Effects of storage conditions on results for quantitative and qualitative evaluation of proteins in canine urine

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OBJECTIVE
To investigate effects of storage conditions on the canine urine protein-to-creatinine ratio (UPC) and on SDS–agarose gel electrophoresis (AGE) of urinary proteins.

SAMPLE
Urine specimens from 20 proteinuric (UPC > 0.5) and 20 nonproteinuric (UPC ≤ 0.2) dogs.

PROCEDURES
UPC and SDS-AGE were performed on urine specimens stored at room temperature (20°C) and 4°C for up to 5 days and at –20° and –80°C for up to 360 days; some specimens were subjected to 3 freeze-thaw cycles. Results were compared with those obtained for fresh urine specimens.

RESULTS
UPC was not affected by storage at room temperature or by freezing. A decrease in UPC was observed for specimens from nonproteinuric dogs after 5 days at 4°C (10%) and from both groups after 90 days at –20° and –80°C (≤ 20% and ≤ 15%, respectively). The SDS-AGE profiles revealed no visual changes regardless of duration of storage for specimens stored at room temperature, 4°C, and –80°C, except for 1 profile after 360 days at –80°C. Repeated freeze-thaw cycles did not affect SDS-AGE profiles. Appearance or strengthening of high-molecular-weight bands that could alter interpretation was evident in SDS-AGE profiles after storage at –20°C for ≥ 15 days (31/40 dogs).

CONCLUSIONS AND CLINICAL RELEVANCE
Storage of urine at –20° or –80°C for up to 1 year influenced the UPC without affecting clinical interpretation. Storage of urine specimens at –20°C impaired visual analysis of SDS-AGE. When SDS-AGE cannot be performed on fresh or recently refrigerated urine specimens, storage at –80°C is recommended. (*Am J Vet Res* 2017;78:990–999)

Urinalysis is of central interest for canine nephrology, and proteinuria is considered a key factor affecting the progression of renal diseases and is one of the earliest abnormalities found in some canine familial progressive glomerular diseases.[1–6] Early and accurate recognition of persistent renal proteinuria is essential to provide appropriate patient care. For human medicine, 24-hour urinary protein excretion is the criterion-referenced standard for urinary protein quantification.[7] However, this method is impractical for dogs, and studies[4,8–10] have confirmed that UPC for a randomly collected urine specimen correlates well with daily protein excretion. To provide useful clinical information, knowledge of analytic and preanalytic factors that could affect UPC determination is required. To our knowledge, the effect of storage of canine urine specimens on UPC measurement has been examined in only 1 study.[11] Results of that study indicated that UPC increased after storage for 12 hours at both 20° and 4°C. This change in the UPC was mainly attributable to an increase of urine protein concentration, rather than to a decrease of urine creatinine concentration. Additionally, no significant changes in UPC were detected after storage at –20°C for 3 months.

Qualitative evaluation of urinary proteins also plays a central role in the early diagnosis of renal diseases in humans, although renal histologic evaluation is the criterion-referenced standard for characterizing renal lesions.[12–14] Obtaining renal tissue is invasive and expensive and is not always recommended, especially for dogs with systemic hypertension or severe azotemia or healthy dogs with a moderately elevated UPC.[15,16] Concentrations of urinary markers increase in dogs with various nephropathies before the onset of azotemia, which could be helpful for localizing the origin of the proteinuria.[17–20] Unfortunately, quantitative measurements of urinary

ABBREVIATIONS

AGE	Agarose gel electrophoresis
CV	Coefficient of variation
IRIS	International Renal Interest Society
UPC	Urine protein-to-creatinine ratio
USG	Urine specific gravity

markers are expensive and not routinely available. Determinations of low-molecular-weight (< 60 kDa) and high-molecular-weight (> 80 kDa) urine proteins by use of electrophoresis have been used to localize renal lesions in proteinuric dogs.[21–25,a] It was found that results for SDS-AGE, compared with results for examination of kidney biopsy specimens, had good sensitivity for use in identifying glomerular and tubular lesions in dogs.[24] Electrophoresis of urine proteins is a noninvasive, inexpensive, and commercially available method for characterizing proteinuria. Additionally, in a recent study[20] of dogs, there was a high correlation between SDS-AGE qualitative findings and quantitative measurements of urine albumin and IgG.[20] Because protein bands in gels may be used for identification purposes[26] and interpretation of results of electrophoresis of urine proteins is based on visual assessment, it is important to know whether the urine SDS-AGE pattern can be affected by storage conditions. In human medicine, intensity of the albumin band decreased and 2 low-molecular-weight bands appeared on patterns obtained for urine specimens stored at –20°C for 12 months, but no obvious change was detected for specimens stored at 20°C for 24 hours or at –70°C for 12 months.[27,28] To our knowledge, no information is available regarding the effect of storage condition on results for electrophoresis of canine urinary proteins.

Because storage conditions might introduce substantial variations in quantitative and qualitative results, they should be taken into account before designing a clinical study that involves the use of stored urine specimens. Therefore, the objective of the study reported here was to assess the effects of storage temperature and duration as well as repeated freeze-thaw cycles on results for electrophoresis (SDS-AGE) of urinary proteins and the UPC in specimens obtained from proteinuric and nonproteinuric dogs.

