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**CURRENT GENETICS OF EQUINE CUTANEOUS
ASTHENIA**

(Review of Literature)

DIPLOMA WORK

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1. INTRODUCTION

1.1 Overview

Cutaneous asthenia is a group of inherited congenital skin disorders that has been reported in a variety of species, characterised by skin hyperextensibility. In humans this condition is known as Ehlers-Danlos syndrome (EDS) which has been classified into several subtypes based on clinical, biochemical and genetic characteristics. EDS is the descriptive term for a heterozygous group of inherited connective disorders which form as a result of mutations on the genes responsible for the encoding of collagen fibres (Borges, *et al.*, 2004; Litschauer, *et al.*, 2010). The characterisation of the disease is quite difficult in veterinary medicine and the condition has also been described as hyperelastosis cutis, dermatosporaxis, zonal dermal separation, Ehlers-Danlos-like syndrome, and cutaneous asthenia. This connective tissue dysplasia of thin, fragile, hyperelastic skin occurs rarely in equine clinical practice and was first described in young Quarter horses in 1978 by Lerner and McCracken as Hereditary equine regional dermal asthenia (HERDA). Since this original report, there has been many more studies and reports of HERDA in quarter horses, while the disorder has been less commonly described in other horse breeds. The clinical signs of this disease are generally recognised in the first few years of life, usually associated with the onset of saddle training and present as solitary or multiple areas of loose, fragile skin on the dorsum, which is slow to heal and a tendency to scar. This condition almost exclusively affects Quarter horses but cases in other breeds have also been reported (Rendle, *et al.*, 2008; Marshall, *et al.*, 2011).

1.2 Genetic disorders of Quarter horses

Johann Gregor Mendel's work established the mechanisms of genetic inheritance which has been improved on immensely, and modern genetic studies have expanded to allow us to determine the genetic cause of almost all pre-existing genetic disorders of the horse. The

tools are now available to rapidly identify the genetic basis of a new genetic disease through the sequencing of the horse genome (Penedo and Ferraro, 2005).

The recent advances of whole genome sequencing and genetic variant analysis of horses have accelerated the pace of genetic discovery (Finno, *et al.*, 2009; Doan, *et al.*, 2012). Previously the identification of genetic diseases in horses has been quite difficult due to their long gestation period, single parturition, dispersion of horses once weaned yielding a lack of records, and the presence of numerous diseases with delayed onset of manifestation. The study of genetic diseases in horses was limited pending the development of equine genome maps and the complete sequencing and assembly of a Thoroughbred mare genome became publicly available in 2007. This accomplishment was furthermore advanced when the entire genome of a Quarter horse mare was sequenced by next-generation sequencing in 2012. This increase in the catalogue of genetic variants for use in equine genomics will aid in future studies of genetic disorders of horses (Doan, *et al.*, 2012).

Cutaneous asthenia also known as hyperelastosis cutis (HC) or hereditary equine regional dermal asthenia (HERDA), is inherited as an autosomal recessive connective tissue disorder reported to be found in Quarter horse lineage tracing to the stallion “Poco Bueno” and his sire “King”. This condition has also been reported in other breeds including; an Arabian cross bred mare, a warmblood Hanoverian foal, a Thoroughbred gelding, and a Hafflinger horse. For this disorder to effect the progeny of an equine mating it must inherit one copy of the defective recessive HERDA gene from both its sire and dam. These horses are asymptomatic carriers of the defective gene while offspring that inherit two copies of the genetic defect become clinically affected (Swiderski, *et al.*, 2006). Tryon, *et al.*, 2009 estimated carrier frequency to be at 3.5% within the Quarter horse breed, and with the prevalence of the gene to be highest (28.3%) amongst the cutting horse subpopulation.

1.3 Cutaneous asthenia

Frequently foals and young horses are presented to veterinarians with skin lesions ranging from trauma, environmental problems, management issues, skin infections, or hereditary skin disorders. There are also certain genetic disorders associated with specific breeds that present with characteristic clinical signs that can therefore be recognised and affiliated with such

disorders (Schott, *et al.*, 2005). Horses affected with cutaneous asthenia do not usually show clinical symptoms until they are on average, 1.5 years of age and this onset of clinical signs is typically associated with initial saddle training. However, it has been described in a 6 week old foal and in another case from a horse bred in the UK where no saddling or trauma was connected with appearance of clinical symptoms (Rendle, *et al.*, 2008; Marshall, *et al.*, 2011). The common signs evident involve loose hyperextensible skin, seromas, haematomas, wounds and sloughing skin on the back and sometimes the neck and legs. Affected skin has demonstrated to be slow to heal and typically leads to extensive atrophic dermal scarring. Histopathology typically shows thinning of the dermis and irregularities in the size and shape and staining of collagen fibres usually arranged in clusters in the deep dermis (White, *et al.*, 2004; Borges, *et al.*, 2005). Diagnosis of HERDA can be concluded based on history, age, clinical signs, histology of skin biopsies, and pedigree analysis. However, definitive diagnosis can only be confirmed when molecular evidence proves the missense mutation of the cyclophilin B (PPIB) equine gene (Tryon, *et al.*, 2007). Currently, the treatment of horses with HERDA is limited to careful management, avoidance of ridden activity, and reducing the risk of further trauma. Typically affected horses are unsuitable for breeding or riding and can become simple pleasure horses but most often, owners choose to euthanize due to the severity of lesions and economic loss associated with this disease. This review describes the current genetic background involved in Horses affected with HERDA.

1.4 Materials and Methods

The aim of this review is to provide a synopsis of the current genetics of cutaneous asthenia or hereditary equine regional dermal asthenia (HERDA) in horses. The objective of this survey was to research and discuss this genetic disorder in detail including the clinical signs, aetiology, pathogenesis and manifestation of the disease, the diagnosis, and to provide a critical review of the genetic background and recent advances in genetic testing and the current situation of equine genomics. The method of data collection used for this evaluation has mostly comprised of articles, journal reports, books, internet sources, and Quarter horse association forums.

2 AIM OF STUDY

2.1 Definition of HERDA

Hereditary equine regional dermal asthenia (HERDA) is a devastating genetic skin disorder predominantly found in subpopulations of the American Quarter horse breed. This inherited connective tissue dysplasia is caused by a homozygous recessive mutation that weakens collagen fibres, compromising the adhesive ability of the skin. HERDA affects male and females in equal proportions and typically manifests within the first few years of life. There is no effective treatment for this disorder, and although careful management can protect against the characteristic skin wounds, usually horses are euthanized due to severity of the lesions and poor quality of life.

