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Short Paper

SDS-PAGE analysis of urinary proteins in dogs with heartworm disease

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Summary

The aim of the study was to describe the urinary electrophoretic pattern of dogs with heartworm disease. Urine samples from 15 heartworm-infected and 15 healthy dogs were taken. Urinary specific gravity, urinary protein concentration and the urine protein/creatinine (U P/C) ratio were determined. Urine proteins were fractionated using SDS-PAGE. Results showed statistically significant differences for the U P/C ratio ($P<0.05$) but not for USG and urinary protein concentrations ($P>0.05$) between groups. Urinary protein SDS-PAGE analysis showed eight distinct bands in the urine of heartworm-infected dogs. The presence of proteins exclusively found in the urine of infected dogs suggests renal damage, even in cases of light proteinuria, indicating that SDS-PAGE is a sensitive method for the identification and characterisation of renal proteinuria in dogs with heartworm disease.

Key words: *Dirofilaria immitis*, SDS-PAGE, Proteinuria

Introduction

Heartworm disease (HD) is hyper-endemic in the Canary Islands (Montoya-Alonso *et al*., 2006; Montoya-Alonso *et al*., 2011).

HD affects many organs causing both acute and chronic inflammatory lesions in kidneys (Carretón *et al*., 2011). Azotaemia and albuminuria are seen in infected dogs (Atkins, 2010).

The analysis of urinary proteins is useful for the diagnosis and treatment of kidney disease; the different renal lesions show typical molecular weight urinary protein patterns (Bazzi *et al*., 1997). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is the preferred method for the study of renal proteinuria when low-and high-molecular weight proteins are present (Temmler and Nolte, 1995).

The aim of this study was to describe the urinary electrophoretic pattern of dogs with HD.

Materials and Methods

Urine samples were taken from 30 dogs, analysed for *Dirofilaria immitis* antigens using a commercial ELISA kit to detect circulating antigens (Anigen Rapid CHW Ag 2.0 Test Kit, Bio Note Inc., Gyeonggi-Do,q Korea).

Group 1 was formed by 15 healthy dogs negative for antigens of *Dirofilaria immitis*, without circulating microfilariae or signs of HD.

Group 2 consisted of 15 dogs positive for circulating *Dirofilaria immitis* antigens, with signs consistent with class 1 to 3 HD (Di Sacco and Vezzoni, 1992). These

animals did not show clinical signs of *Dirofilaria repens*.

Blood samples for haematology and for serum blood urea nitrogen (BUN) and creatinine were taken.

Urine samples were centrifuged for 5 min at 200 g. The sediment was examined, and part of the supernatant was frozen at -80°C until the electrophoresis analyses. The rest of the supernatant was used to determine the urinary specific gravity (USG) and protein concentration by pirogallol red and the molibdate method (Pupkova and Prasolova, 2007). The urine protein/ creatinine ratio (U P/C) value was also determined.

Urine proteins were fractionated by SDS-PAGE using 12.5% polyacrylamide gels following the procedure described by Laemmli (1970), with a Mini Protean III Cell system (Bio-Rad, Hercules, CA, USA). In each gel a molecular weight marker was included (Precision Plus Protein Kaleidoscope® with molecular weights from 250 kDa to 10 kDa) along with one urine sample from a healthy dog and seven from dogs infected by *Dirofilaria immitis*. The amount of protein loaded was 5 µg per sample. Gels were stained using the Coomassie method and were analysed with a gel scanner densitometer (Ultroscan XL, Pharmacia LKB Biotechnology Inc., Piscataway, NJ, USA). The graphic representations and molecular weights of the bands for each lane were obtained using the Ultroscan GSX software (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ, USA).

Means and standard deviations of urinary and molecular weight parameters were performed using the statistical software SPSS statistical package (version 17.0 for Windows). For statistical evaluation, the Chi-square test and a (2 × 2) contingency table were used. The correlation parameters were: electrophoretic patterns with gender and age.

