The Journal of Veterinary Medical Science

NOTE

Internal Medicine

Serum iron concentration is a useful biomarker for assessing the level of inflammation that causes systemic symptoms in bovine acute mastitis similar to plasma haptoglobin

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J. Vet. Med. Sci.
82(10): 1440–1444, 2020
doi: 10.1292/jvms.20-0388

Received: 24 June 2020
Accepted: 20 July 2020
Advanced Epub:
29 July 2020

ABSTRACT. The aim of present study was to evaluate the precision of plasma haptoglobin (HPT), serum iron (Fe) and plasma transferrin (Tf) concentrations as biomarkers of the severity of acute mastitis (AM) in cows. Fourteen Holstein Friesian cows with AM were divided into severe (n=8) and mild groups (n=6) based on systemic and local inflammation, and 12 healthy cows were also enrolled as controls. As a result, significant changes were observed in plasma HPT and serum Fe concentrations. The proposed cut-off points for plasma HPT and serum Fe concentrations for the severity of AM in cows based on receiver operating characteristic analyses were >10.3 μg/ml and <49.0 μg/dl, respectively. No significant difference was observed in the plasma Tf concentration.

KEY WORDS: acute mastitis, cow, haptoglobin, iron, transferrin

Acute mastitis (AM) is major source of economic loss in the dairy industry [25]. *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*) are well known to induce severe inflammation in AM, and cause moderate to severe clinical mastitis in dairy farms [8]. Therefore, accurate evaluation of the severity is important to determine the appropriate treatment for AM in cows and to reduce production loss. A biomarker that reflects the severity of AM may be useful for the evaluation of host response to inflammation in cows with AM.

Haptoglobin (HPT), one of the positive and major acute phase proteins (APP), is mainly synthesized in the liver. Positive APP in hepatocytes is stimulated by pro-inflammatory cytokines [16]. As a 100-fold increase in the circulating level of HPT in response to stimuli is observed in ruminants [16], the concentration of serum HPT is widely accepted as an inflammatory marker for AM in cows [5, 7, 10, 19]. On the other hand, iron (Fe) is known to play many roles in enzymatic activity, and is an essential trace element for the host and pathogen [20]. The Fe concentration decreases rapidly in response to inflammation and this can be explained as a host defense mechanism [3]. A low-Fe environment, which is a consequence of Fe sequestration in the body to control bacterial proliferation, is essential for the bacteriostatic system to operate in the body [29]. Changes in the blood Fe concentration were reported to be useful as biomarkers of inflammatory disease, including in cows with AM [2, 6, 12]. In our previous studies, decreases in serum Fe concentrations were observed in not only acute coliform mastitis [23, 28] and endotoxemia [28], but also after minor surgery with inflammation such as dehorning [27]. In addition, we revealed that hepcidin, which is essential for systemic Fe homeostasis, was induced by inflammation in cows, as in humans [28]. As mentioned above, Fe homeostasis may reflect the severity of inflammation in cows; therefore, inflammation may also affect the levels of blood transferrin (Tf), which plays an important role in Fe transport [4, 16]. Tf, a negative APP, is mainly synthesized in the liver. This pro-inflammatory cytokine causes a decrease in the synthesis of negative APP in hepatocytes [16]. Although Tf is generally considered to be a minor APP in ruminants [16], a previous study reported decreases in Tf levels in buffalo calves experimentally infected with *Salmonella Dublin* [22].

Thus, the utility of circulating levels of HPT and Fe as biomarkers of the severity of AM in cows was previously reported. In addition, the blood Tf concentration, which is related to Fe homeostasis, may also be a biomarker in cows with AM. However, no study has compared the accuracy of these biomarkers. The aim of present study was to evaluate the precision of plasma HPT, serum Fe and plasma Tf concentrations as biomarkers of the severity of AM in cows.

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All procedures were performed in accordance with the Good for the Care and Use of Laboratory Animals of the School of Veterinary Medicine at Rakuno Gakuen University (Approval#: VH18C10) and the National Research Council [18]. Twenty-six Holstein Friesian cows kept at a commercial dairy farm were enrolled in this case-control study. Fourteen of the 26 cows were diagnosed with AM presented based on the clinical findings and milk test using the modified California Mastitis Test (PL tester, Nippon Zenyaku Kogyo, Fukushima, Japan) by veterinary practitioners. These cows were then classified into those with clinical findings based on systemic inflammation into the severe group (n=8), and those with only local inflammation of the udder and no general findings were classified into a mild group (n=6). General clinical findings in the severe group were as follows: anorexia, inhibition of rumen motility, paralysis, hyperthermia or hypothermia [39.6°C (38.3–40.7)], high pulse rate and hyper salivation. Therefore, cows in severe group needed to receive not only local antibiotic treatment but also systemic antibiotic therapy. On the other hand, cows in mild group who received only local antibiotic treatment were clinically normal except for local inflammation of the udder, as assessed by attitudes, appetite and body temperature [38.6°C (38.5–38.8)]. The remaining 12 cows without AM were assigned to the control group and kept at the commercial farm in the same area.

