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Research Article

Comparison of Two Different Measurement Techniques and Three Sample Materials for Bovine Haptoglobin in Early Postpartum Holstein Friesian Dairy Cows

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Abstract

This research was conducted to compare two measurement techniques and three sample materials for the analysis of bovine haptoglobin (Hp), an acute-phase protein, that can potentially be used to detect excessive postpartum inflammation in dairy cows on both farm and individual levels. Serum Hp concentrations of 99 fresh lactating Holstein Friesian dairy cows from 10 farms in the northeastern region of Germany were assessed using both pooled and individual serum samples. A colorimetric measurement on a chemical autoanalyzer (CA) was compared to an enzyme-linked immunosorbent assay (ELISA)-test kit. Additionally, Hp levels in pooled serum, lithium heparinized (LH) plasma and ethylenediaminetetraacetic acid (EDTA) plasma samples were compared. Mean serum Hp levels varied significantly between farms. Pooled serum samples showed high correlations with the mean of individual samples. Hp (ELISA) concentrations were on average 40% lower than Hp (CA) concentrations. Dilutions interfered with the analytical precision of the ELISA. Compared to serum, Hp (CA) concentrations were 2.5 and 2.3 times higher in LH plasma and EDTA plasma, respectively, which was not observed with the ELISA. In conclusion, Hp differentiates farms regarding the inflammatory state of transition cows. Pooled samples of farms can be used for this purpose. Laboratories should specify different reference values for Hp when using colorimetric measurements on a CA or ELISA-test kits, respectively. Furthermore, dilutions need to be prepared with maximum precision. Finally, serum should be the preferred sample type for colorimetric Hp measurements.

Keywords: *Acute-Phase Protein; Haptoglobin; Transition Dairy Cow; Inflammation; Clinical Laboratory Diagnosis*

Abbreviations

ANOVA: Analysis of Variance; APP: Acute-Phase Protein; CA: Chemical Autoanalyzer; EDTA: Ethylenediaminetetraacetic Acid; ELISA: Enzyme-Linked Immunosorbent Assay; Hp: Haptoglobin; IL: Interleukin; LH: Lithium Heparin; LSD: Least Significant Difference; Max: Maximum; Min: Minimum; P: Probability Value; PBS: Phosphate Buffered Saline; SD: Standard Deviation; TNF: Tumor Necrosis Factor

Introduction

The synthesis of acute-phase proteins (APP) is enhanced by pro-inflammatory cytokines (e.g. IL-1-β, IL-6 and TNF-α) as a part of the body's acute phase response to different noxa, such as stress, pain, tissue injury, and infectious agents [1]. Positive APP accumulate in the

blood and function in eliminating the respective noxa [2]. In the last decades, researchers have found that different positive APP vary in importance among different mammalian species [3]. In bovine pathology, one of the most important APP frequently used in clinical diagnostics is the hemoglobin scavenger protein haptoglobin (Hp) [4].

Hp is associated with different diseases such as mastitis [5,6], metritis [7,8], claw lesions [9,10], enzootic pneumonia [11,12] and others. However, clinically healthy animals exposed to stress e.g. due to transportation [13] or crowding in a foreign environment [14] also show increased serum concentrations of Hp. Some authors have reported increased Hp concentrations in apparently healthy multiparous dairy cows early after parturition compared to dry cows or cows in other lactation stages [15,16]. This phenomenon most likely results from different stressors present around calving, such as stress, pain, tissue lesions, bacterial contamination of the uterus and negative energy balance, which have been linked to increased Hp concentrations in apparently healthy primiparous cows [17,18]. Regardless of parity, the early postpartum blood Hp concentration does not necessarily represent a physiological reaction of the innate immune system to transition challenges, but might be used to detect excessive postpartum inflammation [19] in both individuals and groups of fresh lactating cows.

