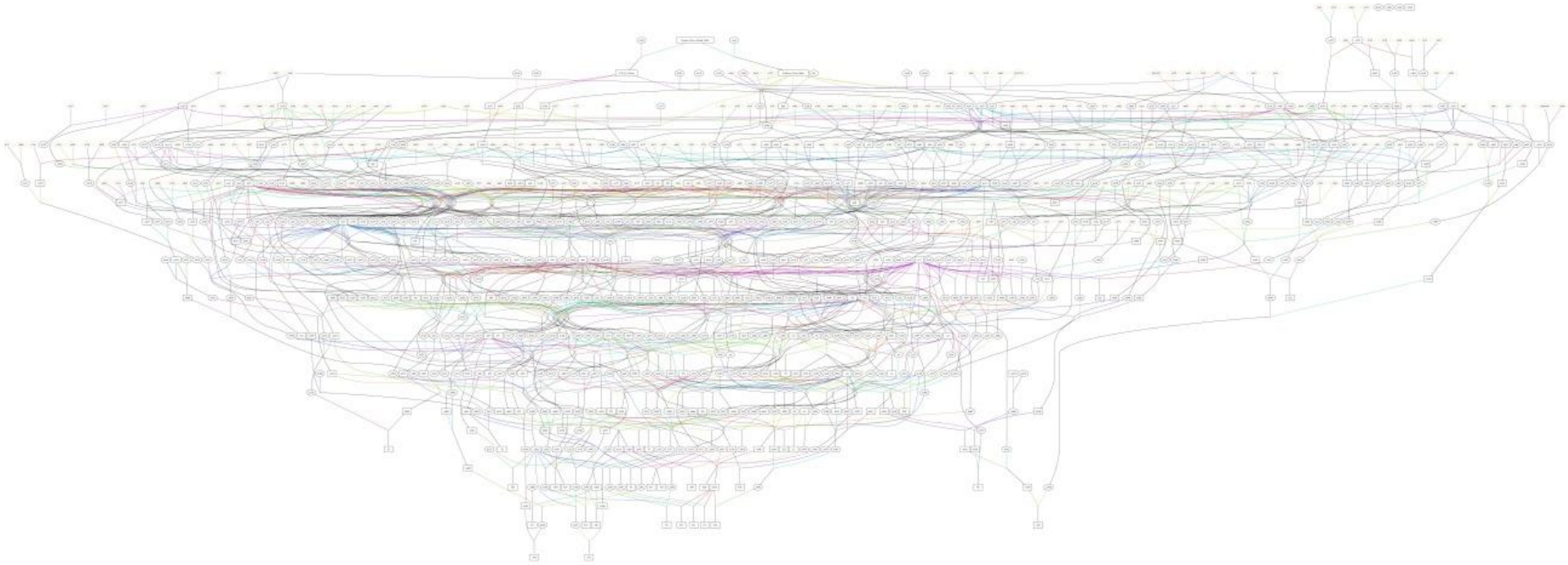


Figura 4. Heredograma de 33 touros coletados para avaliação da frequência alélica da mutação *APAF*1.



ANEXO II

unesp UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"

fmvz - unesp
Faculdade de Medicina Veterinária e Zootecnia
Campus de Botucatu

ATESTADO

Atesto que o Projeto "Frequência alélica da mutação APAF1 em em bovinos da raça Holandesa (Holstein-friesian) no Brasil" **Protocolo CEUA 0008/2019** , a ser conduzido por Lukas Garrido Albertino, responsável/orientador José Paes Oliveira Filho, para fins de pesquisa científica/ensino - encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal - CONCEA.

Finalidade	PESQUISA CIENTÍFICA
Vigência do projeto	06/03/2019 a 28/02/2021
Nome Comum / Espécie / Linhagem	BOVINA / BOS TAURUS / Leite
Raça	Holandês
Nº de animais machos	0
Nº de animais fêmeas	0
Nº de animais sexo indefinido	200
Peso médio de animais machos	1.200
Peso médio de animais fêmeas	600
Peso médio de animais sexo indefinido	0
Idade	03 ano(s) e 0 mes(es) e 0 dia(s).
Procedência	Bovinos Leiteiros de fazendas no Paraná

Projeto de Pesquisa aprovado em reunião da CEUA em 11/02/2019

JOSÉ NICOLAU PRÓSPERO PUOLI FILHO
Presidente da CEUA da FMVZ, UNESP - Campus de Botucatu

Faculdade de Medicina Veterinária e Zootecnia
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ANEXO III

UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"