Results

Group 1 presented plasmatic BUN and creatinine, USG, urinary protein concentration and U P/C ratio in ranges.

Plasmatic values from group 2 were: BUN of 37.9 ± 27.5 mg/dl and creatinine of 1.15 ± 0.65 mg/dl. Urinalysis showed USG values of 1.019 ± 0.018, urinary protein concentration was 57.69 ± 60.84 mg/dl, and U P/C value was 1.25 ± 1.63. There were no statistically significant differences for USG values and urinary protein concentrations between groups ($P>0.05$), but there were statistically significant differences in the BUN, creatinine, and U P/C ratio between groups ($P<0.05$).

Urinary proteins SDS-PAGE analysis of group 1 showed four distinct bands. The bands located in the molecular weight ranges of 100-110 kDa and 60-70 kDa were observed in all the urines. The bands located in the molecular weight ranges of 30-40 kDa and 10-20 kDa were observed in 26.66% (n=4) and 33.33% (n=5).

In group 2, 80% of dogs (n=12) presented eight distinct bands in electrophoresis (Table 1), located in the molecular weight ranges of 120-130 kDa (20%, n=3), 80-90 kDa (46.66%, n=7), 60-70 kDa (93.33%, n=14), 50-60 kDa (80%, n=12), 40-50 kDa (80%, n=12), 30-40 kDa (40%, n=6), 20-30 kDa (33.33%, n=5) and 10-20 kDa (26.66%, n=4) (Figs. 1 and 2). The results obtained in this research are not statistically significant ($P>0.05$).

Discussion

Previous studies have reported alterations in the glomerular function and proteinuria in dogs with HD (Atkins, 2010).

There are no previous reports of urinary proteins using the SDS-PAGE method in dogs with HD. In this study, the 12 positive

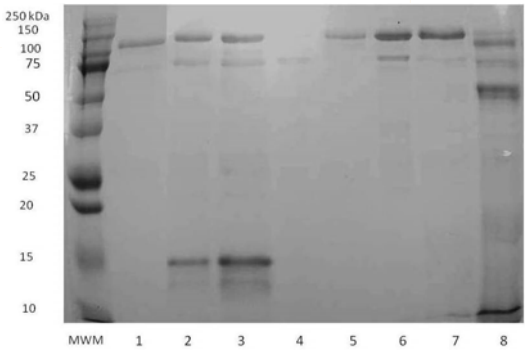

Fig. 1: SDS-PAGE analysis of urinary proteins in dogs with HD. MWM (molecular weight marker). Lane 1: Healthy dog, and Lines 2 to 8: Urines form dogs with HD

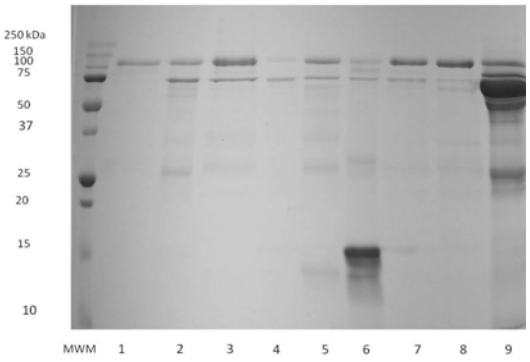

Fig. 2: SDS-PAGE analysis of urinary proteins in dogs with HD. MWM (molecular weight marker). Lane 1: Healthy dog, and Lines 2 to 9: Urines form dogs with HD

Table 1: U P/C and electrophoretic pattern observed in the urine of dogs with *Dirofilaria immitis*. Note how the electrophoretic pattern similar to healthy dogs occurs in urine with a low U P/C. In contrast, the urine with greater U P/C have glomerular or mixed pattern. Finally, half of dogs in class I disease have a electrophoretic pattern similar to healthy dog

