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Partial deletion of the *LAMA3* gene is responsible for hereditary junctional epidermolysis bullosa in the American Saddlebred Horse

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Summary

Laminin 5 is a heterotrimeric basement membrane protein integral to the structure and function of the dermal–epidermal junction. It consists of three glycoprotein subunits: the $\alpha 3$, $\beta 3$ and $\gamma 2$ chains, which are encoded by the *LAMA3*, *LAMB3* and *LAMC2* genes respectively. A mutation in any of these genes results in the condition known as hereditary junctional epidermolysis bullosa (JEB). A 6589-bp deletion spanning exons 24–27 was found in the *LAMA3* gene in American Saddlebred foals born with the skin-blistering condition epitheliogenesis imperfecta. The deletion confirms that this autosomal recessive condition in the American Saddlebred Horse can indeed be classified as JEB and corresponds to Herlitz JEB in humans. A diagnostic test was developed and nine of 175 randomly selected American Saddlebred foals from the 2007 foal crop were found to be carriers of the mutation (frequency of 0.026).

Keywords epidermolysis bullosa, Herlitz JEB, horse, *LAMA3*, laminin.

Introduction

Epidermolysis bullosa (EB) includes a number of heritable disorders affecting the integrity of the skin and mucosa that are characterized by blistering and fragility, especially in areas subject to frictional stress such as the oral cavity and limbs. This disease has been identified in several species, including cattle (Leipold *et al.* 1973), dogs (Nagata *et al.* 1997, Capt *et al.* 2005), cats (Baker & Lyon 1983) and horses (Shapiro & McEwen 1995). In humans, EB is classified as intradermal, junctional or intraepidermal based on immunological or electron microscopic examination (Fine *et al.* 2000). The junctional form involves blistering within the lamina lucida of the basement membrane zone. Junctional epidermolysis bullosa (JEB) has been associated with mutations in any one of the three genes (*LAMA3*, *LAMB3*, *LAMC2*) that encode the polypeptide subunits that comprise the laminin 5 molecule. Laminin 5 is secreted by keratinocytes and is involved in cell adhesion, motility and proliferation. In particular, it is associated with the anchoring filaments connecting the hemidesmosomes to the lamina densa of the dermal–epidermal junction (Rouselle *et al.* 1997).

Epitheliogenesis imperfecta (EI) is a term that has been used to describe congenital epithelial defects in animals including the horse. In horses, although this recessive condition has been described primarily in draft breeds (Kohn *et al.* 1998), it has also been reported in light horse breeds such as the American Saddlebred Horse (Fig. 1) (Lieto *et al.* 2002). Spirito *et al.* (2002) reported that the disease in American Belgian horses is caused by a mutation in the *LAMC2* gene encoding the $\gamma 2$ subunit of the laminin 5 molecule. A cytosine insertion in exon 10 (AY082802.1:c.1571_1572insC) results in a premature termination codon (PTC) producing an ineffectual $\gamma 2$ molecule. This confirms that EI in the Belgian horse is more appropriately characterized as hereditary JEB. In the American Saddlebred Horse, evidence was found for the EI locus residing on horse chromosome 8 in a linkage disequilibrium study (Lieto & Cothran 2003). It is to this chromosome that the *LAMA3* gene has been mapped (Milenkovic *et al.* 2002). Using the reported cDNA sequence of the horse *LAMA3* gene (Milenkovic *et al.* 2005), we designed exon-specific PCR primers for the 38 *LAMA3* exons and performed an exon scan in an affected foal, an obligate carrier and a mixed-breed unaffected horse. This study reports the causative mutation for EI in the American Saddlebred Horse as well as the current gene frequency based on random samples from the 2007

background history on EB

background into on EI

Cytosine insertion produces stop codon in Amer. Belgains

linkage disequilibrium found LAMA3 on Ch. 8

exon scan used to find cause on ASB affected foal, carrier, and unaffected crossbreed

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Figure 1 Saddlebred foal with epitheliogenesis imperfecta now defined as a form of JEB. Photo courtesy of the American Saddlebred Horse Association.

foal crop. The mutation occurs in the *LAMA3* gene, thus genetically redefining EI in this breed as JEB.

