RESEARCH ARTICLE

Urinary Neutrophil Gelatinase-Associated Lipocalin, Cystatin C and Clusterin as Biomarkers for Acute Kidney Injury in Cattle with Tropical Theileriosis

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A B S T R A C T

Schizont-infected nucleated cells and anemia-induced hypoxia may cause acute kidney injury (AKI) in cattle with tropical theileriosis. In most cases, serum creatinine (sCr) fails to detect early kidney injury. Recently, urine biomarkers such as neutrophil gelatinase-associated lipocalin (NGAL), Cystatin C (CysC), and Clusterin (Clu) have outperformed sCr in identifying AKI in various animal species. However, these markers have not been tested in cattle for AKI detection. This study investigated AKI in cattle with tropical theileriosis using serum and urinary NGAL, CysC, and Clu and compared them to standard renal function markers such as sCr, urine protein to creatinine (uPC) ratio and urine-specific gravity (USG). Seventeen cattle were infected with *Theileria annulata* (Th) and ten were controls (CTRL) in this investigation. The urinary NGAL (uNGAL) in the Th group was considerably (P<0.001) greater than that in the CTRL group. uCysC increased significantly in the Th group compared to CTRL group (P<0.001). The Th group had higher sClu than the CTRL group (P<0.001). There was a significant difference (p=0.005) in sCr concentrations between Th and CTRL groups, but the values were within the reference range in both groups. Th group had a higher uPC ratio than CTRL (p= 0.002), whereas USG was lower in Th group than in CTRL group (P<0.001). These results suggested that cattle with tropical Theileriosis have acute tubular injury.

INTRODUCTION

Tropical theileriosis is an acute lymphoproliferative tick-borne disease of cattle caused by *Theileria* (*T.*) *annulata*. The disease has a wide geographical distribution, ranging from China and India to the Mediterranean coastal regions of Europe and Africa. The life cycle of *T. annulata* begins with the inoculation of sporozoites to the host by the infected vector tick during the feeding process. This is followed by macroschizont, microschizont, and merozoite stages in the macrophages and B lymphocytes. After being released from ruptured cells, merozoites invade the red blood cells and develop into the piroplasm stage (Nene and Morrison, 2016; Ahmed *et al.*, 2008).

The schizont stage of *T. annulata*, which affects macrophages and B-lymphocytes, is principally responsible for the clinical signs of tropical theileriosis (Nene and Morrison, 2016). Schizont-infected nucleated cells rapidly spread to non-lymphoid organs and induce pathological changes in several organ systems. Cattle with tropical theileriosis may experience acute kidney injury (AKI), as a result of the degenerative effects of schizont-infected and cytokine-producing nucleated cells on the kidneys (Forsyth *et al.*, 1999). Furthermore, immune-mediated hemolytic anemia has the potential to cause kidney injury over the course of the disease (Agina *et al.*, 2021). Although the occurrence of kidney injury in tropical theileriosis has been demonstrated in histopathological examination of kidneys in experimental studies, there is conflicting data about the detection of AKI using circulating creatinine concentrations in cattle with tropical theileriosis (Col and Uslu, 2007; Dede *et al.*, 2014; Ma *et al.*, 2020).

The determination of circulating creatinine concentration has been acknowledged for many years as a key component in the diagnosis of AKI (Makris and Spanou, 2016). However, there are substantial drawbacks to using circulating creatinine concentration to identify the kidney injury. Due to its delayed reaction during the early

stages of AKI, the fact that creatinine is a measure of renal function rather than renal injury, and the effect of many non-renal variables such as age, muscle mass, and hydration level, creatinine is an unreliable marker for identifying AKI (Devarajan, 2010). In recent years, novel kidney dysfunction and injury biomarkers, including neutrophil gelatinase-associated lipocalin, cystatin C and clusterin have been studied in humans, dogs and cats for the early detection of AKI (Yerramilli *et al.*, 2016). Unlike sCr, these markers are released into the blood and urine shortly after kidney injury (Konukoglu, 2018). In particular, the urinary concentration of these biomarkers has been recognized as a sensitive indicator of AKI in dogs (Monari *et al.*, 2020). Recently, the serum and urine concentrations of NGAL and CysC have been evaluated in horses with AKI (Siwińska *et al.*, 2021). NGAL and CysC have also been studied in dogs with *Babesia rossi* infection and in horses naturally infected with *T. equi* (Ahmadpour *et al.*, 2020; Defauw *et al.*, 2020). Serum CysC concentrations have been found to be related to sCr concentrations and parasitemia in horses infected with *T. equi* (Ahmadpour *et al.*, 2020). Increased urinary NGAL concentrations have also been reported in *Babesia rossi* infected dogs and these increases have been linked with kidney injury (Defauw *et al.*, 2020). In addition, urinary NGAL and Clu concentrations were observed to be considerably higher in dogs with AKI than in healthy dogs (Zhou *et al.*, 2014). In that study, NGAL and Clu have shown high sensitivity and specificity for the detection of AKI in dogs.