LBMCV

Faculdade de Medicina Veterinária e Zootecnia Campus de Botucatu

FREQUÊNCIA ALÉLICA DA MUTAÇÃO APAF1 EM BOVINOS DA RAÇA HOLANDESA (HOLSTEIN-FRIESIAN) NO BRASIL

O touro Pawnee Farm Arlinda "Chief" foi um dos principais reprodutores na história do gado Holandês (Holstein-friesian) que gerou mais de 2 milhões de descendentes e um lucro de U$ 30 bilhões de dólares na indústria leiteira. Entretanto, foi-se descoberto nesse touro, e em seus descendentes, um haplótipo do cromossomo 5 (HH1) associada a uma mutação no gene *APAF1*, uma molécula importante na cascata apoptótica do citocromo C e diretamente relacionada ao desenvolvimento do sistema nervoso central (SNC) durante a embriogênese. Essa mutação causa abortos espontâneos entre o 60° e 200° dia de gestação e causou um prejuízo de mais de U$ 420 milhões de doláres ao mercado leiteiro.

O objetivo do projeto de Mestrado do seguinte aluno é verificar a frequência alélica da mutação no gene *APAF1*, responsável pela diminuição da eficiência reprodutiva em bovinos da raça holandesa, em uma população de bovinos holandeses brasileira. Baseando-se na população de animais dos estados de São Paulo e Paraná, serão selecionados 200 animais de origem pura (P.O.) para coleta de amostras de sangue, pelo ou sêmen. Para cada animal selecionado, será aplicado um questionário com perguntas que possam identificar e caracterizar a propriedade, bem como o rebanho, e perguntas em relação aos índices reprodutivos dos animais.

Será realizada a extração do DNA genômico através de kits comerciais específicos, seguindo as instruções dos fabricantes, e o DNA extraído será utilizado para realização da técnica de PCR, para a amplificação do segmento alvo contendo a mutação *APAF1*. As reações que gerarem produtos com banda única, e com o tamanho correto do fragmento amplificado, serão armazenadas em freezer -20°C para posterior purificação do produto de PCR e sequenciamento.

Tal enfermidade não foi relatada ou estudada no Brasil, por isso a importância deste trabalho, pois a mesma merece nossa atenção, como diagnóstico clínico e diferencial de outras enfermidades que causam baixa fertilidade, devido ao uso de sêmen e animais importados na reprodução de nosso rebanho, dessa maneira, favorecendo a ocorrência da enfermidade em nosso país.

Lukas Garrido Albertino
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lukas.garrido@unesp.br

ANEXO IV

UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"

LBMCV

fmvz - unesp
Faculdade de Medicina Veterinária e Zootecnia
Campus de Botucatu

TERMO DE CONSCIENTIZAÇÃO

Eu, _____,
CPF_____, autorizo **LUKAS GARRIDO ALBERTINO**, CPF **426.836.808-64**, Mestrando do Programa de Pós Graduação em Medicina Veterinária da Universidade Estadual Paulista "Julio de Mesquita Filho", campus Botucatu, sob orientação do **PROF. DR. ADJ. JOSÉ PAES DE OLIVEIRA FILHO**, a coletar amostras de animais de minha propriedade que encontram-se localizados na propriedade intitulada (nome da propriedade, cidade, estado)
_____
para o projeto intitulado "**FREQUÊNCIA ALÉLICA DA MUTAÇÃO APAF1 EM BOVINOS DA RAÇA HOLANDESA (HOLSTEIN-FRIESIAN) NO BRASIL**".

Estando ciente de as amostras coletas tem como fim de pesquisa e não reprodução ou comercialização e que informações, tais como nome do animal, número de registro, nome da propriedade de origem e nome do proprietário, não serão divulgados em meio científico.

De acordo,

_____

_____/_____/_____

Lukas Garrido Albertino
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lukas.garrido@unesp.br

ANEXO V

FREQUÊNCIA ALÉLICA DA MUTAÇÃO APAF1 EM BOVINOS DA RAÇA HOLANDESA (HOLSTEIN-FRIESIAN) NO BRASIL

Animal (nome/registro):				**Amostra:**	
Sexo: Fêmea	**Idade:**		**Raça:** Holandesa (Holstein-friesian)		
Propriedade atual (nome/cidade/UF):					
Nº de animais na propriedade atual:					
Propriedade de origem:					
Progenitores:	**Pai (nome/registro)**				
	Mãe (nome/registro)				
Pai:	( ) Nacional	( ) Importada	**País de origem:**		
Mãe:	( ) Nacional	( ) Importada	**País de origem:**		
Método de concepção:	( ) Inseminação artificial		( ) Monta natural		
	( ) Transferência embrião		( ) Fertilização in vitro		
Protocolo vacinal: MARQUE "X" NAS VACINAS REALIZADAS					
( ) Brucelose	( ) Febre aftosa		( ) Clostridioses e Botulismo		
( ) Raiva	( ) IBR/BVD		( ) Leptospirose		
( ) Outras:					
Produção diária (litros):		**Produção última lactação (litros):**			
Número de concepções:		**Número de partos:**			
Data do último parto:		**Intervalo entre partos:**			

