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Urinary calcium assessment and its relation with age, sex and *Encephalitozoon cuniculi* serological status in otherwise healthy pet rabbits

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ABSTRACT

Background Urinary calcium excretion by rabbits is set proportionately higher than that of other species, giving the rabbit urine its characteristic cloudy appearance. During pregnancy, lactation and growth, or when metabolic disorders interfere in the renal excretion of calcium, it is reported that less calcium is excreted and the urine appears clear. The quantification of the calcium concentration in the urine of rabbits may be of importance in the management of diseases that affect calcium metabolism, especially in kidney disease; however, reference intervals for urinary calcium concentration (UC) are unreported in pet rabbits.

Methods Prospective data from 63 healthy pet rabbits were evaluated. An inhouse modified Arsenazo III technique, without acidifying the urine, was used to determine the reference intervals for UC and UC:creatinine ratio. Urinary protein:creatinine ratio, UC, UC:creatinine ratio, urinary gamma-glutamyl transferase concentration (UG), UG:creatinine ratio, phosphorus, plasma total calcium, plasma creatinine, urea and ionised calcium were assessed according to age, sex, neutered status, urine collection method and *Encephalitozoon cuniculi* IgG status in healthy rabbits.

Results The reference intervals for UC and UC:creatinine ratio were 4.2–31.1 mg/dl (1.03–7.78 mmol/l) and less than 0.3, respectively. The results showed that none of these variables was influenced by the parameters evaluated, except for the phosphorus value, which was higher in pet rabbits aged up to 3 years compared with those over 3 years, suggesting that published reference values for plasma phosphorus in rabbits should be adjusted according to age.

Conclusion The modified Arsenazo III technique described in the present work and the established reference values for UC and UCC in pet rabbits could be useful in the management of kidney disease in rabbits. The results showed that *E cuniculi* IgG seropositivity has no influence on the variables of renal interest in otherwise healthy rabbits.

INTRODUCTION

Kidney disease (KD) in pet rabbits occurs frequently; retrospective studies have estimated its prevalence to be 3–9 per cent,[1 2] which suggests that the prevalence in rabbits is higher than that in dogs and cats (0.5–1.5 per cent).[3]

Unfortunately, naturally occurring KD in pet rabbits is a relatively unknown disease that has not been well characterised.[4–7] There are several conditions reported in the literature that spontaneously affect the kidneys of laboratory and pet rabbits, although the bibliography referring to laboratory rabbits is much more extensive. These conditions can be grouped into congenital diseases (unilateral renal agenesis, misshapen kidney, fused or horseshoe kidney, ectopic kidney, congenital hydronephrosis, renal cortical cysts and polycystic kidneys), infectious and parasitic diseases (pasteurellosis, staphylococcosis, yersiniosis, necrobacillosis and encephalitozoonosis), neoplastic diseases (embryonal nephroma or nephroblastoma, renal carcinoma, lymphosarcoma, leiomyoma, and metastatic neoplasia), and non-specific inflammatory, degenerative and proliferative diseases (acute and chronic renal failure, urolithiasis and other obstructive uropathies, nephrotoxicity, papillary necrosis and cortical infarction, hydronephrosis, fatty infiltration, focal interstitial nephritis, renal fibrosis, glomerulonephritis, suppurative nephritis, kidney abscesses, pyelonephritis, pyelitis, amyloidosis, kidney mineralisation, metastatic mineralisation, pigmentation, tubular basophilia, tubular dilatation, tubular cast formation, perivascular cuffing, and renal cysts).[1 8–12] Clinical signs for renal disease can vary depending on the cause and degree of renal insufficiency. Clinical signs that have been reported in rabbits include dysuria, polydipsia, anorexia, dehydration and abnormal kidney size. Biochemistry and haematological values, cardiovascular parameters, diagnostic imaging, and urine chemistry results may also vary based on the cause of renal disease. Common findings may include anaemia,

azotaemia, hypercalcaemia, hypophosphataemia or hyperphosphataemia, hypertension, osteosclerosis, metastatic or dystrophic calcifications, renal echographic alterations, proteinuria, and isosthenuria or hyposthenuria.[1 4 5 10–17] The classic markers employed in dogs and cats in the management of KD are urea, creatinine, urine protein:creatinine ratio (UPC), systolic blood pressure, and more recently symmetrical dimethylarginine.[7] To the best of the author's knowledge, studies have not been published on the relationship between any of these parameters and pet rabbits suffering from confirmed KD except for azotaemia, which may be observed in pet rabbits suffering from severe KD.[4]