Contrary to previous studies covering different animal species, there is no data on NGAL, CysC, and Clu in cattle with AKI. Given the unfavorable prognostic effect and inconsistent reports of AKI in cattle with tropical theileriosis, it is important to investigate AKI in this population using more sensitive kidney injury and dysfunction markers. In the present study, the objective was to evaluate the serum and urine concentrations of NGAL, CysC, and Clu and to compare them to traditional renal function markers including serum and urine concentrations of creatinine and protein, and urine-specific gravity in cattle with tropical theileriosis.

MATERIALS AND METHODS

Animals: This research was conducted between April 2021 and September 2021 at Firat University Veterinary Teaching Hospital with the ethical approval of Firat University Local Ethics Committee of Animal Experimentation (20/08/2020, 2020/11). Cattle that were presented to the Firat University Veterinary Teaching Hospital and fulfilled the inclusion criteria formed Theileriosis group (Th). The inclusion criteria for the Th group included the microscopic detection of intraerythrocytic piroplasms of *T. annulata* in peripheral blood smear, a decreased hematocrit (HCT), absence of concurrent disease, and a negative urine culture. Based on their history, clinical symptoms and hematological and serum biochemical data, concurrently diseased animals were excluded. The control group (CTRL) was formed with cattle that were found to be healthy based on physical examination, had normal hematocrit and normal serum biochemistry profile, no intraerythrocytic Theileria

piroplasms in peripheral blood smear and negative urine culture.

Sample collection and storage: For hematological and serum biochemical analysis, jugular venous blood samples were separately collected into tubes containing EDTA and clot activator (BD Vacutainer, Plymouth, UK), respectively. Whole blood samples were tested for HCT and white blood cell counts (WBC) immediately after collection in the Th groups and within one hour in the CTRL group. Before centrifugation, serum tubes were kept at room temperature for no longer than one hour. After that, the serum samples were divided into three 1 mL aliquots and stored at -80°C until further analysis. A sterile urine container was used to collect urine samples. Urine samples were obtained by catheterization using sterile catheters. Urine samples were then separated into three portions. The first portion was transferred to the Firat University Faculty of Veterinary Medicine, Department of Microbiology for urine culture within one hour. The second portion was used for physical examination and determination of USG. The last portion of urine was centrifuged and divided into three 1 ml aliquots. Thereafter, aliquots were stored at -80°C until further analysis. Both stored serum and urine samples were analyzed within 6 months.

Hematological and biochemical analysis: An automated hematology analyzer (BC-5000Vet, Mindray, Shenzhen, China) was used to calculate the HCT and WBC. The semiquantitative heat precipitation method was employed to measure the plasma fibrinogen levels. An automated biochemistry analyzer (AdviaXPT, Siemens Healthcare Diagnostics, Malvern, PA, USA) was used to measure urinary creatinine and protein, as well as the serum biochemistry profile, which included total protein, albumin, glucose, total calcium, phosphorus, GGT, ALP, urea-nitrogen, and creatinine. Serum and urine NGAL, CysC, Clu, and serum haptoglobin (HPT) were measured with commercially available cattle-specific ELISA kits (Bioassay Technology Laboratory, Shanghai, China). Urine-specific gravity was determined using a hand-held refractometer (Atago Master-Sur/Nα, Atago Co. Ltd. Tokyo, Japan). Additionally, the mean intra-assay coefficient of variation (CV%) of the NGAL, CysC, Clu and Haptoglobin ELISA kit was calculated based on the duplicate measurement of seven different samples. In order to calculate the intra-assay CV for urine protein and urine creatinine measurements, five consecutive measurements of each analyte were performed on a sample.