FREQUÊNCIA ALÉLICA DA MUTAÇÃO APAF1 EM BOVINOS DA RAÇA HOLANDESA (HOLSTEIN-FRIESIAN) NO BRASIL

*RESPONDA AS QUESTÕES ABAIXO MARCANDO "X" OU POR ESCRITO

1 - Quais são os principais problemas reprodutivos da propriedade?		
( ) Baixa taxa de prenhez	( ) Morte embrionária	( ) Absorção fetal
( ) Baixa taxa de concepção	( ) Abortos espontâneos	( ) Mumificação fetal
( ) Outros:		
2 - Qual é a média de prejuízo anual da propriedade decorrente dos problemas reprodutivos listados acima?		
3 - Qual é a taxa de prenhez anual da propriedade?		
4 - Qual é a média de intervalos entre partos da propriedade?		
5 - Qual é a taxa de concepção anual da propriedade?		
6 - Qual é a produção de leite da propriedade?		
Diária:		/litros de leite
Mensal:		/litros de leite
Anual:		/litros de leite

FREQUÊNCIA ALÉLICA DA MUTAÇÃO APAF1 EM BOVINOS DA RAÇA HOLANDESA (HOLSTEIN-FRIESIAN) NO BRASIL

7 - Este animal tem maior intervalo entre partos do que os demais animais?			
( )	SIM	( )	NÃO
8 - Este animal tem dificuldade em emprenhar?			
( )	SIM	( )	NÃO
9 - Este animal tem dificuldade em emprenhar em um acasalamento específico*?			
( )	SIM	( )	NÃO
Qual cruzamento seria esse?			
*SE POSSÍVEL, ENVIAR AMOSTRAS DO ANIMAL UTILIZADO NO CRUZAMENTO			
10 - Este animal já teve quadros de morte e absorção embrionária?			
( )	SIM	( )	NÃO
11 - Este animal já teve quadros de morte fetal?			
( )	SIM	( )	NÃO
12 - Este animal já teve quadros de abortos espontâneos?			
( )	SIM	( )	NÃO
13 - Este animal já teve quadros de fetos mumificados?			
( )	SIM	( )	NÃO
14 - O que foi feito para diagnosticar a causa da perda fetal?			
( ) Teste para Brucelose	( ) Teste para Leptospirose	( ) Teste par IBR/BVD	
( ) Não foi investigada	( )Outros:		
15 - Os episódios de absorção embrionária, morte fetal, abortos espontâneos e fetos mumificados ocorreram entre o 60º e 200º dia de gestação?			
( )	SIM	( )	NÃO
16 - Os fetos abortados ou mumificados apresentavam má formação craniofacial e/ou crescimento exarcebado do cérebro?			
( )	SIM	( )	NÃO

ANEXO VI

FREQUÊNCIA ALÉLICA DA MUTAÇÃO APAF1 EM BOVINOS DA RAÇA HOLANDESA (HOLSTEIN-FRIESIAN) NO BRASIL

Animal (nome/registro):			**Amostra:**	
Sexo: Macho	**Idade:**		**Raça:** Holandesa (Holstein-friesian)	
Propriedade atual (nome/cidade/UF):				
Nº de animais na propriedade atual:				
Propriedade de origem:				
Progenitores:	**Pai (nome/registro)**			
	Mãe (nome/registro)			
Pai:	( ) Nacional	( ) Importada	**País de origem:**	
Mãe:	( ) Nacional	( ) Importada	**País de origem:**	
Método de concepção:	( ) Inseminação artificial		( ) Monta natural	
	( ) Transferência embrião		( ) Fertilização in vitro	
Protocolo vacinal: MARQUE "X" NAS VACINAS REALIZADAS				
( ) Brucelose	( ) Febre aftosa		( ) Clostridioses e Botulismo	
( ) Raiva	( ) IBR/BVD		( ) Leptospirose	
( ) Outras:				
Número de coletas (semanal):				
Número de paletas produzidas (anual):				
Número de paletas vendidas (total):				

FREQUÊNCIA ALÉLICA DA MUTAÇÃO APAF1 EM BOVINOS DA RAÇA HOLANDESA (HOLSTEIN-FRIESIAN) NO BRASIL

Número de descentes:	**Total:**	
	Fêmeas:	**Machos:**
Número de rebanhos atendidos (total):		