Materials and Methods

Sample

Urine specimens were obtained from 20 proteinuric client-owned dogs and 20 nonproteinuric client- or staff-owned dogs. This prospective study was conducted at the Internal Medicine consultation of the National Veterinary School of Toulouse between October 2012 and June 2014. Informed consent was obtained from the owners of all dogs included in the study, and the study design was approved by the regional ethical committee.

Proteinuric dogs were eligible for the study if they had renal proteinuria and required a urinalysis as part of the diagnostic evaluation. Dogs were proteinuric (UPC > 0.5), as defined by the IRIS staging system.[6] Additionally, it was required that plasma creatinine concentration was measured within 1 week after inclusion, and urine specimens containing ≥ 500 mg of protein/L were obtained to facilitate qualitative visual analysis of gel electrophoresis. Nonproteinuric (UPC ≤ 0.2) dogs, as defined by the IRIS staging system,[6] between 1 and 8 years of age were eligible for inclusion in the study. Dogs had to be healthy as determined on the basis of the medical history and results of a complete physical examination, urinalysis, CBC, and biochemical analysis (creatinine, glucose, albumin, total protein, alanine aminotransferase, alkaline phosphatase, sodium, potassium, chloride, and bicarbonates). Dogs with bacteriuria, an inflammatory urine sediment (defined as > 5 WBCs/hpf [40X magnification]), or gross hematuria (defined as > 250 RBCs/hpf) were excluded from the study because these abnormalities may affect the UPC.[29]

Specimen collection and processing

Urine specimens were collected (day 0) via cystocentesis with ultrasonographic guidance by use of a 0.6 X 25-mm needle[b] and 10-mL syringe[c] or free catch from the midstream phase of micturition, because natural voiding does not affect the UPC when the sediment is not inflammatory.[30] At least 10 mL of urine was collected from each dog and immediately placed in a 15-mL conical tube.[d]

Urine specimens were processed within 2 hours after collection. They were centrifuged[e] at 250 X *g* for 5 minutes. Supernatant was harvested and placed into seventeen 1.5-mL plastic tubes[f] (15 aliquots of 500 µL and 2 aliquots of 1,000 µL). The remaining supernatant and sediment were used for direct microscopic urinalysis.

One 500-µL aliquot was used immediately for UPC determination and SDS-AGE. The other aliquots were stored at various temperatures. A set of three 500-µL aliquots was stored at room temperature (20°C), and another set of three 500-µL aliquots was stored at 4°C; 1 aliquot from each set was analyzed on days 1, 2, and 5. The remaining 500-µL aliquots were frozen at –20°C (n = 4) and –80°C (4); 1 aliquot for each freezer temperature was analyzed on days 30, 90, 180, and 360. In addition, the two 1,000-µL aliquots were frozen at –20° and –80°C and analyzed on day 15.

The influence of repeated freeze-thaw cycles was assessed on the two 1000-µL aliquots. After they were thawed for analysis on day 15, they were refrozen and subsequently thawed and analyzed on day 90 and 360. These aliquots were stored at the respective storage temperatures between analyses.

Urinalysis

Urinalysis was performed on fresh specimens within 2 hours after collection. Urinalysis was performed in accordance with the Clinical and Laboratory Standards Institute recommendations.[31] A urinary dipstick test[g,h] was performed immediately on fresh urine specimens. The USG was determined with a handheld refractometer,[i] which was calibrated daily with distilled water. Bacteriuria was defined as the presence of free or intracellular bacteria observed microscopically on dried, stained, centrifuged preparations of urine sediment. Supernatant was used to

suspend the remaining sediment for microscopic examination of a wet preparation. Numbers of RBCs, WBCs, spermatozoa, epithelial cells, casts, and crystals were expressed as the mean count/10 hpfs.

UPC and SDS-AGE

The UPC was determined, and results of SDS-AGE were obtained for each of the 17 aliquots on the previously described days of the study. Batch analysis of samples was performed (3 batches for the study). Frozen specimens were allowed to thaw for approximately 30 minutes at room temperature and homogenized in a vortex device before analysis. Urine creatinine concentration was measured by use of the Jaffé method[j]; urine specimens were diluted (1:40) before analysis to satisfy linearity requirements. Urinary protein concentration was measured by use of a pyrogallol red method[k] and an analyzer.[l] Two control urines[m,n] were used for calibration and quality control analysis, which were performed before each batch analysis. Methods and calculation of mean bias, interassay CV, and total error observed were performed as recommended elsewhere.[32] Total error observed was calculated as 2CV + bias.

After the UPC had been determined, SDS-AGE was performed by use of nonreducing conditions with a semiautomated system[o] in accordance with the manufacturer's recommendations. Briefly, 80 µL of the final urine supernatant was mixed with 20 µL of an SDS–bromophenol blue solution provided by the manufacturer. An aliquot (5 µL) of this mixture was loaded on agarose gels (agarose concentration, 50 g/L), and migration, staining with acid-violet, and drying were conducted with the semiautomated system. The limit for visible bands was 15 mg/L. Urine supernatants that contained > 1.5 g of protein/L and > 2 g of protein/L were diluted (1:1 and 1:2, respectively) in distilled water. Each gel contained 5 wells; 4 wells were filled with urine specimens, and the fifth was filled with a molecular marker solution[p] from the manufacturer that contained lysozyme (14.3 kDa), triose-phosphate isomerase (26.6 kDa), bovine albumin (66 kDa), and human IgG (150 kDa).

