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Urinalysis and determination of the urine protein-to-creatinine ratio reference interval in healthy cows

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Background: There are no reference intervals for urinalysis in cattle.

Hypothesis/Objectives: Characterize the urine of healthy cows, establish urine protein-to-creatinine ratio (UPC) reference intervals, and test possible differences among dairy and beef cattle, age groups, or stage of lactation.

Animals: Seventy-seven dairy and 74 beef 2.5 to 17 year-old cows of different breeds housed mainly in free stall.

Methods: In this prospective study, urine specimens were collected by catheterization. Complete urinalysis was performed within 1 hour including specific gravity, dipstick evaluation, visual urine pH evaluation with 0.3 pH unit graded strips, and microscopic evaluation of the sediment. Urinary protein and creatinine concentrations and protein electrophoresis were determined on frozen aliquots.

Results: Overall reference intervals were 1.020 to 1.045 for USG, 7.0 to 8.7 for pH, and 0.04 to 0.25 for UPC; because of differences in creatinine concentration, UPC was lower in beef (0.04-0.14) than in dairy (0.05-0.25) cows and in the latter in dry than lactating cows. With dipstick evaluation, most analytes were absent except for blood, ketone, and protein in 24.7, 16.0, and 64.7% of cases, respectively. Microscopic evaluation revealed less than 3 red blood cells, leukocytes, and epithelial cells in 84, 99.3, and 100% cows, respectively. No band was observed at electrophoresis, except in 1 case at MW ~66 000.

Conclusions and Clinical Importance: Creatininuria is higher in beef than dairy cows and proteinuria is likely more efficiently characterized by protein concentration than by UPC.

KEYWORDS

cattle, creatininuria, pH, proteinuria, UPC, Urinalysis, USG

1 | INTRODUCTION

Urinalysis is 1 of the most useful diagnostic tools to monitor animal health as it is rapid, cheap, and readily available in routine practice. It is not only an essential component in the diagnosis of urogenital diseases, but is also a major tool in the diagnosis of kidney, liver, and metabolic diseases. In cattle clinical practice, urine is often collected by spontaneous voiding, even if the most suitable collection method for bacterial examination, to avoid contamination and obtain a urine specimen rapidly, is bladder catheterization.

Urinary dipsticks are easy to use and provide information that has been more or less validated, but the physiologic alkaline urine pH of cattle, for example, leads to a large number of false positive dipstick results for proteinuria. Many research studies or case reports have

not confirmed proteinuria detected by dipsticks in cattle.[1–6] Positive results therefore need to be confirmed by semiquantitative tests such as the nitric-acid test (Heller's test) or sulfosalicylic-acid test,[7] or by quantitative determination of proteinuria by urine protein-to-creatinine ratio (UPC), routinely used in small animal practice.[8] The few references concerning normal urine in cattle state that "protein is not detected in urine"[9] or that "normal urine contains only small amounts of proteins,"[10] but no primary references are given to support these statements. The commonly used literature search engines do not provide any adequately sourced reference intervals for adult cattle urine analytes, including, the microscopic examination of urinary sediment.[11,12]

Considering this lack of scientific knowledge about urinalysis in healthy cattle, the aims of this study were therefore to characterize the urine of healthy adult dairy and beef cows, to establish UPC reference intervals according to the American Society of Veterinary Clinical Pathology (ASVCP) recommendations,[13] and to identify possible differences between dairy and beef cattle. The hypothesis was that urine from adult cattle did not differ from that of other species.

2 | MATERIALS AND METHODS

The protocol of this prospective study was approved by the "Science et Santé Animale" ethics committee (N°115) (SSA-2015-002). All the breeders signed a consent form.

2.1 | Reference sample group, inclusion, and exclusion criteria

Based on recommendations from the Clinical and Laboratory Standards Institute[14] and the ASVCP,[13] 156 healthy adult cows were initially selected, that is, 78 dairy and 78 beef cows. A maximum of 5 cows at 1 of 3 stages of lactation, namely early lactation (0-90 days in milk), mid lactation (90-180 days in milk), and dry period, was included from each herd. Sampling was conducted from December 2015 to June 2016.

2.2 | Selection of herds

To be as representative as possible of the different French husbandry systems, the 32 herds were selected from 3 different geographical areas (West, Center, and South-West of France). They were high-yielding dairy cows fed predominantly on corn silage and beef cows fed exclusively on grass and hay, all with water ad libitum. All herds were free from bovine leukemia virus.

2.3 | Selection of cows

The health status of each cow was checked by a complete questionnaire regarding current health status, food intake, medical history over the past 2 months, and, for dairy cows, individual and herd milk production. The exclusion veriteria were medical treatment during the previous 4 weeks and any abnormality during the complete physical

examination performed before sampling, which included rectal temperature, respiratory, cardiac, and ruminal assessments. The crude protein content of the feed and cow body weight were not taken into account for inclusion, exclusion, or partitioning purposes.