Urine culture: Blood agar with defibrinated sheep blood was used to directly inoculate 100 µL of the aseptically collected urine sample. It was then incubated for a further 24-48 hours at 37°C in both aerobic and 10% CO2 environments.

Statistical analysis: Data were analyzed using statistical software (SPSS 22, IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). Following the Shapiro-Wilk test to determine data normality, the paired sample t-test was used to compare within-group variables, while the independent sample t-test or Mann-Whitney U test was used to analyze between-group variables. The

Spearman's correlation test was used to ascertain the relationship between the variables in cattle with tropical theileriosis. A p-value of 0.05 was accepted to demonstrate a statistical difference.

RESULTS

The current study was conducted on 27 cattle, 17 of which were assigned to the Th group and the remaining 10 to the CTRL group. Simmental (n = 19; 70.4%) and Brown Swiss (n = 8; 29.0%) were the breeds of cattle involved in this study. Cattle in the Th and CTRL groups had median ages of 25.5 months (6-114 months) and 35.5 months (12-67 months), respectively. The mean intra-assay CV values were 8.54%, 4.34%, 6.95% and 2.76% for NGAL, CysC, Clu and haptoglobin, respectively. In addition, the Advia XPT system revealed CV values of 3.63% (mean = 6.34 mg/dL, standard deviation = 0.23 mg/dL) and 1.29% (mean = 72.26 mg/dL, standard deviation = 0.96 mg/dL) for urinary protein and creatinine measurements, respectively.

Table 1 summarizes the results of hematological and serum biochemistry analyses in the Th and CTRL groups. Hematocrit and WBC count were considerably lower (P<0.001) in Th group cattle compared to the CTRL group. In serum biochemistry analysis, serum total protein and albumin concentration were below the lower reference limit in the Th group and decreased significantly compared to the CTRL group (P<0.001).

Table 2 shows results for renal function parameters and kidney injury and dysfunction markers in Th and CTRL groups. The mean of sCr concentration of the Th group was lower than that of the CTRL group (p = 0.005), although the mean sCr concentrations of both groups were within the reference range. The Th group showed significantly (p=0.003) higher urine protein (uPro) concentrations compared to the CTRL group. Similarly, urine protein to creatinine (uPC) ratios were also greater (p=0.002) in the Th group than in the CTRL group (Fig. 1). When compared to the CTRL group, the USG was significantly lower (P<0.001) in the Th group (Fig. 2). The urine NGAL (uNGAL) of the Th group was significantly (P<0.001) higher than both the serum NGAL (sNGAL) of the Th group and the uNGAL (P<0.001) of the CTRL group (Fig. 3A). When compared to the CTRL group, uCysC increased considerably (P<0.001) in the Th group (Fig. 3B). uClu did not differ between groups, while sClu in the Th group was greater (P<0.001) than sClu in the CTRL group and uClu in the Th group (P<0.001) (Fig. 3C).

Fig. 4 displays correlation plots, correlation coefficients and statistical significance between several variables in cattle with tropical theileriosis. uCysC and uNGAL showed a strong positive correlation (r=0.877; P<0.001). uPC showed positive correlation with uCysC (r=0.515; P<0.05) and uNGAL (r=0.622, P<0.01).

DISCUSSION

The current study assessed AKI in cattle with tropical theileriosis using serum and urine concentrations of novel kidney injury and dysfunction markers including NGAL, CysC and Clu as well as standard renal function indices like creatinine, urea nitrogen and urinary protein - creatinine

Table 1: Hematology and biochemistry results for cattle with tropical theileriosis (Th) and control (CTRL) groups.

	Th (n = 17)	CTRL (n = 10)	P-value	Reference Range
Hematology				
HCT (%)	16.6±4.9	34.2±5.9	<0.001	22-33
WBC (x 10³/ μL)	4.6 ±3.7	10.7±2.3	<0.001	4.9-12
Biochemistry				
Total Serum Protein (g/dL)	6.1 ±1.2	8.0±0.7	<0.001	6.74-7.46
Serum albumin (g/dL)	2.7±0.4	3.8±0.3	<0.001	3.03-3.55
Fibrinogen (mg/dL)	633±293	620±199	0.899	200-700
Glucose (mg/dL)	60±16	49±7	0.052	45-75
Total Calcium (mg/dL)	8.6±0.7	9.6 ±1.1	0.003	9.7 ±12.4
Phosphorus (mg/dL)	5.7±1.5	7.4±1.7	0.012	5.6-6.5
GGT (U/L)	62±44	26±11	0.017	15-39
ALP (U/L)	55±34	94±33	0.007	0-488
Haptoglobin (ng/mL)	109.86±7.88	78.21±5.36	0.009	