Disease events associated with excessive inflammatory processes during the periparturient period of dairy cows have a negative impact on milk yield, milk composition and reproductive performance in the following lactation, which further harms the economic efficiency of dairy farms [20,21]. Therefore, the early detection of cows at risk for excessive postpartum inflammation could facilitate appropriate monitoring and treatment of affected animals, thereby potentially minimizing economic loss for dairy farms over time. However, when it comes to the use of laboratory parameters in veterinary clinical diagnostics, both practitioners and researchers need to rely on recommendations for suitable measurement techniques and sample types. In the past decades, different measurement techniques for bovine Hp, such as colorimetric assays [22-24], enzyme-linked immunosorbent assays (ELISA) [25,26] and modern biosensor assays [27,28] have been developed and reveal different measurement results [29], which might confuse practitioners using APP as diagnostic markers. Furthermore, serum and some plasma variants have been found to differ in their APP concentration before [30], which is critical to interpreting measurement results. This study was conducted to 1) assess Hp concentrations in early postpartum dairy cows on different farms using both pooled and individual serum samples, 2) compare two common measurement techniques for the analysis of Hp and 3) compare Hp levels in serum, heparinized plasma and EDTA plasma.

Materials and Methods

The experimental procedures reported herein were conducted with the approval of the Federal State Office of Occupational Safety, Consumer Protection and Health (animal care protocol number: 2347-A-3-1-2018).

Animals and farms

A total of 100 Holstein Friesian dairy cows (22 primiparous, 78 multiparous) from 10 commercial dairy farms were included in this trial between March to April 2018. Farms were selected based on their herd size (≥ 1000 cows), geographic location (within the north-eastern region of Germany), housing (free stall) and consistent recording of animal health, fertility and milking traits. Farm sizes ranged from a total number of 1074 to 2638 cows per farm, with an average of 1506 cows. The average annual milk yield ranged from 7432 to 11982 kg, with an average of 10351 kg. Sample size was calculated for a comparative study of different APP related to health parameters [31]. From each farm, 10 cows within 0 - 8 days p.p. were selected based on their current health state and recent calving history: Only cows that had experienced a light calving process (no stillbirth, twin birth or dystocia, no assistance at calving) and displayed no signs of disease immediately prior to sampling were enrolled in the study. This study excluded cows that could not stand or walk, had high fever (> 40°C), or a recent history of milk fever or retained fetal membranes according to the definitions by Kelton., *et al* [32]. At the time of sampling, cows were on average 4.08 days p.p. (ranging from 0 to 8 days, SD: 1.99), 3.62 years old (ranging from 2 to 9 years, SD: 1.27) and within their 2.39th lactation (ranging from 1st to 5th lactation, SD: 1.15).

Sampling

From each cow, an experienced veterinarian collected three blood samples from the coccygeal vessel using an open blood collection system. The first was collected into a plain tube for separation of serum. The second and third were collected into vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) and lithium heparin (LH), respectively, for separation of plasma (tubes by SARSTEDT AG and Co. KG., Germany).

Blood samples were allowed to clot for approximately 2h. Plain and anticoagulant coated tubes were centrifuged for 10 minutes at 3500 g and 2800 g, respectively. Pooled samples were prepared for each farm and sample type. This process involved pipetting equal volumes (1 ml) of the 10 individual samples into a common pooling tube (10 ml). These pooled serums, LH and EDTA plasma samples as well as all individual serum samples were subsequently divided into aliquots and stored at -24°C until analysis. Additionally, one individual serum sample from each farm was randomly chosen for blinded double determination in the laboratory in order to assess the analytical precision of the respective measurement method. One sample was excluded from the study due to visible hemolysis, which reduced the total number of individual samples to 99 (total n = 139 (including 10 samples for double determinations and 30 pooled samples).

Laboratory haptoglobin measurements

Serum concentrations of Hp were determined in individual serum samples using two different measurement techniques: a colorimetric measurement on a chemical autoanalyzer (hereinafter referred to as CA) (Cobas 8.000 C 701 Autoanalyzer, Roche Diagnostics, Switzerland) using "PHASE"™ Haptoglobin Assay Cat. No. TP-801 (Tridelta Development, Ireland) and a commercially available ELISA-test kit (BIO-X-Diagnostics S.A., Rochefort, Belgium). The colorimetric measurement, first described by Owen., *et al*. [22] is based on the peroxidase activity of hemoglobin, which normally is being inhibited by a low test-pH, but can be preserved by Hp, if present in the specimen. The extinction read at 600 - 630 nm is directly proportional to the amount of Hp in the sample. The CA has a lower detection limit of 0.005 g/l, whereas the detection range for the ELISA is 0.05 - 0.36 g/l. Samples that contained more than 0.36 g/l Hp were diluted using standardized PBS provided by the manufacturer and the ELISA measurement repeated thereafter. The mean intra and inter assay coefficient of variation, declared by the respective laboratory, were 1.2% and 5.3% respectively for the CA and 2.9% and 16.2% respectively for the ELISA.

