Haptoglobin and lactate dehydrogenase measurements in milk for the identification of subclinically diseased udder quarters

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ABSTRACT: Diagnosis of subclinical mastitis is of increasing importance and appropriate detection methods are needed. Both haptoglobin (Hp), an acute phase protein in cattle, as well as lactate dehydrogenase (LDH), an ubiquitous enzyme, can be successfully used to detect clinical mastitis. The present paper describes quantification of Hp and LDH in milk samples from healthy and subclinically diseased udder quarters. Hp was analysed in the laboratory using an ELISA. The activity of LDH was measured in raw milk directly in the milking parlor. Both parameters were suitable to distinguish between sterile samples and bacteriologically positive samples. The ability to differentiate between minor and major pathogens was better for Hp in skim milk than for LDH in raw milk. Hp and somatic cell count (SCC) as well as LDH and SCC were positively correlated (r = 0.8 and r = 0.76, respectively). Subclinical mastitis was defined as follows: SCC > 100 × 10^3 cells/ml and bacteriological positive findings in two out of three weekly samples. Sensitivity and specificity were above 85% for Hp and above 81% for LDH. Using a less rigid classification to define mastitis, i.e. SCC > 200 × 10^3 cells/ml and two out of three weekly samples bacteriologically positive, sensitivity for Hp improved (89%) and remained unchanged for LDH. Both parameters are useful parameters for the diagnosis of subclinical mastitis. LDH activity in raw milk was less sensitive and specific than Hp but the method described herein offers the opportunity to measure LDH activity directly in the milking parlor and might therefore be suitable for on-line system developments.

Keywords: haptoglobin; LDH; milk; diagnosis of subclinical mastitis

Subclinical mastitis is of particular importance in dairy herds. Reneau and Packard (1991) summarized that 70 to 80% of mastitis losses were due to subclinical mastitis. Besides microbiological testing, somatic cell count (SCC) in milk is often used to define subclinical mastitis (Nielen et al., 1995; Deluyker et al., 2005; Gronlund et al., 2005). In addition to established parameters, acute phase proteins (APP) are discussed as being useful in the diagnosis of mastitis. Serum amyloid A (SAA) and haptoglobin (Hp) are major APP in bovines (Eckersall and Conner, 1988) in blood and milk. The use of SAA and Hp determination in the diagnosis of clinical mastitis has been demonstrated in several studies (Eckersall et al., 2001; Gronlund et al., 2003; Nielsen et al., 2004). The ability of SAA and Hp measurements in milk to detect clinical mastitis (sensitivity) was 93% and 86%, respectively (Eckersall et al., 2001). Misclassification of healthy quarters did not occur (specificity 100%) as described by Eckersall et al. (2001). APP are mainly synthesised in the liver. However, a mammary associated isoform has been described for SAA (McDonald et al., 2001; Molenaar et al., 2002). Hp mRNA has been reported to be present in mammary gland homogenates (Hiss et al., 2004). The mammary synthesis indicates close relations between milk APP concentration and mammary health and therefore APP in milk are promising candidates to detect even slight health disturbances. Besides APP, enzymes e.g. N-acetyl-β-D-glucosaminase and lactate dehydrogenase (LDH) are also potential parameters for the diagnosis of mastitis (Pyorala, 2003). Chagunda et al. (2005)

developed a statistical model for the detection of mastitis based on LDH activity. Sensitivity and specificity were 76.5% and 97.7%, respectively for diagnosing clinical mastitis. Lipperheide et al. (1995) and Larsen (2005) described different assays for the determination of LDH activity in raw milk opening the possibility to abandon laborious pretreatment of milk samples. The origin of LDH in mastitic milk is attributed to leucocytes (Kato et al., 1989) and also to epithelial cells from the udder (Bogin et al., 1977; Zank and Schlatterer, 1998). However, the significance of damaged epithelial cells to LDH activity in milk is not clarified.

In this study we aimed to calculate the diagnostic value of Hp and LDH in naturally occurring subclinical mastitis using SCC and microbiological testing to define subclinical mastitis.

