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FULL PAPER

Acute phase proteins as biomarkers of urinary tract infection in dairy cows: diagnostic and prognostic accuracy

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Abstract

The aims of this study were to investigate the level of acute phase proteins in dairy cows with urinary tract infection (UTI) and to evaluate their diagnostic and prognostic value. Eighty-four lactating cows with clinical and laboratory evidence of UTI and 15 healthy controls were included in this study. Serum samples were evaluated for the levels of Haptoglobin (Hp), serum amyloid A (SAA), fibrinogen (Fb), α1-Acid glycoprotein (AGP), total protein, and globulin. The diagnostic and prognostic performance of each parameter was evaluated by estimating the area under receiver operating characteristics curve (AUROC). *Escherichia coli* and *Corynebacterium* spp. were the primary bacteria associated with UTI. The levels of serum Hp, SAA, Fb, AGP, total protein, and globulin were significantly higher in UTI cows. Successfully treated cows (n = 51) had lower levels of Hp, SAA, AGP, total protein, and globulin than non-responsive cows. Overall, Hp, SAA, Fb, and AGP showed comparable diagnostic accuracy (AUROC ranged from 0.93 to 0.98). Both Hp and SAA showed high accuracy in predicting treatment response (AUROC > 0.95), whereas Fb level was of no prognostic value (AUROC = 0.48). From this study, acute phase proteins levels can be used as markers for UTI in cows and higher levels of Hp, SAA and AGP are related to poor treatment response.

Key Words: Urinary tract Infection, Cows, Acute phase proteins, Diagnostic, Prognostic accuracy.

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Introduction

Few literatures are available concerning urinary problems in cattle compared to other species. The sources of urinary tract infection (UTI) are varied and may be related to ascending infection from the urachus in neonatal animals, which can involve the urinary bladder, ureter and kidney[35].

Another pathway for UTI in animals is through the vulva, which may play an important role as a site of entry of several urinary infections in bovine species. Moreover, septic catheterization, post-calving disorders and bacteremia, may also act as predisposing factors for UTI in cattle[1].

Bovine UTI most frequently occurs as a consequence of ascending infection with certain bacterial pathogens including *Corynebacterium renale*, *Corynebacterium cystidis*, *Corynebacterium pilosum*, or *Escherichia coli*[27,21,37,36].

UTI may also elicit vascular damage to the urinary bladder and decrease kidney function competence, with subsequent disturbances in protein, acid-base, water and solute homeostasis and in the excretion of final metabolic products. When the kidneys can no longer regulate body fluids and solute composition, renal failure occurs and consequently the loss of affected animals[7,8]. Additionally, mortality and involuntary culling rates can be as high as 47% among lactating cows with UTI[36].

Examination of a urine sample is one of the most important diagnostic aids that help in the diagnosis of causes and site of UTI in animals[25]. Although, a urine culture is very essential to determine the type of bacterial infection[26], culture results take at least two days, with subsequent delay in the onset of treatment. As early treatment is necessary to prevent further damage to the urinary tract, there is a need for a rapid diagnostic tool to allow therapeutic intervention at the proper time.

Acute phase proteins (APPs) are plasma proteins which increase or decrease in concentration in response to infection, inflammation and internal or external challenges. Monitoring changes in APPs levels have been shown to give valuable diagnostic and prognostic information during infection and inflammation[9]. APPs are considered highly sensitive in detecting subclinical infections or inflammation; however, they are not specific for certain disease, as they can be raised in many diseases and inflammatory conditions[4]. The magnitude and duration of the acute phase response reflect the infection severity and underlying tissue damage[15]; therefore, APPs can serve as good indicators of prognosis and treatment outcome.

In addition to their immunomodulatory role, APPs also have antibacterial activity. Bovine SAA acts by opsonizing a range of Gram negative and Gram-positive bacteria including *E. coli*[30,22]. Additionally, Hp exerts bacteriostatic effect by binding free haemoglobin, thus depriving bacteria, such as *E. coli* from iron required for their growth[8].

Little is known about the diagnostic and prognostic value of APPs in naturally occurring UTI in dairy cows, which is the main goal of this study.