The metabolism of calcium in rabbits is different from that of other domestic species and could play an important role in the management of KD in pet rabbits.[12 18] In contrast to other domestic species, nephrectomised laboratory rabbits do not develop renal secondary hyperparathyroidism and show increased ionised calcium if their dietary calcium-phosphorus ratio is appropriate.[19] Harcourt-Brown[4] observed osteosclerosis and metastatic calcifications associated with hyperphosphataemia and *Encephalitozoon cuniculi* seropositive status in rabbits with naturally occurring severe KD; however, the findings for azotaemia and raised serum total calcium were not consistent. In rabbits, the quantity of calcium absorbed in the intestines and the total and ionised calcium concentrations in blood are greater than that in other domestic mammals. This relative calcium overload is mainly excreted by the kidneys in the form of calcium precipitated salts, giving the rabbit urine its characteristic cloudy appearance.[12 18] The renal excretion of calcium in rabbits is more efficient than that in other domestic mammals. In dogs, the fractional excretion of calcium has been established as 0.15±0.13 per cent,[20] but rabbits are more adaptable, reaching 6 per cent when rabbits are fed on high-calcium diets.[21] The concentration of calcium in the rabbit urine is proven to be directly proportional to that of the concentration of calcium in the diet and in blood.[21–23] Because high concentration of calcium in urine has been related to nephrocalcinosis, and hypercalcaemia has been associated with metastatic calcifications, osteosclerosis, nephrocalcinosis and severe KD,[4 22 24–27] the concentration of calcium in urine could be relevant in the management of KD and in other conditions that affect calcium metabolism in rabbits. In the present study, the objectives were to determine a reference interval for the concentration of calcium in urine (UC) and the urine calcium:creatinine ratio (UCC) in pet rabbits, and to evaluate the influence of age, sex, neutered status, urine collection method and *E cuniculi* IgG status on certain variables of renal interest.

MATERIALS AND METHODS

Animals

The present study was developed as an observational cross-sectional study in which prospective data from 85 healthy pet rabbits over a period of one year (2016) were analysed. Informed consent was obtained from the owners of all the rabbits included in the study. The inclusion criteria were rabbits that showed no signs of illness on physical examination, medical histories that reported no health problems, and when radiographs and/or abdominal ultrasounds were performed they did not reveal any concerning alterations. Twenty-two rabbits were excluded from the study because they did not meet one or more of the inclusion criteria and a healthy status could not be assured. Urinalysis, blood biochemistry and/or ionised calcium assessments were obtained in the rabbits included in the study; the samples were obtained in each rabbit at the same visit to the clinic at a random time between 09.00 and 21.00.

Blood data collection and *E cuniculi* serology

Blood samples for some blood biochemistry values (urea, creatinine, total calcium and phosphorus) assessments were obtained from one saphenous vein in separator lithium heparin tubes, were centrifuged immediately after extraction and analysed within two hours with an automated analyser (Chemray-120; Rayto, Shenzhen, Guangdong, China). For ionised calcium measurement, a sample of at least 70 µl of blood from the other saphenous vein was obtained with a gasometry syringe (1 ml/25 iu dry balanced heparin; Westmed, Tucson, Arizona, USA) and immediately analysed in a gasometer (ABL80Basic FLEX; Radiometer, Brønshøj, Copenhagen, Denmark).

The serological status (presence or absence of IgG) against *E cuniculi* was evaluated (*Encephalitozoon cuniculi* Carbon Immunoassay; Medicago, Uppsala, Uppland, Sweden) in 53 of the 63 healthy rabbits. For detection of IgG antigen the plasma was diluted 1:40 as the manufacturer recommends. The rabbits were considered seropositive when probe reaction was observed (title ≥1:40) and seronegative when that reaction was not observed (title <1:40).