After gels had dried, color copies were made to facilitate further comparison. Gels and copies were immediately visually analyzed separately by 2 investigators (MLT and RL). Only qualitative analyses of migration patterns were performed because of the low accuracy of unidimensional urine protein electrophoresis to quantify protein fractions.[33] Gels were stored in the dark in document cases for 360 days and were reevaluated at the end of that period. Bands that migrated the same distance as the bovine albumin and human IgG standards were considered to be albumin and IgG bands, although the products were not definitely verified. The other bands were characterized as low-molecular-weight bands or high-molecular-weight bands on the basis of their position relative to the albumin band.

Statistical analysis

To enable investigators to better evaluate stability of urine proteins, results for proteinuric and nonproteinuric groups were analyzed separately because minimal changes may be more relevant at a low UPC rather that at a high UPC. Differences between the proteinuric and nonproteinuric groups for age, plasma creatinine concentrations, and urinary variables obtained at day 0 (USG and the mean number of casts, spermatozoa, WBCs, RBCs, epithelial cells, and crystals/10 hpfs; urine protein and creatinine concentrations; and UPC) were analyzed by use of the Mann-Whitney *U* test. Effect of age, sex, and plasma creatinine concentration on results of the initial analysis were assessed with a general linear model. Normality for all variables measured on day 0 was assessed with the Anderson-Darling test.

For each storage temperature, influence of the storage duration on urine protein and creatinine concentrations and UPC was assessed by use of the Dunnett test, with results for day 0 serving as the control values. For specimens that were subjected to repeated freeze-thaw cycles, specimens stored for the same duration at the same temperature and that were not subjected to freeze-thaw cycles were used as control specimens. To assess differences that were not considered attributable to analytic variation, UPC values obtained at the various time points were compared with the UPC on day 0 ± (2.77 × CV). The mean CV determined for the batch analysis was used for these calculations. Therefore, an actual change was identified when the UPC values obtained for specimens after storage at various temperatures for various durations were greater than the UPC on day 0 ± (0.238 × UPC on day 0).

All tests were performed with computer software.[q] For all analyses, values of $P < 0.05$ were considered significant. All numerical results were reported as median and range because none of the tested variables was normally distributed.

Effects of storage temperature and duration on results of SDS-AGE were visually assessed by comparing all profiles with that on the color copy for the fresh specimen obtained on day 0. For specimens that were subjected to repeated freeze-thaw cycles, profiles obtained with specimens stored for the same duration at the same temperature and that were not subjected to freeze-thaw cycles were used as control specimens. The presence of any modification (appearance of bands, disappearance of bands, or increases or decreases in the intensity of bands) was recorded.

Results

Sample

All recruited dogs met the inclusion criteria. The nonproteinuric group consisted of 10 males (5 castrated and 5 sexually intact) and 10 females (5 spayed and 5 sexually intact); there were 5 mixed-breed dogs, 3 Beagles, 3 Australian Shepherds, and 1 each of 9 other

breeds. The proteinuric group consisted of 15 males (4 castrated and 11 sexually intact) and 5 females (3 spayed and 2 sexually intact); there were 3 Yorkshire Terriers, 2 Labrador Retrievers, 2 mixed-breed dogs, and 1 each of 13 other breeds. Proteinuric dogs (median, 10 years; range, 1 to 14.5 years) were significantly ($P < 0.001$) older than nonproteinuric dogs (median, 2.5 years; range, 1 to 7.5). Proteinuric dogs were affected by various diseases, including neoplasia (n = 6), hyperadrenocorticism (3), leptospirosis (2), leishmaniasis (2), immune-mediated diseases (3), acute pancreatitis (2), chronic kidney disease (1), and cervical spondylopathy (1). The plasma creatinine concentration at the time of inclusion in the study was within the laboratory reference interval (< 133 µmol/L) for all dogs, except for 2 (a neutered male dog with a pheochromocytoma [148 µmol/L] and a male dog with recently diagnosed [3 days before study inclusion] leptospirosis [589 µmol/L]). Plasma creatinine concentration did not differ significantly ($P = 0.52$) between groups.

Collection of urine specimens

All specimens, except for 3, were collected by use of cystocentesis. Collection via free catch was used for medical reasons for 2 dogs (a spayed female dog with immune-mediated thrombocytopenia and a male dog with acute pancreatitis) and for convenience reasons for a healthy male dog. Urinalysis revealed no evidence of inflammation or hematuria in any of the specimens.

Urinalysis

Mean bias, interassay CV, and total error observed were calculated for UPC determination **(Table 1)**. Urinalysis revealed significant differences between the proteinuric and nonproteinuric groups for several variables **(Table 2)**.

Urine protein and creatinine concentrations and UPC

Day 0—Urine protein and creatinine concentrations and the UPC in specimens analyzed on day 0 were summarized (Table 2). Specimens from proteinuric dogs had a significantly higher urine protein concentration ($P < 0.001$), UPC ($P < 0.001$), and number of casts/10 hpfs ($P = 0.001$) and a significantly lower USG ($P < 0.001$), urine creatinine concentration ($P < 0.001$), and number of WBCs/10 hpfs ($P = 0.011$). The UPC was not significantly affected by sex ($P = 0.42$) or plasma creatinine concentration ($P = 0.59$).