2.4 | Urine specimen collection and analysis

Urine specimens were collected by catheterization after careful washing, using a 20 mL syringe (Injekt, B Braun, Melsungen, Germany) and a sterilized catheter, by the same investigator (NH). Urine specimens were processed by a Board-certified specialist in veterinary clinical pathology (CT). Within 1 hour and after macroscopic examination, the urine was aliquoted into 5×1.5 mL plastic tubes (Safe Lock, Eppendorf, Le Pecq, France) which were centrifuged at $250g$ for 5 minutes (GustoMini centrifuge, Fisher brand, HeathroScientific, Illinois). The supernatants from 4 tubes were transferred into 4 appropriately labeled 1.5 mL plastic tubes (Safe Lock; Eppendorf) and stored at 4°C until frozen at −80°C within 24 hours. The urine in the 5th tube was used for urinalysis. Approximately 100 μL of urine supernatant was retained for resuspension of the sediment by gentle mixing. Approximately 35 μL of the suspension was transferred to a glass slide (Menzel-Gläser, Brunswick, Germany) and covered with a standard 22×22 mm coverslip (Menzel-Gläser).

2.5 | Urinalysis, UPC, and electrophoresis

The complete urinalysis included a urine specific gravity (USG) determination (Portable Refractometer, Zuzi, ATC) calibrated between each analysis, a 10-variables dipstick evaluation (Combur-Test strips, Cobas, Roche diagnostic, France), a visual urine pH evaluation with 0.3 pH unit graded 6.5-10.0 strips (MColorpHast, Merck KGaA, Darmstadt, Germany), and microscopic evaluation (Nikon, E200, Tokyo, Japan) of the sediment. The mean numbers of granular casts and crystals were determined from 10 low-power microscopic fields. The mean numbers of epithelial cells, red blood cells, and white blood cells were determined from 10 microscopic fields under high power. An arbitrary semiquantitative count of bacteria on a 0/1/2 scale was performed by the same person (CT). Results were recorded on separate forms for each animal.

Six to 12 months later, 1 frozen urine aliquot from each cow was thawed for approximately 30 minutes at room temperature and homogenized. Urinary protein and creatinine concentrations were measured by pyrogallol red (U/CSF Protein, Thermofisher Scientific, Waltham, Massachusetts) and enzymatic (Creatinine Enzymatic, Thermofisher Scientific) methods respectively, using an automated analyzer and the manufacturer's reagents (Indiko, Thermofisher Scientific). Calibration and quality controls were performed with the manufacturer's solutions (sCal and MAS Urichem Track solutions, Thermofisher Scientific). The interassay precision coefficients of variation (CVs) were <3.2 and <3.7% for creatinine and proteins, respectively, and the corresponding biases were <−3.6 and 1.4%.

Sodium dodecyl sulfate agarose gel electrophoresis (SDS-AGE) was performed on 18 random urine specimens, irrespective of the UPC, and on the 4 urine specimens with the highest protein concentrations.

TABLE 1   Distribution of cows used for the determination of healthy bovine urinalysis

Production	Number Total	Lactation stage			Diet		Age (y)	
		St1	St2	St3	CS	H	Mean	Range
Dairy	77	25	30	22	49	28	5.6	2.5-13
Beef	74	25	27	22	30	44	7.1	4-17

Abbreviations: H, hay; CS, corn silage; St1, 1-90 days in milk; St2, 90-180 days in milk; St3, dry.

All electrophoreses were performed with a semiautomated system (Hydrasys, Sebia Italia SRL, Italy). Eighty microliters of the urine supernatant were mixed with 20 μL of the additive provided by the manufacturer (Hydragel 5 proteinuria, Sebia Italia SRL). Five microliters of the treated urine were loaded on the gels (agarose 50 g/L), and the migration, staining with acid-violet and drying, were conducted with the semiautomated system. Protein identification was visual, based on the manufacturer's molecular markers solution (Molecular mass control, Sebia Italia SRL) containing lysozyme (14.3 kDa), triosephosphate isomerase (26.6 kDa), bovine albumin (66 kDa), and human IgG (150 kDa).

2.6 | Statistical analysis

All demographic data and results were recorded on Excel spreadsheets (Microsoft, Redmond, Washington). Descriptive statistics, Spearman correlations, Passing-Bablok agreement curves, and difference diagrams were obtained with Analyse-It (Leeds, United Kingdom). Reference intervals were determined with Reference Value Advisor[15] using the nonparametric method, except when n <40 in which case the robust method was adopted. The 90% confidence intervals (CIs) were calculated by the nonparametric method when n >120 and by a bootleg methods in other cases. Outliers were identified by visual inspection of the histograms and Tukey's test. Values for the deleted outliers are given in the table footnotes. All variables were tested for possible effects of the following co-variables by ANOVA using Systat 13 (Systat Software, San Jose, California): presence of urine ketones, type of production (beef versus dairy), stage of lactation (beginning versus middle versus dry), breeding farm (1-32), and age. When the observed differences were statistically significant, the relevance of partitioning was based on Harris & Boyd's z test.[16]

3 | RESULTS

3.1 | Specimens included and demography of cows

Five urine specimens were discarded because of turbidity, the presence of traces of blood, or of numerous bacteria on microscopic examination.