HCT: Hematocrit; WBC: White Blood Cell; p values lower than 0.05 is considered significant.
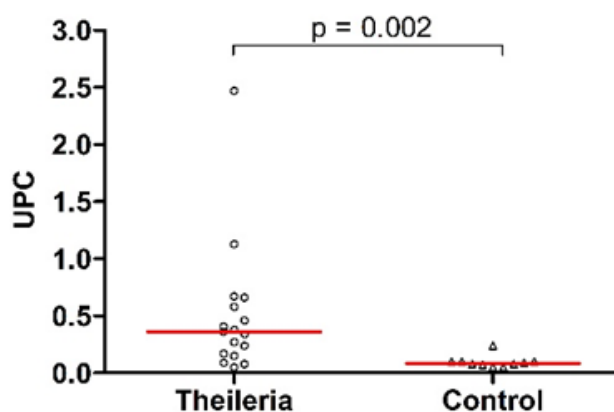
Fig. 1: Scatterplot displays uPC ratios in cattle with tropical theileriosis and in healthy cattle. The red horizontal bars indicate the median in each group. P< 0.05 is considered significant, uPC = Urine Protein to Creatinine.
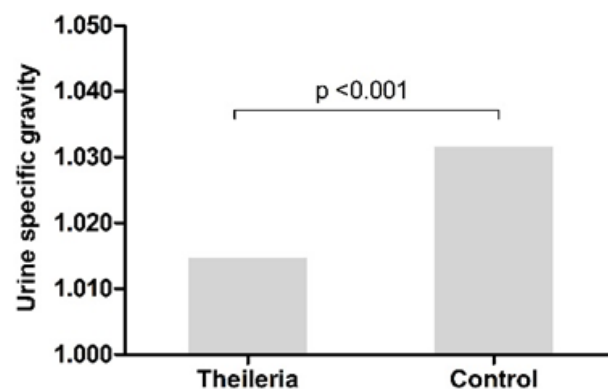
Fig. 2: Barplot displays mean of USG values in cattle with tropical theileriosis and in healthy cattle. P< 0.05 is considered significant, USG = Urine Specific Gravity.
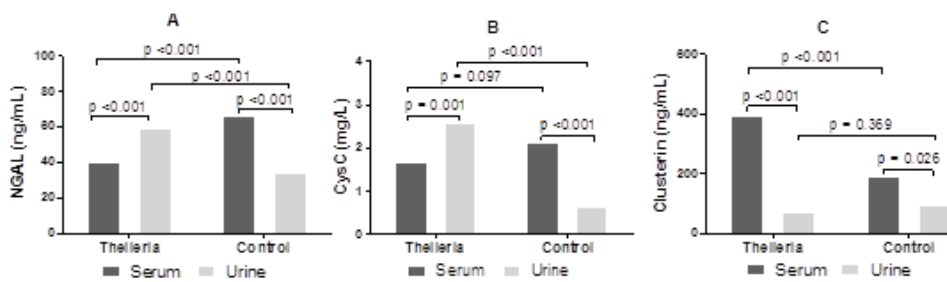
ratios in cattle with tropical theileriosis. To the authors, this is the first study to investigate the serum and urinary concentrations of NGAL, CysC and Clu in cattle with tropical theileriosis. The results of this study revealed that cattle with tropical theileriosis had higher uNGAL and uCysC concentrations, as well as a higher uPC ratio but sCr concentrations in both cattle with tropical theileriosis and healthy cattle were within the reference range.

NGAL, a member of the lipocalin family, is detectable at very low concentrations in circulation and urine in a healthy state due to continual low-rate release by different

Table 2: Renal function parameters and kidney injury and dysfunction biomarkers for cattle with tropical theileriosis (Th) and control (CTRL) groups.