Materials and methods

Animals: All the animal procedures used in this study were performed according to the guidance of the Animal Ethics Committee of College of Veterinary Medicine and Animal Resources, at King Faisal University, Saudi Arabia. A total of 103 lactating cows from a private farm in the eastern region of Saudi Arabia were initially enrolled in this study over two year period (September 2012 to August 2014). Cows were divided into two groups based on clinical, biochemical, and bacteriological examination. The first group (n = 15) consisted of clinically healthy cows with normal urine picture and negative bacterial culture. The second group involved cows with a clinical picture similar to UTI (n = 88). Clinically affected cows had anorexia, decreased

milk production, dysuria, stranguria, pollakiuria, blood-tinged urine and abdominal pain. Rectal examination of affected animals revealed severe pain sensation during bladder palpation and resistance to the examination. No clinical abnormalities were found in other parts of the urinary tracts of examined cows. The UTI cows were confirmed by an abnormal urine picture and a positive bacterial culture. Cows that had signs of UTI without positive bacterial culture were excluded from the study (n = 4).

Physical examination of cows: The study cows were fully examined according to the methods described by Rosenberger[28] which included general behavior and condition, auscultation of the heart, lungs, rumen and intestine, measurement of heart rate, respiratory rate and rectal temperature, swinging auscultation and percussion auscultation of both sides of the abdomen, and rectal examination.

Haematological and Biochemical analysis: Blood samples were collected from the jugular vein into Plain and EDTA vacutainer tubes from all cows. Plasma and serum were obtained from blood samples and stored at −20°C until analysed.

Determination of total protein, albumin, globulin, blood urea nitrogen (BUN) and creatinine: The serum samples were tested using an automated biochemical analyser (VetScan VS2, Abaxis, Northern California, USA) to determine the concentration of total protein, albumin, globulin, blood urea nitrogen (BUN) and creatinine.

Determination of acute phase proteins: Hp was measured with a commercially available colorimetric assay kit (Tridelta Development Plc, Wicklow, Ireland) according to the manufacturer's instructions. SAA was measured using ELISA kits (Tridelta Development Plc, Wicklow, Ireland). The analytical sensitivities of these tests in plasma have been determined as 0.3 μg/ml for SAA and 0.0156 mg/ml for Hp by the manufacturer. Fibrinogen was measured by heat precipitation-refractometry method[7]. α1-Acid glycoprotein was measured with a commercially available SRID kit (Tridelta Development Plc, Wicklow, Ireland, Cat. No. TP-805B), according to the manufacturer's instructions. The antisera in the agar gel reacts specifically and exclusively with Bovine AGP. Bovine AGP concentrations within a range of 50 to 1,500 μg/ml was detected according to the sensitivity of the kits.

Urinalysis: Urine samples were obtained via aseptic catheterization and immediately assessed for color, transparency and odor. The urine samples were similarly tested using a strip test (Combur^9-Test, Roche). Smears of the urine sediment were stained with Gram's stain and examined microscopically. Urine samples were cultured bacteriologically on blood agar, nutrient agar and MacConkey agar followed by incubation for 48 h at 37°C. Bacterial species identification was determined using VITEK2 Compact, Biomerieux, France. Antibacterial sensitivity tests were carried out using the standard methods of the US National Committee for Clinical Laboratory Standards[33]. Two different technicians who were blind to the results of each other and case control classification conducted bacterial identification and APPs measurements.

Treatment protocol: The cows with UTI received one of the following antibiotic therapies for 10–21 days according to the results of sensitivity tests, amoxicillin (Betamox LA, Norbrook) 15 mg per kg, IM every 48 h (n = 48), Sulfadiazine (200 mg/ml) and Trimethoprim (40 mg/ml) (Norodine 24, Norbrook) (n = 20) 1 ml/16 kg bodyweight daily by intramuscular injection and Ceftiofur (Excenel RTU Pfizer) 2.2 mg/kg, IM (n = 16). Moreover, all diseased cows received flunixin meglumine (Finadyne, Schering-Plough Corporation, USA) 1.1 mg/kg body weight IV for three days. Additionally, they were treated for 2 to 3 days with 10 l of dextrose-Saline (Dextrose 5% and Saline 0.9%) administered IV in a slow drip.

The cows with UTI were further categorized into two groups according to the response to treatment (the treatment based on urine culture and sensitivity tests for isolated bacteria and selection of the proper antibiotics), the success group (n = 51) and the other failure one (n = 33). Treatment success was based on disappearance of clinical signs, clinical examination of cows and negative urine culture.