The 151 cows finally included were Prim'Holstein (n = 57), Charolaise (n = 24), Blonde d'Aquitaine (n = 22), Limousine (n = 21), Montbeliarde (n = 10), Brune des Alpes (n = 5), Normande (n = 5), Salers (n = 5), and Aubrac (n = 2) breeds. The demographic characteristics of the 151 cows are listed in Table 1.

Despite the large age range (2.5-17 years), 95% of the cows were 2.5-11 years old. The median age of the 74 beef cows was 1.5 years higher than that of the 77 dairy cows (ANOVA, P = .04). Most cows (n = 125) were housed free-stall and a few in tie-stall (n = 26). Approximately half of the cows received a dry mostly hay-based feed (n = 72), and the other half received a moist feed based on corn and grass silage (n = 79).

Quality controls for the measurements of urine protein and creatinine indicated interassay precision CVs of <3.2 and <3.7% for creatinine and proteins, respectively, and corresponding biases of <−3.6 and 1.4%.

3.2 | Macroscopic examination of urine

All urines were straw yellow, most often clear and in some cases mildly turbid. The odor was faintly aromatic.

3.3 | USG and urine pH

Descriptive statistics and the reference intervals for USG measured by refractometry and the pH measured with precision strips are given in Table 2, and the corresponding histograms are shown in Figure 1. The overall reference intervals were 1.020 to 1.045 for USG and 7.0 to 8.7 for pH.

Urine specific gravity estimated by refractometry was moderately but significantly higher in cows receiving dry feed (P = .04) and moderately higher in the presence of ketone bodies (P = .03). The mean difference between specimens with no ketone bodies and with 2 or 3+, was 0.005. Ketonuria intensity had no significant effect on USG (Tukey's honestly significant difference [HSD] test P > .05). The USG measurements obtained by refractometry were not correlated with the urine test strips results (Spearman's r = 0.01), for which 83.7% were equal to 1.000.

The measurements with precision strips indicated that none of the covariables had any effect on pH; its distribution was different from Gaussian and could not be transformed into Gaussian by the usual transformations. The pH measurements obtained by routine urine test strip were higher than by precision strip and the correlation was weak (Spearman's r = 0.51). The median (urine test strip − precision strip) difference was 0.7, ranging from −0.5 to 1.3 with 50% of the differences between 0.7 and 0.9.

TABLE 2 Descriptive statistics and reference intervals of urine specific gravity (USG) by refractometry and urine pH measured with precision strips in healthy dairy and beef cows

	N	Mean (SD)	Median (range)	Normality P	LLRI (90% CI)	ULRI (90% CI)
USG	150[a]	1.030 (0.006)	1.030 (1.015-1.050)	.001 (.000)	1.020 (1.015-1.022)	1.045 (1.041-1.050)
pH	147[b]	8.2 (0.4)	8.3 (6.6-8.7)	.000 (.000)	7.0 (6.5-7.4)	8.7 (8.7-8.7)

Abbreviations: CI, confidence interval; Normality, Anderson-Darling test, untransformed and Box-Cox transformed values between parentheses; LLRI, lower limit of the reference interval; ULRI, upper limit of the reference interval.
[a]One result missing.
[b]Four results missing.

3.4 | Urine test strip chemical analysis

The results of all analyses are given in Table 3 (1 specimen was lost). Most analytes were absent or rarely present, and in the latter case only at low concentrations. Only the blood and protein pads frequently gave positive results, in 24.7 and 64.7% of the cases, respectively. A moderately high percentage (16%) of ketone positive urines was also observed. No effect of the covariables on the test strip results was apparent.
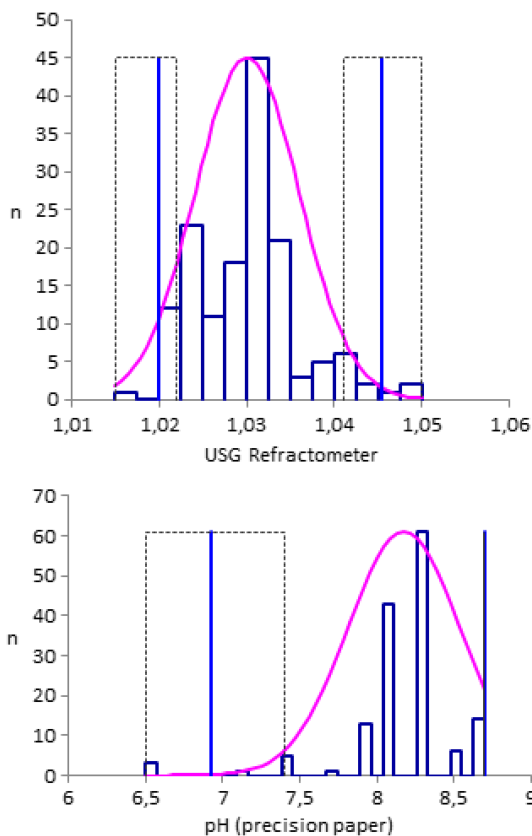

FIGURE 1 Histograms of urine specific gravity (USG) by refractometry and urine pH by precision strips in healthy dairy and beef cows (pink line is the fitted distribution; vertical lines and dashed lines are reference limits and their 90% confidence intervals)

3.5 | Microscopic examination of urine

The percentages of cases corresponding to the mean recorded counts are presented in Table 4.

