



Simões, P.B.A., Campbell, M., Viora, L., Gibbons, J., Geraghty, T.E., Eckersall, P.D. and Zadoks, R.N. (2018) Pilot study into milk haptoglobin as an indicator of udder health in heifers after calving. *Research in Veterinary Science*, 116, pp. 83-87.(doi:[10.1016/j.rvsc.2017.05.024](https://doi.org/10.1016/j.rvsc.2017.05.024))

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Deposited on: 07 July 2017

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Original Article¹

Pilot study into milk haptoglobin as an indicator of udder health in heifers after calving

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Abstract

Mastitis, inflammation of the mammary gland, is often caused by intramammary infection with bacterial organisms. It impacts on dairy cattle welfare, production, udder health and longevity in the herd. Current detection methods for mammary inflammation and infection all have limitations, particularly for on-farm diagnosis of non-clinical mastitis after calving. Acute phase proteins have been suggested as alternative early indicators of the disease and can potentially be used as cow-side test with results in real time. In this study, milk haptoglobin concentrations were investigated over the first week postpartum to explore haptoglobin's potential as indicator of udder health in dairy heifers. Haptoglobin concentration was highest on day 3 of lactation, and was positively correlated with somatic cell count, a commonly used marker of inflammation ($r_s = 0.68$). Haptoglobin level was also associated with bacteriological culture results, a key indicator of infection status, whereby median haptoglobin concentration on day 3 and 5 was higher in quarters that were infected at calving than quarters that were non infected at calving. Sensitivity and specificity of haptoglobin concentration as indicator of infection were low, both for lenient and strict culture-based definitions of intramammary infection (57 or 60% and 61 or 63%, respectively). Although haptoglobin was a poor biomarker for intramammary infection with coagulase negative staphylococci in heifers during the first week after calving, it may have value as an indicator of major pathogen infections, particularly in large scale dairy herds where pre-partum heifers are managed off-site.

Keywords: Heifer; Biomarker; Intramammary infection; Haptoglobin; Postpartum; Somatic cell count

Introduction

Mastitis, inflammation of the mammary gland, is one of the most common diseases affecting dairy cattle. It is well recognized as a serious production problem and a costly disease (Halasa et al., 2007) due to the direct effect on milk yield, composition and processing proprieties (Auldist et al., 1996), and the impact on animal welfare and its association with a higher culling risk (De Vliegher et al., 2012).

In recent years, the intensification and technological progress in dairy farming has resulted in major changes to herd management and milking methods. Large herds often rely on off-site contracted heifer-rearing units to raise their replacement animals. Information regarding growth rate and reproductive performance of heifers in such units is frequently shared with the main herds, but udder health status and hygiene history are largely unknown. The transfer of these heifers to the main herd generally occurs during the last months of gestation. This transfer coincides with major physiological changes related to the approach of parturition and beginning of the lactation, which is recognized as a period of increased susceptibility to udder health problems (De Vliegher et al., 2012; Sordillo, 2005). Damage to the mammary tissue during this period may result in long term adverse effects on udder health and milk yield, which affect dairy farming profitability and sustainability (De Vliegher et al., 2012).

For decades, udder health has primarily been evaluated using detection of mammary inflammation, e.g. through somatic cell counting, or intramammary infection, e.g. using culture-based methods. Modern advances in proteomics have led the way to the discovery of biomarkers that have the potential to detect udder health problems earlier, ‘in-line’, faster, less expensively and with higher sensitivity (Viguiet et al., 2009). Acute Phase Proteins (APPs) are a group of biomarkers with potential as indicators for udder health (Pyörälä et al., 2011). These proteins are mainly produced in the liver and concentrate in blood as a result of the acute phase response, a cascade of events that occurs after an inflammatory processes is triggered, for example by bacterial invasion of the mammary gland (Petersen et al., 2004). APP are recognized as integral players in the mammary gland’s innate immunity, responding

non-specifically to pathogenic invasion (Cray et al., 2009). Their concentration in milk from healthy animals is negligible but it is significantly elevated in milk from cows with clinical or subclinical mastitis, increasing the interest in their ability to differentiate healthy from infected quarters (Eckersall et al., 2001, 2006; Grönlund et al., 2005). The fact that there is local expression of Hpt mRNA in mammary gland tissue, rather than influx of systemically produced Hpt, adds to the potential specificity of Hpt as marker of udder health (Eckersall et al., 2001; Hiss et al., 2004).

