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A METHOD FOR JUNCTIONAL EPIDERMOLYSIS BULLOSA DIAGNOSTICATION IN DRAFT HORSES**

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Abstract: Junctional Epidermolysis Bullosa (JEB) is an inherited disease which causes skin lesions in newborn foals and results in large areas of skin loss. The mutation responsible for the disease is a cytosine insertion in the LAMC2 gene, which results in absent expression of the laminin γ2 polypeptide chain of laminin 5. JEB is inherited as an autosomal recessive trait (*Spirito et al.* 2002, *Milenkovic et al.* 2003, *Spirito et al.* 2002). Our objective was to develop an easy and efficient method for correctly identifying the normal homozygous and heterozygous carrier horses for the JEB trait. We analyzed a population of Romanian Draft Horses using a set of primers which amplify a fragment from the LAMC2 gene possibly containing the insertion. The number of allele peaks depends on whether the horse tested is a heterozygote (carrier) or homozygote (normal or JEB affected). Results suggest that the genetic test will be useful in identifying horses which are heterozygous for the JEB trait and foals with JEB.

Key words: Romanian Draft Horse, JEB, carrier, diagnostication.

Introduction and Literature Review

Epidermolysis Bullosa (EB) is a heterogeneous group of mechano-bullous disorders characterized by fragility of the skin and the mucous membranes. The junctional form of EB, JEB, is characterized by blister formation within the lamina lucida of the basement membrane zone and by an autosomal recessive pattern of inheritance. In the severe Herlitz variant, H-JEB, tissue cleavage results from the mutations in one of the three genes (*LAMA3*, *LAMB3* or *LAMC2*) (*Aumailley et al*. 1998, *Korge et al.* 1996, *Pulkkinen et al.* 1999). These are encoding the three subunits (a3, b3 and g2) of the extracellular adhesion ligand laminin 5 associated with the hemidesmosome-anchoring

lament complexes. Likewise, cases of EB have been described in different species, such as sheep (*Bruckner-Tuderman et al.* 1991), dogs (*Palazzi et al.* 2000), cats (*Olivry et al.* 1999), mice (*Colognato et al.* 1999), and rats (*Brenneman et al.* 2000).

In horses, JEB is an inherited disease that causes skin lesions in newborn foals and results in large areas of skin loss. The disease was first discovered in Belgian draft horses. Different areas of the body were affected, in particular the limbs with recurrent loss of the hooves. The affected foals died or were euthanised. The phenotype of the affected foals suggests a condition in horses similar to H-JEB in humans. Affected foals were born with skin blistering, skin and buccal ulceration followed by the loss of the hooves, as ascertained by a veterinarian and confirmed by histological examination (*Milenkovic et al.* 2003). The affected skin showed disjunction of the epidermis from the underlying dermis at the dermal-epidermal junction.

In France, a type of lethal junctional EB was described for the first time in the Trait Breton and Trait Comtois draft horses and the limited family data are compatible with a recessive mode of inheritance (*Goureau et al.* 1989).

The mutation associated with the clinical signs of JEB in Belgian and French draft horses has been identified and is linked to the γ2 subunit of the laminin-5 gene (*Spirito et al.* 2002, *Milenkovic et al.* 2003, *Spirito et al.* 2002). The mutation is a cytosine insertion in the genomic nucleic acid sequence of affected horses at position 1368 of the laminin γ2 encoding polynucleotide, a frame shift, and a premature termination codon (*Spirito et al.* 2002, *Milenkovic et al.* 2003, *Spirito et al.* 2002). This results in an absent expression of the laminin γ2 polypeptide. An autosomal recessive mode of inheritance of this mutation has been verified (*Spirito et al.* 2002, *Milenkovic et al.* 2003, *Spirito et al.* 2002).

Material and Methods

In our research we analysed a population of Romanian Draft Horses. The isolation of genomic DNA from fresh blood was performed with Wizard Genomic DNA Extraction Kit (Promega).

We used one set of primers which amplify a fragment from the LAMC2 gene possibly containing the insertion. The forward primer was labelled with 6-FAM dye.

PCR was performed in a GeneAmp 9700 PCR System (AppliedBiosystems). The reactions were carried out in 25-μl final volume containing PCR Buffer, $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each primer, 0.5 units of AmpliTaq Gold DNA Polymerase, diluted DNA (50 ng per reaction) and nuclease-free water. PCR amplifications were performed in 0.2 ml

tubes using 30 cycles with denaturation at 95 ºC (30 s), annealing at 57 °C (30 s) and extension at 72 °C (45 s). The first denaturation step was performed at 95 ºC (10 min) and the last extension took 10 min. at 72 °C.

PCR products were loaded with the GeneScan-500 ROX Internal Size Standard (AppliedBiosystems) into one of the ABI PRISM 310 DNA Genetic Analyzer (AppliedBiosystems).

The results were analyzed with the GeneScan 3.1.2. Software (AppliedBiosystems) which assigns a base pair size for each signal. GeneScan data can then be exported directly to Genotyper 2.5.2. Software (AppliedBiosystems) for automated genotyping.

Results of Investigations and Discussion

Our objective was to develop an easy and efficient method for correctly identifying the normal homozygous and heterozygous carrier horses for the JEB trait.

PCR was performed on DNA samples with fluorescently labeled primers designed to amplify the region containing the mutation. The mutation is a single base insertion, and thus, carriers have a PCR product that is one base longer than the
normal allele. The single base difference is detected by analysis of the PCR products on an ABI 310 DNA Genetic Analyzer.

An amplified fragment for a normal horse had 169 bp. The conditions for PCR were selected so that the two primers could amplify the DNA from normal, carrier and JEB affected horses.