None of the covariables had any significant effect on urine microscopic observations ($P > .05$). A moderate correlation was observed between the number of RBC under microscopy and the visual estimation of "Blood" from the urine test strip (Spearman's $r = 0.58$). Positive test strip results (4 traces and 1 4+) were obtained in 5 cases when no RBC were observed under microscopic examination. Few WBC were identified in 24.7% of the specimens, whereas the test strips were positive in only 3 cases: 2 had scores of 0.1 and 0.3, and 1 was negative on cytological examination. Correlations between the RBC and WBC observations were very weak (Spearman's $r = 0.24$). WBC were observed in 11/70 cases when no RBC was observed, and RBC were observed in 47.8% of cases when no WBC was observed.

3.6 | Urine protein and creatinine concentrations, UPC

Four results were considered as outliers for urine protein concentration based on visual inspection of the histogram and Tukey's test, and were therefore excluded from the UPC calculations.

Results before and after partitioning are given in Table 5, with an overall UPC RI of 0.04 to 0.25. The distribution of UPC was statistically different from Gaussian and could not be transformed into Gaussian with any of the usual processes. The type of production, diet, and stage of lactation had a significant effect on the UPC values, the highest values being approximately 2 times lower in beef than in dairy cows ($P < .001$, thus warranting different reference intervals, as confirmed by Harris and Boyd's test [$z > z*$]) (Table 5 and Figure 2). Although statistically significant, the effect of the diet did not necessitate separate reference intervals (Harris and Boyd's test $z < z*$). Tukey's HSD test revealed a significant effect of the stage of lactation in dairy but not in beef cattle, with higher values of UPC at stages 1 and 2 of lactation than in dry cows.

None of the covariables had a significant effect on urine protein concentration (Table 5). Urine creatinine concentration was approximately 2 times lower in dairy than in beef cattle ($P < .001$) (Table 5).

TABLE 3 Urine test strip results for urine specimens from 150 dairy and beef cows

Variable	Negative	1+	2+	3+	4+
Leukocytes	147	3	0	0	0
Blood	113	18	9	4	6
Proteins	53	82	15	0	0
Glucose	150	0	0	0	0
Ketones	126	15	7	2	0
Urobilinogen	150	0	0	0	0
Nitrites	150	0	0	0	0
Bilirubin	147	1	2	0	0
pH	6	7	8	9	
	1	2	10	137	
Specific gravity	1.000	1.005	1.010	1.015	>1.015
	125	14	4	4	3

Urine creatinine concentration increased significantly with age, but this effect was limited as the difference between the 25% younger and 25% older cows was not significant (Mann-Whitney's test, $P = .17$). A very strong correlation and hyperbolic relationship was observed between urine creatinine concentration and UPC (Spearman's $r = 0.90$) (Figure 3). Creatinine concentration was more strongly correlated with USG by refractometry in beef than in dairy cows (Spearman's r were 0.44, 0.47, and 0.76 in the whole reference sample group, dairy cows, and beef cows, respectively).

A weak correlation was noted between protein detection by test strips and urine protein concentration (Spearman's $r = 0.58$). The latter increased significantly according to the number of "+" results of the strip (ANOVA, $P = .001$). There was no correlation between the protein test strip results and UPC (Spearman's $r = 0.05$), the latter not being different according to the test strip results (ANOVA, $P = .47$) (Figure 4).

3.7 | Urine protein electrophoresis

The median duration of urine storage before SDS-AGE analysis was 15 months, (minimum 12 months and maximum 24 months). Storage duration was not correlated with any SDS-AGE finding. No band was observed in the 22 gels except in 1 case characterized by a high UPC (0.27) and protein concentration (503 mg/L), in which a band of approximately 66 kDa was observed (Figure 5).

4 | DISCUSSION

This study reports experimental results for urinalysis in healthy dairy and beef cows, including routine on-field analyses, cytology, reference values for protein determination, and investigation of the possible effects of type of production, breed, lactation stage, parity, and nutrition as covariables.

4.1 | Reference sample group and methods

This experiment was carried out in a large reference sample group selected according to international recommendations for the de novo establishment of reference intervals[13,14,17] with nonparametric determination of reference limits and their 90% CIs, except if compartmenting meant that the groups contained fewer than 40 animals. Moreover, the statistical methods used to determine the reference intervals could not be applied to all variables (notably test strip results and cytology), as most of the obtained values were semiquantitative.