Statistical analysis: Given the small size of the control group and non-normally distributed markers in cows with UTI, each blood biomarker was assessed using non-parametric analysis (Wilcoxon Mann-Whitney) at $P < 0.05$ to compare the data between cases and controls, and between cows with treatment success or failure. The diagnostic and prognostic value for each of Hp, SAA, Fb, and AGP was determined by estimating the area under receiver operating characteristics curve (AUROC), where each of the APPs (index test) was evaluated against case control status of cows as defined by clinical symptoms and bacterial isolation. The AUROC indicates the overall accuracy of the tested parameter. Based on the ROC, the best cut-offs points to predict presence of UTI were determined. The difference in the AUROC among measured APPs was compared using a non-parametric method[6]. In order to estimate the combined diagnostic potential of APPs, logistic regression analyses were performed with case control status as dependent variable and measurement of APPs levels as independent variables. All analyses were done using Stata version 13 (Stata Corp, College Station TX, USA)

Results

Clinical picture of UTI in cows

Cows with UTI showed anorexia, decreased milk production, dysuria, stranguria, pollakiuria, blood-tinged urine and abdominal pain. Rectal examination of affected animals revealed severe pain sensation during bladder palpation and resistance to the examination. There were no clinical abnormalities in other parts of the urinary tracts of examined cows.

Urine analysis findings

Analysis of urine samples from the UTI group showed proteinuria, hematuria and pyuria. Bacteriological examination of urine samples from the UTI group revealed presence of a single organism in 72 cows and mixed cultures in 12 cows. The isolated bacteria were *E. coli* (n = 45) *Corynebacterium* spp. (n = 33), *Proteus* spp. (n = 11) *and Streptococcus* spp., (n = 7).

Hematological and biochemical findings

The levels of Hp, SAA, Fb, AGP, albumin and globulin were much higher in cows with UTI compared to healthy ones. On the other hand, there were non-significant changes in the levels of urea and creatinine in UTI cows compared to healthy ones. In addition, the magnitude of increase in APPs levels was stronger for Hp and SAA compared to AGP and Fb (Table 1).

Table 2 shows the variables according to treatment success or failure. The response to treatment was significantly ($P < 0.05$) associated with the levels of Hp, SAA, AGP, total protein, and globulin. Additionally, Fig. 1 shows an increase in the proportion of non-responsive cases with higher levels of each of Hp, SAA, and AGP, but not with Fb.

Spearman's correlation analysis showed a high negative ($r \geq -0.77$, $P < 0.001$) correlation between treatment success and the levels of Hp, SAA, total protein, and globulin. Additionally, moderate negative correlation was found between treatment success and AGP (Table S1). Finally, there was a weak correlation between bacterial species and treatment response. Regardless of bacterial species, cases with treatment failure showed significantly higher values of Hp, SAA, and AGP compared to successfully treated ones (data not shown). The diagnostic accuracy of different APPs is shown in Table 3, overall, Hp,

Table 1. Descriptive results and univariate analysis of blood biomarkers in cows with clinical diagnosis of UTI and in healthy cows

Variable[a]	Cows with UTI (n = 84)			Healthy cows (n = 15)			*P-value*[b]
	Mean	Median	Range	Mean	Median	Range	
HP (g/l)	4.32	3.51	0.13–10.23	0.12	0.14	0.00–0.15	0.0001
SAA (μg/ml)	90.48	80.17	22.55–166.25	22.51	23.15	16.54–24.23	0.0001
Fibrinogen (g/l)	11.35	13.22	3.54–17.44	3.54	3.54	2.10–5.20	0.0001
AGP (μg/ml)	313.46	291.41	240.15–402.46	237.00	236.25	220.36–259.36	0.0001
Total proteins (g/dl)	7.34	6.80	6.50–8.90	6.71	6.70	6.50–6.90	0.0178
Albumin (g/dl)	2.68	2.70	2.40–2.90	2.71	2.70	2.50–2.90	0.4018
Globulin (g/dl)	4.42	4.00	3.50–5.60	3.72	3.70	3.30–4.30	0.0004

[a]Hp: Haptoglobin; SAA = Serum Amyloid A; Fb = Fibrinogen; AGP = α1-Acid glycoprotein, Min = Minimum, Max = Maximum
[b]*P*-value resulting from non-parametric Wilcoxon Mann-Whitney test

Table 2. The description of the variables depending on the success or failure with the treatment

