EARLY DETECTION OF CHRONIC PROGRESSIVE LYMPHOEDEMA SUSCEPTIBILITY IN BELGIAN DRAUGHT HORSE STALLIONS BY MEANS OF ELISA

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INTRODUCTION

Chronic progressive lymphoedema (CPL) is a detoriorating disease affecting several large draught horse breeds, including Belgian draught horses (De Cock *et al.*, 2003; Ferraro, 2001). Systemic failure of the lymph system and the skin elastic network are a causative factor (De Cock *et al.*, 2006a; De Cock *et al.*, 2006b). A genetic component is suggested (De Cock *et al.*, 2006a; Geburek *et al.*, 2005). Cutaneous changes and deformations of the lower limbs result from reduced lymphatic drainage. Clinical signs may appear early (less than 2 yrs) and mostly aggravate through life, often ending with severe disability justifying euthanasia (De Cock *et al.*, 2003; Ferraro, 2001). Secondary infections often affect disease progression and complicate diagnosis (De Cock *et al.*, 2003; Geburek *et al.*, 2005). Since distinct symptoms may only appear after introduction into breeding programs, early detection of susceptible animals would tremendously help genetic selection.

Elastin (ELN) turnover in healthy individuals is low and only elicits basic formation of antibodies (anti-ELN antibodies/AEAb's) to tropo-ELN (ELN precursor) and to alpha(α)-ELN (ELN breakdown product) (Colburn *et al.*, 1992). Some diseases are characterized by an altered ELN metabolism and are associated with increased serum AEAb's, detectable with an enzyme-linked immunosorbent assay (ELISA). Examples in humans are scleroderma (Colburn *et al.*, 1992), systemic lupus erythematosus (Colburn *et al.*, 2003) and polymyalgia rheumatica (Colburn *et al.*, 2006). In draught horses, severity of clinical CPL symptoms was shown to correlate with the level of anti-α-ELN Ab's (van Brantegem *et al.*, 2007b). Microscopic skin abnormalities are present before the appearance of the first clinical signs (De Cock *et al.*, 2009). In the present study, it was hypothesized that levels of AEAb's against α-ELN in young horses (without distinct clinical symptoms) may indicate CPL susceptibility. We investigated AEAb levels in young Belgian draught horse stallions and investigated the correlation of these with clinical signs later in life. Furthermore, the effect of age on anti-α-ELN Ab's was studied.

MATERIAL AND METHODS

Animals and sample collection

At timepoint 1 (T1), 16 privately owned Belgian draught horse stallions (max. 3yrs) were examined and radiographs of the 4 lower limbs were taken (Faculty of veterinary medicine, Ghent University, Merelbeke, Belgium). All animals were in good health, except for those with skin lesions associated with CPL. At least 2 years later (T2), possible CPL-status was re-evaluated clinically (Table 1). Additionally, 20 stallions (> 3yrs) were tested (no clinical examination at T1), to investigate the influence of age on anti-α-ELN Ab's. Therefore, stallions were divided into age categories (cat 1: 1.5≤x<2yrs cat 2: 2≤x<2.5yrs, cat 3: 2.5≤x<3yrs, cat 4: 3≤x<6yrs, cat 5: ≥6yrs). Plasma from a foal that had not received collostrum served as a negative control. Animal information (studbook number and date of birth) was kindly provided by the Royal Belgian draught horse studbook.
The 4 limbs of all stallions were examined clinically (inspection and palpation) and radiographs (15/16 young stallions and 19/20 older stallions) were analysed, searching for CPL associated lesions (skin thickening, skinfolds, nodules, ulceration, scaling, …). Veterinary examination included inspection and palpation of the limbs, from the knee/hock, gradually moving down to the hooves. Radiographic analysis includedcounting of skinfolds and nodules and a visual evaluation of skin thickness (Fig. 2). An average score for each horse was calculated from the 4 limbs for clinical examination and radiograph analysis.
Blood was collected at official horse meetings around T1, from the left jugular vein in EDTA coated 9ml venosafe tubes (Terumo Europe n.v.) using a 20Gx1.5" needle (Vacutainer®, BD). Collected blood was immediately cooled on ice for transport. Afterwards, samples were centrifuged (10min, 1614g, Juan CR412, VWR). Plasma was aliquoted and stored at -20°C until used.