MATERIAL AND METHODS

Animals and sampling

126 quarters from German Holstein cows, milked in an automated milking system (Leonardo, WestfaliaSurge Deutschland GmbH, Boenen, Germany) were sampled consecutively three times in weekly intervals. The cows were in their first to 7th lactation (mean: 3.2); average milk yield was 8 831 kg. To be able to include sufficient animals with subclinical mastitis, cows were preselected on the basis of their milk SCC, i.e. half of the cows selected had elevated milk SCC (> 100 × 10^3 cells/ml) for at least two months whereas the other half had SCC within the normal range. In total 378 foremilk samples were collected aseptically from 126 quarters being free from clinical mastitis. Heat, pain, redness, swelling and abnormal milk were regarded as signs of clinical mastitis. Before sampling, the first strippings of milk were discarded, teat ends were then disinfected with 70% alcohol and allowed to dry. Milk samples for bacteriological analyses were taken into sterile vials and transported refrigerated to a commercial laboratory (Veterinar Analysezentrum, Geesthacht, Germany) were the analyses were carried out according to the recommandations of the German Veterinary Society about isolation and identification of pathogens of mastitis (DVG, 2000). Isolates were identified using the API 20 Strep identification system (bioMerieux, Nurtingen, Germany). For the determination of SCC milk samples were preserved with 400 µg bronopol (2-bromo-2-nitro-1,3-propanediol; Sigma-Aldrich, Taufkirchen, Germany) per ml and analysed by the dairy laboratory Hochwald (Thalfang, Germany) using a Fossomatic cell counter (Fossomatic 5500, Rellingen, Germany). The upper limit of detection was 10 000 × 10^3 cells/ml.

A third sample without additive was frozen and stored at –20°C for Hp analyses.

Lactate dehydrogenase (LDH) activity

LDH activity was determined spectrophotometrically (340 nm) in raw milk using dry chemistry according to Lipperheide et al. (1995). Raw milk samples were analysed directly in the milking parlor with the analyser DT 60 II (Ortho-Clinical Diagnostics, a Johnson & Johnson Company, Neckargemund, Germany) at 37°C. Values were multiplied with the correction factor 0.38 according to the manufacturer's instructions. Intra and inter assay coefficients of variation were below 8%. The lower limit of detection was 38 U/l.

Haptoglobin (Hp) assay

Milk Hp concentrations were determined using a competitive ELISA as described previously (Hiss et al., 2004). Milk samples were defatted before use. In modification of the original protocol, the micro titre plates (EIA plate 9018, Corning Costar, Cambridge, MA, USA) were coated with a pool prepared from bovine sera (0.1 µl in 100 µl of 50mM $NaHCO_3$, pH 9.6) containing 3.5 mg/ml Hp instead of purified Hp. The standard used herein was another pool of bovine serum (1.8 mg/ml) calibrated against a standard obtained from the European Union Concerted action on the standardization of animal APP (QLK5-T-1999-0153). Milk samples were diluted 1:5 up to 1:1 000 according to their Hp content. The lower limit of detection was 0.35 µg/ml for milk samples. Intra and inter assay coefficients of variation were below 10%.

Statistical analysis

SPSS 12.0 for Windows was used. Data for LDH activity and haptoglobin concentrations were not normally distributed and this could not be remedied by logarithmic transformation. Therefore

the median values with minima and maxima are presented. The non-parametric Kruskal-Wallis test and the Mann-Whitney test were used to compare Hp concentrations and LDH activities in different pathogen groups. An alpha adjustment for multiple comparisons was made. Although quarters were repeatedly sampled all milk samples were used for this interpretation without consideration of quarter and cow to get considerable numbers of cases. The use of less sensitive non-parametric tests does not eliminate but minimises potential tampering if statistical assumptions are not satisfied (Danzer, 1989). Potential relationships between SCC, Hp and LDH were evaluated by calculating the coefficients of correlation (Spearman's rank correlation).