In our experiment successful amplification yields one or two allele peaks with an expected size of 169 and (or) 170 bp. The number of allele peaks depends on whether the individual tested is a heterozygote (carrier) or homozygote (normal or JEB affected). If we test a normal horse we must obtain just one peak at 169 bp. If we analyze a homozygous affected horse we also obtain just one peak, but at 170 bp. If the horse is a heterozygous carrier we must obtain two peaks at 169 and 170 bp because one allele is normal and the other one contains the insertion. We did not find any carrier or affected horses among the Romanian Draft Horse population analysed.

In Figure 1 the profile for a heterozygous carrier and for a homozygous normal horse are shown.

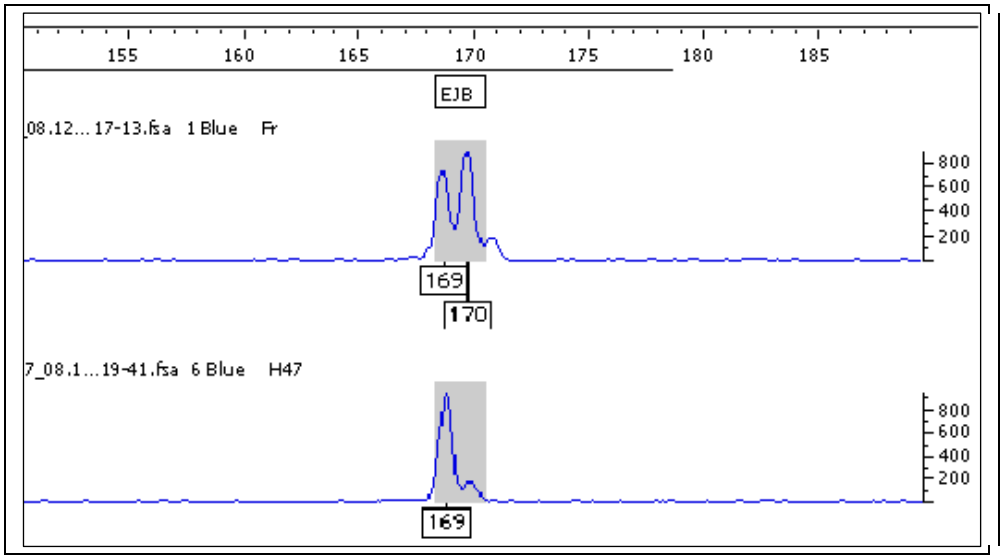

Figure 1. Genotyper software analysis of PCR amplification product for a heterozygous carrier and for a normal homozygous horse
Slika 1. Genotipizirajuća softverska analiza proizvoda PCR amplifikacije za heterozigotnog nosioca i normalnog homozigotnog konja

Conclusions

The identification of the causal mutation of JEB is of great importance to draft horse breeders. A rapid, simple genotyping method by DNA amplification from blood or hair samples detecting normal and mutated fragments is now available for genetic tests. The identification of healthy carriers for the mutation allows the development of different breeding strategies. Population reproductions can be conducted, avoiding matings between carriers to obtain unaffected foals. Alternatively, breeder associations may decide to eradicate the mutation by preventing all carriers to reproduce.

In conclusion, this study proposes a rapid molecular test for the identification of healthy carriers which will help the breeders to eliminate the disorder from their populations.

Consequently, the investigation of possible occurrences of the affected gene among other draft horse populations from other countries and among other breeds will be of interest.

Metoda za dijagnostikovanje *Junctional Epidermolysis Bullosa* kod radnih konja

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Rezime

Junctional Epidermolysis Bullosa (JEB) je nasledna bolest koja izaziva ozlede kože kod novorođene ždrebadi u vidu gubitka velikih površina kože. Mutacija koja je odgovorna za ovu bolest kod radnih konja je identifikovana. Umetanje citozina u LAMC2 gen, rezultira u izostajanju ekspresije laminin γ2 polipeptidnog lanca laminina 5. JEB se nasleđuje kao recesivna osobina autozoma. Naš cilj je bio razvoj efikasne i jednostavne metode za tačnu identifikaciju normalnih nosilaca homozigota i heterozigota za JEB osobinu kod konja.

Analizirali smo populaciju rumunskih radnih konja koristeći set prajmera koji pojačavaju fragment iz LAMC2 gena koji možda sadrži umetak. Uslovi za PCR su odabrani tako da dva prajmera mogu da pojačaju DNK kod normalnih konja, nosilaca i konja kod kojih se manifestovala ova bolest - JEB. Broj pikova alela zavisi od toga da li je testirani konj heterozigot (nosilac) ili homozigot (normalni ili konj sa manifestacijom JEB). Ako testiramo normalnu životinju trebalo bi da dobijemo samo jedan pik/vrh na 169 bp. Ako analiziramo homozigotnog konja, sa manifestacijom bolesti, takođe dobijamo samo jedan pik/vrh ali na 170 bp. Ako je konj heterozigotni nosilac moramo dobiti dva pika/vrha na 169 i 170 bp zato što je jedan alel normalan a drugi sadrži umetak.

Rezultati ukazuju da će genetski test biti koristan u identifikovanju konja heterozigotnih na JEB osobinu i ždrebadi sa JEB. Identifikacija zdravih nosilaca mutacije omogućava razvoj različitih odgajivačkih strategija. Mogu se raditi reprodukcije populacije izbegavajući parenja između nosilaca kako bi se dobilo zdravo potomstvo. Ili, udruženja odgajivača mogu doneti odluku da iskorene mutaciju sprečavanjem reprodukcije životinja koje su nosioci.

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