Effects of storage time and temperature and repeated freeze-thaw cycles—The UPC could not be determined on day 5 for 4 specimens (1 proteinuric and 3 nonproteinuric) stored at 4°C because of technical issues. Distributions of the UPC after storage at various temperatures and for various durations were plotted **(Figure 1)**. Urine protein concentration, urine creatinine concentration, and UPC after storage at various temperatures and for various durations, compared with values for samples on day 0, were summarized for each group (Table 2).

Urine protein and creatinine concentrations and the UPC for specimens from both groups were not affected by storage at room temperature or by repeated freeze-thaw cycles at –20° and –80°C. Urine creatinine concentration for specimens from both groups

Table 1—Interassay CV, mean bias, and total error observed (TE_{obs}) for the urinary protein concentration, urinary creatinine concentration, and UPC for control solutions used for calibration and quality control before each batch analysis.

	Protein				Creatinine				UPC			
Batch	Target (mg/L)	CV (%)	Bias (%)	TE_{obs} (%)	Target (mg/L)	CV (%)	Bias (%)	TE_{obs} (%)	Target (mg/L)	CV (%)	Bias (%)	TE_{obs} (%)
1	341	2.7	1.2	6.6	652	6.2	0.6	13.0	0.52	5.7	0.01	11.4
2	246	6.3	2.0	14.6	660	7.8	1.0	16.6	0.37	9.3	0.03	18.6
3	227	8.0	1.1	17.1	651	8.6	1.3	18.5	0.35	10.9	0.01	21.8
Mean	271	5.7	1.4	15.9	654	7.5	1.0	16.0	0.41	8.6	0.02	17.3

The TE_{obs} was calculated as 2CV + bias as reported elsewhere.[32]

Table 2—Median (range) values for urinalysis of fresh specimens (day 0) obtained from 20 nonproteinuric and 20 proteinuric dogs.

Variable	Nonproteinuric	Proteinuric	*P* value*
USG	1.040 (1.016–1.050)	1.019 (1.006–1.046)	< 0.001
RBCs (mean No./10 hpfs)	1.9 (0–20.0)	2.4 (0.1–20.0)	0.370
Epithelial cells (mean No./10 hpfs)	0.3 (0–10.1)	1.7 (0–13.5)	0.060
WBCs (mean No./10 hpfs)	0.2 (0–2.5)	0 (0–4.9)	0.011
Spermatozoa (mean No./10 hpfs)	0 (0–20.0)	0 (0–10.0)	0.631
Casts (mean No./10 hpfs)	0 (0–3.0)	0.5 (0–3.2)	0.001
Crystals (mean No./10 hpfs)	0 (0–3.2)	0 (0–0.2)	0.107
Protein (mg/L)	206 (133–703)	1,116 (556–3,888)	< 0.001
Creatinine (mg/L)	2,475 (975–4,955)	619 (307–3,162)	< 0.001
UPC	0.09 (0.05–0.16)	1.82 (0.50–7.92)	< 0.001

*Values were considered significant at $P < 0.05$.