2.2 Clinical signs and Histopathology of HERDA

The clinical signs of HERDA can present anytime between two months and six years old but typically manifests by the age of two most notably associated with the introduction of saddle riding. The most common signs are extremely fragile hyperelastic thin skin that tears easily and exhibits slow poor diminished healing. Separation is noted between the superficial and deep dermis in affected areas resulting in loosely attached skin that does not return to its original position after manipulation. Seromas progressing to haematomas and ulcers with sloughing skin express the extensive nature of HERDA characteristic wounds (*Figure 1*). Lesions may be solitary or multiple and present most frequently along the dorsal region, and less often on the legs. Impaired healing of wounds results in extensive dermal scarring and poor quality easily damaged skin. Pain has been observed when handling the affected skin in some cases with seromas and subcutaneous haematomas appearing shortly after manipulation. Skin lesions have proved to be of no association with parturition and are not a consequence of surgical interference. All the affected horses described in research had no difficulty in the birthing process and no associated skin lesions or wounds were ever documented. Male horses affected with HERDA displayed no skin lesions or clinical signs after castration and only manifested the disease symptoms many months or even a year later (White, *et al.*, 2004).

A case in Austria described a quarter horse stallion affected with HERDA that suffered from recurrent laminitis in the last year of his life. Radiographs of both forelegs displayed subluxation of both coffin joints and it was suggested this abnormal loading of the bone was due to collateral ligament hypermobility or laxity as a result of disturbed collagen structure associated with HERDA. No other cases of laminitis or joint disorders have been described in horses with HERDA, nevertheless, joint hypermobility is frequently reported in human EDS patients, and there have also been cases of dogs with EDS-like symptoms displaying joint hypermobility (Litschauer, *et al.*, 2010). One could hypothesize that although this clinical symptom has not been described in HERDA cases in Quarter horses, it could have some association or connection with their athletic performance traits of flexibility and agility.

Recent studies have demonstrated ocular findings, including alterations in corneal thickness, and incidence of corneal ulcers associated with HERDA affected horses, thus suggesting that there are other defects associated with HERDA not limited to the skin (Mochal, *et al.*, 2010). Research from 2013 documented the presence of metastatic squamous cell carcinoma consistent with Marjolin's ulcers in association with chronic non-healing wounds in HERDA affected horses (Badial, *et al.*, 2013). While Bowser, *et al.*, 2013, concluded that the HERDA cyclophilin B mutation results in global alterations of the biomechanical properties of tissues rich in fibrillar collagen even when clinical symptoms are not evident, and thus propose that the HERDA phenotype is not limited to the integument.

Histological changes in the skin of horses affected with hyperelastosis cutis are not remarkable; however recurring common findings in the deep dermis have been described. Thinning of the deep dermis with irregularities in the size, shape, and staining affinity of the collagen fibres have been demonstrated histopathologically (*Figure 2*). A reduced amount of dermal collagen, fragmentation and disorientation of these fibres, and many others with abnormal red-stained centres in trichome-stained sections were also described. Seldom, the collagen fibre arrangement has been described as loose with thin fibres in the superficial and periadnexal dermis (*Figure 3*). Collagen fibres with foci of degeneration were found to be arranged in clusters and separated by clear spaces from the superficial dermis. This characteristic lesion where the upper and lower sections of the deep dermis separate is known as "zonal dermal separation" (Brounts, *et al.*, 2001). Control horse tissue samples were characterised by displaying thick and long, evenly distributed collagen fibres. The zonal dermal separation (ZDS) has previously been defined as a linear zone of loose collagen clusters located in the middle of the deep dermis, with fluctuating separation of collagen

bundles and lacking dermal density. White, *et al.*, 2004, hypothesized that the ZDS can only be distinguished with deep biopsies. Almost all the research studied documented the same method for biopsy sample evaluation. Skin specimens, either 6mm punch or incisional wedge were obtained from lesions on the dorsum, lateral neck, withers, croup, abdominal area, and regions on the legs. Similar skin biopsies were taken from same areas on control horses of same breed and age. Tissue specimens were placed in neutral-buffered 10% formalin, routinely processed and stained with haematoxylin and eosin (H&E), Masson trichrome (MT), and acid orcein-Giemsa (AOG). Furthermore, the presence or absence of inflammation, trauma, and fibrosis were evaluated for every sample. Findings described no statistical difference between the healthy control and the horses with HERDA in regards to the presence of trauma and fibrosis. In addition, there was no significant difference observed between HERDA affected horses and the healthy control for both immunohistochemistry and electron microscope examinations (White, *et al.*, 2004).

In another study published by Grady *et al.*, 2009, the biomechanical and molecular characteristics of affected and unaffected skin of HERDA horses were investigated and the work demonstrated a greater reduction in skin tensile strength in horses in the former group. The lack of tight collagen bundling and large spaces throughout the collagen matrix was suggested to be the cause of the many failure sites observed in HERDA samples during this tensile strength analysis. Elasticity and energy to failure of skin was significantly lower in affected horses compared to unaffected horses, with no statistical differences noted between dorsal trunk samples and extremities or abdomen samples. The conclusion was drawn that biomechanical properties of HERDA are not regionally confined to specific locations of the horse's skin as affected individuals possessed uniformly weaker skin across varying sample regions. It has been suggested that exposure to environmental conditions such as biting insects, ultraviolet light, and trauma may be partially responsible for the presentation of HERDA lesions in specific locations. As the dorsum is more exposed to UV light than other areas it would be subjected to greater damage and so would exacerbate the symptoms of HERDA in this region (Grady *et al.*, 2009).

Corneal samples were also taken for histological examination in research done by Mochal *et al.*, 2010, where after a tissue was stained, a portion was fixed in McDowell fixative, rinsed, and dried before mounting on aluminium stubs with carbon plates and coated with gold-palladium for viewing on a scanning electron microscope (SEM) at 5kV. Results demonstrated that corneal thickness is significantly less in horses with HERDA than that of

control horses and the SEM examination revealed zones of disorganised collagen fibrils in corneas of HERDA affected horses and not those horses lacking the mutated gene. During the four year research it was also apparent the occurrence of ulcers was much more significant in horses with HERDA in comparison to those control horses.