Materials and methods

Sample preparation

10 EI affected ASB

2 JEB affected Belgains

10 obligate carriers ↓ DNA

other samples from hair

Archived DNA samples stored at the University of Kentucky Equine Parentage Lab from 10 EI-affected American Saddlebred foals and two JEB-affected Belgian foals were used in this study. DNA was isolated from archived serum samples from 10 obligate carriers using the PureGene DNA Isolation Kit (Gentra Systems). All other samples used in this study from American Saddlebreds or other breeds were hair sample submissions to the University of Kentucky Equine Parentage Lab for routine genotyping. DNA was extracted from three to five hair bulbs by placing the bulbs in 50 μl of a solution containing 850 μl/ml deionized water, 100 μl/ml GeneAmp 10× PCR buffer (500 mM KCl, 100 mM Tris–HCl, pH 8.3; Applied Biosystems), 50 μl/ml 25 mM $MgCl_2$, 50 μl/ml 1% SDS and 5 μl/ml 20 mg/ml proteinase K. The hair bulbs and solution were incubated at 60 °C for 1 h, 97 °C for 45 min and then stored at 4 °C until use.

Exon scan

Genomic DNA from an affected foal, an obligate carrier mare and a mixed-breed unaffected horse were used for the exon scan. DNA was diluted in Tris–EDTA buffer to 25 ng/μl. The horse *LAMA3* sequence (AF533668) was aligned to the human sequence for chromosome 18 (AC010754) using BLAST, and the intron/exon structure of horse *LAMA3* was determined to develop exon-specific primers for exons 1–18. The subsequent availability of the horse genome map (http://www.genome.ucsc.edu/cgi-bin/hgGateway) allowed immediate exon-specific primer design for the remaining exons (19–38). To create exon-specific primers, a BLAT search (http://www.genome.ucsc.edu/cgi-bin/hgBlat?command=start) was performed using the horse *LAMA3* cDNA sequence containing exons 19–38 to obtain surrounding intron sequence. Primers were designed using PRIMER DESIGNER 4.0 (Scientific & Educational Software) and included, when possible, at least 100 bp of flanking intronic sequence.

Each 25-μl PCR reaction contained 25 ng genomic DNA, 0.25 mM each dNTP, 2.5 μl GeneAmp 10× PCR buffer, 2.5μl of 25mM MgCl2 solution, 0.2 U AmpliTaq polymerase (Applied Biosystems) and 1.0 pmol each primer, and was brought to volume with deionized water. The standard PCR conditions for exon amplification were 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s, with a 10-min extension at 72 °C and a final holding temperature of 4 °C on a PTC 200 DNA Engine (MJ Research). Products were visualized on 1% agarose gels and cleanup was performed using the Exo-SAP-It kit (USB). All products were sequenced for mutation analysis using an ABI 377 DNA Sequencer and the Big Dye Terminator Cycle Sequencing kit V1.1 (Applied Biosystems) per manufacturer's directions. Electropherograms were analysed for mutations visually and using the Vector NTI ALIGN X software.

Exon 10 of the *LAMC2* gene was amplified using genomic DNA from the 10 EI-affected Saddlebred foals, one Saddlebred obligate carrier, two JEB-affected Belgian foals and a normal non-saddlebred horse as described by Spirito *et al.* (2002). The exon was amplified and sequenced using the primers 5′-TGTTACTCAGGGGATGAGAA-3′ (forward) and 5′-CTGGGGGCAGTTATTGCAC-3′ (reverse) to detect the presence of the c.1571_1572insC mutation.