The cows with multiple quarter infections were excluded from this study. Quarter mastitis milk samples were collected when clinical mastitis was first observed. All quarter milk samples were stored at −20°C until culturing. The quarter milk samples were measured as follows. First, 10 μl of each milk sample was plated on a 5% sheep blood agar plate (Nihon Becton, Dickinson and Co., Tokyo, Japan). The plates were incubated for 24 hr at 37°C and visually examined. If bacterial colonies were not evident, plates were incubated for an additional 24 hr at 37°C and reexamined. Bacterial colonies were identified using standard microbiological procedures, including Gram staining, and catalase and coagulase testing, in accordance with previously published guidelines [17].

Venous blood samples were collected by jugular venipuncture from all cows upon the first medical examination. Blood samples were stored in heparin-coated vacuumed tubes and plain tubes, and then centrifuged for 15 min at 1,500 × g using a standardized procedure to harvest plasma and serum. Plasma HPT concentrations were measured by ELISA using a commercial HPT ELISA kit (Bovine Haptoglobin ELISA, Immunology Consultants Laboratory, Inc., Portland, OR, USA). In this study, the serum Fe concentrations were measured by the 2-nitroso-5-(N-propyl-N-sulphopropylamino) phenol (nitroso-PSAP) method using an auto-analyzer (LABOSPEC 003, Hitachi Medical Co., Tokyo, Japan) at an OD of 753 nm with a commercial kit (N-assay L Fe-H Nittobo, Nitto Boseki, Co., Ltd, Tokyo, Japan). The nitroso-PSAP method for measuring serum Fe was described previously [9]. The Tf concentration in plasma was also measured by ELISA using a commercial Tf ELISA kit (Bovine Transferrin ELISA, Immunology Consultants Laboratory, Inc.). In addition, serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and γ-glutamyl transpeptidase (GGT) concentrations were measured using an automatic biochemical analyzer (DRI-CHEM 3500V, FUJIFILM Corp., Tokyo, Japan).

Statistical analysis was conducted using Excel Toukei 2010 (SSRI, Osaka, Japan). Data are expressed as the median and range. The Steel-Dwass test was employed for comparison among groups. In addition, receiver operating characteristic (ROC) curves were used to characterize the sensitivity and specificity of each parameter of AM severity-associated changes. The optimal cut-off point for a test was calculated by the Youden index [1]. The Youden index (J) is defined as the maximum vertical distance between the ROC curve and the diagonal or chance line, and is calculated as J=maximum [sensitivity+specificity-1]. The cut-off point on ROC curves that corresponds to J is taken as the optimal cut-off point [1]. The significance level was set at $P<0.05$.

In the severe group, all of the mastitis milk samples demonstrated positive growth for *K. pneumoniae* (6/8), *E. coli* (1/8) and *Truperella pyogenes* (1/8). On the other hand, the distribution of isolated species in the mild group was as follows: *Staphylococcus aureus* (3/6), *Streptococci* (1/6) and *Corynebacterium bovis* (1/6). The percentage of negative culture samples were 16.7% (1/6) in the mild group. In the control group, no mastitis pathogens were isolated from all milk samples.

The plasma HPT, serum Fe and plasma Tf concentrations, and other biochemical parameters (AST, ALT and GGT) in cows with AM are summarized in Table 1. In the present study, the plasma HPT concentration in the severe, mild and control groups were 64.7 (10.3–130.9), 0.4 (0.2–11.2) and 0.3 (0.1–1.0) μg/m*l*, respectively. The plasma HPT concentration in the severe group was higher than those in the mild ($P<0.01$) and control ($P<0.01$) groups. However, there was no significant difference in plasma HPT concentration between the mild and control groups. Similarly, significant differences in serum Fe concentrations were observed among the groups. The cows with AM in the severe [29.0 (8.0–49.0 μg/d*l*)] group had lower serum Fe concentrations than those

Table 1. the levels of haptoglobin, iron, and transferrin, and values of liver function in cows with acute mastitis