A report on a 6 week old warmblood colt in Australia demonstrated some significant differences compared to those previously described in Quarter horses with HERDA based on both clinical and histopathological findings. Shortly after an uneventful birth from a multiparous mare, the foal presented with a skin laceration on the left flank, cranial to the stifle and a skin tear involving the perineum between the hind legs was also noted. Over the next few weeks multiple lacerations and haematomas in various locations were treated and some wounds persisted while others healed with abnormal scar formation. Follow up examinations described numerous haematomas and open wounds over the extremities and hyperextensible skin over the cheeks and flanks that remained stretched after handling. Due to extensive lesions and their severity, the foal was euthanized on the diagnosis of cutaneous asthenia and immediate skin biopsy specimens were obtained for further analysis and a mane hair sample was sent for genetic evaluation. The foal was found to be negative for the HERDA associated mutated gene, demonstrating a difference between this case of cutaneous asthenia in a warmblood foal compared to the breed-specific disorder HERDA found in quarter horses. Previous reports of cutaneous asthenia in warmblood foals in the literature describe the occurrence of lesions in similar locations, particularly the stifle area. This suggests that this skin disorder may also appear to be a regional condition in warmblood breeds, or that it might just be a result of recumbent foals inflicting increased pressure and friction on little protected areas such as those described in these cases (Marshall *et al*, 2011).

2.2 Aetiology

The genetic defect responsible for this disease was described by Tryon, *et al*, 2007, as being a mutation in the equine genome cyclophilin B (PPIB) where a Gly-6 to Arg-6 substitution at codon 115 was identified. The exact mechanism by which this mutation causes the disorder is unknown; however, it is believed the gene plays a significant role in the triple helix folding of collagen. Boudko, *et al.*, 2012, suggest that the disease phenotype is caused by a distortion in the structure and flexibility of the N-terminal tail, altering interactions with other endoplasmic reticulum-resident chaperones and foldases, or even eliminating binding-competent

conformations. Studies indicate that cyclophilin B (CypB) mutation results in disturbance and modifications in collagen fibre assembly, folding and organisation which alters the biomechanical properties of fibrous tissues even without the appearance of clinical symptoms (Bowser *et al*, 2013). Ishikawa *et al*, 2012, describe how the causative mutation occurs in the 6th residue of the mature protein and that CypB acts as a chaperone for nuclear translocation of proteins. The results of this work is consistent with previous reports that the HERDA CypB gene is not as efficient in catalysing the procollagen folding within the endoplasmic reticulum of fibroblasts and that the resulting delay in folding can lead to various alterations of the collagen alpha chains.

Currently the aetiology of cutaneous asthenia in warmblood horses is unknown; however, it is not caused by the same mutation responsible for HERDA in quarter horses or those horses that share quarter horse lineage. Presently, cutaneous asthenia is diagnosed in warmblood horses based upon history and clinical signs of the disorder. Pending further studies on this disease or pedigree analysis of warmbloods (or other horse breeds) is completed, the avoidance of breeding affected animals should be maintained in order to reduce the risk of additional spreading of this devastating disorder (Marshall, *et al.*, 2011).

3. GENETICS OF HERDA

3.1 Current development of equine genetics

An enormous development in the genetic community was accomplished in 2007 when the work of mapping and sequencing the entire horse genome was completed, and a new frontier in equine genetics had been opened. To date, this data has been exploited to provide some very influential tools that can be used to define simple and more complex inherited diseases in horses. At present, a widely available *genetic DNA test* has been developed to detect a single nucleotide polymorphism (SNP) mutation that is responsible for HERDA. This diagnostic test allows for identification of those horses that are affected or carriers of the disorder.

A genetic disorder results from the inheritance and expression of an abnormal mutation in genetic material, fundamentally an alteration in the DNA sequence of a cell's genome. There are numerous DNA sequence differences in horses that are responsible for various characteristics such as coat colour, confirmation, and performance traits; however, some are unfortunately mutated genes for pathogenic disorders. Alterations in genetic material that result in such diseases are due to mutations that modify an important protein in the body. Ultimately, hereditary diseases have developed from original mutation in a germ cell, which results in descendants that carry such mutations in every cell. Every individual species contains unique gene variants known as alleles that are defined by the DNA sequence or the phenotype. Individuals with two identical alleles are identified as homozygous, while those with two different alleles are heterozygous. The way a phenotype manifests from the genotype expresses the mode of inheritance of a genetic disease, so if an animal displays the phenotype while possessing two copies of the allele it is called a recessive trait. An animal with a dominant trait for the disease is seen when only one copy of the affected allele is required for the horse to have and express the disorder. Animals with a dominant trait for the disease express one normal allele and one mutated allele and a heterozygous phenotype is seen. Therefore, on the basis of inheritance a mutation can be classified into heterozygous when one allele is mutated or homozygous when both parent alleles have mutated. In addition, the mode of inheritance can be multigenic or polygenic where multiple genes interact with each other to create a phenotype or many small genes of individual effect cause

the phenotype, respectively. The outcomes for various crosses for recessive and dominant traits are shown in *Table 1* and *2*, assuming het means heterozygous for the mutant allele and hom means homozygous for the mutated allele (Bannash, D., 2008).

Table 1: The results for various crosses for a recessive trait (Bannash, D., 2008)

Crosses	Results
Unaffected X unaffected	100% unaffected
Carrier X Carrier	50% carrier, 25% affected, 25% unaffected
Affected X affected	100% affected
Unaffected X Carrier	50% unaffected, 50% carrier
Carrier X affected	50% carrier, 50% affected
Unaffected X affected	100% carrier

Table 2: The results for various crosses for a dominant trait (Bannash, D., 2008)

Crosses	Results
Unaffected X affected	100% Unaffected
Affected (het) X Affected (het)	75% affected (50% het, 25% hom), 25% unaffected
Affected (het) X Affected (hom)	100% affected (50% het, 50% hom)
Affected (hom) X Affected (hom)	100% affected
Unaffected X Affected (het)	50% affected (het), 50% unaffected
Unaffected X Affected (hom)	100% affected (het)

Alterations of the DNA sequence in a gene can occur in many different ways and can cause various different effects depending on where they occur and whether they affect the function of essential proteins. The many different forms of mutations can be classified based on structure, small or large scale, whether they alter functions, or if they are spontaneous or induced mutations. Point mutations, insertions and deletions represent small scale mutations. A point mutation involves the exchange of a single nucleotide for another. Point mutations occurring in the protein coding region of a gene can either be missense or non-missense

mutations. A missense mutation, such as the one in HERDA, entails the change in a single DNA base pair that results in the substitution of one amino acid for another, while a non-missense mutation instead of substituting amino acids, it prematurely signals the cell to stop building the protein resulting in shortened protein with abnormal function. Insertion mutations encompass the addition of one or more nucleotide resulting in an abnormally functioning gene. A deletion mutation modifies the number of DNA base pairs by either removing a few nucleotides in a gene (small deletion mutation) or by removing an entire genome or several neighbouring genes (large deletion mutation), also resulting in proteins with altered function. A duplication mutation consist of a nucleotide aberrantly copied one or more times. Subsequently, insertion, deletion, and duplication mutations represent a frameshift mutation by shifting the groups of three DNA base pairs and changing the code for amino acids, ultimately shifting the genes reading frame and significantly altering the gene product. Large scale mutations include any alteration in the structure or arrangement of chromosomes, such as amplifications, inversions, and translocations.