For LDH activity and haptoglobin concentration, cut-off values were defined as highest specificity (probability of a negative result by a healthy quarter) and concomitantly highest sensitivity (probability of a positive result by a diseased quarter) using maximum Youden-Index (sensitivity + specifity – 1) (Perkins and Schistermann, 2005). Mastitis (diseased quarter) was defined according to the recommendation of the German Veterinary Society (DVG, 1994), i.e. SCC > 100 × 10^3 cells/ml and two out of three weekly samples positive for bacteriological findings. In addition, a second less rigid definition with an SCC threshold of 200 × 10^3 cells per ml and also two of three weekly samples tested bacteriologically positive. As quarters were repeatedly sampled cut-offs were not only calculated for the three-week-period but also separately for every week to check possible bias.

A *P*-value of 0.05 was considered significant.

RESULTS

Bacteriology and somatic cell count

79 samples were negative for bacterial growth. In 299 cases pathogens were detectable: 70 *Corynebacterium bovis (C. bovis)*, 60 coagulase-negative Staphylococci (CNS), 45 mixed infections, 29 *Streptococcus* spp. (other than *Streptococcus agalactiae*, *S. dysgalactiae* and *S. uberis*) 35 mixed infection including *Staphylococcus aureus* (in combination with either CNS, *C. bovis* or *Streptococcus* spp.), 49 *Staphylococcus aureus* (*S. aureus)* and three *Escherichia coli (E. coli)*. In eight samples more than two pathogens were isolated and were therefore regarded as contaminated and excluded from the study. Mixed infection comprised two different pathogens (*Streptocccus* spp., CNS or

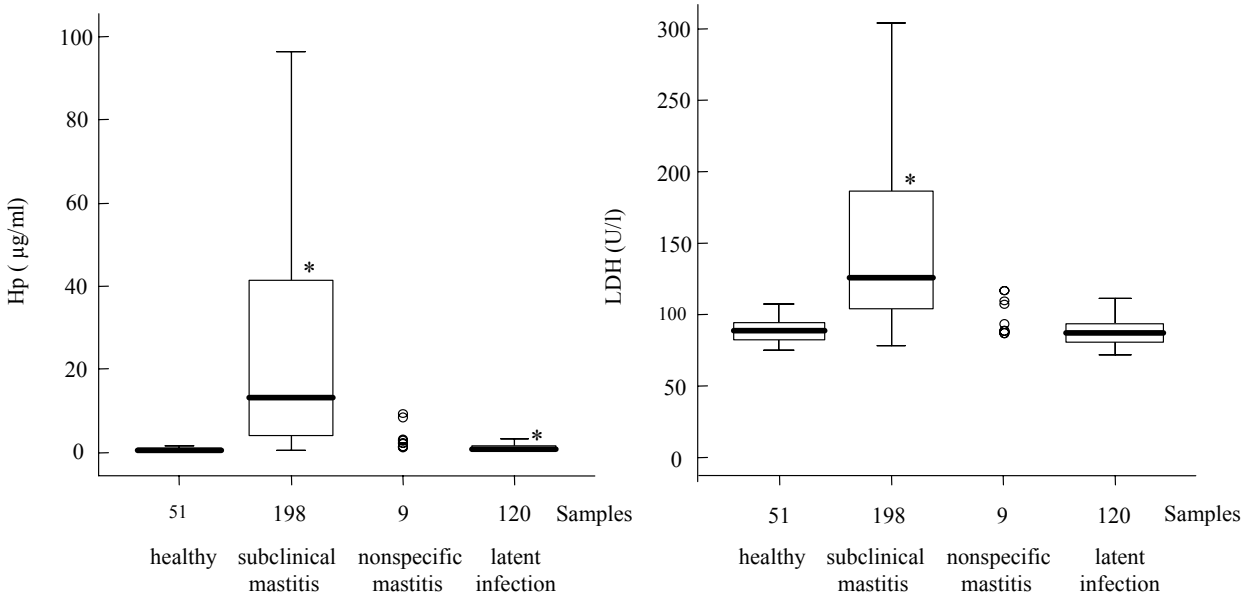

Figure 1. Hp concentrations in skim milk and LDH activities in raw milk in healthy udder quarters and different cases of mastitis. For the definition of mastitis 100 × 10^3 cells/ml were used as SCC limit (plus two out of three samples bacteriologically positive). The plots show the medians (line within box), 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers). Samples were demonstrated regardless of the quarter and the cow