Variable[a]	Success cases (N = 51)			Failure cases (N = 33)			*P-value*[b]
	Mean	Median	Range	Mean	Median	Range	
HP (g/l)	2.36	2.55	0.13–4.50	7.34	7.540	0.15–10.23	0.0001
SAA (μg/ml)	60.94	62.25	22.55–142.12	136.14	144.21	23.66–166.25	0.0001
Fb (g/l)	11.64	13.22	3.54–17.44	10.88	13.22	3.54–17.12	0.7832
AGP (μg/ml)	284.33	290.14	240.15–301.25	358.49	377.25	240.36–402.46	0.0001
Total proteins (g/dl)	6.72	6.70	6.50–7.10	8.30	8.20	7.50–8.90	0.0001
Albumin (g/dl)	2.69	2.74	2.40–2.90	2.68	2.70	2.40–2.90	0.8986
Globulin (g/dl)	3.86	3.90	3.50–4.90	5.27	5.30	4.80–5.60	0.0001

[a]Hp: Haptoglobin; SAA = Serum Amyloid A; Fb = Fibrinogen; AGP = α1-Acid glycoprotein, Min = Minimum, Max = Maximum
[b]*P*-value resulting from non-parametric Wilcoxon Mann-Whitney test

SAA, Fb, and AGP showed comparable diagnostic accuracy (AUROC ranged from 0.93 to 0.98), however, Fb had the lowest sensitivity (74%) and Hp had the lowest specificity (73%) at the selected cut-off points. Using a logistic regression model, the diagnostic accuracy of various combinations of Hp, SAA, AGP, and Fb were tested. The highest diagnostic performance was obtained for the combination of Hp, AGP and Fb (AUROC = 0.98, Sensitivity (Se) = 0.98, Specificity (Sp) = 0.87).

Both Hp and SAA showed high degree of accuracy in predicting treatment outcome (Table 4 and Fig. 2), at the selected threshold (AUROC = 0.95 & 0.96, respectively). In addition, AGP showed moderate degree of accuracy (AUROC = 0.85). On the other hand, Fb level is considered worthless for discrimination between treatment success and failure (AUROC = 0.48). Comparison of the AUROC revealed significant difference between Fb and each of Hp, SAA and AGP ($P < 0.001$). There was no evidence of difference in the AUROC among Hp, SAA, and AGP, however, both Hp and SAA showed better sensitivity and specificity than AGP.

Discussion

Little is known about diagnostic and prognostic value of acute phase proteins in cases of UTI in dairy cows. The clinical signs presented by diseased cows are consistent with the clinical

picture described by other researchers[36,26,34].

E. coli and *Corynebacterium* spp. were the most dominant bacterial species isolated from cows with UTI. Our results agree with earlier studies[35,36]. Infection by *E. coli* and *Corynebacterium* spp. can be acquired externally through contaminated environment or internally from bacteria colonizing urogenital epithelium[20]. Additionally, both *E. coli* and *Corynebacterium* spp. have pili, which facilitate bacterial attachment to urogenital epithelium and colonization of the urinary tract[36]. Although, no significant association was observed between bacterial species and treatment outcome ($P = 0.25$), the proportion of treatment failure was higher among cases with *E. coli* infection (Table, S2).

The synthesis and role of APPs may differ depending on the animal species. In bovine, both Hp and SAA are major APPs, whereas AGP and Fb act as moderate and minor APPs, respectively[9]. In cows with UTI, there was a significant increase in Hp, SAA, AGP, and Fb levels. Moreover, the magnitude of increase was greater for both Hp and SAA when compared to AGP and Fb. These results agree with earlier studies reporting elevation in APPs levels during infection or inflammation, compared to very low or undetectable levels in healthy animals[15,23].

The elevated levels of APPs in cows with UTI could be attributed to their role in the body's defence against infection or inflammation. As indicated earlier, APPs act by opsonization of many pathogens, scavenging of toxic substances, and binding to metabolites released from cellular degradation, thus preventing their use by pathogens[3]. Elevated levels of APPs have been previously reported, as with SAA in UTI in cats[29] and mice[12]. Additionally, elevated levels of APPs have been associated with other bacterial infections including bacterial bronchopneumonia in calves[11], *Mannheimia haemolytica* in calves[13], naturally occurring and experimentally induced mastitis[16,24], metritis[5] and lameness[31].