Urinalysis performance

In all neutered and entire female rabbits, urine samples were collected by manual expression of the urinary bladder on a surface cleaned without quaternary ammonium disinfectants or chlorhexidine as recommended by the dipstick manufacturer. In entire male rabbits, the urine samples were obtained via ultrasound-guided cystocentesis. Urine samples (n=43) were centrifuged at 3900 g for 45 seconds (StatSpin VT, Iris Sample Processing; Beckman Coulter, Brea, California, USA). In the supernatant, the urine protein, UPC, urine gamma-glutamyl transferase (UG) and the urine gamma-glutamyl-transferase:creatinine ratio (UGC) were calculated as described in previous studies.[5 6] The UC was determined in the supernatant using the Arsenazo III reaction without acidifying the urine, and the UCC was calculated by dividing the UC value with the previously obtained urinary creatinine concentration. In the supernatant, the urinary specific gravity content was determined by

refractometry. Urinary sediment was evaluated via light microscopy (40 x) without staining the urine.

Statistical analysis

The 63 healthy rabbits were grouped by age (≤3 years v >3 years), sex, neutered status, sampling method and *E cuniculi* IgG status. Reference Value Advisor was utilised to assess statistical differences among all biochemistry and urine chemistry analytes.[28]

The normality of the data distribution was first assessed with the Shapiro-Wilk test. Significant differences between urine collection method and urine chemistry results were assessed using the software OpenStat V.11.9.08. Student's *t* test was applied. A value of P<0.05 was considered to be significant.

Two different measurements in each of 13 randomly selected urine samples from 13 rabbits seronegative to *E cuniculi* IgG were employed to assess the feasibility of the modified Arsenazo III method for UC measurements in pet rabbits. Repeatability and reliability were defined in the same software as follows. Hoyt's reliability coefficient and Cronbach's alpha reliability coefficient were calculated, and then an analysis of variance (ANOVA) was performed from the paired measurements in 13 randomly selected urine samples. Both reliability coefficients evaluated the internal consistency of the test and were considered acceptable, good or excellent if their values were greater than 0.7, 0.8 or 0.9, respectively. Student's *t* test (P<0.05 indicates significance) was applied to evaluate the repeatability of the method by assessing statistically significant differences in paired measurements. The correlation coefficient (r) was also calculated using Student's *t* test to determine the repeatability of the method, and r values of 0–0.29, 0.3–0.49, 0.5–0.69, 0.7–0.89 or 0.9–1 were considered negligible, low positive, moderate positive, high positive or very high positive correlations, respectively.

RESULTS

The study animals lived in the province of Madrid (Spain) and consisted of six entire females, 19 neutered females, 15 entire males and 23 neutered males. The age of the rabbits ranged from five months to 9.5 years, with 39 (62 per cent) aged up to 3 years and 24 (38 per cent) more than 3 years. The bodyweight of the rabbits was between 700 g and 2975 g. Urinalysis, blood biochemistry and ionised calcium assessments were obtained using 43, 62 and 47 rabbits, respectively. The breed of the rabbits was not recorded.

In table 1, the reference intervals obtained here (90 per cent confidence) for UPC, UC, UCC, UG, UGC, phosphorus, plasma total calcium, plasma creatinine, urea and ionised calcium values are shown. The urine specific gravity ranged from 1024 to 1062, and no sediment analysed was considered as active.

The *E cuniculi* serology resulted in 18 (34 per cent) positive and 35 (66 per cent) negative results. After verifying the normal distribution of the data, the observed differences in the UPC, UC, UCC, UG, UGC, phosphorus, plasma total calcium, plasma creatinine, urea and ionised calcium values in relation to the *E cuniculi* serology results, age, neutered status or sampling method (cystocentesis v manual expression of the urinary bladder) were not significant (P>0.05). The phosphorus value was the only evaluated variable that was age-related (P=0.0412) (tables 2 and 3). Because the difference between the number of *E cuniculi* seropositive and seronegative individuals could influence the Student's *t* test, a one-way ANOVA was also performed, demonstrating that the observed differences were not significant.