The reference sample group was constituted to represent the average adult cow population of France with equal percentages of dairy and beef cows of different breeds, at various stages of lactation, and from different breeding systems. A maximum of 5 animals was sampled per farm to avoid a possible "farm effect," but this also prevented any investigation of such an effect. The healthy status of the animals was based on the complete history and clinical examination of each animal. The decision to include ketone-positive urine specimens in the study can be questioned. However, these specimens were included because none of the ketone-positive animals showed clinical signs at the time of sampling or during the 2 weeks after urine collection, and also because the presence of ketones in the urines had no effect on any of the analyses (except a very slight effect on USG).

The same analytical methods were used for urinalysis in this study as in other species. These methods have some limits: (1) no bovine urine control specimen is available so we had to use human control specimens for urine proteins and creatinine measurements; (2) clinical refractometers have not been validated for bovine urine; in this case again, there was no gold standard to determine the accuracy of the measurements; (3) we did not measure urine pH with a pH-meter but relied on an extended scale precision test strip previously used for calf urine and showing good correlation with the pH-meter measurement ($r = 0.89$)[18] because we thought that it might be useful to obtain more precise measurements with a 0.3 pH unit scale than with the routine urine test strips which have a 1 pH unit scale; we also considered that using a pH strip would be closer to routine practice than referring to a laboratory for pH measurement, even though portable pH-meters

TABLE 4 Percentage of observations of microscopic analytes in urine from healthy dairy and beef cows. Scores: see Material and Methods

Mean score	0	0.1-3.0	3.1-10.0	>10	
RBC	46.7	37.3	6.7	9.3	3.3% ≥50
WBC	75.3	24.0	0.7	0	Maximum = 20
Crystals	100	0	0	0	
Hyaline casts	94.0	6.0	0	0	Maximum = 1.3
Epithelial cells	90.3	9.7	0	0	99.4% ≤1.6, Maximum = 3
Bacteria	97.3	2.7	0	0	Maximum = 3

TABLE 5 Descriptive statistics and reference intervals of UPC, urine creatinine and urine protein concentrations in healthy dairy and beef cows

	Units	n	Mean (SD)	Median (range)	Normality	LLRI (95% CI)	ULRI (95% CI)
UPC	-	147	0.105	0.085	0.000	0.039	0.252
			(0.055)	(0.029-0.309)	(0.013)	(0.029-0.046)	(0.221-0.309)
		77 dairy	0.139	0.128	0.011	0.050	0.284
			(0.056)	(0.046-0.309)	(0.899)	(0.046-0.071)	(0.235-0.309)
		55 dairy	0.155	0.147	0.013	0.082	0.299
		St1 and St2	(0.052)	(0.081-0.309)	(0.809)	(0.081-0.210)	(0.245-0.309)
		22 dairy	0.093	0.087	0.077	0.043	0.191
		St3	(0.034)	(0.046-0.183)	(0.737)	(0.036-0.053)	(0.149-0.248)
		70 beef	0.068	0.068	0.002	0.035	0.135
			(0.021)	(0.029-0.141)	(0.188)	(0.029-0.041)	(0.105-0.141)
Creatinine	mg/L	147	1631	1423	0.000	458	3617
			(866)	(360-4662)	(0.629)	(360-535)	(3404-4662)
		77 dairy	1085	999	0.000	387	2520
			(500)	(360-2963)	(0.708)	(360-471)	(2005-2963)
		55 dairy	910	943	0.315	371	1721
		St1 and St2	(327)	(360-1875)	(0.332)	(360-456)	(1370-1875)
		22 dairy	1524	1435	0.274	623	3026
		St3	(589)	(730-2963)	(0.860)	(549-762)	(2474-3631)
		70 beef	2232	2111	0.035	1017	4037
			(783)	(954-4662)	(0.573)	(954-1124)	(3605-4662)
Proteins	mg/L	147[a]	134.5	139.0	0.000	67	211
			(29.2)	(54-241)	(0.000)	(54-81)	(167-241)

Abbreviations: CI, confidence interval; LLRI, lower limit of the reference interval; Normality, Anderson-Darling test on untransformed and Box-Cox transformed values between parentheses; St1, 0-90 days in milk; St2, 90-180 days in milk; St3, dry period; ULRI, upper limit of the reference interval.