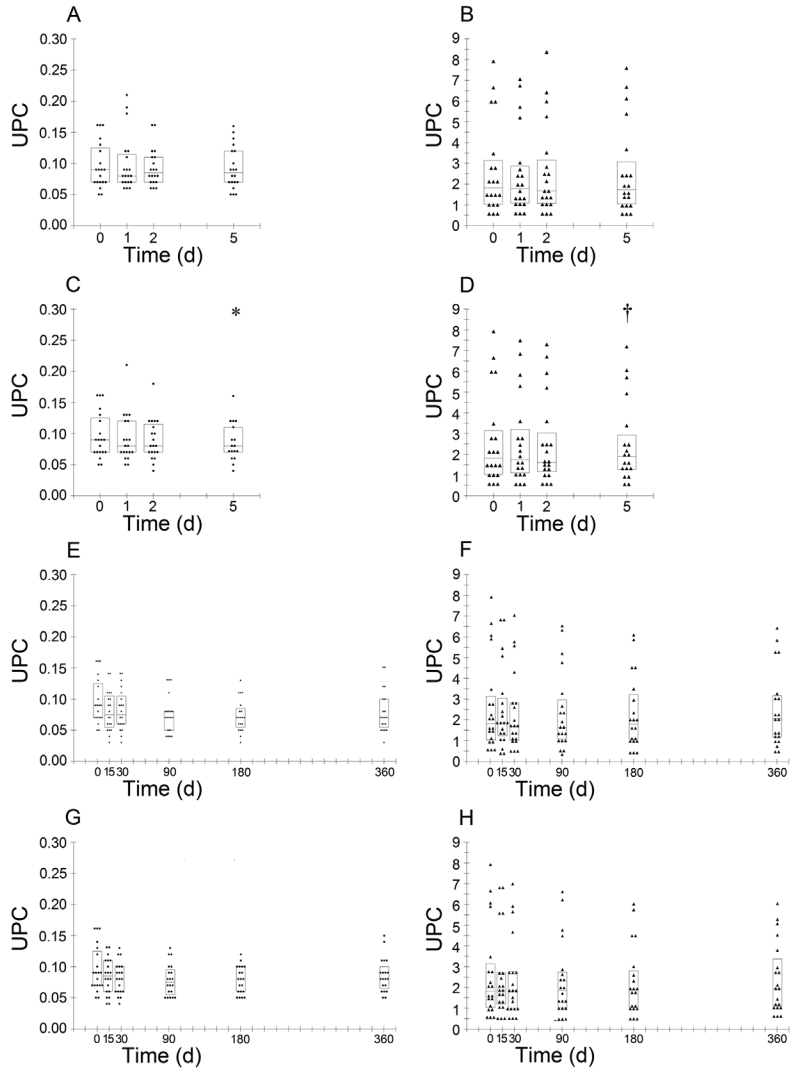

Figure 1—Box plots of the UPC for urine specimens obtained from 20 nonproteinuric (UPC ≤ 0.2 [A, C, E, and G]) and 20 proteinuric (UPC > 0.5 [B, D, F, and H]) dogs and stored at room temperature (20°C [A and B]), 4°C (C and D), –20°C (E and F), and –80°C (G and H). Day of urine collection was designated as day 0. Each symbol represents results for 1 dog. For each box, the bottom and top represent the 25th and 75th percentiles, respectively, and the horizontal line represents the median. *Represents results for only 17 dogs. †Represents results for only 19 dogs.

was not affected by storage at –20° and –80°C. Urine protein concentration and the UPC were not affected for specimens of proteinuric dogs after storage at 4°C.

The UPC decreased marginally, but significantly, for specimens from nonproteinuric dogs after storage at 4°C for 5 days and storage at –20° and –80°C for > 15 days, whereas the UPC only decreased significantly for specimens from proteinuric dogs stored at –20°C for 30 to 180 days and at –80°C for 90 to 180 days. The UPC differed significantly for several urine specimens stored at various temperatures and for various durations; these differences were mainly associated with a decrease of the UPC for specimens stored at –20°C and, less frequently, at –80°C **(Table 3)**.

A moderate, but significant, decrease of the urine protein concentration for specimens from nonproteinuric dogs was observed after storage at 4°C for 5 days and after storage at –20° and –80°C for > 15 days. In contrast, the urine protein concentration for specimens from proteinuric dogs only decreased significantly after storage at –20° and –80°C for 180 days.

A mild, but significant, decrease of the urine creatinine concentration for specimens from proteinuric dogs was observed after storage at 4°C for 5 days. In contrast, a mild, but significant, increase of the urine creatinine concentration for specimens from nonproteinuric dogs was observed after storage at 4°C for 2 days.

Three dogs had changes in the UPC, dependent on the temperature and duration of storage, that would have resulted in a change in status (ie, the UPC crossed a defined threshold [0.2 or 0.5] for the IRIS staging system).[6] For one of these dogs, the UPC on day 0 was 0.16, but the UPC increased to 0.21 after specimen storage at room temperature or 4°C for 1 day. For the second dog, the UPC on day 0 was 0.5, but it decreased to 0.49 after specimen storage at room temperature for 1 day; 0.49 and 0.49 after storage at 4°C for 1 and 5 days, respectively; 0.38, 0.43, 0.33, 0.38, and 0.41 after storage at –20°C for 15, 30, 90, 180, and 360 days, respectively; and 0.45, 0.41, and 0.48 after storage at –80°C for 30, 90, and 180 days, respectively. For the third dog, the UPC on day 0 was 0.62, but it decreased to 0.46 and 0.47 after specimen storage at –20°C for 15 days and storage at –80°C for 30 days, respectively.

SDS-AGE

Day 0—No bands were evident on SDS-AGE profiles of specimens from 10 nonproteinuric dogs. A low-intensity, low-molecular-weight (35-kDa) band was observed only on profiles of specimens from sexually intact male dogs (5/5 nonproteinuric dogs and 10/11 proteinuric dogs; **Figure 2**). A low-intensity albumin band was observed on 8 of 20 profiles of specimens from nonproteinuric dogs. For specimens obtained from the proteinuric dogs, 11 of 20 had concomitant low-molecular-weight, albumin, and high-molecular-weight bands; an albumin band, albumin band and low-molecular-weight band, and albumin band and high-molecular-weight band were apparent on the SDS-AGE patterns of specimens from 2, 2, and 5 dogs, respectively. Method of urine collection and hypercreatininemia were not associated with distinctive migration patterns.