*P < 0.05 compared to healthy quarters

C. bovis). SCC in bacteriologically negative samples ranged from 4×10^3 cells/ml to $1\,512 \times 10^3$ cells/ml (median = 40×10^3 cells/ml). Median SCC in infected quarters was 237×10^3 cells/ml (minimum 6×10^3 cells/ml and maximum > $10\,000 \times 10^3$ cells/ml).

LDH activity and Hp concentration

LDH activity was low in foremilk samples in which no bacterial growth could be observed (median = 89 U/l, range 75 U/l to 144 U/l). Samples being positive for growth of pathogens exhibited higher LDH activity compared to samples without bacterial growth ($P < 0.01$; median = 105 U/l, range 72 U/l to 700 U/l).

Hp concentration in milk was detectable in 340 out of 370 samples. 30 samples were below the limit of detection (0.35 µg/ml) which was then used as value for statistical comparisons. In sterile samples Hp concentration ranged from 0.35 µg/ml to 16 µg per ml (median = 0.7 µg/ml, detectable 63 out of 79). Median Hp concentration in samples with pathogens was 4.4 µg/ml (range 0.35 µg/ml to 974 µg/ml, $P < 0.01$ compared to sterile samples), whereupon Hp could be detected in 277 out of 291 samples. LDH activities and Hp concentrations for the different pathogens are presented in Table 1. SCC and Hp as well as SCC and LDH were positively correlated ($r = 0.8$, $P = 0.01$ and $r = 0.76$, $P = 0.01$, respectively). The correlation between Hp and LDH was 0.7 ($P = 0.01$).

The evaluation of the 3-week-period revealed 17 out of 126 quarters as being healthy. 66 quarters were subclinically diseased as they comply with the requirements of the German Veterinary Society (SCC > 100×10^3 cells/ml and culture-positive in two out of three samples). The remaining quarters were either culture-positive (40 quarters with latent infection) or showed SCC > 100×10^3 cells/ml (3 quarters with nonspecific mastitis). Hp concentrations and LDH activity in these groups

Table 1. LDH activities (raw milk) and Hp concentrations (skim milk) from infected udder quarters and quarters without bacterial growth. Samples were demonstrated regardless of the quarter and the cow

	n	LDH (U/l) Median (range)	P^1	P^2	Hp (µg/ml) Median (range)	P^1	P^2	SCC (10^3/ml) Median (range)
Sterile	79	89 (75–144)		0.001	0.7 (0.35–16.0)		0.001	40 (4–1 512)
C. bovis	70	92 (75–245)	n.s.	0.001	1.85 (0.35–85.0)	0.001	0.001	70.5 (6–2 249)
Mixed infection	45	104 (72–700)	0.001	0.001	2.4 (0.5–150.2)	0.001	0.001	136 (12–10 000)
CNS	60	98 (73–700)	0.006	0.001	3.1 (0.35–576.0)	0.001	0.001	167 (9–4 171)
Streptococcus spp.	29	246 (76–700)	0.001	n.s.	4.4 (0.5–974.0)	0.001	0.001	405 (32–10 000)
Mixed infection + *S. aureus*	35	103 (74–165)	0.006	0.001	4.8 (0.35–232.4)	0.001	0.001	335 (8–8 804)
S. aureus	49	167 (82–680)	0.001		39.6 (0.35–304.8)	0.001		1,741 (16–10 000)
E. coli	3	284 (175–396)	not tested	not tested	81.0 (59.0–184.0)	not tested	not tested	8 861 (1,658–10 000)

[1]compared to sterile samples

[2]compared to *S. aureus*

n.s. = not significant

Mixed infection = includes two different pathogens (*C. bovis*, CNS or *Streptococcus* spp.); mixed infection + *S. aureus* = means *S. aureus* in combination with either *C. bovis*, CNS or *Streptococcus* spp.