For the assessment of the modified Arsenazo III method (table 4), Hoyt's and Cronbach's alpha reliability coefficients indicated good internal consistency. The correlation between the paired measures was highly positive, and P value was >0.05, which indicated that the differences between the paired measures were not significant. The 13 randomly selected paired samples for the modified Arsenazo III method were considered comparable according to previous results that showed that the UC and UCC

Table 1 Reference intervals obtained (90% confidence) for UPC, UC, UCC, UG, UGC, phosphorus, plasma total calcium, plasma creatinine, urea and ionised calcium values

	Reference interval (90% confidence)
Biochemistry (n=62)	
Urea mg/dl (mmol/l)	19–61 (6.78–21.78)
Creatinine mg/dl (µmol/l)	0.7–1.4 (61.88–123.76)
Calcium mg/dl	11.1–14.5
Phosphorus mg/dl (mmol/l)	2.1–4 (0.68–1.29)
Urinalysis (n=43)	
Urine creatinine mg/dl (mmol/l)	73–361 (6453–31,912)
Urine proteins mg/dl (g/l)	21–53 (2.1–5.3)
UPC	<0.3
UG (iu/l)	19–123
UGC	<0.6
Urine calcium mg/dl (mmol/l)	4.2–31.1 (1.03–7.78)
UCC	<0.3
Others (n=47)	
Ionised calcium (mmol/l)	1.56–1.87

UC, urinary calcium concentration; UCC, urine calcium:creatinine ratio; UG, urinary gamma-glutamyl transferase concentration; UGC, urine gamma-glutamyl transferase:creatinine ratio; UPC, urine protein:creatinine ratio.

Table 2 Evaluation of age-related differences in the values of certain laboratory variables between rabbits over three years of age and rabbits aged three years or younger

Variables compared	n	Group: ≤3 years Mean±sd	Group: >3 years Mean±sd	P value (Student's *t* test)
Urea mg/dl (mmol/l)	23	43.74±9.55 (15.62±3.41)	38.04±11.14 (13.58±3.98)	0.0570
Creatinine mg/dl (mmol/l)	22	0.96±0.21 (84.86±18.56)	0.95±0.2 (83.98±17.68)	0.9246
UPC	13	0.20±0.08	0.22±0.07	0.1276
UC mg/dl (mmol/l)	15	15.67±7.41 (3.92±1.85)	17.28±7.54 (4.32±1.89)	0.5604
UCC	14	0.10±0.08	0.12±0.08	0.3754
UG (iu/l)	14	53.78±27.53	47.43±21.68	0.5555
UGC	14	0.3±0.11	0.33±0.13	0.4031
Phosphorus mg/dl (mmol/l)	22	3.12±0.44 (1.01±0.14)	2.85±0.4 (0.92±0.13)	0.0412*
Calcium mg/dl (mmol/l)	20	12.96±0.91 (3.24±0.23)	12.94±0.79 (3.23±0.2)	0.9382
Ionised calcium (mmol/l)	14	1.73±0.07	1.71±0.06	0.5823

*P<0.05 was considered significant.
UC, urinary calcium concentration; UCC, urine calcium:creatinine ratio; UG, urinary gamma-glutamyl transferase concentration; UGC, urine gamma-glutamyl transferase:creatinine ratio; UPC, urine protein:creatinine ratio.

values were not affected by the *E cuniculi* serology results, age, neutered status or sampling method.

DISCUSSION

The UPC and plasma concentrations of urea and creatinine are important indicators in the management of KD in other species.[3] In pet rabbits, azotaemia, raised total calcium levels and hyperphosphataemia have been observed in severe KD,[4 19] and experimental hypercalciuria in laboratory rabbits is associated with nephrocalcinosis.[24] According to the results obtained in the present study, UPC, UC, UCC, UG, UGC, phosphorus, plasma total calcium, plasma creatinine, urea and ionised calcium values were not dependent on the *E cuniculi* serological status in healthy rabbits, which is consistent with previous observations in seropositive non-azotaemic rabbits with normal UPC values.[5] Rabbits with severe KD may be IgG seropositive for *E cuniculi*; however, this may not indicate *E cuniculi* as the cause of renal insufficiency. IgG titres status has not been shown to be linked to acute or symptomatic infections, whereas positive IgM titres are associated with acute infections.[2 29] The results obtained in the present study also suggest that IgG seropositivity to *E cuniculi* may not present clinical measurable renal implications in otherwise healthy rabbits.