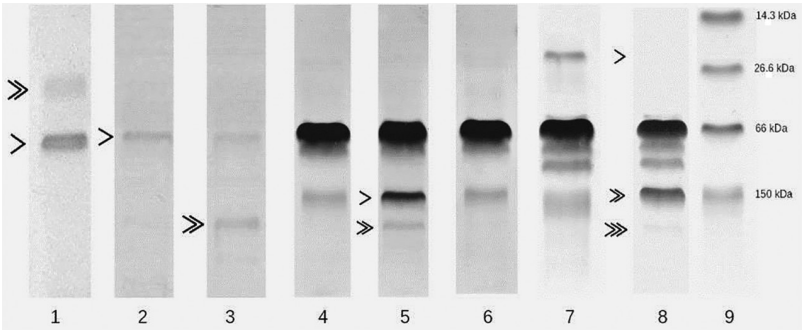

Figure 2—Images of SDS-AGE gels for urine specimens obtained from proteinuric and nonproteinuric dogs and stored at various temperatures for various durations. Day of urine collection was designated as day 0. Lanes 1 and 2 represent urine specimens on day 0 from a nonproteinuric sexually intact male dog and a neutered male dog, respectively. Notice the faint albumin bands (arrow) for both dogs and a low-molecular-weight band (double arrowhead) only for the sexually intact dog. Lane 3 is the urine specimen from the neutered male dog in lane 2 after the specimen was stored at –20°C for 30 days. Notice the faint high-molecular-weight band (double arrowhead) and concurrent mild decrease in intensity of the albumin band. Lanes 4, 5, and 6 are the urine specimen from a proteinuric female dog at day 0, after storage at –20°C for 15 days, and after storage at –80°C for 15 days, respectively. Notice the faint high-molecular-weight band (double arrowhead), which has the same molecular weight as for the specimen in lane 3, and a strengthening in the intensity of the IgG band at 150 kDa (arrowhead). In lane 6, notice the absence of modification of the SDS-AGE pattern, compared with that for the specimen at day 0 (lane 4). Lanes 7 and 8 represent a urine sample from a proteinuric neutered male dog at day 0 and after storage at –20°C for 180 days, respectively. The pattern on day 0 has a mixed (glomerular and tubular) pattern. In lane 8, notice the appearance of the faint high-molecular-weight band (triple arrowhead) with the same molecular weight as in lanes 3 and 5, a strengthening of the intensity of the IgG band at 150 kDa (double arrowhead), and disappearance of the low-molecular-weight band evident on day 0 (arrowhead); this would be interpreted as a glomerular pattern. Lane 9 represents molecular weight markers.

Effects of storage conditions on visual interpretation of SDS-AGE profiles

All specimens, except for 1, had no visible changes on any profile after storage at room temperature, 4°C, or –80°C or after 1 or 2 repeated freeze-thaw cycles when specimens were stored at –80°C. A faint high-molecular-weight band was visible only on the profile for the specimen from 1 nonproteinuric dog after storage at –80°C for 360 days.

A high-molecular-weight band (> 150 kDa) or strengthening of the intensity of the IgG band (or both) was observed on profiles of specimens obtained from 13 proteinuric and 18 nonproteinuric dogs after specimen storage at –20°C for > 15 days. Although a high-molecular-weight band appeared in the profile of a urine specimen from a hypercreatininemic neutered dog after specimen storage at –20°C, the migration pattern for the specimen from a male dog with leptospirosis did not change over time after storage at a similar temperature. For 2 of 3 specimens obtained via natural voiding, a > 150-kDa band appeared or had a strengthening of intensity. The high-molecular-weight band on profiles of specimens from proteinuric dogs was thick, whereas it was faint and scarcely visible on profiles of specimens from nonproteinuric dogs (Figure 2).

A pattern was detected for specimens from 9 dogs (5 nonproteinuric and 4 proteinuric dogs). The appearance or strengthening in intensity (or both) of bands with a molecular weight ≥ 150 kDa was associated with a decrease in the intensity of a band located at a lower molecular weight.

Gel staining faded over time. There was complete disappearance of less intensely stained bands after storage in the dark for 360 days **(Figure 3)**.

Discussion

The study reported here revealed that UPC for proteinuric and nonproteinuric dogs was not significantly affected by storage of urine specimens at room temperature and 4°C for up to 3 days or by freeze-thaw cycles. The UPC for urine specimens stored at –20° and –80°C decreased slightly with time, mainly because of a decrease in urine protein concentration. This decrease was less noticeable and observed later when urine specimens were stored at –80°C. The SDS-AGE profiles for urine specimens from proteinuric and nonproteinuric dogs were only affected by storage at –20°C; the appearance or strengthening of the intensity of bands with a molecular mass ≥ 150 kDa was observed on SDS-AGE profiles for 31 of 40 (78%) dogs after storage of urine specimens for 15 days. To our knowledge, the study reported here was the first in which the effects of storage conditions on visual assessment of electrophoresis of urinary proteins of dogs have been assessed.

In the present study, UPC remained stable when urine specimens were stored at room temperature for 5 days and at 4°C for 3 days. These findings differ from those of another study[11] in which investigators found an increase in UPC after storage for 12 hours at room temperature or 4°C. Additionally, in that other study,[11] no significant changes in UPC were detected after storage at –20°C for 3 months. Differences between that study and the study reported here may be explained by the interlaboratory variability regarding measurement of the UPC as well as by the design of the present study in which proteinuric and nonproteinuric dogs were assessed separately.[34]

In the present study, the urine protein concentration decreased mildly but significantly with time when urine specimens were stored at –20°C and less noticeably when specimens were stored at –80°C, which lead to a concomitant decrease of the UPC. This decrease of urine protein concentration might have been attributable to the denaturation of urine proteins during storage. Urine creatinine might be more stable than urine proteins during storage because concentrations of urine creatinine for nonproteinuric and pro-

