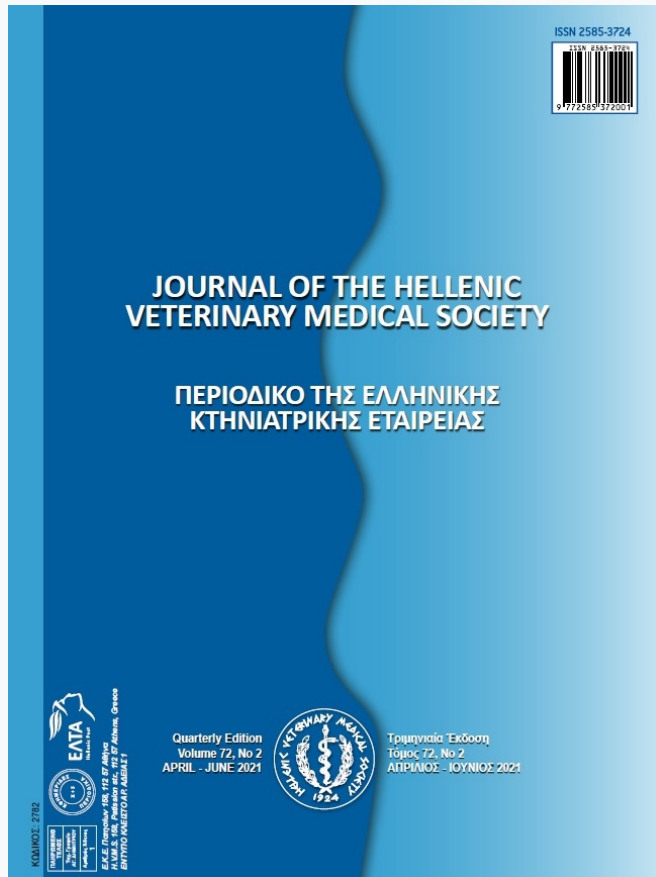


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R.D. BAKA, C.K. KOUTINAS, L.V. ATHANASIOU, Z.S. Polizopoulou

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## Correlation of a quantitative and a semi-quantitative method for proteinuria detection in chronic kidney disease in dogs

R.D. Baka<sup>1</sup>, C.K. Koutinas<sup>2</sup>, L.V. Athanasiou<sup>3</sup>, Z.S. Polizopoulou<sup>1</sup>

<sup>1</sup>Diagnostic Laboratory, Faculty of Veterinary Medicine, School of Health Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece

<sup>2</sup>Clinic of Companion Animals, Faculty of Veterinary Medicine, School of Health Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece

<sup>3</sup>Department of Medicine, Faculty of Veterinary Medicine, University of Thessaly, Karditsa, Greece

**ABSTRACT:** Proteinuria can arise in various physiologic and pathologic conditions. Persistent proteinuria without any abnormalities detected in urine sediment is indicative of chronic kidney disease and has great diagnostic value as it is used for the categorization of the patient on IRIS (International Renal Interest Society) staging system. There are several techniques for urine protein measurement including the semi-quantitative/qualitative (urine dipstick, sulfosalicylic acid turbidimetric test and Heller's reaction test) and the quantitative tests (urine protein to creatinine ratio (UPC) and microalbuminuria assay). The purpose of this study was to correlate the semi-quantitative Heller's reaction test for proteinuria detection, with the UPC in urine samples from 89 dogs with chronic kidney disease. The non-parametric *Spearman's correlation coefficient* was used to correlate Heller's reaction test with UPC in urine samples from dogs with chronic kidney disease in proteinuria detection. Correlation analysis revealed a statistically significant positive and moderate correlation between the Heller's reaction test and UPC ( $r(89)=0.510$ ,  $p<0.0001$ ) which was slightly improved when  $USG>1010$  ( $r(72)=0.541$ ,  $p<0.0001$ ) (urine specific gravity). Heller's reaction test might be a useful alternative to detect proteinuria when UPC is not available in the clinical setting, however it cannot be used interchangeably with UPC for the IRIS sub-staging of chronic kidney disease (CKD).

**Keywords:** dog, Heller's reaction test, proteinuria, UPC, urinary system

Corresponding Author:  
Rania D. Baka, 11, StavrouVoutyra str., 54627 Thessaloniki, Greece  
E-mail address: raniadbaka@gmail.com

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## INTRODUCTION

Proteinuria can result from a variety of proteins excreted or lost into the urine. Albuminuria, the presence of albumin in urine, is the main contributor to overt proteinuria and has the greatest clinical significance in dogs and cats as it seems to be a multifactorial and frequent finding (Sink and Weinstein, 2012; Grauer, 2011; Lyon et al., 2010). Proteinuria can arise in several different physiologic and pathologic conditions, but persistent proteinuria associated with inactive urine sediment is consistent with chronic kidney disease (Grauer, 2011). Detection of proteinuria includes both screening routine analysis and use of advanced laboratory methods (Sink and Weinstein, 2012). Detection and measurement of proteinuria is essential, in order to classify the patients using the IRIS staging system (International Renal Interest Society), to administer the appropriate medication, to prevent further kidney damage and to establish the prognosis.