A blinded double determination was performed from one randomly selected individual serum sample of each farm in order to determine the analytical precision.

Besides the individual serum samples, pooled samples of serum, LH plasma and EDTA plasma from each farm were analyzed for Hp using both measurement methods.

Statistical analysis

All data were analyzed using IBM SPSS Statistics version 26 for Windows (IBM Corp., Armonk, New York). Data are presented as mean ± standard deviation of the mean (SD), unless otherwise indicated.

Descriptive statistics were performed aiming at simply ranking farms depending on Hp concentrations in pooled and individual samples, and at assessing Hp levels obtained from different measurement methods and sample materials. A one-way analysis of variance (ANOVA) with post-hoc-testing (LSD) was performed to test for significant differences in Hp concentrations between farms. Pearson's correlation coefficient and Wilcoxon's test were used to examine the relationship between Hp concentrations in pooled and individual serum samples and between the two measurement methods. Bland-Altman-Plots [33] were created to further assess and explain differences in Hp concentrations found by the CA and the ELISA-test kit, respectively. Differences between the three sample materials were examined

using Friedman's test and their relationships were assessed using Pearson's correlation coefficient and univariable linear regression analysis suppressing an intercept. Differences and correlations were considered significant if $P < 0.05$.

Results

Descriptive statistics

The mean serum Hp concentration of all farms found in individual serum samples analyzed with the CA was 0.68 ± 0.43 g/l. The ELISA-test kit revealed a mean individual serum Hp concentration of 0.41 ± 0.36 g/l. In pooled samples, the mean Hp (CA) concentration was 0.74 ± 0.25 g/l and the mean Hp (ELISA) concentration was 0.47 ± 0.24 g/l (Table 1).

Serum Hp concentrations determined with both the CA and the ELISA test kit revealed significant differences among farms ($P = 0.004$) [31].

Correlation among pooled and individual samples

Hp (CA) and Hp (ELISA) concentrations measured in pooled serum samples of each farm showed highly significant correlations ($P < 0.001$) with the arithmetic mean of the individual serum samples (Table 1) [31].

Comparison between chemical autoanalyzer and ELISA

Hp concentrations determined by the ELISA-test kit in individual serum samples were on average 40% lower than those determined by the CA (Table 1) but the results were still closely related ($r = 0.855$, $P < 0.001$; Figure 1). Blinded double determinations (duplicate samples) revealed more precise results of the CA compared with the ELISA-test kits (Figure 2a and 2b; intra assay CV (CA): 1.75%, intra assay CV (ELISA): 9.26%, calculated from n = 10 duplicate samples with the root mean square method [34]). Comparing the results of non-diluted and diluted samples it becomes obvious that the process of dilution impairs the analytical precision of the ELISA: In non-diluted samples (Figure 3a) the mean of the difference is 0.22 g/l, the 95% confidence interval ranges from 0.03 - 0.41 g/l and does not include the zero line. In diluted samples (Figure 3b) the 95% confidence interval is much larger, ranging from -0.24 to 0.94 g/l and it does include the zero line.