	Th (n = 17)	CTRL (n = 10)	P-value	Reference Range
Renal Function Parameters				
sCr (mg/dL)	1.08±0.22	1.34 ±0.19	0.005	1.0-2.0
sUrea (mg/dL)	19.6±7.1	11.9±2.6	0.003	20-30
uPro (mg/dL)	36.1 (2.4-240)	7.9 (2.2-26.1)	0.003	
uCr (mg/dL)	75.3 (31.0-441)	86.2 (22.6-188)	0.824	
uPC ratio	0.36 (0.05-2.47)	0.09 (0.05-0.24)	0.002	0.04-0.25
USG	1.015±0.002	1.032±0.003	<0.001	1.020-1.045
Kidney Injury and Dysfunction Biomarkers				
uNGAL (ng/mL)	59.15±15.18	34.25±6.44	<0.001	NA
sNGAL (ng/mL)	40.22±11.52	66.87±14.58	<0.001	NA
P-value	<0.001	<0.001		
uCysC (mg/L)	2.59±0.68	0.67±0.38	<0.001	NA
sCysC (mg/L)	1.68±0.74	2.13±0.46	0.097	NA
P-value	0.001	<0.001		
uClu (ng/mL)	74.02±62.62	94.69±44.13	0.369	NA
sClu (ng/mL)	393.18±153.18	191.69±87.15	<0.001	NA
P-value	<0.001	0.026		

s: serum; u: urine; Cr: Creatinine; Pro:Protein; uPC: Urine Protein to Creatinine; USG: Urine Specific Gravity; NGAL: Neutrophil Gelatinase Associated Lipocalin; CysC: Cystatin C; Clu: Clusterin; NA: Not Available. p values lower than 0.05 is considered significant.

Fig. 3: Barplots display means of urinary and serum NGAL (A), CysC (B), Clu (C) concentrations in cattle with tropical theileriosis and in healthy cattle. P<0.05 is considered significant; NGAL = Neutrophil-Gelatinase Associated Lipocalin; CysC = Cystatin C; Clu = Clusterin.
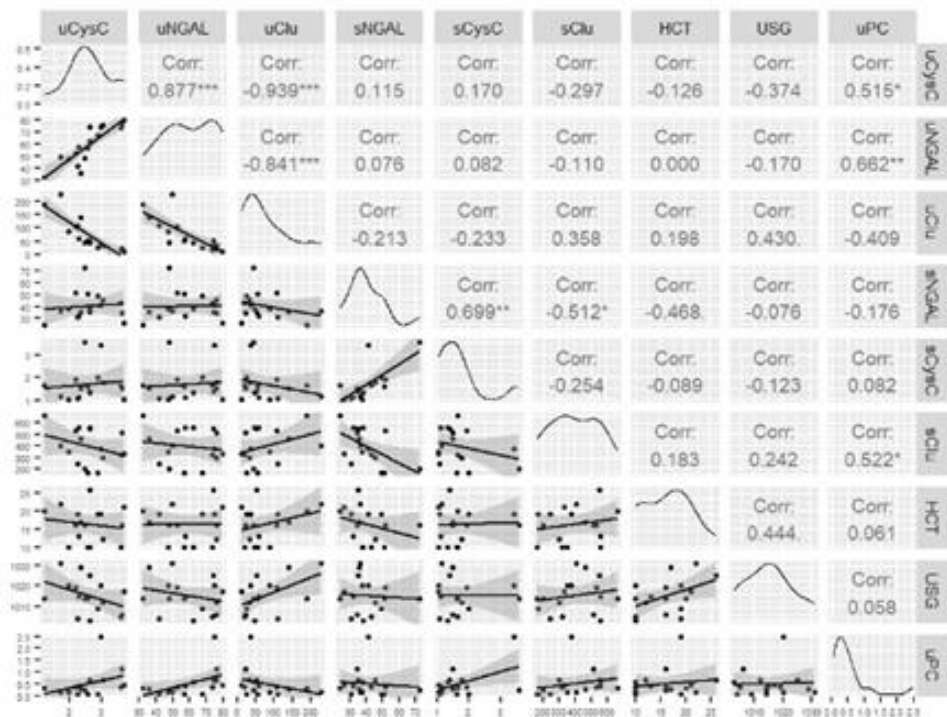
Fig. 4: Correlogram for some variables in cattle with tropical theileriosis. *P<0.05; **P<0.01; ***P<0.001.