Table 2. Sensitivities and specificities to separate healthy and subclinically diseased udder quarters by using Hp or LDH. Quarters were classified subclinically diseased when SCC > 100 × 10^3 cells/ml and two out of three weekly samples were bacteriologically positive. The second definition used was SCC > 200 × 10^3 cells/ml and two out of of three weekly samples tested bacteriologically positive. Cut-offs for the three-week-period were given

	Cut-off	Sensitivity (%)	Specificity (%)	Youden Index
Hp (SCC 100 000 cells/ml)	2.2 µg/ml	85	92	0.77
Hp (SCC 200 000 cells/ml)	2.7 µg/ml	89	92	0.81
LDH (SCC 100 000 cells/ml)	99.5 U/l	81	86	0.67
LDH (SCC 200 000 cells/ml)	103.5 U/l	80	87	0.67

are demonstrated in Figure 1. For the calculation of cut-off values only healthy quarters and subclinically diseased quarters were used. Calculated cut-off values ranged from 1.75 µg/ml in the third week to 2.7 µg/ml in the second week for Hp in skim milk. Regarding the 3-week-period (samples were treated as being independent) the cut-off was 2.2 µg/ml for Hp. For LDH in raw milk the cut-offs ranged from 98.5 U/l in the first week to 102.5 U/l in the second week. The cut-off for the 3-week-period was 99.5 U/l.

Using the mastitis classification defining 200 ×10^3 cells/ml as SCC limit (plus two out of three samples bacteriologically positive), the cut-off values ranged from 1.75 µg/ml to 2.7 µg/ml and 98.5 U/l to 109 U/l for Hp in skim milk and LDH in raw milk, respectively. The 3-week-period revealed 2.7 µg/ml as the cut-off for Hp and 103.5 U/l for LDH. In the following only the cut-off for the 3-week-period will be used for Hp and LDH. Sensitivity, specificity and Youden indices for both mastitis definitions are summarised in Table 2.

DISCUSSION

Bacteria isolated from the samples included minor and major pathogens as repeatedly reported (Poutrel and Rainard, 1982; Reneau, 1986; Sargeant et al., 2001). Reports on milk Hp concentrations subjected to different pathogens are limited. *S. aureus* and *Streptococcus uberis* have been shown to increase milk Hp concentration in clinical mastitis (Gronlund et al., 2003; Pedersen et al., 2003). The only major pathogens isolated in this study were *S. aureus* and *E. coli*. Gronlund et al. (2003) analysed APP in acute and chronic *S. aureus* mastitis and the Hp concentration ranged from < 1.2 µg/ml to 323 µg/ml in acute cases. The range for chronic *S. aureus* mastitis was < 1.2 µg/ml to 28 µg/ml. Hp concentrations from our own study are in the range described by Gronlund et al. (2003). However, the authors used a different ELISA and it is not clear whether concentrations are directly comparable. *E. coli* was isolated in our study from three samples only. Therefore no statistics was performed although the numerical Hp values were highest in *E. coli* samples. Hp concentrations in mastitis caused by minor pathogens are not well described. The results of this study show that Hp medians were different in subclinical mastitis caused by minor and major pathogens and that it is also possible to distinguish between sterile samples and samples being positive for minor pathogens. These differences are remarkable all the more because only subclinical cases were used in our study. Gronlund et al. (2005) analysed APP in chronic subclinical mastitis and Hp concentrations ranged from < 0.3 µg/ml to 358 µg/ml in samples being positive for udder pathogens (*S. aureus, Streptococcus uberis, Streptococcus dysgalactiae,* CNS, *E. coli*). Due to a small numbers of cases the authors did not perform statistics in relation to different udder pathogens.