Further classification of mutations is based on the effect on function, or by the affected phenotype and whether the mutation is spontaneous or induced. The mutations causing genetic disorders are spontaneous and are always expressed from genetic material and differing from congenital diseases which are present from birth but may not be caused by a genetic abnormality.

The aim of diagnosing genetic diseases is to identify afflicted and carrier horses of the disorder and distinguish them from healthy animals. A pedigree analysis is executed to determine if a genetic disorder is present and to identify the mode of inheritance, while also enabling to establish the probability of whether the prospective parents are homozygous for the normal gene, or carriers of the defective trait and thus provides the ability to make predictions about breeding combinations and their outcome, hence allowing the possibility to eliminate the inherited defect from the breed entirely. The mutation that causes the autosomal recessive disorder HERDA was deduced using genome association analysis followed by homozygous mapping based on the horse genome sequence (Bannash, D., 2008).

The horse genome was assembled and released to the public in 2007. This long process entailed obtaining the DNA sequence in small fragments which are then assembled into longer portions called contigs, followed by linking into supercontigs and assigned chromosomal locations. After the initial study of the Thoroughbred mare, "Twilight" chosen

for the whole genome sequence, other breeds were selected for additional sequence analysis. The DNA sequence differences identified between these horses are known as *single nucleotide polymorphisms (SNPs)* with approximately two million identified by researchers. These SNPs are extremely important genetic markers for disease association and are valuable tools for future genetic disease studies in the horse. The possibility of mapping and identifying many more diseases is now more feasible as a consequence of this new data and technology and it enables scientists to understand genetic diseases at a molecular level, ultimately giving both breeders and veterinarians the tools to eliminate inherited genetic diseases (Bannash, D., 2008).

After considerable research into the genetics of HERDA and with the arrival of trustworthy DNA screening it is now possible to decisively conclude those horses afflicted with the disease and those who are carriers of the defective gene. Concurring with this extensive research it is apparent that Quarter horses diagnosed with the disease, either affected or a carrier have been genetically linked to the famous stallion “Poco Bueno”, his sire “King”, and his full brother “Old Grandad”. Consequently, as a result of selective inbreeding, horses inherit the disorder in one of two ways; firstly, both parents can be carriers, each with a normal dominant gene and a recessive HERDA gene, or one parent can be a carrier while the other parent is affected by the mutated gene. As the condition is caused by a recessive gene, it means that both sire and dam must possess the gene in order to produce an afflicted foal and even in this case the disease may not necessarily manifest. Under the laws of genetics and mode of inheritance, breeding of carrier horses have a 25% chance of producing affected offspring, 25% will be genetically normal and 50% of the foals are expected to be carriers but not affected.

The American Quarter Horse Association (AQHA) was established in 1941, and at present being the largest breed registry in the world is hugely invested in its breed and has dedicated vast amount of time, money and resources into the genetic research of the hereditary disorders associated with Quarter horses. The AQHA’s equine research committee was formed in 1960 and has since led the way in the developing world of equine health and research. To support and assist breeders making informed decisions regarding their breeding program, the AQHA offers a five panel genetic test at reasonable cost to determine if an American Quarter horse is a carrier or affected by one of the five genetic disorders, including: Glycogen branching enzyme deficiency (GBED), Hyperkalemic periodic paralysis (HYPP), Malignant hyperthermia (MH), Polysaccharide storage myopathy (PSSM), and HERDA. Severe

consequences occur in the succession of these diseases onto new generations causing both unnecessary pain and suffering to the horse while breeders suffer emotional, financial, and economic loss. With no prevention or treatment for this debilitating disease the use of genetic testing to identify HERDA should help reduce or eliminate the possibility of further producing infected horses in the future. The DNA test carried out using a hair or blood sample, can diagnose affected animals before clinical signs are detected, so there is no reason why animals with the disease should ever be bred to reproduce. Ultimately, the breeding goal should be founded on reducing the frequency of the disease allele or to eliminate the occurrence of the disease entirely and this could be achieved by not breeding horses with dominant disease traits or those carrying recessive traits.

3.2 Heritability

HERDA is an autosomal recessive skin disorder most notably documented in Quarter horses; however, there have been some cases of its presence in other breeds too such as the American Paint Horse and the Appaloosa. Reports of this disease occurring in a Thoroughbred gelding, a cross-bred Arabian mare, a Hanoverian foal have also been documented. Within the Quarter horse population, the prevalence of the disease dominates the cutting horse subpopulation. These athletes are bred for their speed, agility and calm disposition and most often have come from similar popular performance winning stallion lineage. Affected horses with the gene mutation are found to trace back to a specific *stallion* “*Poco Bueno*” or possibly one of his ancestors. There are four known Quarter horse that were carriers of HERDA and that have produced at least one affected foal. These infamous stallions including; “Dry Doc”, “Doc O Lena”, “Great Pine”, “Zippo Pine Bar”, all share similar pedigree tracing back to “Poco Bueno” via his son or daughter, “Poco Pine” and “Poco Lena”. “Poco Bueno” was one of the most influential sires in Quarter horse breeding history and has sired over 400 foals. This popular pedigree line has been utilized by cutting horse breeders over many generations resulting in a gene pool of carriers with this recessive trait.