cell types (Singer *et al.*, 2013). Circulating NGAL is freely filtered by the glomerulus, and vast amounts of filtered NGAL are absorbed by tubules unless there is concurrent tubular injury. Immediately following kidney injury, renal production and urine secretion of NGAL increases dramatically (Peacock *et al.*, 2013). Previous transcriptomic research found that *Ngal* gene was highly expressed in the kidneys during the early stages of AKI (Yuen *et al.*, 2006). Furthermore, NGAL release was abundantly induced in the kidneys following ischemic and nephrotoxic injury in animal models (Mishra *et al.*, 2004). In the present study, uNGAL concentrations were found to be increased regardless of sNGAL concentrations in Theileria-infected cattle. This data could indicate increased NGAL expression in the distal tubules as a result of injury or decreased tubular reabsorption of urine NGAL as a result of proximal tubular injury (Devarajan, 2010). Similar to our findings, parvovirus-infected dogs had higher uNGAL

than healthy dogs, but there was no increase in plasma NGAL in infected dogs (van den Berg *et al.*, 2018). Siwińska *et al.* (2021) also reported that horses at risk of AKI due to colic had greater uNGAL concentrations but not increased sNGAL compared to horses that were not at risk for AKI. In contrast to our findings, that study also revealed that sNGAL concentrations increased along with uNGAL concentrations in horses with AKI. The simultaneous increase in serum and urine NGAL may be the result of enhanced NGAL mRNA expression in non-renal organs during AKI, resulting in the release of NGAL into circulation. In addition, the release of NGAL by neutrophils and the impaired clearance of NGAL due to a decreased GFR caused by AKI may contribute to an increase in circulating NGAL levels (Devarajan, 2010). In our study, an increase in uNGAL independent of sNGAL may be due to subclinical renal injury.

Cystatin C is a low-molecular-weight protease inhibitor that is involved in intracellular protein catabolism and is released into the plasma at a constant rate by all nucleated cells in the body. It is considered to be a superior marker to sCr to assess early AKI (Bagshaw and Bellomo, 2010). In critically ill dogs, serum CysC increased more rapidly than creatinine and indicated early AKI more accurately than sCr (Paes-Leme *et al.*, 2021). Serum and urinary CysC concentrations have been found to be higher in horses with AKI (Siwińska *et al.*, 2021). In the same study, increased uCysC concentrations were also reported in horses with colic regardless of sCysC concentration. Circulating CysC is almost completely filtered by the glomerulus, reabsorbed by the renal tubules, and then catabolized, but it does not undergo tubular secretion. In physiological states, urine concentrations of CysC are therefore detectable only in trace amounts, and any increase in uCysC is attributed to renal tubular dysfunction. (Bagshaw and Bellomo, 2010). Similar to NGAL, increases in uCysC concentrations independent of sCysC concentrations in the present study suggested that tubular injury occurred in *T. annulata* infected cattle, but that glomerular function was not compromised. In another study, the authors investigated the changes in sCysC concentration in horses infected with *T. equi* (Ahmadpour *et al.*, 2020). In that study, horses infected with *T. equi* demonstrated increased sCysC concentrations. In addition, there was a substantial positive association between sCysC concentration and parasitemia rate. In contrast to that study, our findings revealed no association between parasitemia and CysC concentrations in serum or urine. This could possibly be explained by the detrimental effects of schizont-infected and cytokine-secreting nucleated phagocytes on renal tubules rather than the severity of parasitemia and anemia (Forsyth *et al.*, 1999).

Clusterin is a multifunctional glycoprotein and performs numerous functions, including lipid transport, cell stress protection, cell aggregation, sperm maturation, anti-apoptosis, cellular function protection in kidney damage, and complement activation (Jones and Jomary, 2002). Increased uClu has been identified as a potent indicator of drug-induced proximal tubular injury in rats and an increase in its urine concentrations precedes histological abnormalities in the kidneys and an increase in sCr (Dieterle *et al.*, 2010). In addition, uClu and uNGAL have been reported to be increased in gentamicin-induced