A urine dipstick colorimetric test is the typical first-line screening test for the detection of proteinuria and/or albuminuria (Stockham and Scott, 2008). The protein test methodology used for dry reagent strips is able to detect albumin better than other proteins (Strasinger and DiLorenzo, 2008). However, false positive reactions for protein are common and limit the test's utility (Grauer et al., 2004). Semi-quantitative tests are frequently used to confirm positive reactions for protein on a urine dipstick test (Grauer, 2011; Lyon et al., 2010). The most commonly reported semi-quantitative measuring methods are the urine dipstick and the sulfosalicylic acid test (SSA) (Sink and Weinstein, 2012). Heller's reaction test is a turbidimetric test, quite simple to perform using a test tube, a small amount of nitric acid and equal amount of urine sample (Figure 1), which should be laid slowly over the nitric acid layer (Figure 2). The two liquids (nitric acid and urine) should not be mixed during the procedure (Figure 3). Heller's reaction test is a qualitative method (Figure 4). The test is positive when urine reacts with nitric acid and a white ring of variable thickness forms in the tube (Medaille and Brien-Marshall, 2008). The quantitative methods include urine protein to creatinine ratio (UPC) and microalbuminuria assays (Sink and Weinstein, 2012). The UPC has become the gold standard test for detecting proteinuria and should be run on any patient with evidence of proteinuria tested by urine dipstick or positive SSA (Herley and Langston, 2012). The UPC, performed on a single random urine sample, is

closely correlated to the 24-hour urine protein quantification (Le Vine et al., 2010; Adams et al., 1992; Monroe et al., 1989).



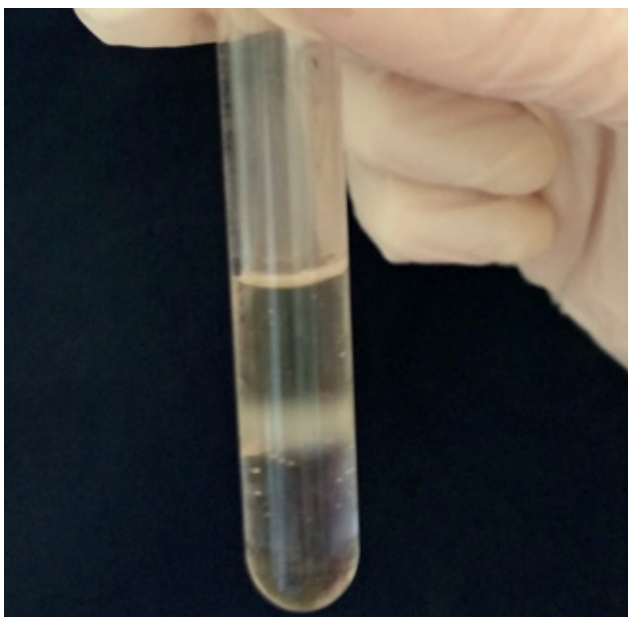
**Figure 1.** Materials needed for Heller's reaction test (tube, nitric acid, urine sample).



**Figure 2.** Put a small amount of nitric acid (approximately 1ml) in the tube.



**Figure 3.** Add equal amount of urine in the tube by layering the urine sample above the nitric acid, caution should be given in order not to mix the samples.



**Figure 4.** Urine proteins react with nitric acid and the white ring appears (positive Heller's reaction test).

Despite the availability of tests detecting proteinuria, occasionally quantitative methods, which are more precise, cannot be performed in a clinical setting. In these cases, a semi-quantitative test could be used as an alternative practical method to detect proteinuria.

The purpose of this study was to correlate the semi-quantitative Heller's reaction test and the quantitative UPC test in detecting proteinuria in 89 dogs with chronic kidney disease (CKD) and to evaluate the utility of a semi-quantitative test when UPC is not available.