Table 1. Scoring table for the veterinary examination of CPL in Belgian draught horses (severity of lesions and affected region from the coronary band up to the fetlock joint)

Score	Class	Lesions	Affected region of limb
1	No	None	None
2	Mild	Mild swelling limb(s) One/two skinfolds rear pastern region	Under fetlock joint
3	Moderate	Moderate swelling limbs Several skinfolds (+nodules) rear pastern region	Including fetlock joint[2]
4	Severe	Severe swelling limbs Several thick skinfolds+nodules rear pastern region (+dorsal)	Above fetlock joint[2]
5	Extreme	=score 4 but with additionaldorsal lesions, impaired movement, bad odour, exsudate	Above fetlock joint

Statistical analysis

Pearson correlation coefficients were calculated and a general linear model (including CPL scores at T2, age at T1 and COD) was fitted using SAS9.3. (SAS Institute Inc.) with statistical significance set at $P<0.05$.

Enzyme linked immunosorbent assay

The performed ELISA was a modified version of the method described by Van Brantegem *et al.* (2007). Wells of a polystyrene microtiterplate (MaxiSorbTM, Nunc) were coated with 120µl antigen dissolved in a 0.05M carbonate buffer (pH9.6) at a concentration of 40µg/ml. The antigen used was bovine α-ELN (Elastin Products Company, Inc.). The plates were covered and incubated overnight at room temperature (RT). Next morning, plates were washed with an automatic plate washer (SkanWasher, Skatron Instruments A.S.) 3x with 300µl PBS-Tween (PBS-T= Tween 20® 0.05%, Sigma Aldrich) and used immediately. To prevent aspecific binding, 250µl PBS-T+1% bovine serum albumine (BSA) was added to each well and incubated for 1h at RT. The plates were washed (3x, 300 µl PBS-T) and incubated with 100µl of test serum (1/80 diluted in PBS-T+1%BSA)(1h at RT). After that, the plates were washed again (3x, 300 µl PBS-T), incubated with alkaline phosphataselabeled antibodies (goat anti-equine IgG H+L) (Southern Biotech Ass. Inc.), 1/1000 diluted in PBS-T+1% BSA (1h at RT). The plates were washed (3x, 300 µl PBS-T) and 100µl of p-nitrophenylphosphate diluted in 0.2M Tris buffer (1mg/ml) (Sigmafast™, SigmaAldrich) was added (30min at 37°C in the dark). Finally, the plates were measured spectrophotometrically at 405nm (optical density/OD) using an automated plate reader (Victor™, PerkinElmer Inc.). Intra- (CV1) and inter-assay (CV2) coefficients of variation were calculated to determine the test procedure reproducibility, measuring 6 replicates of the NC per plate and 4 separate assays. To correct for variations between plates, results were standardized as corrected OD (COD). COD=(ODsample-ODnegative control)/(1-ODnegative control).

RESULTS AND DISCUSSION

Animals

The average period between T1 and T2 for young stallions (n= 16) was 2.3±0.4 yrs (range 2 to 3) and 2.5±0.3 yrs (range 2.2 to 3) for older stallions (n= 20). Animal information (age and CPL scores) per age class (young and old) is listed in Table 2. The average and maximum age of older stallions was quite low. Since stallions were sampled at official stallion inspections, a certain trend was observed: the lower the age, the more stallions were included in that age class. In general, stallions are known to show worse CPL symptoms compared to mares (De Keyser *et al.*, 2010) while stallions are mostly kept for breeding. Since lesions are progressive, a large number of stallions is culled early. Both young and older stallions showed mild to moderate CPL symptoms at T1 and T2, although clinical scores of older stallions were slightly but not significantly higher (Table 2). The latter corresponds to the described disease progression. Interestingly, radiographic analysis results in higher CPL scores compared to a clinical evaluation in younger horses (<

3yrs), although both methods were significantly correlated (R=0.63, P=0.011). Presumably, very mild lesions which are not yet visible or palpable, can be distinguished radiographically.