U P/C	Heartworm class desease	Electrophoretic pattern
0.12	1	Mixed glomerular/tubular
0.13	1	Similar to healthy dog
0.35	1	Similar to healthy dog
0.12	1	Mixed glomerular/tubular
0.9	2	Mixed glomerular/tubular
0.11	2	Mixed glomerular/tubular
1.9	2	Glomerular
2.9	2	Glomerular
0.79	2	Glomerular
1.2	2	Glomerular
0.21	3	Glomerular
0.2	3	Similar to healthy dog
5.9	3	Mixed glomerular/tubular
1	3	Glomerular
2.98	3	Glomerular
Mean: 1.254, and SD: 1.609		

dogs showed bands of proteins not observed in urine of healthy dog. 58.33% (n=7) were proteins of high- and medium-molecular weight, that were previously described in dogs with glomerular damage (Zini *et al*., 2004). On the other hand, 41.66% (n=5) presented proteins of high-medium- and low-molecular weight, which corresponds to a mixed pattern (Zaragoza *et al*., 2003a).

Bands identified in the ranges of 100-110 kDa and 60-70 kDa in the group 1, may correspond to the molecular weight of transferrin and albumin, respectively, as previously described by Schultze and Jensen (1989); furthermore, no individual urine sample from the heartworm-infected dogs showed the presence of these bands. This can be attributed to transferrin decrease in blood concentration during inflammatory processes, decreasing the renal excretion of this protein (Gallardo *et al*., 2008).

The band located in the molecular weight range 30-40 kDa was identified only in 25% from group 1; however, in group 2 this band was identified in 40% of the urines (n=6). The molecular weight of this band is concordant with that of α1-microglobulin (27 kDa) (Lulich and Osborne, 1990). Further, the increase in the renal elimination of this protein is associated with tubular damage (Yanagisawa *et al*., 1983).

The band located in the molecular weight range 10-20 kDa, observed in 33.33% (n=5) of the urines from group 1 and 26.66% (n=4) of the urines from group 2, coincides with the molecular weight of β2-microglobulin (12 kDa) and lysozyme (14.40 kDa). Both proteins have been previously identified in healthy dogs (Nabity, 2011).

Viable microphilariae caused lymphocytic-plasmacytic infiltration on the interstitial cells of renal medulla. They also produced membranous and membrano-proliferative glomeruli lesions (Pasca *et al*., 2012). In our study, the bands observed in the urine from dogs with HD are located in the ranges 120-130 kDa, 80-90 kDa and 50-60 kDa, which correspond with proteins of high- and medium-molecular weight. Urinary proteins in dogs with glomerular dysfunction consist mainly of albumin and high molecular weight proteins (Lulich and Osborne, 1990). The present study demonstrates that dogs with HD have a glomerular proteuniria principally.

The band located in the molecular weight range 20-30 kDa was observed only in the urine from group 2 in 33.33% (n=5). This band may correspond to a short chain of the IgG, previously identified (Zaragoza *et al*., 2003b) in dogs with renal disease caused by leptospira.

A good correlation exists between the histopathological findings in dogs with renal disease and the results obtained by the electrophoretic analysis of proteinuria (Zini *et al*., 2004), therefore the presence of bands of proteins exclusively found in dogs with

HD suggests the presence of renal damage, even in cases of light proteinuria.

The principal limitation of the study was that we did not perform techniques to identify specific protein in urine, such as mass spectrometry, western-blot, 2-D electrophoresis. Nevertheless, we provide important information in dogs with HD that can be considered a preliminary result. Our intention is to establish a research which may use the frozen urine of these animals, because freezing did not alter the concentration of urine proteins (Zhou *et al*., 2006).

In conclusion, we found that the majority of animals with HD have glomerular proteinuria. The presence of protein bands found only in dogs infected by *Dirofilaria immitis* suggests that SDS-PAGE is a useful and sensitive method for the identification and characterisation of renal proteinuria in dogs with HD, even when they show a light proteinuria and renal biochemical parameters within the normal concentrations.

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