## MATERIALS AND METHODS

This retrospective study involved the medical records of 89 dogs admitted to the Companion Animal

Clinic of Aristotle University from May 2011 to December 2014. Urine samples from 89 canine patients with chronic kidney disease were included in this study. Patients with clinical (dysuria, pyuria, hematuria, pyometra, vaginitis, prostatitis) or laboratory evidence (active urine sediment, positive urine culture test) of lower urinary or genital tract infection were excluded from the study. Urine samples collected via free catch using a sterilized container or cystocentesis were included for analysis. For those dogs, whose urine was collected via free catch, appropriate cleaning of the external genitalia was performed prior to voiding. A complete urinalysis was performed in all samples including measurement of urine specific gravity, urine dipstick colorimetric test, Heller's reaction test, microscopic evaluation of urine sediment, urine protein/creatinine ratio (UPC) and urine culture. Urinalyses were performed in the Companion Animal Clinic, UPC measurements were performed in the Diagnostic Laboratory, School of Veterinary Medicine, Aristotle University and the urine cultures were performed in a private veterinary diagnostic laboratory (Vet Analyseis Lab, Larisa, Greece). Urine specific gravity was measured in a refractometer (American Optical Leica TS Meter, American Optical Co, Massachusetts USA) compensated for temperature. The urine dipsticks used were the Combi Screen 10 SL PLUS, Analyticon, Germany. Urine protein/creatinine ratio was measured in Vital Lab Flexor E, (Spankeren, The Netherlands) spectrophotometric analyser. The coefficient of variation (CV) (%) at room temperature for mean urine protein concentration of 8.8, 19.2 and 82.4 mg/dL was 9.5, 4.35 and 3.49, respectively while for the mean urine creatinine concentration of 20.64, 41.80 and 64.60 mg/dL it was 8.2, 4.6, and 9.98, respectively. UPC was performed in the supernatant urine samples (after centrifugation). Both urine analyses and Heller's reaction test were done within 15 minutes from sample collection while UPC measurement was completed 2 hours after sample collection.

Due to the nature of the Heller's reaction test (qualitative), we adapted the quantification of the test (Athanasίου et al., 2014) in order to study the correlation between the two methods. The quantification of Heller's reaction test was based on the thickness of the white ring. In the negative test no white ring formation was observed. Positive tests were graded using a 4-scale classification system based on the height of the white ring measured with a ruler. According to this system, Heller +1 was defined as white ring of 1mm, Heller +2 as a white ring of 2mm, Heller +3 as a white ring of 3mm and Heller >+3 as a 4mm white ring (Figures 1-4).



**Figures 1-4.** Procedure of Heller's reaction test.

Due to the nature of the variable Heller (discrete measurements), the non-parametric *Spearman's correlation coefficient* was used in order to study the correlation between UPC and Heller concentrations. In order to assess a better correlation between the two methods, the dogs were subdivided in two groups based on urine specific gravity (USG), the first group of dogs with  $USG > 1010$  and the second group with  $USG \leq 1010$ . Since 1010 was the mean value of isosthenuria, it was set as the cut-off value.

Statistical analyses were performed using the R environment (R core team, 2013, Vienna, Austria).

## RESULTS

The study population consisted of 89 patients belonging in 18 different breeds. The majority of animals (42/89)(47.1%) were mixed breed dogs. Thirty-six (40.4%) were middle-aged dogs (>5-10 years old for small breeds and >3-7 years old for large breeds), 24/89 (26.9%) were young adult dogs and 26/89 (29.2%) were old dogs. Only 3 young (<1 year) dogs (3.3%), diagnosed with chronic kidney disease, were enrolled in the study. The median age of the population was 66 months (5.5 years) (range from 5 months to 204 months). Most of the study population was male intact dogs (49/89)(55%), followed by 20 female neutered (22.4%), 15 female intact (16.8%) and 5 male castrated dogs(5.6%). Table 1 shows the results of Heller's reaction test in the study population. Table 2 shows the results of UPC ratio after categorization of cases as non-proteinuric (UPC: 0-0.2), borderline proteinuric (UPC:0.21- 0.5) and proteinuric (UPC>

0.5), according to the IRIS substaging system (IRIS staging of CKD modified 2019). Based on the results, 8/89 dogs (8.9%) had a negative Heller's reaction test (no proteinuria). Based on UPC measurement, 7/89 dogs (7.8%) did not have proteinuria. The remaining 82/89 dogs (92.1%) had either borderline proteinuria (7/89)(7.8%) or prominent proteinuria (75/89) (84.2%) (UPC>0.5) (Table 2). The median UPC value was 3.67 (range from 0 to 61.5).