The ROC analyses showed no significant

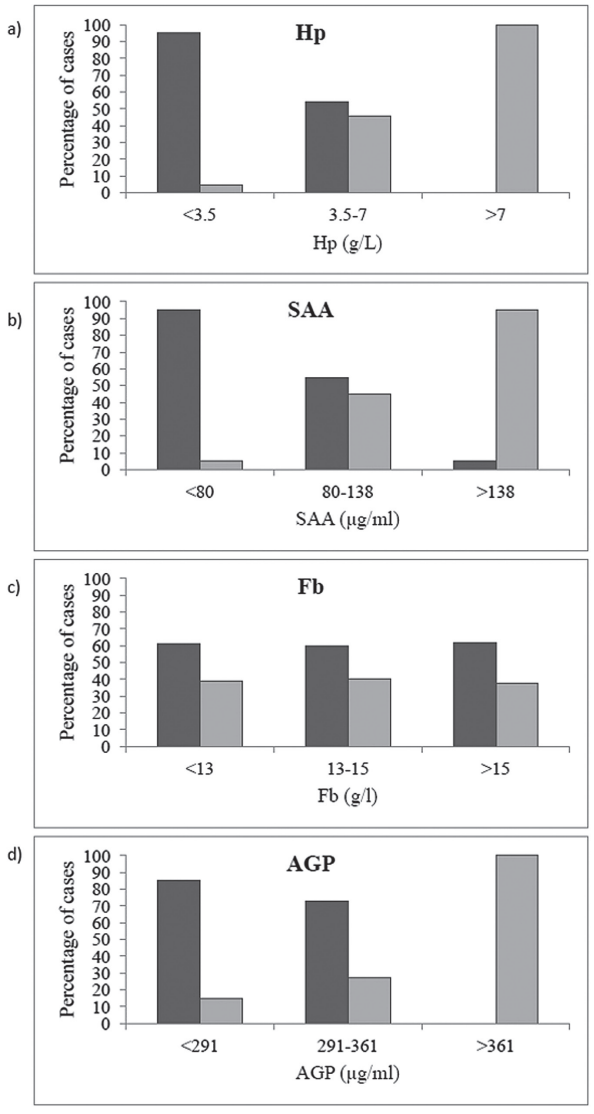

Fig. 1. Percentage of cases with treatment success (dark gray bars) or failure (light gray bars) at different percentiles (< 50%, 50–75%, and > 75%) of acute phase proteins [Haptoglobin (Hp), Serum Amyloid A (SAA), Fibrinogen (Fb) and α1-Acid glycoprotein (AGP)] levels among UTI group of cows.

differences in the AUC (0.93–0.98) among APPs used in this study, indicating comparable overall diagnostic performance, although AGP and Hp exhibited more sensitivity, and SAA and Fb more specificity. A logistic regression model combining Hp, AGP, and Fb yielded a greater accuracy compared to each individual or other APPs combination. These results support earlier recommendation of using APPs profile involving

Table 3. Test characteristics of acute phase proteins (APP) for diagnosis of UTI in cows

Variable[a]	Threshold	Sensitivity (%)	Specificity (%)	Correctly classified%	AUC (95% CI)
Hp	> = 0.15	92	73	89	0.95 (0.91–0.99)
SAA	> = 24	89	87	89	0.96 (0.93–0.99)
Fb	> = 4.70	74	87	76	0.93 (0.87–0.99)
AGP	> = 250	94	80	92	0.98 (0.95–1.00)

[a]Hp = Haptoglobin; SAA = Serum Amyloid A; Fb = Fibrinogen; AGP = α1-Acid glycoprotein; AUC = Area under the curve

Table 4. Test characteristics of acute phase proteins (APP) for prognosis of UTI in cows

Variable[a]	Threshold	Sensitivity (%)	Specificity (%)	Correctly classified%	AUC (95% CI)
Hp	> = 4.5	94	98	96	0.95 (0.89–1.00)
SAA	> = 120	94	98	96	0.96 (0.90–1.00)
Fb	> = 12	63	31	42	0.48 (0.35–0.61)
AGP	> = 300	82	90	87	0.85 (0.73–0.96)

[a]Hp = Haptoglobin; SAA = Serum Amyloid A; Fb = Fibrinogen; AGP = α1-Acid glycoprotein; AUC = Area under the curve