↑ cDNA labeled C insertion

Identification of the deletion

forward exon 23 reverse exon 28

The DNA fragment containing the deletion site was amplified using the forward primer for exon 23, 5′-TCAGATAGCAGCGTCTCATA-3′, and the reverse primer for exon 28 5′-GGAAGAAGGCCAAGTCTTAC-3′ (Table 1). Eleven primer pairs were designed to walk down this fragment through the putative deletion start point. Each pair was designed to amplify a product of approximately 500 bp to facilitate sequencing. Primer pairs were designated as walking primer pairs (WPP) 1 to 11 (Table 2). The last forward primer to successfully amplify a product in the affected foal (WPP9, 5′-GAGGCATGTCGATCCTAGGT-3′) was run with the reverse primer for exon 27 (5′-AGCTCCATAGCGTTACAGTG-3′). This product was sequenced in the affected foal and carrier to pinpoint the deletion breakpoint (EU812567).

To verify the mutation in additional samples, the PCR was adjusted to use the forward primer from WPP10 (5′-GTGCCAACCACTGTGCATTA-3′), which is in intron 23, with both the reverse primer from WPP10 (5′-AGGAAGACTCTGAGCAGTAG-3′), to detect the normal form of

Table 1 Exon-specific primers for the region of *LAMA3* containing the deletion.

Exon	Sequence (5′–3′)	Amplification in affected foals?
Exon 23		Yes
For	TCAGATAGCAGCGTCTCATA	
Rev	GTGCATGTTCTCAAAGTCC	
Exon 24		No
For	AGGAGGCAGCTGAAGCTGAG	
Rev	AAAGTGTTTTGGGCGCTGAC	
Exon 25		No
For	ATAGCTCTGAACCGCTGTGA	
Rev	TACCCTGTTGGCTAACACTG	
Exon 26		No
For	AGTAGAGAGTGCAGTGTTTC	
Rev	GAGCCAGTTCCCCATAACAC	
Exon 27		No
For	CTGTTTCTTCCCGAGGCTAC	
Rev	AGCTCCATAGCGTTACAGTG	
Exon 28		Yes
For	GGCTGAAGAGAAGATGTCAC	
Rev	GGAAGAAGGCCAAGTCTTAC	

the gene, and the reverse primer from exon 27, to detect the deletion (Fig. 2). The 25-μl reaction contained 25 ng genomic DNA, 0.25 mM each dNTP, 2.5 μl GeneAmp 10× PCR buffer, 2.5μl of 25mM MgCl2 solution, 0.2 U AmpliTaq polymerase, 1.0 pmol WPP10 forward primer and 0.5 pmol each reverse primer (WPP10 and exon 27), and was brought to volume with deionized water. PCR conditions were the same as those used for the exon scan.

Determination of frequency

Random hair samples from 175 American Saddlebreds were obtained by selecting every fifth sample of the 2007 American Saddlebred foal crop coming into the laboratory for routine genotyping. In addition, 31 Tennessee Walking Horse samples were similarly selected for testing. Seventy other horses from 13 different breeds were also screened for the deletion. Frequency was determined for the deletion allele by direct counting within each breed sample set. Frequency of expected occurrence of homozygous-affected animals was calculated using the equation for Hardy–Weinberg equilibrium.

Results

Exon scan

unaffected expressed all exons

All 38 exons were successfully amplified in the obligate carrier and normal horse control. However, in the affected foal, all exons amplified with the exception of exons 24, 25, 26 and 27 (EU704008). Using the exon 23 forward primer, which was the last exon to successfully amplify in the foal, and the exon 28 reverse primer, a product 5027 bp long was produced with DNA from the obligate carrier and affected foal (EU812567). This is in comparison to the expected product size of 11 616 bp in a normal horse according to the horse genome sequence (BK006617). We confirmed that this 5027-bp product corresponded to *LAMA3* sequence by successfully amplifying and sequencing exon 23 using this product as template. The larger expected product (11 616 bp) containing the normal sequence from exon 23 through exon 28 in the carrier and normal control horse was not visible on the gel or did not amplify under the PCR conditions used.