[a]Four outliers deleted: 321, 451, 503, and 557 mg/L.

have been used in cattle practice[19]; (4) the test strip and USG determinations were done on the urine supernatant; and (5) reading the dipsticks was visual (by the same operator to reduce variability), which has been shown to be inferior to automated reading.[1]

4.2 | USG and pH

The reference interval for USG determined in this study was similar to those reported in textbooks[10,20] and did not vary with the different covariables, notably cow nutrition. Most of the urines were moderately concentrated so that routine urine refractometers are likely well adapted to bovine urine and, unlike feline urine, do not require special USG scales. Relevant validation of the refractometer measurements of USG would require USG to be correlated with osmolality, as done for instance in canine urine.[21] Moreover, the test strip measurements of USG were poorly correlated with the refractometer measurements, as in other species, thereby supporting the recommendation to ignore this pad and systematically use a refractometer, as already reported for dog, cat and cattle urine.[1]

Most data on cow urine pH have been obtained during the first days of lactation when an anionic diet is recommended to prevent

milk fever and leads to acidic to neutral urine.[22] The pH reference interval in this study was similar to the ones cited in textbooks (7-8.5)[10,20] and in 2 studies on 57 healthy cows (6.9-8.7 with a portable and a benchtop analyzer)[19] and on 139 Holstein and Jersey cows (8.03 ± 0.13).[23] In the first study, the urine test strip results were well correlated with the pH-meter measurements ($r = 0.89$), whereas in the present study their correlation with the pH precision strip was weak ($r = 0.5$). As in calves, the results obtained with urine test strips were higher than those obtained with precision strips, and the difference was slightly greater (mean of 0.70 versus 0.25 in calf urine). These results confirm the practical benefit of employing the precision pH strip already used in calves (see above).

4.3 | Urine test strip and microscopic evaluation

Except for pH and specific gravity measurements, urine test strips are designed for the detection and semiquantitative evaluation of "abnormal" constituents in human urine, that is, analytes which are absent (or present at concentrations below the limit of detection) in the urine of healthy people. They are routinely used for urinalysis in animals but, to the best of our knowledge, have not been validated, except on some
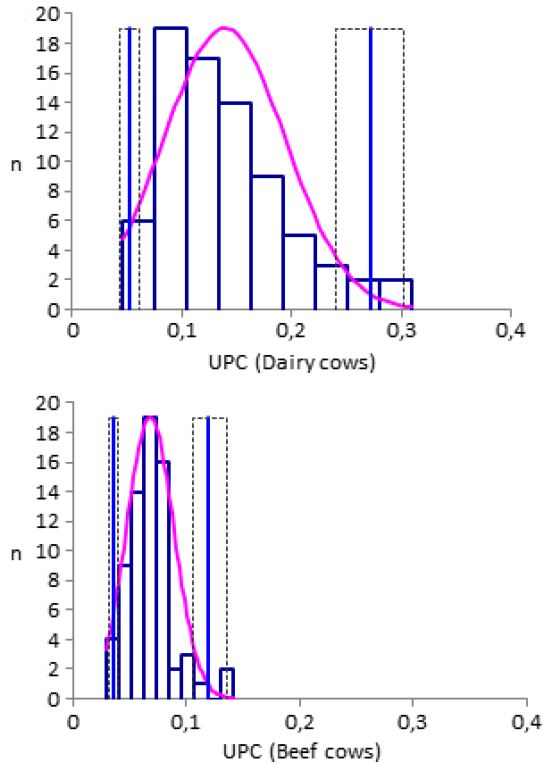
FIGURE 2 Comparison of urine protein-to-creatinine ratio (UPC) histograms in healthy dairy and beef cows (pink line is the fitted distribution; vertical lines and dashed lines are reference limits and their 90% confidence intervals)

occasions in canine urine.[21,24] As regards bovine urine, only 1 study on 100 diseased cows compared the protein pad of a test strip with quantitative measurements (see below).[1] As test strips are designed to detect abnormalities, it is not surprising that all or almost all results were negative for glucose, leukocytes, urobilinogen, bilirubin, and
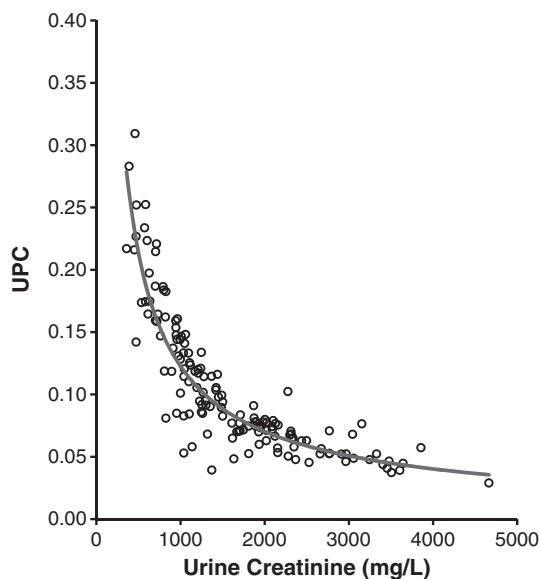