AKI in Beagle dogs, and both biomarkers had a high sensitivity for determining AKI (Zhou *et al.*, 2014). Contrary to previous reports uClu did not increase in *T. annulata* infected cattle. Furthermore, uClu showed substantial negative correlations with uNGAL and uCysC. The lack of an increase in uClu and its negative correlation with uNGAL and uCysC may be associated the extent and the duration of the kidney injury in cattle infected with *T. annulata*. In humans uNGAL, uCsyC and uClu have been evaluated for early diagnosis of diabetic kidney injury and the prediction of microalbuminuria (Zeng *et al.*, 2017). In that study, uNGAL and uCysC have increased prior to the onset of microalbuminuria, but uClu has increased after the onset of microalbuminuria. Interestingly, cattle infected with *T. annulata* exhibited a remarkable increase in sClu rather than uClu. Previous studies have demonstrated that sClu can be increased by a variety of pathologic processes such as complement activation and inflammation (Kuleš *et al.*, 2014; Minamijima *et al.*, 2022). A proteomic study conducted on horses with colitis revealed that serum Clu was upregulated approximately two times higher in horses with colitis than in healthy horses (Minamijima *et al.*, 2022). The authors of that study argued that Clu upregulation in horses with colitis may be related to the oxidative stress and inflammation that occurs during colitis. Another proteomic study suggested that serum Clu was upregulated in *Babesia canis* infected dogs and that this overexpression reflected complement activation (Kuleš *et al.*, 2014). Although *T. annulata* infected cattle had greater serum HPT concentrations as an indicator of inflammatory status compared to healthy cattle, the absence of correlation between sClu and serum HPT concentrations indicated that increased sClu concentrations could not reflect a systemic inflammatory response.

In the present study, sCr concentrations in *T. annulata*-infected cattle were lower than in healthy cattle, but sCr concentrations in both groups were within the reference ranges. In a previous study, increased sCr concentrations were reported in *T. annulata* infected compared to healthy cattle (Col and Uslu, 2007). In another study, the sCr concentrations in *T. annulata* infected cattle were comparable to those of healthy cattle. (Dede *et al.*, 2014). Similar to previous reports, in the present study sCr concentrations failed to document kidney injury in *T. annulata* infected cattle. Unlike sCr, USG and uPC ratio showed kidney injury in cattle with *T. annulata*. The failure to concentrate urine is an early indicator of renal tubular injury (Parrah *et al.*, 2013). Additionally, the assessment of the uPC ratio in spotted urine samples permits estimation of glomerular or tubular injury in the kidney (Grauer, 2011). In the present study, uPC ratios were considerably higher in cattle infected with *T. annulata*. In addition, significant positive correlations between uPC, uNGAL and uCysC support that tubular injury occurs in *T. annulata* infected cattle. Furthermore, USG was lower in *T. annulata* infected cattle than in healthy cattle in our study. The established reference intervals for uPC ratio and USG in dairy cattle were 0.04 to 0.25 and 1.020 to 1.045, respectively (Herman *et al.*, 2019). On the basis of this reference interval, 64.7% of cattle infected with *T. annulata* exhibited proteinuria, while 70.6% of infected cattle had decreased USG in the present study. Katsoulos *et al.* (2020) established an uPC ratio cut-off value of 0.19 to distinguish

cattle with and without kidney injury. In the current study, 70.6% of cattle infected with *T. annulata* and 10% of healthy cattle exhibited an elevated uPC ratio based on that cut-off value. Proteinuria can originate from non-renal or renal abnormalities. Non-renal causes of proteinuria generally result from lower urinary tract inflammation or hemorrhage (Grauer, 2011). Non-renal proteinuria is not the case in our study as we formed study groups based on the negative urine cultures and normal urine sediment findings. On the other hand, renal proteinuria can be originated from glomerular and tubulointerstitial pathologies. In conjunction with the uCysC and uNGAL results, the increased uPC ratio could indicate tubular injury in *T. annulata*-infected cattle. Furthermore, moderate increases in uPC ratios are consistent with proteinuria originating from renal tubules.

The main conclusions of this study are that AKI develops in cattle during Theileriosis and that urinary dysfunction and injury markers, including uCysC, uNGAL, uPC ratio, and USG are more effective than sCr in identifying AKI in this population. Despite the fact that this study produced significant findings, there are still certain issues that require further investigation. First off, this study was designed as a cross-sectional study. Therefore, we did not examine how these urine markers changed following therapy. Second, the number of cattle in the control group was insufficient to establish reference ranges for serum and urine NGAL and CysC concentrations.

Authors contribution: KCT conceived and designed the study. KCT, PFPD, SB, and ZY executed the study and analyzed the serum and urine samples. KCT analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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