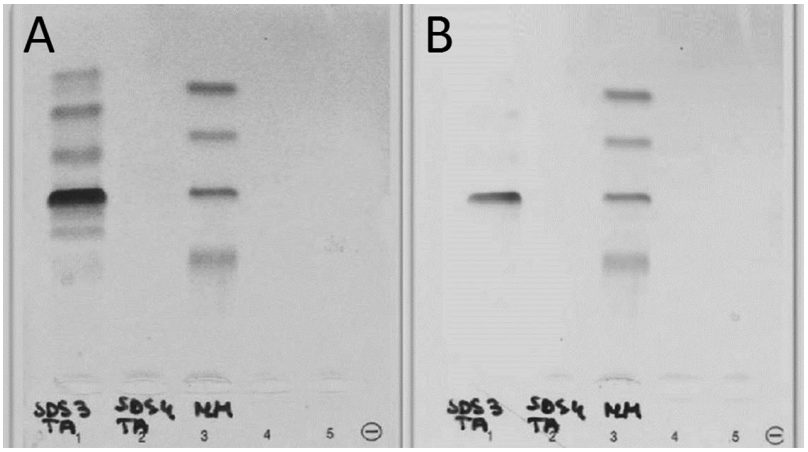

Figure 3—Images of an SDS-AGE gel for a urine specimen on day 0 (A) and after the gel was stored in the dark in a document case for 360 days (B). Notice that there is considerable fading of the staining over time and the disappearance of some bands.

teinuric dogs were significantly affected only after storage at 4°C for 2 and 5 days, respectively. The fact that SDS-AGE patterns obtained for specimens stored at –20°C were altered, with a decrease in the intensity of some protein bands, may further support the hypothesis that there was protein denaturation or alteration. Bacterial consumption may also have explained the decrease in protein concentration. However, most of the specimens were collected via cystocentesis and immediately stored at –20°C. Bacterial metabolism is inhibited at –20°C; thus, this hypothesis seems unlikely. Additional sources of variation include the analyzer, batch of reagents used, and skill of the technical staff. To minimize the possible influence of these factors, all measurements were performed by the same operator (FP) by use of the same analyzer. However, because the present study extended over several months, 3 batches of reagent were used, which might have been responsible for some of the observed variation. Indeed, the interassay CV increased during the study period and might have contributed to the variation. However, no information regarding acceptable interassay CV and total error observed for urine protein and creatinine analyses are available for veterinary medicine, and the CV and total error observed obtained in the present study were well below the acceptable values recommended in human medicine for these measured urinary analytes.[35] Therefore, it appeared unlikely that the increase in CV would have contributed to the observed variations. Additionally, most of the differences between serial UPC values did not reach the maximal analytic difference.

Variations in UPC associated with storage conditions might lead to an incorrect categorization (as determined by use of the IRIS staging system) of urine specimens with a UPC close to the cutoff values (ie, 0.2 and 0.5). Indeed, in the present study, the substage for a few dogs changed over time when urine specimens were stored at different temperatures. This finding is in agreement with those of another study[24] in which the analytic variability was higher at low urine protein and creatinine concentrations. In the present study, 3 dogs for which the initial UPC was close to an IRIS threshold were misclassified several times. One dog initially categorized as nonproteinuric was classified as borderline proteinuric 2 times, whereas 2 other dogs initially classified as proteinuric were subsequently classified as borderline proteinuric 4 and 12 times, respectively. However, no dog initially classified as proteinuric was subsequently classified as nonproteinuric. Therefore, these variations might have reflected only the analytic variability because relatively few specimens reached the maximal analytic difference. Additionally, these variations were not considered clinically relevant and were unlikely to affect medical decisions, especially in view of the biological variability of the UPC for dogs.[36,37]

Both potential sources of erroneous interpretation (ie, analytic and biological variability) should be kept in mind when a dog is evaluated on the basis of a single UPC determination. Pooling several urine specimens collected from the same dog on different days has been suggested[37] to reduce individual variability of the UPC. When pooling of urine specimens is used, investigators should take into account the storage temperature and delay between specimen collections to prevent further misinterpretation of the UPC. Furthermore, sequential measurements to monitor the course of a disease or efficacy of treatment should be conducted by the same laboratory to minimize overestimation or underestimation of the UPC attributable to interlaboratory variability.[34]

Initial qualitative evaluation of urinary proteins revealed that albumin was the most predominant band, albeit faintly stained, in the pattern for a urine specimen obtained from a healthy dog, which is considered a physiologic finding provided that the dog's UPC is consistently < 0.2.[24,38] Additionally, a 25- to 30-kDa band was observed in most of the SDS-AGE profiles for urine specimens obtained from male dogs. This band in healthy dogs has been identified as the major secretory protein of the prostate gland, arginine esterase.[26] However, we cannot exclude that the 25- to 30-kDa band observed for 10 of 11 proteinuric male dogs had a tubular origin. Although this hypothesis seems less likely given the absence of identification of this band for the remaining proteinuric dogs, gel-based protein extraction and identification would be necessary to conclude its origin.

The SDS-AGE profiles were not modified by storage of urine specimens at room temperature and 4°C or by repetitive freeze-thaw cycles. These findings

are similar to those reported for a study[28] on human urine, whereby SDS-PAGE patterns for urine specimens were not affected by prolonged exposure at room temperature and multiple freeze-thaw cycles. Additionally, SDS-AGE profiles obtained for urine samples stored at –80°C for up to 365 days were not modified for 39 of 40 dogs, which is consistent with results of another study[27] with human urine in which no visual change in SDS-PAGE was observed for urine specimens stored at –70°C for 12 months.