By examining many pedigrees and the linking of horses within the Quarter horse population, scientific investigations have established HERDA as a heritable disease and clarified its mode of inheritance. Tryon, *et al.*, 2005, estimated the mean heritability value of 0.38 as a strong indication that inheritance plays a significant role in the prevalence of this disease. It is worth noting that although the Quarter horse breed is presently vast and diverse, a relatively small

quantity of horses were used to establish the breed, and most modern Quarter horses can detect one or more of these foundation lineages in their pedigrees. Furthermore, selection has been used quite effectively to produce such distinct successful subpopulations as performance athletic horses for cutting, reining, or racing events. Selective inbreeding increases the probability that the same allele is transmitted to an offspring by both sire and dam, compared to a case of random out bred mating population. The more often a population is inbred, the greater the inbreeding coefficient and the larger the likelihood of bringing together recessive alleles, thus increasing the incidence of the disease. As the carrier frequency of HERDA appears relatively low in Quarter horse population, including those subpopulations with the highest incidence of disease, by availing of the modern genetic tools, there is a chance for future selection strategies to reverse the prevalence of the disease.

The Australian Quarter Horse Association has implemented regulations for obligatory HERDA testing and they imply that a horse must be negative for the HERDA gene in order to be eligible to register with the association. The laws effective since 2008 state that any new foundation stock must test negative for HERDA before they can be accepted into the foundation recording system, and only official AQHA HERDA testing kits can be used with test results being analysed from licenced veterinary laboratory facilities will be accepted. Any imported horses or new registering horses with pedigrees not carrying “Poco Bueno” lineage do not require testing.

3.3 The causal candidate gene for HERDA

Published research has described and verified the prevalence of the mutation in *peptidyl-prolyl cis-trans isomerase B (PPIB)* which encodes *cyclophilin B (CycB)*, to be the causal candidate gene for carriers and affected horses of HERDA, where the gene alters chaperone functions including folding of collagen proteins and post translational modifications of fibrillar collagen. The proposed CycB mutation has shown to disrupt collagen folding making it a probable cause for the histological characteristics seen in HERDA. A homozygous mapping approach was taken to map the locus of an affected horse, since the identification of a common ancestor on both sides of the pedigree could now be identified. A whole genome scan was used to locate regions that overlapped by descent in affected individuals on the q arm of *ECA1 (Equus caballus autosome 1)* (closest to marker *AHT58*). Single nucleotide

polymorphisms (SNPs), with fine-structure mapping were used to further refine the region to 2.5 Mb. A non-synonymous mutation in exon 1 of equine PPIB was revealed by sequence analysis. As a consequence of the SNP segregating in association with HERDA, it can be utilised for identifying carriers and affected horses, while also used for screening foals to detect the evidence of the disease. A further study involving a group of 1079 unaffected Quarter horses from the UC Davis Veterinary Medical Hospital and Genetics Laboratory were screened for the mutation and 38 samples were found to be heterozygous for the mutated gene, demonstrating a **3.5% carrier frequency**, which supported their preceding estimates of 1.8-6.5%. The discovery of unique SNPs in the critical region was the first stride toward developing a conclusive genetic test for HERDA. From 64 horses affected with HERDA, four DNA markers were detected to be homozygous in all samples, and one of these markers, the c.115 Gly > Arg missense mutation in PPIB is the only polymorphism unique to families segregating for HERDA and only those horses affected were homozygous (Tryon, *et al.*, 2005, 2007).

The genetic test originally organised and performed by the UC Davis Veterinary Genetics Laboratory in California USA is described in the article by Tryon, *et al.*, 2007. Plucked tail hair samples were submitted for DNA analysis along with a complete history and pedigree of the horse and AQHA registration number. Initially primers were used to amplify a portion of the cyclophilin B gene which contains an informative single SNP. A 250bp fragment was generated using an unlabelled forward primer and a fluorescently labelled reverse primer. This fragment was digested by Earl restriction endonuclease following amplification. This Earl digestion site was cut at 46bp from the termination of the forward primer. The unique SNP in HERDA genomes introduces a second site 67bp from the original Earl site. However, a study in the prevalence of the mutation gene among a Quarter horse population in France (1.6%) reported to be approximately half of number of carriers reported in the USA (3.5%) (White and Bourdeau, 2010).

Current research by Doan, *et al.*, 2012, available in BioMed Central's open access journal BMC Genomics describes the use of **next-generation sequencing** to map variation in the genome of a Quarter horse mare. Through analysis of genetic variants associated with specific traits a comparison between a Thoroughbred and the Quarter horse's genome was completed and over 3 million variants were found. The Quarter horse's genome was found to be enhanced for variants in genes involved in sensory perception, signal transduction and the immune system. This study identified genetic variants including 3.1million single nucleotide

polymorphisms (SNPs), 193 thousand insertion/deletion polymorphisms (INDELs), and 282 copy number variants (CNVs) in the genome of a Quarter horse mare, have been identified and analysed. When scientists searched for disease causing mutations and performance traits associated variants, they discovered the mare was heterozygous for a c.115G>A mutation in the PPIB gene which is responsible for HERDA and two SNPs associated with racing endurance, which originates in Thoroughbreds.

This work involved *whole genome sequencing*, alignment and identifying a new genome sequence. A Quarter horse mare owned by Texas A&M University was used for the study where DNA was obtained from a single blood sample. From a generated small insert library comprising approximately 270 base-pair (bp), 14 lanes of 75bp paired-end sequencing was performed. The sequencing reactions yielded a total 61,310,282,925 bases of DNA. This was then trimmed and mapped to the reference Thoroughbred genome where reads were aligned and chromosomes assembled to receive sequence coverage. Sequences mapped to the chromosome assembly was analysed to identify genetic variants. A functional annotation clustering analysis of the genetic variants was executed and their impact on gene structure and function was studied. The genomic sequence was further reviewed for mutations and polymorphisms associated with known diseases and various traits in horses.

This research was the first entire genome sequencing of a Quarter horse to be published, and the only equine genome sequenced by using next generation sequencing. Thus, analysis and identification of these genetic variants in an individual horse has provided valuable information for future equine genetic studies (Doan, *et al.*, 2012).