5027 bp long in carrier and affected

11,616 bp in normal, unaffected

Eleven primers pairs, designated WPP1–11, were designed beginning at the 5′ end of exon 23 to walk along the gene towards the putative deletion breakpoint between intron 23 and exon 28 (Table 2). Each primer pair covered approximately 0.5 kb and the predicted products overlapped by approximately 250 bp. Products from WPP1 to WPP9 amplified in the affected foal. WPP10 and WPP11 did not amplify a product in the foal. WPP9 for was run with exon 27 rev and exon 28 rev. A product of 768 bp was successfully obtained using the exon 27 rev primer, and the sequence of this product was used in a two-way BLAST against the normal horse genomic sequence to identify the deletion breakpoint. A deletion was confirmed, beginning with sequence 4 bp into exon 24 and extending 41 bp beyond exon 27 (Fig. 2a). There is an ACT overlap between the two breakpoints, where the last three bases prior to the deletion are ACT, and the last three bases of the deleted segment are ACT (Fig. 2a). The deletion comprises 6589 bases according to the horse genome map and supplemental sequencing by the authors of a gap in intron 25 (EU780072). The deletion effectively eliminates four exons (24, 25, 26 and 27) from the *LAMA3* chain. Thus a predicted 169 amino acid residues would be missing from the translated protein (residues 1032–1201).

deletion of 6589 bases ↓ exons 24–27 from LAMA3

Sequencing of the remaining *LAMA3* exons revealed at least five other SNPs in *LAMA3*, only one of which was homozygous in all affected foals and heterozygous in all obligate carriers (data not shown). This base substitution occurred in exon 10 but did not cause a change in the amino acid. It was also found in numerous other breeds of horses in the heterozygous state. It is interesting that no horse, including the two JEB-affected Belgian foals, was found to be homozygous for this exon 10 c.1178A>G SNP (AF533668.1) other than the 10 EI-affected Saddlebred foals.

Verification of the mutation

Primer WPP10 for, which is in intron 23, was used in a multiplex PCR with two reverse primers: WPP10 rev and exon 27 rev. WPP10 rev is 3′ to the exon 24 breakpoint (Fig. 2b). This amplification results in a 284-bp product in those horses possessing the deletion and a 599-bp product

affected did not express exons 24, 25, 26, 27

Table 2 Primers used to walk the *LAMA3* sequence from intron 22 through exon 25.

Name	Sequence (5′–3′)	Gene location	5′ nt position[1]	Amplification in affected foal?
WPP 1				Yes
For	ACACTCGCGGTGATGGTCCTT	I22	771	
Rev	GGTGGCATGTTCTCAAAGTC	I23	1161	
WPP2				Yes
For	TCAGACCACTGTGGATAGAG	E23	841	
Rev	AAGAATGGCCTCTAGGAACC	I23	1434	
WPP3				Yes
For	TTGCCAGGCTGGGCCAGATT	I23	1352	
Rev	CTGAAGATGCAGTGTGACTC	I23	1792	
WPP4				Yes
For	ACAGACCTGGTCCAGCTCAC	I23	1673	
Rev	GCAGGAGCCTTGTCTATCTT	I23	2243	
WPP5				Yes
For	GCTCCCAAGGGCAAGGTTTA	I23	2035	
Rev	CCTTGCCATGGATCATCCTC	I23	2476	
WPP5				Yes
For	GACAAGGCTCCTGCGCTCAA	I23	2249	
Rev	GGCAATAGTCGCTGTCTTCC	I23	2783	
WPP7				Yes
For	GAGACCTTGAGGACAAGTAG	I23	2430	
Rev	CCACTAATCACCGTGCTGAG	I23	2948	
WPP8				Yes
For	GACTGCTCAGGCTGCAGGAT	I23	2738	
Rev	CTCGCAATCACAGTCACTCT	I23	3183	
WPP9				Yes
For	GAGGCATGTCGATCCTAGGT	I23	3023	
Rev	CCGACTGAAGAAGAGCTAGA	I23	3662	
WPP10				No
For	GTGCCAACCACTGTGCATTA	I23	3505	
Rev	AGGAAGACTCTGAGCAGTAG	I24	4085	
WPP11				No
For	TCCTAGTGCGATACAAGCTG	E24	3754	
Rev	GTCTTCACGAGCCACGCTTA	E25	4257	