FIGURE 3 Relationship between urine protein-to-creatinine ratio (UPC) and urine creatinine concentration
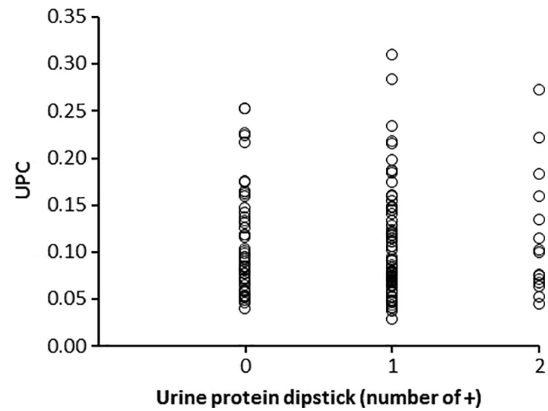

FIGURE 4 Distribution of urine protein-to-creatinine ratio (UPC) in healthy dairy and beef cows according to results from the protein pad of urine test strips (dipstick proteinuria as number of +)

nitrites in this study on healthy cows. However, some of the specimens were positive for blood and proteins, which is discussed below.

The "blood" pad on urine test strips is highly unspecific as it detects peroxidase-like activities of hemoproteins such as hemoglobin, myoglobin, and microbial hemoproteins, and is subject to false positive reactions with oxidant drug residues, bleach, or detergents.[25–27] None of these unspecific reactions were likely in this study, as none of the cows showed signs of muscle disease which could result in myoglobinuria, and new sterile plastic tubes were used for the analyses. Moreover, the limit of detection of the pad is low, about 5 RBCs/μL of urine. Although it is difficult to convert the microscopic examination results into an RBC concentration, the 3 cells/hpf that we chose arbitrarily are likely to correspond to negative test strip results. This could account for the relatively high proportion of positive specimens, similar to the number of specimens with >3 cells/hpf detected by microscopy. In our opinion, this moderate percentage of positive specimens in healthy cows likely results from minor trauma during urinary catheterization. In some cases, it might be caused by RBC hemolysis in the urine, which could lead to positive test strip results even though no RBCs were observed by microscopy. The moderate correlation between the test strip results and cytology and the 5 positive test strip results (4 traces and 1 4+)
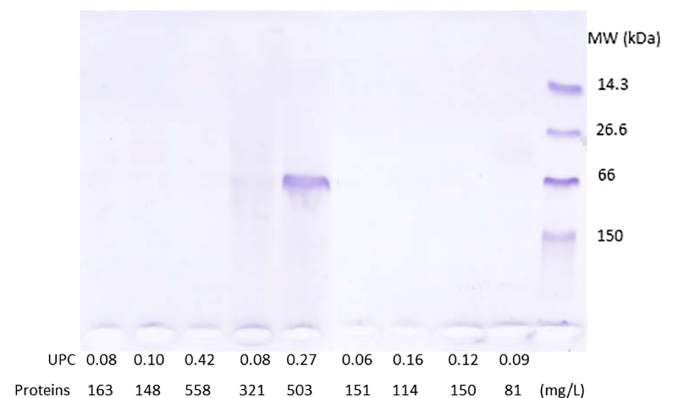

FIGURE 5 Examples of sodium dodecyl sulfate agarose gel electrophoresis (SDS-AGE) in urines from healthy cows

when no RBC were observed by microscopy highlights the caution needed when interpreting urine test strip blood pads in bovine medicine.

The surprising absence of an effect of lactation stage or breed on the ketone test strip results could be explained by the possible ruminal origin of ketone bodies. In ruminants, ketone bodies are continuously used as an energy source by the extrahepatic tissues, the rumen and liver being primary producers of ketone bodies. In the fed state, the ruminal epithelium of healthy cows is the primary source of circulating ketone bodies, which could explain the positive ketone results obtained in the healthy cow.[28,29] The presence of low-level ketones in urine should therefore not be considered as "abnormal" and had no influence on the other dipstick variables, except for a minor effect on USG.

The leukocyte detection pad on urine dipsticks is based on the activity of leukocyte esterases present in human granulocytes. This pad is reported to be insensitive in canine and feline urine[30,31] and to give numerous false negatives, whereas it very frequently gives false positives in feline urine,[32,33] maybe because of the excretion of cauxin a feline-specific carboxyl esterase which is also known to cause false positive protein results.[34] The performance of the leukocyte pad in bovine urine has not been investigated, but the dipstick pad was shown to react with neutrophils on bovine uterine cytobrushings with a very low rate of false negatives and a good correlation with the number of leukocytes.[35,36] It was also reported to give positive results in 12/17 cases of pyelonephritis but to be negative in the 5 other cases even though macroscopic pyuria was observed.[37] In the present study, 99% of the cases had no or fewer than 3 WBC/hpf at microscopy, so such a low concentration of WBC would probably be below the detection limit of the leukocyte pad. The 3 dipstick positive cows had microscopic leukocyte scores ≤0.3.

For the microscopic examination, scores <5 elements/microscopic field are usually considered "normal" for cells, casts and cysts in human,[38] or canine urine.[39] In this study, most results were <3 elements/microscopic field. We therefore suggest that, in bovine urinalysis, a threshold of <3 elements/microscopic field or cells, hyaline casts, and crystals would be more suitable.