Further research into the interaction of the mutated PPIB gene with endoplasmic reticulum-resident proteins was carried out. The study describes the crystal structures of wild-type and mutated Cyclophilin B (CypB) involved in the triple helix folding of collagen, which catalyses the slow cis-trans isomerization of multiple proline residues in the collagen chain. CypB is involved in construction of transient multi-protein complexes similar to other endoplasmic reticulum chaperones, and assist individual protein activities on nascent chains with the associated (HSP47), binding immunoglobulin protein (BiP), ER protein 5 (ERp5), calreticulin, and calnexin. This work described a difference in the N-terminal labile end of the mutated gene that is important in complex formation with ER-resident chaperones and foldases. Any variances observed in this research were attributable to the HERDA mutation Gly6 > Arg6. All N-terminal residues of the mutated CypB displayed a strong electron

density, while the Gly6 > Arg6 mutation in the CypB appears to provide further stability in the N-terminal region also. Ultimately this research showed evidence that the missense mutation in the CypB has two consequences; firstly it enhances the affinity in the binding of the polyacidic region in calreticulin, and second, it can slow, limit, or even eliminate binding-competent conformations by considerably distorting the structure and flexibility of the N-terminal tail. These two consequences have a synergistic effect on the general protein-domain binding, which may cause deviations in the repertoire and mode of interactions with other proteins. Evidence shows that the disease phenotype is due to alterations in the arrangement and flexibility of the N-terminal tail, modifying interactions with other ER-resident chaperones and foldases (Boudko, *et al.*, 2012).

Ishikawa, *et al.*, 2012, suggest that the mutation changes the protein-protein interactions of CypB resulting in less effective catalysis of the rate-limiting step in collagen folding. In the region of the mutation, opposite the catalytic domain of CypB, small structural changes are noted. The interaction of CypB with P-domain of calreticulin is disrupted by the mutation, resulting in the formation of the P3H1 CypB cartilage-associated-protein complex, and possibly other proteins ultimately disturbing the biosynthesis of procollagens. The work confirms a missense mutation of the equine PPIB gene responsible for HERDA consistent with previous reports that utilised homozygous mapping to reach this deduction. The previously established autosomal recessive pattern of inheritance associated with inbreeding reflects the transmission of the defected gene.

4. CURRENT LITERATURE

4.1 Most recent literature findings

Bowser, *et al.*, 2013, presented research of tensile properties in collagen-rich tissues in HERDA affected Quarter horses. Scientists previously hypothesised that tendons, ligaments, and large vessels, similar to skin, are rich in fibrillar collagen and thus will also have abnormal biochemical properties and alterations in horses with HERDA. The research indicated that the CypB mutation in HERDA results in major alterations in collagen assembly, folding and fibril organisation. These modifications alter the mechanical properties of tissues rich in collagens, even in the absence of clinical symptoms. Highly significant decrease in tensile strength, energy to failure, and elastic modulus were identified in the skin of horses with HERDA. The magnitude of the difference in biomechanical properties between the HERDA and control skin tested varied significantly depending on the region sampled. The most extreme differences were noted in the skin from the dorsum, which might explain why lesions are more frequent in this region. The decreased tensile strength of tendinoligamentous structures observed in HERDA affected horses, highlights the significance of quantifying biomechanical properties of tissues from these carriers. This may contribute an advantage in disciplines requiring extremes in flexibility and lateral movements, such as the cutting sport. The findings concluded that type I and/or type III collagen-rich tissues, and other fibrillar collagens, are significantly weaker and abnormal in HERDA affected horses compared to those lacking the mutated gene, and therefore, suggesting the HERDA phenotype is not limited to the integument.

Studies completed by Badial, *et al.*, 2013, described findings of metastatic squamous cell carcinoma (SCC) in necropsy examinations of two Quarter horses diagnosed with HERDA. Marjolin's ulcers in humans, is a chronic condition of non-healing ulcers or scars connected with the development of SCC, and similar lesions have been documented previously in horses suffering from chronic ulcer and infiltrating squamous cell carcinoma. This research is the first to detect an association of SCC and poor healing wounds from horses with HERDA

lesions. The first horse, a 4 year old mare was donated for research and spent six additional years at the hospital before she was euthanized due to chronic lesions and her progressive deterioration (*Figure 4*). The second case described an 8 year old mare, also donated for research, experienced two pregnancies while hospitalized, and was euthanized at 10 years of age. Both horses exhibited the typical characteristic lesions and were confirmed to have the HERDA gene. It was suggested that the chronic inflammation and repeated wounds on fragile skin endured by the HERDA affected horses predisposed to malignant SCC, similar to Marjolin's ulcers in humans. Further to this, SCC in horses, attributable to prolonged exposure to ultraviolet light has been documented, and thus, it was hypothesised that overexposure to heat and sunlight could be a causing factor in lesions associated with HERDA. In addition, the deterioration and progressive decline in health status that led to the euthanasia of both horses, was postulated to be as a result of metastasis. Histopathological and necropsy examinations demonstrated a local infiltration of the tumor and metastasis to the regional inguinal lymph node and the lungs. As cyclophilin B is a contributing factor of the regulation of T lymphocytes adhesion to an inflammatory site, and this gene occurs as a missense mutation in HERDA horses, which may lead to a malfunction of T cells and immune surveillance failure, thus possibly causing the metastasis in the thoracic cavity. Immunohistochemistry examinations using Ki-67 and p53 biomarkers were used to distinguish between benign and malignant processes and evaluate the proliferation index and expression of SCC. Results demonstrated values much higher than previously reported in both humans and cattle with ocular SCC that were associated with very poor prognosis. In conclusion, the development of SCC at lesion sites or pulmonary metastasis in these HERDA horses may be a result of combining factors such as sensitivity to UV light, chronic inflammation and wounds, and T cell suppression and alterations in dermal collagen due to the CypB mutation responsible for HERDA (Badial, *et al.*, 2013).

5. DISCUSSION AND CONCLUSION

5.1 Contributing factors to the current HERDA situation in horses.

With the recent development of equine genome mapping and the complete sequencing of the whole horse genome, the discovery of different equine genetic mutations will continue to expand. At present, there are still many inherited disorders for which the genetic mutation is unknown, nonetheless as the field of equine genomics continues to progress and advance, it is likely that several more loci for single and polygenic traits will be identified (Finno, *et al.*, 2009).

As a result of the research undertaken at UC Davis Veterinary Genetics Laboratory along with similar studies, the causative mutation responsible for HERDA is now known and is easy to identify, track, and screen against this trait. In theory, this disease should be relatively simple to eliminate from the breeding program, however this disorder still appears in varying degree throughout the Quarter horse population. One of the reasons HERDA has manifested more frequently in cutting horse circles than any other is because through the years breeders have tended to utilize the popular “Poco Bueno” bloodlines on both sides of the pedigree. This lineage has proven to be very successful in its discipline whether cutting, barrel racing or reining, and it is theorized this popular line possesses the traits for speed and agility, thus breeders exploited this knowledge to produce potentially winner foals. The famous stallion “Poco Bueno” truly influenced the breed and the sport of cutting horses, and consequently his alleles became proportionately more common, both the good traits and the bad genetic defect mutation. With an ever increasing number of offspring and lineage of horses carrying the defective gene, the likelihood of producing afflicted foals became more prevalent and the genetic disorder that is HERDA became extensive in the equine world. The HERDA allele is segregated within the western performance discipline of cutting where the carrier frequency is 28.3% in cutting horses. This is much greater than the 3.5% carrier frequency in the general Quarter horse population. Horses that are heterozygous for the disease gene are over-used among cutting horses with elite performance, and subsequently a correlation exists today

between the lineage of cutting horses carrying the HERDA allele and performance results in the discipline (Tryon, *et al.*, 2007, 2009; Bowser, *et al.*, 2013).