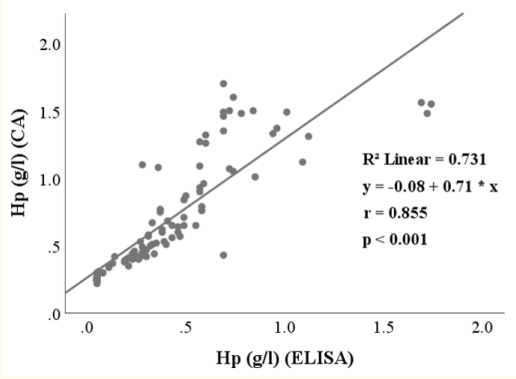

Figure 1: *Scatterplot illustrating the association between Hp concentrations in serum of n = 99 Holstein Friesian dairy cows within 0 - 8 days p. p. determined by an enzyme-linked immunosorbent assay (ELISA)-test kit and a chemical autoanalyzer (CA), respectively.*

Farm	n	Hp [g/l]			
		CA		ELISA	
		IS	PS	IS	PS
1	10	0.53 (0.27)^{ab}	0.51	0.31 (0.24)^{ab}	0.42
2	10	0.38 (0.12)^{a}	0.36	0.21 (0.15)^{a}	0.17
3	9	0.57 (0.43)^{abc}	0.57	0.24 (0.22)^{a}	0.33
4	10	0.63 (0.29)^{abc}	0.74	0.34 (0.16)^{ab}	0.28
5	10	0.80 (0.51)^{bcd}	0.90	0.38 (0.24)^{abc}	0.55
6	10	0.99 (0.47)^{d}	1.10	0.54 (0.22)^{bc}	0.55
7	10	0.44 (0.39)^{a}	0.45	0.18 (0.24)^{a}	0.19
8	10	0.72 (0.39)^{abcd}	0.76	0.44 (0.27)^{abc}	0.54
9	10	0.91 (0.49)^{cd}	1.06	0.82 (0.60)^{d}	0.99
10	10	0.86 (0.49)^{cd}	0.95	0.61 (0.49)^{cd}	0.72
$\bar{x}$		0.68 (0.43)^{A}	0.74 (0.25)^{A}	0.41 (0.36)^{B}	0.47 (0.24)^{B}
r_p ($\bar{x}$ (IS) - PS)	10	0.99		0.96	
P_p ($\bar{x}$ (IS) - PS)		< 0.001		< 0.001	

Table 1: *Haptoglobin (Hp) concentrations determined in n = 99 individual (IS) and n = 10 pooled (PS) serum samples of dairy cows within 0 - 8 d p.p. from 10 farms using two measurement methods (CA = Chemical Autoanalyzer, ELISA = Enzyme-Linked Immunosorbent Assay). Data presented as $\bar{x}$ (SD). ($\bar{x}$ = mean, SD = standard deviation of the mean)*

r_p = Pearson's correlation coefficient, P_p = Probability value of Pearson's correlation

^{a,b,c,d}: Means of different superscript letters within a column differ significantly (ANOVA, post-hoc-test: LSD; P < 0.05).

^{A,B}: Means of different uppercase superscript letters within a row differ significantly (Wilcoxon-test; P < 0.05).

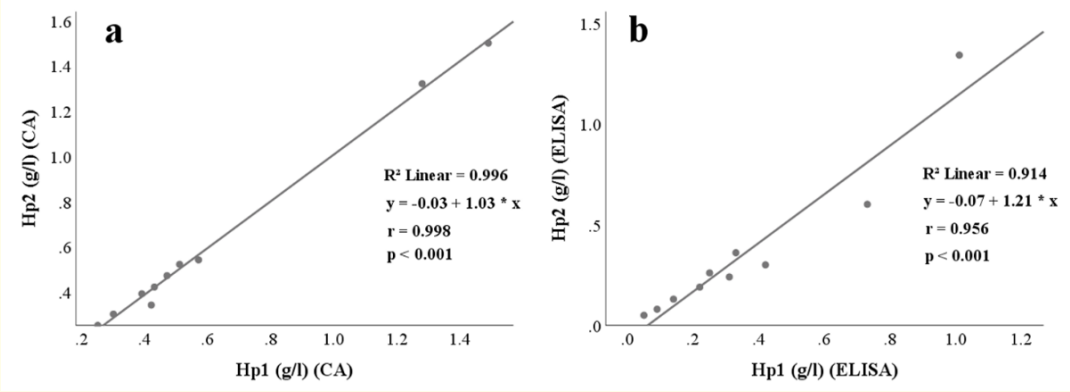

Figure 2: *Scatterplots showing the association between duplicate analyses of Hp using either a colorimetric measurement method on a chemical autoanalyzer (CA) (a) or a commercial enzyme-linked immunosorbent-assay (ELISA) (b), respectively. (n = 10 individual samples of dairy cows within 0 - 8 days p.p. from 10 farms). Intra assay CV: a: 1.75, b: 9.26 (calculated from n = 10 samples with the root mean square square method [34]).*