In the present study, statistically significant differences were not observed in UPC, UC, UCC, UG, UGC, phosphorus, plasma total calcium, plasma creatinine, urea and ionised calcium values in healthy pet rabbits in relation to sex and neutered status or between samples obtained via cystocentesis in entire males and samples obtained via manual expression of the urinary bladder. Phosphorus was the only evaluated variable that was significantly affected by age, and the value was higher in pet rabbits aged up to 3 years. In other species, the plasma concentration of phosphorus is higher in young individuals, which is likely related to bone growth and an increase in renal tubular reabsorption mediated by growth hormone.[30] A literature search failed to identify reports of age-related effects on the phosphorus plasma concentration in rabbits.

The reference interval obtained for the UPC and UGC in the present study (<0.3 and <0.6, respectively) was narrower than the interval reported in previous studies.[5 6 15 16] The animal selection criteria, urine sampling method or unknown causes may have generated these differences. In entire male rabbits asymptomatic for urogenital disease, the author's preliminary observations suggest that elevations may be seen in UPC and UGC values in urine samples obtained by manual expression of the urinary bladder and that these elevations may not be observed if the urine sample of the same animal is obtained via cystocentesis (table 5). Mancinelli and others[6] observed significant differences for UGC values according to neutered status. As a matter of fact, the UPC ratio is generally considered reliable if urine samples are collected by cystocentesis but not by free catch, manual expression or catheterisation.[31] According to the present study, statistically significant differences were not observed between the samples obtained via cystocentesis in entire male rabbits and manual expression of the urinary bladder in female rabbits and neutered male rabbits. In dogs, in agreement with the author's observations in rabbits, statistically significant differences were not observed between UPC values from urine samples obtained by cystocentesis and from samples obtained by free catch.[31] According to the author's observations, in entire male rabbits ultrasound-guided cystocentesis may be advisable when evaluating extrarenal factors that may have an influence on the results of the urinalysis. Physiological proteinuria that appears at puberty has been observed in entire male rats and mice, and this is

Table 3 Evaluation of the influence of the neutered status, urine collection method and *Encephalitozoon cuniculi* IgG presence in certain laboratory variables