[1]Based on GenBank accession BK006617.

in those horses without the deletion (data not shown). Affected foals have only the 284-bp product due to the absence of the WPP10 rev primer binding site, whereas carriers have both the 284- and 599-bp bands (Fig. 2b,c). Homozygous normal animals have only the 599-bp band because the product of the WPP10 for/exon 27 rev primer pair is too large to migrate with the smaller fragments.

Samples from the nine remaining Saddlebred EI foals, 10 obligate carriers, two JEB-affected Belgian foals, 175 American Saddlebreds, 31 Tennessee Walking Horses and 70 horses of 13 other breeds were screened using this test. All 10 affected Saddlebred foals were homozygous for the deletion and all 10 obligate carrier samples were heterozygous. No individuals possessing only the 284-bp fragment were found other than the affected foals (data not shown). Heterozygous individuals were not found in any of the miscellaneous breeds with the exception of one Racking Horse. The deletion has been designated as BK006617:g.3724_10312del6589.

To verify that causative mutations for JEB are distinct in the Belgian horse and the American Saddlebred, exon 10 of the *LAMC2* gene was also amplified using genomic DNA from the 10 EI-affected Saddlebred foals, one Saddlebred obligate carrier, two JEB-affected Belgian foals and a mixed-breed unaffected horse. Both Belgian foals were homozygous for the mutation c.1571_1572insC but all of the affected Saddlebred foals as well as the carrier and normal horse had the normal sequence of five cytosine residues. Conversely, neither of the affected Belgian foals had the 284-bp fragment found in the DNA samples from affected and carrier Saddlebreds.

Estimation of allele frequency in the American Saddlebred breed

Random samples from 175 American Saddlebreds were tested for the mutation. Of these, nine tested heterozygous for the mutation. Based on this sample set, the frequency