**Table 1.** Heller's reaction test in 89 dogs with chronic kidney disease that presented in Companion Animals Clinic from 2011 to 2014

Heller's reaction test	Number of dogs (Total 89)
0	8
+1	10
+2	25
+3	40
>+3	6

**Table 2.** UPC results in 89 dogs with chronic kidney disease that presented in Companion Animals Clinic from 2011 to 2014

UPC	Number of dogs (Total 89)
0-0.2	7
0.21-0.5	7
>0.5	75

Data analysis showed a statistically significant positive and moderate correlation between UPC and Heller's reaction test ( $r(89)=0.510$ ,  $p<0.0001$ ) when all 89 samples were used. The correlation was slightly improved ( $r(72)=0.541$ ,  $p=0.0001$ ) in samples with  $USG>1010$ . In samples with  $USG \leq 1010$  no

significant correlation was observed, ( $r(17)=0.3208$ ,  $p=0.209$ ).

## DISCUSSION

When UPC is not readily available, Heller's reaction test might be a useful alternative to detect proteinuria for urine samples with  $USG>1010$ . Semi-quantitative and quantitative methods have been compared in previous studies, in order to find alternative methods for quick and accurate assessment of proteinuria (Lyon et al., 2010; Garner and Wiedmeyer, 2007; Mardell and Sparkes, 2006; Welles et al., 2006). In a previous study, qualitative, semi-quantitative and quantitative methods have been compared in order to assess albuminuria in urine samples of clinically healthy dogs and cats (Lyon et al., 2010). In particular, the results of urine dipstick, sulfosalicylic acid (SSA) test and UPC were compared to those of an albumin-specific ELISA, to show that albuminuria detection should be interpreted cautiously when urine dipstick, SSA test and UPC are used interchangeably due to the high percentage of false-positive results when trace or greater considered a positive result (Lyon et al., 2010). According to the authors, the false-positive rate decreased when trace reactions were excluded in both analyses (9.3% and 11.5% for the urine dipstick and SSA test, respectively) and the specificity was increased to 98.9% for the urine dipstick and to 99% for the SSA test, when both trace and 1+ reactions were excluded. In contrast with our study, the results of this work had not been influenced by the urine specific gravity, probably because only healthy animals were evaluated; however, they mention that the exclusion of trace reactions can increase the specificity of both methods. In the current study, all urine samples were moderately to severely proteinuric and the low urine patients specific gravity (due to CKD) influenced the correlation of the two methods. The two methods were positively and moderately correlated when  $USG>1010$  and thus Heller's reaction test can be used as a quick alternative in urine samples of dogs with CKD. In another study, there was a good correlation between results of urine dipstick, SSA test and UPC and a point-of-care microalbuminuria immunoassay (Garner and Wiedmeyer, 2007). However, in this study, samples that had mildly positive urine dipstick results (trace or 1+), there was a high false-positive rate (69%), indicating that the use of a urine dipstick to detect albuminuria in dogs had poor diagnostic value at lower protein or albumin concentrations (Garner and Wiedmeyer, 2007). Similar to our findings, cor-

relation of diagnostic methods for proteinuria detection was shown to be more sensitive in urine samples with  $USG>1010$  (Garner and Wiedmeyer, 2007). In our study, no significant correlation was found between Heller's reaction test with UPC, in urine samples with  $USG\leq 1010$ , indicating that in much diluted urine, Heller's reaction cannot estimate proteinuria accurately. The findings of the current study were in parallel with the results of previous studies comparing two different methods of proteinuria detection where a positive correlation of the two methods was demonstrated. As Heller's reaction test is not a commonly-used qualitative test for proteinuria, this is the first study that correlated Heller's reaction test with the quantitative UPC test in dogs with CKD.

Urine samples were collected by cystocentesis or free catch, based on a previous study, in which there was a strong association between UPC values regardless of the sample collection method (Beatrice et al., 2010).

Proteinuria has been incriminated as an independent mediator of progression of renal disease (Walls, 2001; Remuzzi, 1999; Burton and Harris, 1996). There was a study showing that  $UPC>1$  in canine patients with chronic kidney disease at the time of initial diagnosis of CKD can cause uremic crisis and death sooner than in patients with  $UPC<1$  (Jacob et al., 2005). Thus, it is essential to detect proteinuria in canine patients with CKD as soon as possible.

The nature of the study and the usage of urine samples collected via free catch despite the appropriate cleaning of the external genitalia prior voiding were the main limitations.

This was the first study in which Heller's reaction test (a qualitative method), an easy, quick and practical method for proteinuria detection, was correlated with the quantitative UPC method in dogs with CKD. However, it cannot be used interchangeably with UPC especially in diluted urine ( $USG<1010$ ). Nevertheless, it might be a good first-choice test to detect proteinuria in urine samples with  $USG>1010$ , when quantitative UPC method is not available in the clinical setting.

## CONFLICT OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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