Variable	n	Group: EZC-negative Mean±sd	Group: EZC-positive Mean±sd	P value (Student's *t* test)
Urea mg/dl (mmol/l)	27	41.70±10.58 (14.89±3.78)	40.37±9.77 (14.41±3.49)	0.5813
Creatinine mg/dl (mmol/l)	26	0.93±0.19 (82.21±16.80)	0.94±0.19 (83.1±16.80)	0.8766
UPC	18	0.21±0.08	0.23±0.06	0.5680
UC mg/dl (mmol/l)	15	19.04±7.29 (4.76±1.82)	18.17±7.94 (4.54±1.99)	0.7267
UCC	15	0.10±0.07	0.11±0.07	0.5297
UG (iu/l)	18	53.58±25.13	46.27±20.34	0.2969
UGC	19	0.26±0.09	0.3±0.12	0.3427
Phosphorus mg/dl (mmol/l)	20	2.98±0.55 0.96±0.18)	3.13±0.49 (1.01±0.16)	0.4178
Calcium mg/dl (mmol/l)	27	12.94±0.81 (3.23±0.2)	13.01±0.53 (3.25±0.13)	0.7470
Ionised calcium (mmol/l)	13	1.72±0.06	1.73±0.08	0.5705
Variable	**n**	**Group: males Mean±sd**	**Group: females Mean±sd**	**P value (Student's *t* test)**
Urea mg/dl (mmol/l)	25	40.56±10.52 (14.48±3.76)	41.60±11.30 (14.85±4.03)	0.7347
Creatinine mg/dl (mmol/l)	24	0.9±0.17 (79.56±10.03)	0.98±0.23 (86.63±20.33)	0.2372
UPC	15	0.22±0.09	0.21±0.08	0.8312
UC mg/dl (mmol/l)	16	18.09±6.06 (4.52±1.51)	19.7±8.67 (4.92±2.17)	0.5418
UCC	14	0.1±0.07	0.1±0.06	0.7740
UG (iu/l)	14	54.69±28.78	46.03±21.02	0.4465
UGC	14	0.32±0.11	0.26±0.12	0.1762
Phosphorus mg/dl (mmol/l)	23	3.01±0.51 (0.97±0.16)	2.93±0.44 (0.95±0.14)	0.6131
Calcium mg/dl (mmol/l)	22	12.95±0.95 (3.24±0.24)	13.05±0.66 (3.26±0.17)	0.7265
Ionised calcium (mmol/l)	15	1.72±0.06	1.71±0.08	0.6198
Variable	**n**	**Group: neutered females Mean±sd**	**Group: entire females Mean±sd**	**P value (Student's *t* test)**
Urea mg/dl (mmol/l)	6	43.67±9.52 (15.59±3.4)	43±10.83 (15.35±3.87)	0.9206
Creatinine mg/dl (mmol/l)	6	0.95±0.31 (83.98±27.4)	1.09±0.16 (96.36±14.14)	0.4457
UPC	6	0.2±0.09	0.22±0.06	0.5667
UC mg/dl (mmol/l)	6	21.50±6.26 (5.38±1.56)	14.31±7.27 (3.58±1.82)	0.0508
UCC	6	0.09±0.04	0.07±0.04	0.4196
UG (iu/l)	6	43.05±16.35	51.90±26.29	0.4001
UGC	6	0.2±0.07	0.22±0.11	0.6115
Phosphorus mg/dl (mmol/l)	6	3.09±0.29 (1±0.09)	2.91±0.16 (0.94±0.05)	0.3376

Continued

Table 3 Continued

Variable	n	Group: neutered females Mean±sd	Group: entire females Mean±sd	P value (Student's *t* test)
Calcium mg/dl (mmol/l)	6	13.25±0.63 (3.31±0.16)	13.05±0.63 (3.26±0.16)	0.6175
Ionised calcium (mmol/l)	6	1.74±0.08	1.69±0.05	0.2476
Variable	**n**	**Group: neutered males Mean±sd**	**Group: entire males Mean±sd**	**P value (Student's *t* test)**
Urea mg/dl (mmol/l)	15	39±10.43 (13.92±3.72)	40.4±9.97 (14.42±3.56)	0.7262
Creatinine mg/dl (mmol/l)	15	0.98±0.08 (86.63±7.07)	0.9±0.2 (79.56±17.68)	0.7597
UPC	10	0.23±0.05	0.22±0.1	0.8660
UC mg/dl (mmol/l)	11	15.97±7.78 (3.99±1.95)	16.67±4.96 (4.17±1.24)	0.8061
UCC	12	0.09±0.08	0.11±0.08	0.5610
UG (iu/l)	11	46.18±14.42	57.33±33.01	0.2666
UGC	11	0.29±0.08	0.32±0.12	0.4742
Phosphorus mg/dl (mmol/l)	13	2.87±0.53 (0.93±0.17)	3.05±0.54 (0.99±0.17)	0.4033
Calcium mg/dl (mmol/l)	14	12.97±0.87 (3.24±0.22)	12.98±0.91 (3.25±0.23)	0.9839
Ionised calcium (mmol/l)	9	1.71±0.05	1.74±0.07	0.2545
Variable	**n**	**Group: neutered females Mean±sd**	**Group: entire males Mean±sd**	**P value (Student's *t* test)**
Urea mg/dl (mmol/l)	15	43.47±11.54 (15.52±4.12)	40.4±9.97 (14.42±3.56)	0.3112
Creatinine mg/dl (mmol/l)	15	0.96±0.26 (84.86±22.98)	0.9±0.2 (79.56±17.68)	0.3980
UPC	10	0.21±0.09	0.22±0.1	0.8480
UC mg/dl (mmol/l)	11	21.4±8.41 (5.35±2.1)	16.67±4.96 (4.17±1.24)	0.0647
UCC	10	0.11±0.06	0.11±0.08	0.9401
UG (iu/l)	11	45.34±15.46	57.33±31.01	0.2566
UGC	11	0.26±0.11	0.32±0.12	0.2384
Phosphorus mg/dl (mmol/l)	13	2.96±0.52 (0.96±0.17)	3.05±0.54 (0.99±0.17)	0.7352
Calcium mg/dl (mmol/l)	14	13.21±0.58 (3.3±0.14)	12.98±0.91 (3.25±0.23)	0.4532
Ionised calcium (mmol/l)	9	1.73±0.08	1.74±0.07	0.7978
Variable	**n**	**Group: entire females Mean±sd**	**Group: entire males Mean±sd**	**P value (Student's *t* test)**
Urea mg/dl (mmol/l)	6	43±10.83 (15.35±3.87)	43.33±2.94 (15.47±1.05)	0.9354
Creatinine mg/dl (mmol/l)	6	1.09±0.16 (96.36±14.14)	0.86±0.21 (76.02±18.56)	0.1676
UPC	6	0.22±0.06	0.19±0.08	0.6364
UC mg/dl (mmol/l)	6	14.31±7.27 (3.58±1.82)	16.22±3.46 (4.05±0.86)	0.6212
UCC	6	0.07±0.04	0.1±0.04	0.0697