Even today, there still appears to be some breeders that value performance and winning over the horse and the breed as a wholes' health. This is one of the contributing factors as to why the disease is still evident in the present day. Many owners and breeders simply believe that genetic disorders are something that will never affect them or their horse personally and therefore do not seek information on these diseases until it is perhaps too late. The financial and emotional impact of owning a HERDA affected horse can potentially be prevented with easily accessible education on the disease, and the availability of the diagnostic genetic screening test. The science of genetics can aid in removing the uncertainty involved in breeding, resulting in breeder's gaining the knowledge that they are producing well balanced healthy foals. Eventually this action could possibly lead to the eradication of the disease from the breeding program.

5.2 Prevention and control of HERDA

The UC Davis Veterinary Genetics Laboratory (VGL), originally established in 1950s has since been operating as the largest horse parentage testing facility in the world. The VGL was the first laboratory to offer DNA testing to the horse and currently offers numerous diagnostic tests for equine coat colours, genetic diseases, and has an active ongoing research and development program in equine genomics. Since the mapping of the horse genome sequence was completed in 2007, the genetic basis for many hereditary diseases has been discovered and diagnostic tests have been developed and are available to the public. In an effort to control further manifestation of HERDA, it would be extremely beneficial for owners and breeders to become more informed about this genetic disorder in the horse and take the necessary precautionary steps in order to selectively avoid the further production of affected foals. By careful selection of breeding stock and availing of the genetic screening against this disorder, it may be possible to slowly reduce and even eliminate HERDA from the horse population in the future.

Mandatory testing for genetic diseases such as HERDA is not the standard among breed registries around the world, with the exception being the Australian Quarter Horse Association (AQHA). It seems the vast majority of breed associations and organisations do

not support the exclusion of carriers of HERDA or other genetic disorders from their registries and this may be due to their beliefs that it is not wise to completely eliminate a genetic disease from the breed, because in doing so will result in the loss of other beneficial traits and ultimately diminish the genetic pool (Penedo and Ferraro, 2005). To prevent the loss of valuable traits or qualities that a carrier horse may possess, while at the same time solving the issue of the spread of the genetic disease, it is important to note that carriers should not be bred to other carriers or horses homozygous for the mutated gene. Accordingly, careful breeding decisions and disclosure of carrier status on horse certificates should enable breeders to continue to utilise their carrier horse in breeding programs provided they are responsible, and thus, they will not inevitably be affected economically by owning a carrier horse.

Previously, genetic testing was only conducted by breeders if they were aware that a known carrier of the disease was related to the horse being used for breeding. However, this strategy of only testing lines where there is a known number of affected horses already bred is not an entirely effective method, and data confirms that almost all subgroups of American Quarter horse population carry certain disease alleles. Furthermore, the dynamics of breeding has changed with the use of artificial insemination, superovulation, and most recently cloning. With these techniques presently being utilised more often, only a few years are needed for the disease allele to be copied from a popular stallion and passed on to many thousands of horses worldwide.

Devoid of proper genetic screening, the continued breeding of popular carrier horses may therefore lead to the increase production of affected foals in the future, and thus thorough genetic testing of popular sires and dams for HERDA alleles is highly recommended (Tryon, *et al.*, 2009).

5.3 The Future of Genetics in the horse

Modern genetics have the tools to detect and accurately identify an affected horse from a non-infected horse. Routine diagnostic testing is widely available to screen for many genetic disorders and traits, and these tests are very affordable for the average owner or breeder. The greatest challenge presented to the control and reduction of genetic disorders in the species, is the oblivious attitude of breeders or the lack of knowledge by owners on such genetic

diseases. It is the duty of veterinarians to be up to date on current knowledge of genetic diseases, and to provide information and advice on the disorders and risks associated to breeders and owners. As genetic tests are available in horses with hereditary diseases, there can now be less uncertainty in predicting breeding outcomes and owners should avail of this valuable technique, and there is no reason that animals with a disorder should ever be knowingly produced.

It is important that the many breed associations and organisations are educated on genetic diseases and should implement mandatory testing to screen for such disorders prior to registering, in order to preserve the integrity of their breed. Presently some horse registries require testing for certain diseases; such as the AQHA for Hyperkalemic Periodic Paralysis, and approve certain official laboratories to run these tests. However, there is currently no governing body requiring genetic testing for HERDA besides the Australian Quarter Horse Association.

Since the first sequencing and assembly of a horse genome was completed in 2007, the studies in the equine genomic community have advanced immensely. The current catalogue of genetic variants for use in equine genetics has greatly increased with the discovery and addition of novel SNPs, INDELs, and CNVs. These genetic variants will be extremely valuable resources for future research of genetic variation regulating performance traits and diseases in horses.

6. APPENDIX



*Figure 1: (a) Lesions of HERDA on a 1.5 year old filly
(b) Close up showing haematomas (arrows) and ulceration with sloughing skin (arrow head)
(White, *et al.*, 2007)*

(a)



(b)