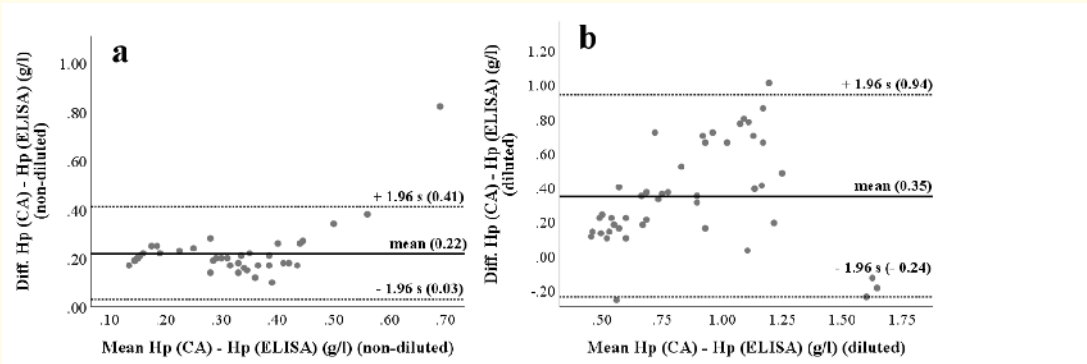

Figure 3: *Bland-Altman-Plots in our submitted manuscript illustrating the differences between the chemical autoanalyzer (CA) and the enzyme-linked immunosorbent assay (ELISA) including only those specimens measurable by ELISA without previous dilution (a), and including all specimens containing high amounts of Hp that needed to be diluted before measurement by the ELISA (b), respectively. --- = upper and lower limit of 95 % confidence interval, — = mean of difference (n = 99 individual serum samples of dairy cows within 0 - 8 days p.p. from 10 different farms.*

Hp measurements in different sample materials

Table 2 shows the concentrations of Hp (CA), and Hp (ELISA) measured in serum, EDTA plasma and LH plasma (pooled samples of each farm). The ELISA-test kit did not reveal any significant differences among the three sample materials tested. The Hp concentrations determined with the ELISA-test kit were significantly lower compared to those determined with the CA in all three sample materials ($P < 0.05$). The colorimetric method showed significant differences between values obtained from serum and plasma samples: Hp concentrations measured in EDTA and LH plasma were 2.3 and 2.5 times higher ($P < 0.001$) than those measured in serum, respectively.

	n		Hp (CA) [g/l]	Hp (ELISA) [g/l]
Serum	10	$\bar{x}$	0.74^{aA}	0.47^{aB}
		SD	0.26	0.25
		Min	0.36	0.17
		Max	1.10	0.99
EDTA plasma	10	$\bar{x}$	1.72^{bA}	0.39^{aB}
		SD	0.63	0.21
		Min	0.66	0.05
		Max	2.53	0.70
LH plasma	10	$\bar{x}$	1.85^{bA}	0.43^{aB}
		SD	0.59	0.08
		Min	0.81	0.12
		Max	2.63	0.71
Friedman-test		P	< 0.001	0.789

Table 2: *Haptoglobin (Hp) concentrations determined in pooled serum (n = 10), lithium heparinized (LH) plasma (n = 10) and ethylenediaminetetraacetic acid (EDTA) plasma (n = 10) samples of n = 99 dairy cows within 0 - 8d p.p. from 10 farms using a colorimetric measurement method on a chemical autoanalyzer (CA).*

$\bar{x}$ = mean, SD = standard deviation of the mean, min = minimum, max = maximum.

P = Probability value of Friedman-test testing differences between sample types.

^{a,b,c}: Means of different lowercase superscript letters within a column differ significantly (Friedman-test; $P < 0.05$).

^{A,B}: Means of different uppercase superscript letters within a row differ significantly (Wilcoxon-test; $P < 0.05$).