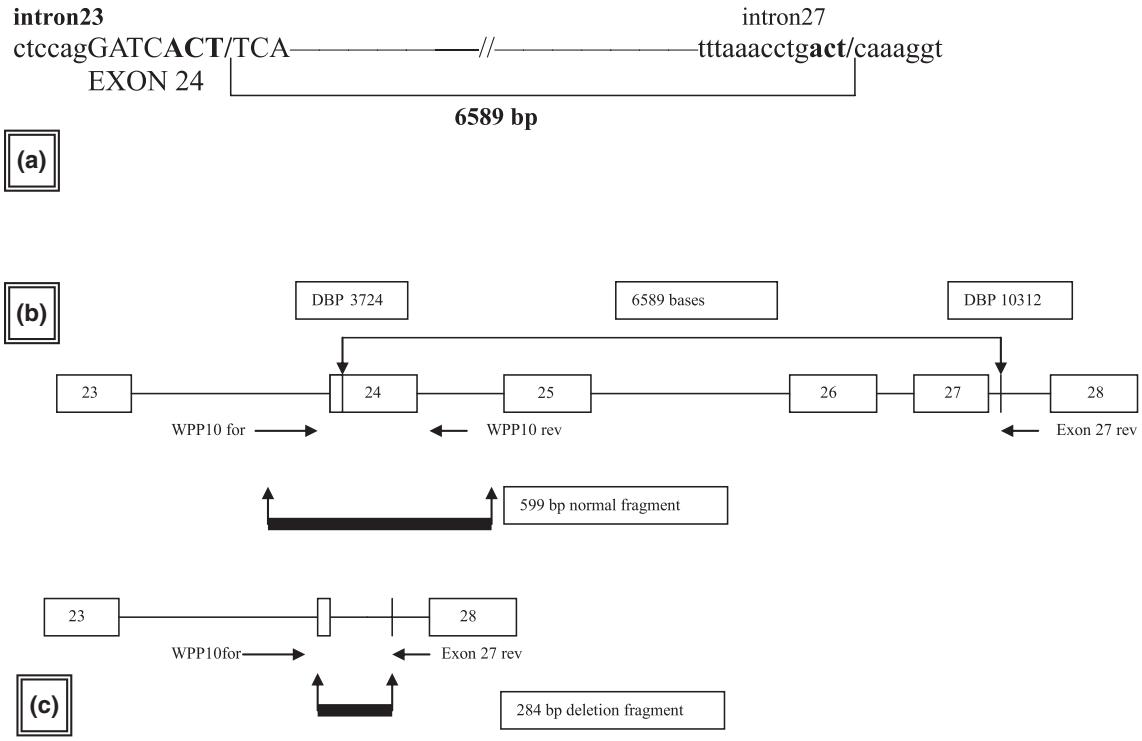

Figure 2 Diagram showing the *LAMA3* deletion. (a) Sequence of the breakpoint (the breakpoint is demarcated by /). Exon sequence is in upper case letters and intron sequence is in lower case. (b, c) Exons and deletion breakpoints (DBP) with the relative positions of the primers used to detect presence of the deletion. In the normal horse (b), the primers amplify exon 24, resulting in a 599-bp fragment. In horses with the g.3724_10312del6589 deletion in *LAMA3* (c), a 284-bp fragment is amplified.

of the *LAMA3* deletion allele is 0.026, with the carrier frequency being 0.051. Assuming Hardy–Weinberg equilibrium, the expected frequency of homozygotes or affected foals was 0.0007.

Discussion

This work demonstrates that the skin disease previously identified as EI in American Saddlebred horses is likely due to a deletion of 6589 bp in the *LAMA3* gene. The deletion spans a region including exons 24–27 and we predict this results in the absence of a functional laminin 5 molecule, causing cleavage within the dermal–epidermal basement membrane at the lamina lucida level. As such, the condition is more appropriately called JEB. A similar condition was described in the Belgian horse based on a different mutation occurring in the *LAMC2* gene (Spirito *et al.* 2002). In this study, two affected Belgian foals were tested and not found to have this *LAMA3* mutation. Conversely, the affected Saddlebred foals with this skin defect did not have the *LAMC2* mutation. In both the Belgian draft horse and the American Saddlebred, the condition occurs as an autosomal recessive trait, which is consistent with the inheritance of JEB observed in humans and other species.

The *LAMA3* gene encodes the α3 polypeptide subunit of laminin 5 and can be alternatively transcribed, resulting in the α3A or α3B subunits. The α3A subunit is the one found primarily in the epithelial tissue whereas α3B occurs primarily in lung and neuroepithelia (Miner *et al.* 1997). Together with the β3 and γ2 chains, the α3A subunit forms the trimeric laminin 5A molecule (Schneider *et al.* 2007). The α3 polypeptide is also found in the laminin 6 and laminin 7 molecules, which are involved in stabilizing the association of laminin 5 with the basement membrane (Champliad *et al.* 1996).