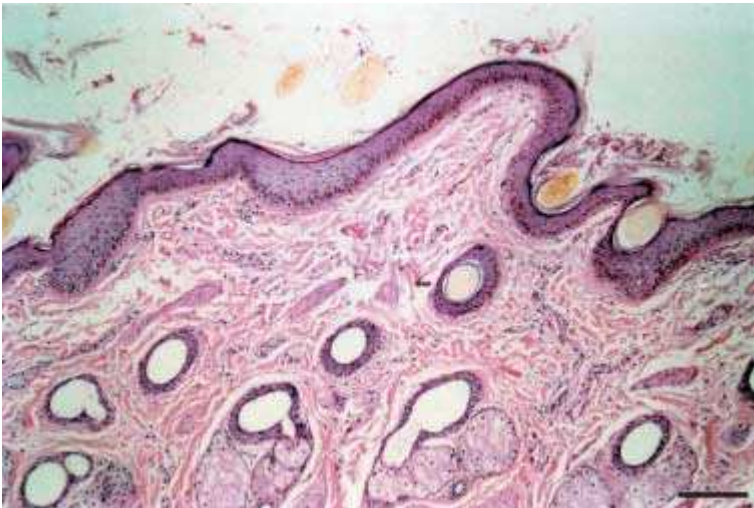
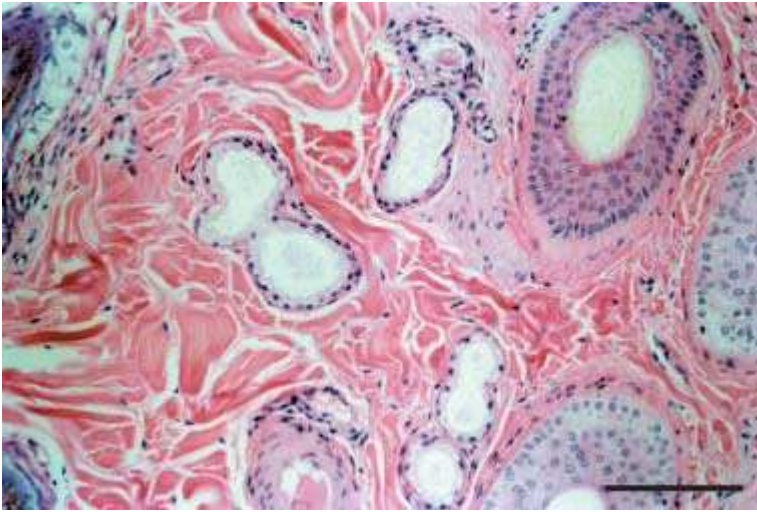


Figure 2: Normal (a) and HERDA affected skin (b).

Note the thin, loose arrangement of collagen fibres in the affected skin compared to a biopsy sample from the same area in a normal horse of same age and breed. H&E. Bar = 50 μ m.

(Borges, *et al.*, 2005).

(a)



(b)

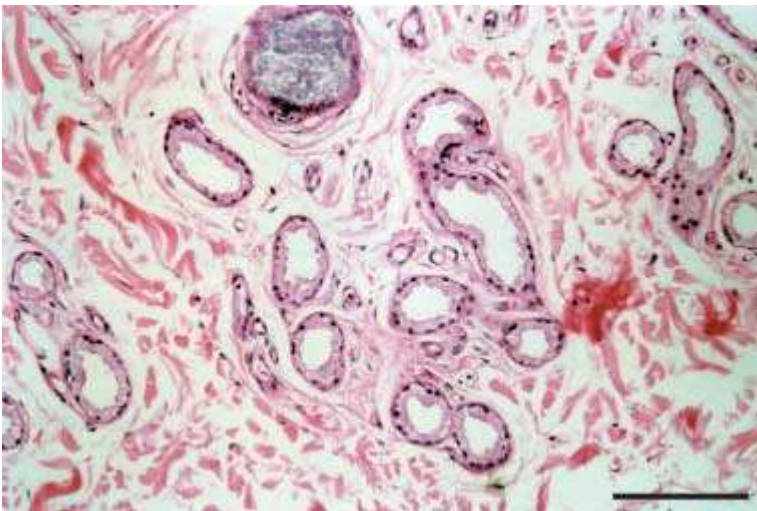


Figure 3: Normal (a) and HERDA affected skin (b).

The peri adnexal dermis of the affected skin displays thinner and paler collagen fibres. Note the increased intercollagen clear spaces. Normal skin biopsies were sampled from same areas of a normal horse of same age and breed. H&E. Bar = 50 μ m. (Borges, *et al.*, 2005).



*Figure 4: Quarter horse mare with HERDA. (A) Atrophic scar on right paralumbar region: mare at 4 years old. (B) In subsequent years, skin tore several times in same area. (C) Progressive weight loss and wound deterioration at the day of euthanasia: mare was 10 years old. (Badial, *et al.*, 2012)*

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8. SUMMARY

Cutaneous asthenia, also known as hyperelastosis cutis is an inherited congenital skin disorder that has been reported in a variety of species, characterised by skin hyperextensibility. This degenerative connective tissue dysplasia of friable, thin, hyperelastic skin was first described in Quarter horses in 1978 by Lerner and McCracken as Hereditary equine regional dermal asthenia (HERDA). The clinical symptoms of HERDA typically present on average, at 1.5 years of age and are frequently associated with the introduction of the saddle and riding. It primarily affects the dorsal region of the body and manifests as extremely loose, hyperextensible, fragile skin that tears easily and upon stretching does not return to its normal position. Single or multiple seromas and haematomas develop leading to chronic slow healing ulcers with sloughing of the skin and atrophic scar formation. This debilitating disease is found predominantly in Quarter horses, with the highest incidence seen in the cutting horse subpopulation, however cases in other breeds have also been reported. HERDA is an inherited autosomal recessive skin disorder that almost exclusively has been found in Quarter horse lineage tracing to the famous stallion “**Poco Bueno**”.

The genetic defect responsible for this disorder is a missense mutation in the equine genome cyclophilin B (CypB) where a Gly-6 to Arg-6 substitution at codon 115 has been identified. It is believed this mutation alters protein-protein interactions of CypB and delays the triple helix folding of collagen. Research has indicated that this CypB mutation is responsible for alterations in diverse tissues containing fibrillar collagen, thus suggesting the HERDA phenotype is not limited to the skin. Since the mapping of the horse genome sequence was completed in 2007, the genetic basis for many hereditary diseases has been discovered and diagnostic tests have been developed and are available to the public. Further to this, the first sequencing of a Quarter horse genome by next generation sequencing in 2012, has led to an increased quantity of genetic variants for use in equine genomics which will provide advantageous resources for future equine genetic studies regarding performance traits and diseases. In an effort to control further manifestation of HERDA, it would be extremely beneficial for owners and breeders to become more informed about this genetic disorder in the horse and take the necessary precautionary steps in order to selectively avoid the further production of affected foals. Mandatory testing for genetic diseases such as HERDA is not the standard among breed registries around the world, with the exception being the Australian Quarter Horse Association (AQHA). Consequently, by availing of the genetic screening against this disorder and by careful selection of breeding stock, it may be possible to slowly reduce and even eliminate HERDA from the horse population in the future.

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