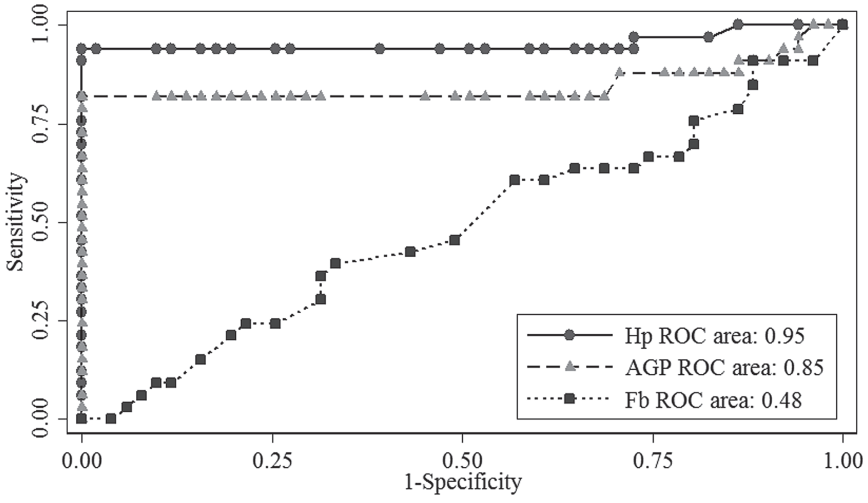

Fig. 2. Receiver operating characteristic plot: comparison of the area under the curve (AUC) for Haptoglobin (Hp), α1-Acid glycoprotein (AGP) and fibrinogen (Fb). Serum amyloid A (SAA) was omitted from the figure for clarity purpose and to close similarity with Hp.

positive major, moderate, and negative APPs[4]. As APPs have different roles and profiles, using major, moderate, and minor APPs can give a more complete picture about the acute phase response in infected animals.

Although APPs are not specific to certain condition, their circulating levels could show the degree of tissue damage and the severity of the response to infection, and thus could be valuable indicators of prognosis and treatment response[23]. In the current study, non-responsive cases had higher levels of Hp, SAA, and AGP compared to successfully treated ones. Additionally, the proportion of UTI cases with treatment failure increased linearly with higher levels of Hp, SAA, and AGP leading to 100% treatment failure when

exceed certain threshold. On the other hand, Fb level was not different among successfully treated and non-responsive cows. The association between elevated levels of Hp, SAA, and AGP and disease severity have been reported in case of mastitis[17,16,10]. According to these authors, Hp, SAA, and AGP were effective in the determination of the severity of infection and in predicting the outcome of mastitis in heifers and cows. Additionally, Hirvonen *et al.*[17] indicated that fibrinogen was a reliable indicator for the presence of bacterial infection, but was not useful as a prognostic indicator for mastitis. In other studies on calves with chronic respiratory diseases, dead and euthanized calves showed higher levels of Hp and SAA compared to those successfully treated[14,32]. Furthermore, studies on calves with bronchopneumonia found that Hp concentration was useful in predicting the number of antimicrobial treatments and in identifying calves that would have required anti-inflammatory[2,18]. Results from this study and the aforementioned studies on mastitis and respiratory diseases confirm the reliability of APPs in predicting treatment outcome associated with bacterial infection.

The prognostic value of APPs was further evaluated using ROC analyses which indicated a high accuracy (AUC $>$ 0.95) of Hp and SAA in predicting response to treatment, followed by AGP with a moderate degree of accuracy (AUC = 0.85). The same could not be said about Fb which recorded a much lower AUC value (AUC $<$ 0.5) in differentiating successfully treated UTI cases from non-responsive ones.

We acknowledge some limitations to our study. Due to non-specific response of APPs, it would be more accurate to include non-infectious inflammatory disease group as well. Although the authors excluded concurrent presence of other clinical conditions by thorough clinical assessment of study cows, the presence of other subclinical infections cannot be ruled out. The presence of concurrent subclinical infections if any may have affected the levels of APPs leading to overestimation of accuracy parameters. These limits would affect the utility of APPs as specific markers for differential diagnosis of UTI, but not as screening test for presence of infection.

From the present study, it could be concluded that acute phase proteins could be used as a supplement to clinical examination for screening, but not for differential diagnosis of UTI in dairy cows. Combination of Hp, AGP, and Fb yielded the highest accuracy. Moreover, the association between higher level of Hp, SAA and AGP and treatment response suggest that these markers can be used as prognostic predictors in cows with UTI.

Competing Interests

The authors declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.14943/jjvr.64.1.57

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