Continued

Table 3 Continued

Variable	n	Group: entire females Mean±sd	Group: entire males Mean±sd	P value (Student's *t* test)
UG (iu/l)	6	51.90±26.29	56.77±35.34	0.8382
UGC	6	0.22±0.11	0.31±0.12	0.2887
Phosphorus mg/dl (mmol/l)	6	2.91±0.16 (0.94±0.05)	3.29±0.59 (1.06±0.18)	0.1822
Calcium mg/dl (mmol/l)	6	13.05±0.63 (3.26±0.16)	12.87±0.82 (3.22±0.2)	0.7393
Ionised calcium (mmol/l)	6	1.69±0.05	1.72±0.08	0.3543

To evaluate whether significant differences occurred in the studied values according to the urine collection method, significant differences between entire males (urine samples were obtained via cystocentesis because of artefacts observed in free-catch samples) and neutered males, and entire females and neutered females, were evaluated.
Urine from entire males was collected via cystocentesis. In the remaining animals, urine was collected by manual expression of the urinary bladder over a clean surface.
None of the P values was considered significant (P<0.05) in this table.
EZC, *Encephalitozoon cuniculi*; UC, urinary calcium concentration; UCC, urine calcium:creatinine ratio; UG, urinary gamma-glutamyl transferase concentration; UGC, urine gamma-glutamyl transferase:creatinine ratio; UPC, urine protein:creatinine ratio.

presumably caused by proteins present in the secretion of the sex glands.[32 33] To the author's knowledge, studies referencing these observations in rabbits are unreported in the literature.

To the author's knowledge, reference values of UC (4.2–31.1 mg/dl (1.03–7.78 mmol/l)) and UCC (<0.3) have not been previously reported for domestic rabbits. The Arsenazo III technique employed here for UC measurements formally required the acidification of the urine sample; however, adequate absorbance and sensitivity were observed at more basic pHs.[34] The satisfactory statistical evaluation of the modified Arsenazo III technique in the present study suggests that it is reliable as a rapid inhouse test. A 24-hour urine collection is considered a superior method of screening for UC than is a spot urine sample. In the clinical setting this technique is not routinely used because of the expense, owner inconvenience and technical difficulty of 24-hour urine collection in animals. Spot urine calcium measurement does not present these inconveniences and has been employed with good results in human beings and animals.[35–37] In rabbits there are no studies on the correlation between calcium measurements taken in urine samples collected over 24 hours and those taken in spot urine samples. Although the repeatability and reliability of the method used in the present study for the measurement of spot UC in healthy pet rabbits were good and the reference values of UC and UCC obtained for pet rabbits are expected to be clinically relevant, as is the case in other species,[35–37] it would be advisable to obtain these reference values in rabbits by evaluating samples collected over 24 hours and spot urine samples.