4.4 | Urine protein-to-creatinine ratio

The urine protein concentration measured in this study (mean, 135 mg/L) was lower than previously reported in 127 healthy Holstein cows[40] but similar to the results obtained earlier in 35 cows.[41] Interestingly no covariable (notably breed and diet) was associated with proteinuria.

In healthy animals, creatinine excretion into the urine depends on the glomerular filtration of plasma creatinine resulting from muscle creatine-phosphate breakdown, with no or limited tubule reabsorption or secretion according to species.[42]

It is thus logical that, owing to the higher muscle mass of beef cows, their plasma creatinine concentration is higher than that of dairy cows (about 2-fold higher, unpublished results), and the urine creatinine concentration is also higher. Similar variations in urine creatinine concentration according to lean muscle mass have also been observed, for

example, in humans[43] and wild-ranging capuchin monkeys.[44] Recovery of muscle mass after lactation may also have accounted for the higher creatininuria observed in dry compared to lactating dairy cows. A similar increase of urine creatinine excretion during lactation has been reported in Holstein and Jersey cows, the former having a higher creatinine concentration.[23] In the latter study, creatininuria was lower (628 ± 246 mg/L) than in our present study in which no breed-related difference was observed. Creatinine excretion in the milk is likely to have been only a very minor factor contributing to the lower creatininuria observed in lactating versus dry dairy cows. Although information about milk creatinine concentration is sparse, it was reported to be almost stable at about 10 mg/L in 2 cows from days 2 to 15 after calving.[45] As the covariables did not influence urine protein concentration and creatinine differed according to the type of production and stage of lactation, the observed variations in UPC were expected to be inversely related to creatininuria. In other species, such as dogs, the relevance of UPC to evaluate proteinuria is based on using the urine creatinine concentration as a means of correcting urine protein concentration for urine dilution concentration. However, the urine creatinine concentration in the cows of this study was a poor indicator of the urine dilution estimated by USG, at least in dairy cows, as shown by the weak correlation observed. Moreover, as urine creatinine concentration has been shown to be the main factor of variation in UPC, the relevance of using this ratio to evaluate urine protein excretion in cattle can be challenged. We suggest that, in cows, a single quantitative measurement of protein concentration would be sufficient. However, UPC could be used to monitor changes in proteinuria in cows in which the muscle mass remains stable and no notable changes in creatininuria are expected. Pending further study of the diagnostic efficiency of this variable in both healthy and diseased animals, including cows with renal diseases and cows with nonrenal diseases, the tentative threshold for bovine urine protein concentration could be the upper limit of the reference interval, that is, 250 mg/L (including the CI of the limit).

The semiquantitative evaluation of proteinuria by urine strip was very poorly correlated with UPC (Figure 4). This finding is consistent with previous studies,[40] which reported a high number of false positive test strip results in cattle.[1] An alkaline urine pH has been suggested as the possible cause of nonspecific staining of the protein pad,[46] as the buffering power of the pad (at about pH = 3) is probably overridden at high pH. This was confirmed in cattle urine as the correlation between the test strip results and the quantitative protein concentration was greater after the exclusion of alkaline urine specimens, pH >7.5.[1] However, only test strips for which the limit of detection is about 0.3 g/L can be used in field practice. Positive results therefore need to be confirmed by a quantitative measurement of proteinuria, especially in urines with a pH <7.[7]

4.5 | Urine protein electrophoresis

The urinary proteins concentration in healthy animals and humans is very low. The kidney glomerulus filters low-molecular weight proteins (MW <69 kDa), which are almost entirely reabsorbed in the tubule, whereas traces of tubule proteins are added after filtration.[42] As a result,

no or only very faint bands are observed during routine urine protein electrophoresis in healthy subjects, the most frequent being albumin, as observed in dogs.[47] When high-resolution techniques, such as electrophoresis with silver staining or proteomics, were applied to bovine urines, they showed that numerous different proteins and peptides could be found.[41,48] However, a routine stain such as the acid violet used in this study has a much higher detection limit, so it is not surprising that an albumin band was clearly observed in only 1 case. The absence of proteins in the other specimens could be due either to very low concentrations in the urines or to prolonged storage of the specimens, that is, longer than the 1 year storage period reported to have no effect on canine urine protein electrophoresis.[47] However, there was almost no trace of smears on the gels, which would be a sign of proteolysis, which was reported to be low at -70°C in human specimens.[49]

5 | CONCLUSION

Two main points of this study are that creatininuria is higher in beef than in dairy cows and that bovine proteinuria is likely more efficiently characterized by protein concentration than by UPC. As the reference intervals were established according to the ASVCP recommendations, they can be applied to similar populations in laboratories using the same techniques or in other cattle or with other techniques after proper transference or validation.[13]

CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

The protocol was approved by the "Science et Santé Animale" ethic committee (N°115) (SSA-2015-002). A consent form was signed by all breeders.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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