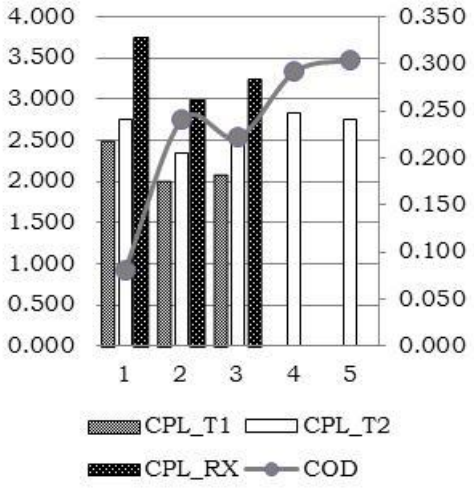

Figure 1. Average CPL score and OD at different age classes.
(CPL_T1 and T2= Average CPL score limbs at timepoint 1 resp. 2, CPL_RX= average CPL score limbs derived from radiographs at T1.
1st Y-axis= CPL score, 2nd Y-axis= COD, X-axis= age categories

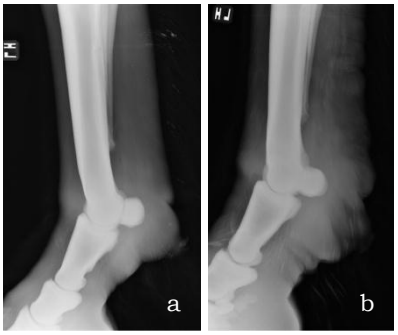

Figure 2. Left hind limb radiographs of (a) a non affected horse and (b) a severely affected horse. Note the difference in skin thickness and the presence of skinfolds

Table 2. Mean ± SD and range of age at T1 and T2, CPL scores and COD for younger (n= 16) and older (n= 20) stallions

Variable	Young	Old
Age at T1	2.4±0.3 (1.6–2.7)	4.3±1.2 (3.4–7.6)
Age at T2	4.7±0.5 (3.6–5.6)	6.7±1.3 (5.6–9.8)
CPL score at T1	2.1±0.6 (1.0–3.0)	
CPL score at T2	2.5±0.8 (1.0–4.0)	2.8±0.9 (1.0–4.0)
[1]CPL RX	3.3±1.1 (1.0–5.0)	2.6±0.9 (1.0–4.0)
[2]COD	0.207±0.168 (0.07–0.601)	0.292±0.237 (0.0760–0.921)

[1]Average CPL score limbs derived from X-rays close to timepoint 1.
[2]Average corrected optical density

ELISA

The raw OD value of the NC was 0.073 (0.066-0.079). The CV1 and CV2 for the NC were 3.0 and 4.9 respectively. Average CPL scores at T1 and T2 were slightly negatively correlated with COD, although this did not reach statistical significance. In older stallions, a slightly positive correlation was found, , although this did not reach statistical significance. Our results are in agree-

ment with a previous study demonstrating that with increasing age, more AEAb's are produced (van Brantegem *et al.*, 2007a). A general linear model showed no significant contribution of COD and age at T1 on CPL scores at T2 (explaining 17% of the variation in CPL score at T2). COD increases with age (Figure 1), which is in agreement with a earlier study in warmblood horses (van Brantegem *et al.*, 2007a).

CONCLUSION

Based on the results in this study, we doubt that CPL susceptibility could be predicted early in life using this ELISA.

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