Table 3 and 4 show the results of the univariable linear regression analysis for Hp concentrations obtained using the two methods and three different sample materials. Close relationships were found between the Hp concentrations determined in different sample types both within and between the two methods (Pearson's correlation coefficient r ≥ 0.795, P < 0.001). The unstandardized regression coefficient B for serum Hp represents a directly calculable difference in the measurement range of the two methods (serum Hp (CA) concentration vs. serum Hp (ELISA) concentration: B = 1.42, meaning that values obtained with the CA are 1.42 times higher than those obtained with the ELISA-test kit). Hp (CA) concentrations in EDTA and LH plasma are 3.3 and 3.5 times higher compared to serum (ELISA), respectively. When using the ELISA-test kit, EDTA and LH plasma Hp concentrations were lower than serum Hp concentrations (B = 0.88 and B = 0.86, respectively), but this difference was not significant (P = 0.789; Table 2).

	Serum (ELISA)			
	B	**SE**	**r_p**	**P_p**
LH (ELISA)	0.86	0.057	0.908	< 0.001
EDTA (ELISA)	0.88	0.084	0.840	< 0.001
Serum (CA)	1.42	0.129	0.828	< 0.001
LH (CA)	3.53	0.313	0.862	< 0.001
EDTA (CA)	3.29	0.318	0.795	< 0.001

Table 3: *Results of a simple linear regression analysis assessing the relationships between haptoglobin (Hp) concentrations measured in serum with a commercial enzyme-linked immunosorbent assay (ELISA)-test kit (**x**) and Hp concentrations determined in other sample materials (LH = Lithium Heparinized Plasma, EDTA = Ethylenediaminetetraacetic Acid Plasma) using either the ELISA or a colorimetric method on a chemical autoanalyzer (CA), respectively (y). n = 10 pooled serum samples of dairy cows within 0-8 days p.p. from 10 farms. Simple linear regression analysis: y = B * x; B = Unstandardized Regression Coefficient, SE = Standard Error of B r_p = Pearson's Correlation Coefficient, P_p = Probability Value of Pearson's Correlation.*

	Serum (CA)			
	B	**SE**	**r_p**	**P_p**
Serum (ELISA)	0.66	0.060.	0.828	< 0.001
LH (ELISA)	0.59	0.031	0.923	< 0.001
EDTA (ELISA)	0.60.	0.033	0.911	< 0.001
LH (CA)	2.48	0.058	0.979	< 0.001
EDTA (CA)	2.33	0.072	0.959	< 0.001

Table 4: *Results of a simple linear regression analysis assessing the relationships between haptoglobin (Hp) concentrations measured in serum with a colorimetric method on a chemical autoanalyzer (CA) (**x**) and Hp concentrations determined in other sample materials (LH = Lithium Heparinized Plasma, EDTA = Ethylenediaminetetraacetic Acid Plasma) using either the CA or a commercial enzyme-linked immunosorbent assay (ELISA)-test kit, respectively (y). n = 10 pooled serum samples of dairy cows within 0-8 days p.p. from 10 farms. Simple linear regression analysis: y = B * x; B = Unstandardized Regression Coefficient, SE = Standard Error of B, r_p = Pearson's Correlation Coefficient, P_p = Probability Value of Pearson's Correlation.*

Discussion

Correlation among pooled and individual samples

In general, pooled samples of each farm showed high correlations with the individual samples (Table 1) [31], underlining their suitability for herd-level analyses of inflammation in transition dairy cows [35]. However, the correlation coefficient for the association between individual and pooled serum samples analyzed with the ELISA-test kit is slightly lower, than the one for the CA. A likely explanation could be the fact that predilutions interfered with the analytical precision of the ELISA-test kit (Figure 3a and 3b).

Comparison between chemical autoanalyzer and ELISA

The fact that the colorimetric method for serum Hp determination revealed about on average 40% higher values compared to the ELISA coincides with Cooke and Arthington's findings [29]. In their study, an APR was triggered in nine mixed-breed steers by vaccination against *Mannheimia haemolytica*. The colorimetric method based on peroxidase activity revealed on average 56% higher Hp concentrations when compared to an ELISA, however, the results from both methods were positively and closely correlated ($r = 0.97$, $P < 0.01$) as in the present study ($r = 0.855$, $P < 0.001$). Thus, both methods are suitable for the measurement of Hp concentrations and hence for the diagnosis of inflammatory reactions in cattle, but different reference values should be declared by laboratories and taken into consideration by the practitioner.