As aforementioned for Hp, data on LDH activity due to different pathogens are also scarce. Kato et al. (1989) reported highest LDH activity in milk samples infected with *S. aureus*. Lower LDH activities were found in samples being positive for *C. bovis* and CNS. However the authors analysed only few samples (3 to 7) and no statistics are given. In our own study LDH activity was different in sterile samples and bacteriologically positive samples. The only exception were quarters infected with *C. bovis*: LDH activity in milk from these quarters could not be distinguished from sterile quarter milk. This finding is not surprising since *C. bovis* is not highly

pathogenic. The priority in the diagnosis of mastitis was lowest for *C. bovis* as assigned by Pitkala et al. (2004). The low median SCC in samples positive for C. bovis (70.5×10^3 cells/ml) support these findings although *C. bovis* may increase SCC as shown by Laevens et al. (1997).

With the exception of *E. coli* samples, highest LDH activity was found in samples containing *Streptococcus* spp. Even *S. aureus* samples showed numerically lower LDH activities compared to *Streptococcus* spp. These results can not be explained the more so as SCC in *S. aureus* is markedly higher ($1\ 741 \times 10^3$ cells/ml) compared to *Streptococcus* spp. (405×10^3 cells/ml). This discrepancy seems also to be reflected in the correlation between LDH and SCC which is slightly less compared to Hp and SCC.

Regarding the three-week-period healthy quarters could be differentiated from quarters suffering from subclinical mastitis with both parameters. However, sensitivity and specificity were better for Hp in skim milk than for LDH in raw milk. Although repeated samples from the same quarters were not basically independent it seems to be more appropriate to use the cut-off from the three-week-period. As bacteria (especially *S. aureus*) were shed intermittently with milk it might be better to evaluate a period and not just a point in time as suggested by the German Veterinary Society and thus not satisfying the statistical assumption of independence.

The first SCC threshold used to define mastitis was in accordance with the German Veterinary Society. However, SCC thresholds are different among authors (Nielen et al., 1995; Chagunda et al., 2005; Deluyker et al., 2005; Gronlund et al., 2005) and detection results depend on the definition of subclinical mastitis (Nielen et al., 1995). The consensus between microbiological testing and SCC differs in accordance to SCC thresholds as shown by McDermott et al. (1982) and by Poutrel and Rainard (1982). For this reason we compared two different SCC thresholds. The differences between 100×10^3 cells/ml and 200×10^3 cells/ml were negligible for LDH cut-off values. Hp sensitivity was slightly improved when a SCC threshold of 200×10^3 cells/ml was used. Concerning the discussion of thresholds, it has to be pointed out that we aimed to maximise sensitivity and specificity concomitantly.

The only threshold described for Hp in subclinical mastitis is given in Gronlund et al. (2005). The authors stated that Hp levels above the detection limit of 0.3 μg/ml indicate an abnormal udder quarter. In our study Hp could be detected in 80% of the samples without bacterial growth and the cut-off value for Hp is 2.2 μg/ml (mastitis definition with the SCC threshold of 100×10^3 cells/ml, three-week-period) and 2.7 μg/ml (mastitis definition with the SCC threshold of 200×10^3 cells/ml, three-week-period). The difference between the threshold described herein and Gronlund et al. (2005) might be explained by a different assay calibration (see above).

Udder quarters with latent infections without elevated SCC could not be distinguished from the healthy quarters using LDH activity. Hp concentrations were lower in healthy quarters compared to latently infected quarters ($P < 0.05$). However, the difference was only marginal (medians of 0.6 μg/ml in healthy quarters versus 0.8 μg/ml in latent infection). The diagnostic value for this purpose is thus doubtful. Non-specific mastitis was diagnosed in only three quarters (nine samples) and therefore no statistics was performed.

Our results suggest that both parameters are useful parameters for the diagnosis of subclinical mastitis. LDH activity in raw milk was less sensitive and specific than Hp but the method described herein offers the opportunity to measure LDH activity directly in the milking parlor and might therefore be suitable for on-line system developments. Hp in skim milk was more sensitive in differentiating between udder pathogens than LDH in raw milk and the diagnostic value (sensitivity and specificity) was higher for Hp. Nonetheless Hp determination is an analysis which has to be performed in a laboratory. The development of methods for on-line APP measurements is claimed since several years (Eckersall et al., 2001; Gronlund et al., 2003) but these methods are not available yet.

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