The calcium content of the diet of the pet rabbits included in the present study was not analysed, but the rabbits were considered healthy according to the inclusion criteria and the diet of the rabbits included in the present study was considered to be representative of the pet rabbit population attended in the clinical setting. Because the concentration of calcium in the rabbit urine is directly proportional to that of the concentration of calcium in the diet and in blood,[21–23] and the reference values obtained for total and ionised calcium are similar to those previously reported,[38–40] the calcium:phosphorus ratio of the diet was expected to be correct in the pet rabbit population included in the present study. Nevertheless, in future studies the reference values of UC and UCC obtained in the present work should be compared with the same reference values obtained in rabbits with strictly controlled diets and in the wild rabbit population.

Table 4 Assessment of repeatability and reliability of the modified Arsenazo III UC testing method in pet rabbit urine samples by statistical analysis of paired measurements in 13 randomly selected urine samples

	n	First testing mean value±sd	Second testing mean value±sd	Student's *t* coefficient (P)	Student's *t* correlation coefficient (r)	Hoyt's coefficient of reliability	Cronbach's alpha coefficient of reliability
UC mg/dl (mmol/l)	13	16.9±8.54 (4.22±2.13)	17.88±7.85	0.5368*	0.77†	0.875 (±)	0.869‡

*If P>0.05, differences between paired measurements are considered not significant.
†r >0.6 and <0.8 are considered as strong correlations between paired measurements.
‡Hoyt's and Cronbach's alpha reliability coefficients >0.8 and <0.9 are considered good.
UC, urinary calcium concentration.

Table 5 Influence of the urine sample collection technique on the urinalysis results for entire male rabbits: an example of three cases

	Rabbit 1		Rabbit 2		Rabbit 3 (suspected kidney disease)		
	ME	ME postorchiectomy	ME	Cystocentesis	ME	Cystocentesis	Reference interval
UPC	1.59*	0.3	1.05*	0.25	1.04*	0.45*	<0.3
UGC	2.07*	0.35	2.4*	0.38	2.24*	1.18*	<0.6
UCC	0.09	0.1	0.16	0.13	0.57*	0.31*	<0.3
USG	1047	1044	1038	1030	1013*	1014*	1024–1062
Sediment	ACC CC	ACC, CC	ACC	ACC	CC, EC, SP*	CC	ACC, CC

*Value over the reference interval (table 2).
ACC, amorphous calcium carbonate; CC, calcium carbonate; EC, epithelial cells; ME, manual expression of the urinary bladder over a clean surface;SP, spermatozoids; UCC, urine calcium:creatinine ratio; UGC, urine gamma-glutamyl transferase:creatinine ratio; UPC, urine protein:creatinine ratio; USG, Urine Specific Gravity
.

Hypocalciuria has been reported in rabbits when metabolic demand for calcium is increased by growth, pregnancy, lactation, anorexia or in rabbits that are on a calcium-deficient diet. Rabbits affected by certain disorders involving calcium metabolism could excrete less calcium, giving the urine a clear appearance.[12 18] Hypercalciuria has been related to nephrocalcinosis, and hypercalcaemia has been associated with metastatic calcifications, osteosclerosis, nephrocalcinosis and severe chronic renal disease in rabbits.[4 22 24–27] Those studies suggest that UC could be relevant in the management of KD and in other conditions that affect calcium metabolism in rabbits. Future studies could include using the modified Arsenazo III technique to measure UC and UCC in rabbits with KD, in rabbits with a high metabolic demand for calcium or in rabbits with any other suspected impairment on calcium metabolism.

CONCLUSIONS

The results obtained in the present study suggest that *E cuniculi* IgG seropositivity, age, sex, neutered status and urine collection method may not have implications for UPC, UC, UCC, UG, UGC, phosphorus, plasma total calcium, plasma creatinine, urea and ionised calcium measurements from otherwise healthy rabbits; however, the phosphorus value was higher in pet rabbits aged up to 3 years compared with those over 3 years, suggesting that published reference values for plasma phosphorus in rabbits should be adjusted according to age.

The data analysed in the present study indicate that UC and UCC, the reference intervals of which were established for the first time in healthy pet rabbits, can be calculated using a modified Arsenazo III technique as a rapid inhouse procedure for pet rabbits.

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