ELISA measurements in the present study resulted in less precise values when necessary dilutions were performed due to high concentration of Hp in a specimen (Figure 3a and 3b). These findings underline the importance of accurate and corrected dilutions to obtain reliable results.

APP measurements in different sample materials

The concentrations of Hp determined with the CA were higher in both plasma variants compared to serum (Table 2-4). The results from the ELISA test kit did not correspond to these findings, as there was no significant difference between the three sample types (Table 2). Thus, we presume, that the sample type somehow influences the colorimetric measurement method based on the peroxidase activity of Hp-bound hemoglobin. Hussein., *et al*. [30] found higher values in plasma compared to serum analyzing the APP ceruloplasmin with a colorimetric assay, however, the reason for this phenomenon remained unclear. Other authors have reported higher values of different chemical analytes in heparinized plasma compared to serum before [36-38], but to our knowledge, this has not yet been assessed for bovine Hp.

Cooke and Arthington [29] used heparinized plasma and compared a colorimetric method and an ELISA test kit for Hp analysis in their aforementioned study. The difference between the two methods was even more pronounced (on average 56% higher values obtained by the CA compared to the ELISA) than in our study (on average 40% higher values). This seems to coincide with our finding that higher Hp concentrations are determined in LH plasma compared to serum when using a colorimetric measurement method. Double determinations showed that the colorimetric measurement is repeatable and precise, hence, internal errors of the method can be neglected. A possible explanation for the discrepancies between the sample materials might be related to the coagulation process in serum tubes, which is inhibited by EDTA and LH. Thus, remaining plasma components in EDTA and LH coated tubes might influence peroxidase activity measured by the analyzer. A similar effect has been found in milk samples containing lactoperoxidase [39], however, it is not clear which substance would have similar effects in plasma. Another probable reason for the differences between serum and plasma could be an interference of the respective anticoagulant itself with the colorimetric measurement technique. Pure heparin has been described to enhance peroxidase-like activity of gold nanoclusters in another study [40], however, this interaction is very specific and it is not clear how LH would possibly interfere with the peroxidase activity detected by the colorimetric Hp assay used in this study. Additionally, the

close linear relationships between the values obtained using both plasma variants and serum reported in this study (Table 3 and 4) lead to the conclusion, that the anticoagulant itself cannot be the cause of the differences, since LH and EDTA would most likely react differently. In fact, we conducted a small companion trial to the present study (data not shown). Hp concentration was measured in both serum and plasma samples previously mixed with EDTA and LH, respectively, using the colorimetric method on the CA. Aqua dest. was used as a control and Hp concentration from all aqua dest. tubes remained below the detection limit of the assay. As in the present study, Hp concentrations measured in both plasma variants were higher compared to serum. Overall, no influence of the anticoagulants was observed.

Hence, the cause of the high Hp values found in plasma samples compared to serum when using the CA might be a certain plasma component expressing peroxidase activity. Further research is needed to find an explanation for this phenomenon.

Conclusion

Serum Hp concentration differentiates dairy farms regarding the inflammatory state of transition cows. Future studies should examine the association of Hp in early postpartum dairy cows to the farm's economic and animal health traits. Practitioners could use pooled samples for status analyses of farms regarding inflammatory processes in fresh lactating dairy cows, but further research is needed to establish reference values for this purpose. For the laboratory analysis of bovine Hp, different suitable methods are being provided by commercial laboratories, however, the practitioner should pay attention to the specific sample requirements of the methods. If necessary, pre-analytic dilutions of the specimens should be prepared with a high level of precision and thoroughness in order to provide reliable results. It can further be concluded that colorimetric methods depending on peroxidase activity should be performed using serum or otherwise interpreted carefully. Specific reference values individually established by each laboratory are essential to avoid misinterpretation in case plasma is being used.

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Conflicts of Interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of this paper.

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