Measurement	Unit	Control (n=12)	Mastitis	
			Mild (n=6)	Severe (n=8)
HPT	(μg/m*l*)	0.3 (0.1–1.0)[a)]	0.4 (0.2–11.2)[a)]	64.7 (10.3–130.9)[b)]
Fe	(μg/d*l*)	135.0 (103.0–212.0)[a)]	118.0 (83.0–156.0)[a)]	29.0 (8.0–49.0)[b)]
Tf	(mg/d*l*)	40.5 (25.2–100.3)	39.7 (24.1–58.8)	47.5 (30.7–74.5)
AST	(U/*l*)	29.0 (15.0–54.0)	30.0 (16.0–55.0)	25.5 (17.0–34.0)
ALT	(U/*l*)	73.0 (57.0–107.0)	74.5 (53.0–108.0)	80.5 (54.0–125.0)
GGT	(U/*l*)	24.5 (15.0–50.0)	26.0 (21.0–37.0)	26.5 (14.0–50.0)

HPT: haptoglobin, Fe: iron, Tf: transferrin, AST: aspartate aminotransferase, ALT: alanine aminotransferase and GGT: γ-glutamyl transpeptidase. a–b: $P<0.01$.

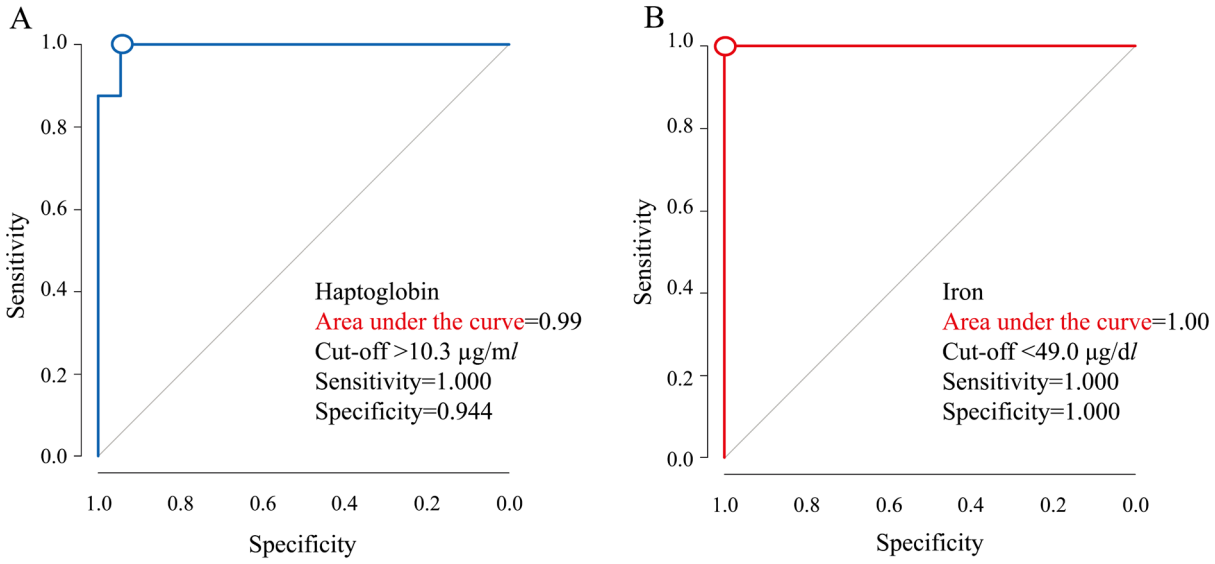

Fig. 1. Receiver operating characteristic curves for plasma haptoglobin (A) and iron (B) concentrations. The optimal cut-off points were calculated by the Youden index (J). Open Circle: Cut-off point.

in the mild [118.0 (83.0–156.0 μg/d*l*), $P<0.01$)] and control groups [135.0 (103.0–212.0 μg/d*l*), $P<0.01$)]. However, there was no significant difference in the Fe concentration between the mild and control groups. In contrast, no significant difference was observed in the plasma Tf concentration among groups; the plasma Tf concentration in the severe, mild and control groups were 47.5 (30.7–74.5), 39.7 (24.1–58.8) and 40.5 (25.2–100.3) mg/d*l*, respectively. The serum biochemical values in cows with AM were also measured, but there were no significant differences in serum AST, ALT or GGT related to liver function among the groups.