In the present study, potentially important qualitative modifications in SDS-AGE profiles attributable to storage of specimens were observed after storage at –20°C for ≥ 15 days for 32 of 40 dogs. Indeed, the appearance of a high-molecular-weight band > 150 kDa, strengthening of the intensity of a 150-kDa band, or both were associated with a variable decrease in the intensity or disappearance of low-molecular-weight bands or the albumin band and was observed for 9 dogs. These observations were not expected because albumin degradation has been reported[27] for human urine stored at –20°C for up to 12 months, whereby a decrease in intensity of the albumin band was concomitant with the appearance of 2 low-molecular-weight bands (identified as products of albumin degradation) for 25% of the human urine specimens. In the present study, there was no appearance of low-molecular-weight bands, but there was a strengthening in the intensity of high-molecular-weight bands, associated with a decrease in the intensity of protein bands of lower molecular weight for some specimens.

Several hypotheses might explain these modifications. New bands might have corresponded to bacterial proteins. Indeed, bacterial contamination of urine is possible even in the absence of any cytologic evidence of bacteriuria.[39] However, because the urine protein concentration did not increase during storage, this phenomenon appeared to be unlikely, unless there was concomitant bacterial consumption. Further studies with the addition of an antimicrobial agent would be needed to investigate this hypothesis. Another explanation might have been the formation of protein aggregates that could have altered migration patterns. Absence of a marked difference in urine protein concentration between aliquots stored at –20° and –80°C supported this hypothesis. The reason this phenomenon would occur only in specimens stored at –20°C and not at –80°C is not clear, although noncovalent binding or a structural modification such as disulfur binding is more likely to occur at –20°C. Collection method (cystocentesis vs natural voiding) may also have been an explanation because urogenital contamination, although minimal, may be associated with the presence of some remainder proteins or cells that could aggregate. However, this appeared to be extremely unlikely given the low number of dogs for which free catch was used (3/10) and the fact that 1 of 3 of the SDS-AGE profiles for the urine samples collected from these dogs did not have any modifications. Finally, we cannot exclude a possible alteration in urine composition attributable to denaturing of the plastic tubes and that some of the observed modifications might have been prevented by the use of different tubes, although this hypothesis appeared to be unlikely on the basis of results for another study[r] recently performed by our research group. Identification of the proteins migrating at > 150 kDa might have provided a better understanding of the mechanism for alteration of urine SDS-AGE patterns for specimens stored at –20°C.

Only 1 SDS-AGE profile had a visible modification, with the appearance of a new high-molecular-weight band after storage at –80°C for 360 days. This observation might have been secondary to a handling error (ie, aliquot previously stored at –20°C erroneously stored at –80°C). Another explanation might have been that the phenomenon accounting for the appearance of new bands for specimens stored at –20°C was not totally inhibited at –80°C and also could have caused effects after a long period of storage (ie > 180 days).

On the basis of results of the present study, storage of urine specimens at –20°C is not recommended because it can lead to misinterpretation of SDS-AGE profiles. Additionally, because gel staining faded over time and some bands disappeared, analyses of the SDS-AGE urinary profile should be performed immediately or the gel should be dried and color photocopied to facilitate subsequent visual analysis.

In the present study, the influence of storage conditions (time and temperature) that mimic conditions commonly occurring in routine practice (eg, shipping specimens to external laboratories for analysis or analysis of refrigerated specimens collected several days previously) or in retrospective studies (eg, analysis of frozen specimens stored for several months after collection) was assessed. On the basis of results of the study reported here, storage of urine specimens at –20°C should be avoided. Determination of UPC and urine SDS-AGE should be performed within a few days after collection with urine specimens stored at a stable room temperature or at 4°C, whereas the recommended temperature for longer periods of storage is –80°C.

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Footnotes

a. Brown JS, Nabity MB, Brock R, et al. Comparison of urine SDS-PAGE with renal histological findings and clinicopathologic data in dogs with renal disease (abstr). *Vet Clin Pathol* 2010;39:556.

b. Neolus, Terumo Europe N.V., Leuven, Belgium.

c. Injekt, B. Braun, Melsungen, Germany.
d. PS-Tube, Greiner Bio-One, Courtaboeuf, France.
e. EBA 3S centrifuge, Andreas Hettich GmbH and Co, Tuttlinger, Germany.
f. Safe Lock, Eppendorf, Le Pecq, France.
g. Aution Stick PA, Scil Animal Care Co, Alfort, France.
h. Pocketchem Analyzer UA, Scil Animal Care Co, Alfort, France.
i. Atago Co Ltd, Tokyo, Japan.
j. Créatinine Monoréactif liquide stable PAE, Kitvia, Labarthe Inard, France.
k. Elitech Microprotein, Seppim, Sées, France.
l. Kbio2, Kitvia, Labarthe Inard, France.
m. Liquicheck urine chemistry control, level 1, Bio-Rad, Marnes-la-Coquette, France.
n. Liquicheck urine chemistry control, level 2, Bio-Rad, Marnes-la-Coquette, France.
o. Hydrasys, Sebia Italia SRL, City, Italy.
p. Molecular mass control, Sebia Italia SRL, City, Italy.
q. Systat, version 13, SPSS Inc, Chicago, Ill.
r. Quignon L. Etude prospective secondaire visant à préciser l'influence de la température de conservation des urines sur le profil électrophorétique des protéines urinaires sur gel d'agarose chez le chien protéinurique. Thèse d'Exercice Vétérinaire. Institut National Polytechnique–Ecole Nationale Veterinaire de Toulouse, 2015.

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