Laminin G domain-like (LG) modules consisting of 180–200 amino acid residues occur at the C-terminus of all five laminin alpha chains (α1–α5) (Timpl *et al.* 2000). It is the five globular LG domains of the α3A chain that are involved in cell adhesion and span the lamina lucida in the basement membrane (McMillan *et al.* 2003). The LG2 and LG3 domains contain binding sites for integrins, specifically α3β1, α6β4 and α6β1 (Aumailley *et al.* 2003). The deletion of exons 24, 25, 26 and 27 reported in this study results in 169 missing amino acid residues (1032–1201) in the α3 molecule. The missing residues comprise 111 amino acids in the LG2 domain (residues 1012–1143) and the first 15 amino acids of the LG3 domain (residues 1186–1301) (Milenkovic *et al.* 2005).

Hirosaki *et al.* (2000) showed that the LG4 and LG5 domains are not critical for cell adhesion but deletion of the LG2 and LG3 domains severely compromised or prevented the adhesion activity of laminin 5. The LG2 domain contains a unique integrin α3β1-binding site and the

conformation of the LG2 domain in conjunction with LG1 and LG3 is important for integrin/laminin interactions. Therefore, we propose that the lack of all but 20 amino acid residues of the *LAMA3* LG2 domain and partial deletion of the LG3 domain in Saddlebred foals homozygous for the *LAMA3* deletion is sufficient to cause clinical JEB. Because altered expression of the laminin 5 molecule is associated with the autosomal recessive disorder known as severe Herlitz JEB in humans (Fine *et al.* 2000) and the nature and extent of the lesions found in affected Saddlebred foals lead to neonatal death (Lieto *et al.* 2002), it is appropriate to conclude that JEB in the American Saddlebred also corresponds to the severe Herlitz form of JEB in humans.

Numerous mutations have been associated with the various forms of EB found in animals and humans. Varki *et al.* (2006) surveyed mutations in the *LAMA3*, *LAMB3* and *LAMC2* genes among 209 human patients with JEB and found that the majority of these mutations (75%) resulted in a PTC. These insertions and deletions involve only one or a few base pairs. Other identified mutations involved splice site or missense mutations. A large insertion was described by Capt *et al.* (2005) in the German Pointer dog, which is a 6.5-kb insertion of repetitive satellite DNA within intron 35 of *LAMA3*, resulting in a mutated mRNA species containing a PTC. This mutation reduced, but did not eliminate, expression of the wild-type α3 chain. The 6589-bp deletion in the *LAMA3* gene described in this study represents the largest deletion yet found to be associated with JEB in humans or animals. The deletion effectively removes four exons critical to the functionality of the α3 polypeptide, rendering horses homozygous for the deletion unable to produce a functional laminin 5 molecule.

According to records at the American Saddlebred Horse Association, this condition was first reported in the American Saddlebred Horse in 1975 (Dede Gatlin, ASHA, personal communication). The condition is recessive and carriers are not affected; therefore, there was an unknown risk of producing an affected foal. Lieto & Cothran (2003) estimated the genotype frequency of EI to be 0.0016 based on five reported affected foals out of approximately 3000 born that year, which is a frequency of 0.04. Discovery of the deletion allowed us to test directly a random sample of the 2007 Saddlebred foal crop for the mutation. In this study, 5.1% of 175 Saddlebred foals were found to be carriers. Consequently, the estimated frequency is 0.026 (2.6%). Preliminary examination of pedigrees of affected foals indicates a possible common ancestor. More extensive pedigree analysis is needed before the mutation event can be pinpointed. Archived Saddlebred samples at the University of Kentucky are only available from 1992 onwards so, if the mutation occurred prior to this, the exact origin may never be known. Because carriers can be valuable performance horses and breeding stock, this test allows them to remain in the breeding population. To prevent the disease, breeders simply need to be certain that carrier mares are not mated to carrier stallions. Of the other breeds surveyed, only one Racking horse was found to be a carrier. Because by definition Racking horses can be part Saddlebred, it is likely the mutation was inherited from a Saddlebred ancestor. Based on the current data, there is no indication that the mutation event occurred prior to establishment of the Saddlebred breed. However, a more extensive sampling of breeds is needed to determine if this mutation exists in other breeds of light horses.

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