ROC curves were used to characterize the sensitivity and specificity of plasma HPT and serum Fe concentrations for the AM severity-associated changes (Fig. 1). The area under the ROC curves (AUC) for plasma HPT concentrations was 0.99. The proposed cut-off point for the plasma concentration of HPT in cows with severe AM based on analysis of the ROC curves was >10.3 μg/m*l*. The sensitivity and specificity of the proposed diagnostic cut-off point for the plasma concentration of HPT were 100.0% and 94.4%, respectively (Fig. 1A). On the other hand, the AUC for serum Fe concentrations was 1.00. The proposed cut-off point for the serum Fe concentration in cows with severe AM based on analysis of the ROC curves was <49.0 μg/d*l*. The sensitivity and specificity of the proposed diagnostic cut-off point for the serum Fe concentration were 100.0% and 100.0%, respectively (Fig. 1B). Therefore, statistical analysis demonstrated no significant difference between the AUC for the plasma HPT concentration and that for the serum Fe concentration (P=0.54).

The novelty of present study is the precision of plasma HPT, serum Fe and plasma Tf concentrations as biomarkers of the severity of AM in cows. In this study, there was no significant difference in diagnostic ability for severe AM in cows between the plasma HPT and serum Fe concentrations. On the other hand, no significant change was observed in the plasma Tf concentration between cows with and without AM under field conditions.

Several factors unrelated to inflammation also alter the blood HPT, Fe and Tf concentrations in cows. Liver function in particular affects the induction and regulation of APP synthesis [16]. Similarly, hepcidin, which is essential for systemic Fe homeostasis, is also affected by liver function [21]. Therefore, in this study, it was necessary to examine liver function. As a result, no significant differences were observed in serum AST, ALT or GGT concentrations among the groups. Therefore, the effects of liver function on the inflammatory markers examined in the present study were minimal.

Several previous studies reported the usefulness of serum HPT [5, 7, 10, 19] and Fe [2, 6, 12, 23, 28] concentrations as biomarkers of the severity of AM in cows. Although the circulating concentrations of APP are related to the severity of the disorder and the extent of tissue damage in the affected animal, accurate timing of sampling is needed to provide diagnostic information [16]. Suojala *et al*. [26] observed changes in serum HPT concentrations at 24 hr after intramammary infection of *E. coli* in cows. Similarly, sampling timing is important for the diagnostic value of serum Fe concentration in cows with AM. Our previous studies demonstrated the rapid response of serum Fe concentration to inflammatory stimuli, such as endotoxin challenge [28] and minor surgery [27], in cows; the reaction times of serum Fe concentrations in cows after endotoxin challenge [28] and minor surgery [27] were 24 and 12 hr after stimulation, respectively. The advantages of blood HPT and Fe concentrations as biomarkers of AM in cows may be a good reaction and their rapid response to inflammation. Indeed, our study demonstrated that plasma HPT and serum Fe concentrations are superior for assessing the severity of AM in cows. However, due to the difficulty of measuring blood HPT levels, the application of HPT assays to veterinary diagnosis is not widespread. On the other hand, measurement of the serum Fe concentration can be easily performed for a low cost, and can be used in clinical cases by veterinary practitioners.

McNair *et al*. [13] reported that the serum concentrations of bovine Tf during acute *Haemophilus somnus* pneumonia

decreased, but these variations remained within the normal range. In our study, cows with AM had no change in plasma Tf, similar to cows with respiratory disease [11, 13]. Moser *et al.* [15] also found that serum Tf concentrations did not change after the administration of endotoxin to calves during a 24-hr observation period. These studies support our current study. Thus, the plasma Tf concentration is not a biomarker of the severity of AM in cows. The plasma Tf concentration is a poor biomarker of AM because of its poor reaction (<2-fold decrease) [16] and slow response [22] to stimuli in ruminants. Experimental studies [13, 22] revealed decreases in serum Tf concentrations 120 hr after infection, which was much slower than the response times of the blood HPT and Fe concentrations [6, 26]. The mechanism of the decrease in plasma Tf concentration in response to inflammation is not the acceleration of degradation, but the suppression of APP synthesis in hepatocytes [16]. In addition, the blood Tf has a long half-life [24]. We hypothesize that the blood Tf concentration as a biomarker of inflammation in cows is more useful under chronic inflammatory conditions, as in humans [14].

In conclusion, we demonstrated that the plasma HPT and serum Fe concentrations are useful biomarkers of the severity of AM in cows. Although the degree of inflammation related to AM is generally evaluated using the value of HPT, which is major inflammatory marker in cows, our study clarified that the Fe concentration is a sufficient substitute for HPT. Of note, the serum concentration of Fe has a further advantage because it can be easily measured for a low cost. In particular, economic efficiency is important for farm animals. Therefore, veterinary practitioners can actively use serum Fe concentrations as a biomarker in clinical cases.

ACKNOWLEDGMENTS. We wish to thank the timely help given by Dr. Otsuka M and Dr. Murakami Y in analyzing the large number of samples. This study was supported by Rakuno Gakuen University Research Fund (2019).

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