Reliable, sensitive and early detection of udder health problems is a crucial component of herd health and productivity management. Increased sophistication of milking systems, technical developments, and specialized, often highly trained staff provide new opportunities for on-farm, real-time udder health diagnostics (Viguier et al., 2009). The concentration of Hpt in milk is easier to measure than the concentration of other APP, and this measurement is possible using colostrum (Thomas et al., 2016b). For SCC, the level at calving and during the first days of lactation is related to heifers' IMI status at calving (Barkema et al., 1999). The wide range of Hpt values that can be observed at or shortly after calving, at least in adult cattle, (Thomas et al., 2016b) may also be indicative of udder health status. Therefore, the aim of this study was to investigate milk Hpt concentrations over the first week postpartum in dairy heifers, and the potential use of Hpt concentration as indicator of udder health.

Material and methods

Sample collection

The study was conducted on a commercial dairy farm with approximately 700 lactating animals on-site, and heifer rearing facilities off-site. Heifers were moved to the lactating cow facility approximately 6 weeks before expected parturition and housed in a heifer group. Shortly before calving, they were moved to a close-up and calving pen, which was also for heifers only. After calving, heifers were moved into the group of 1st lactation animals. All lactating animals were milked three times a day in a 52-point rotary parlour. Twenty-four healthy Holstein-Friesian heifers were followed during the first five days postpartum. Quarter level colostrum and milk samples were collected on the day of calving (day 1), and three (day 3) and five (day 5) days in milk. Samples were obtained from 96 quarters of 24 heifers over four weeks. Sample collection was conducted after teat disinfection with gauze swabs soaked in 70% ethanol and after the first streams of colostrum or milk had been discarded. On the day of calving, the farmer collected two samples from each quarter at first milking, i.e. during the colostrum phase. The first sample was collected into sterile pre-labelled 15 mL Falcon tubes (VWR international Ltd, UK) for bacteriological culture and stored on-farm at -20°C. The second sample was collected into pre-labelled 50 mL Falcon tubes (VWR international Ltd, UK) and split after collection. Approximately 20 mL were transferred into pre-labelled containers supplied by the National Milk Laboratories (NMR Co., Hillington Park, Glasgow) containing milk preservative (2-bromo-2-nitropropane-1,3-diol) and kept refrigerated at 4°C for up to 4 days, for somatic cell count measurement. The remainder of each sample was retained in the original 50 mL tube and stored at -20°C for haptoglobin analysis. On day 3 and 5, milk samples were collected for SCC and Hpt measurement only at the second milking of the day, starting at 11.30 am. The study was approved by the School Research Ethics Committee of the University of Glasgow, School of Veterinary Medicine (Ref31a/14).

Milk haptoglobin measurements

Colostrum and milk haptoglobin concentration was determined by sandwich ELISA procedure, using purified rabbit anti bovine Hpt IgG (Life Diagnostics *Inc.*, West Chester, Pennsylvania, USA) that was conjugated to alkaline phosphatase (Innova biosciences, Cambridgeshire UK) following the manufacturer's instructions, with the assay procedure described previously (Thomas et al., 2015). The lower detection limit of the assay was 0.4 µg/mL.

Somatic cell count measurements

Colostrum and milk samples were analysed at the National Milk Laboratories. SCC was determined by flow cytometry somatic cell counter technology (Fossomatic, Foss, Hillerød, Denmark) within 4 days of sample collection.

Bacteriological culture

All colostrum and milk samples were cultured within three weeks of sample collection, following the standards of the National Mastitis Council (Harmon et al., 1990). Samples were thawed under refrigeration for approximately 15 hours, warmed to room temperature and mixed thoroughly. Then, 0.01 mL of each quarter sample was inoculated onto Sheep Blood Agar 5 % plates (E&O Laboratories Limited, Bonnybridge, Scotland), using calibrated sterile plastic loops (Thermo Scientific Sterilin, UK). All plates were incubated aerobically up to 48 hours at 37°C, and the number of colony-forming unit (CFU) was counted for each bacterial morphotype. Quarters were considered culture-negative if no growth was observed. Samples containing three or more bacterial species were considered contaminated, and were not taken into account to define quarters as infected or non-infected. The remaining culture-positive samples were used to classify quarters as infected using a lenient and strict definition, respectively. For the lenient definition, any quarter with positive culture result in a non-contaminated sample was considered infected. This definition has high sensitivity but

suboptimal specificity, i.e. 85.8% and 75.1% respectively for any organism (Definition A; Dohoo et al., 2011). For the strict definition, only quarters with at least 1000 CFU/mL of the same bacterial morphotype were considered infected. This definition has high specificity but suboptimal sensitivity, i.e. 100% and 35.2% respectively for any organism (Definition C; Dohoo et al., 2011). Definition A is recommended by Dohoo et al. (2011), whereas definition C is preferred by many mastitis experts (Andersen et al., 2010).

Gram stains, oxidase tests (Oxoid Ltd.), and catalase tests were carried out on all cultured bacterial isolates. Further identification was carried out based on the results of these tests. The tube coagulase test with rabbit plasma was used to differentiate coagulase positive and coagulase negative staphylococci, with the former presumed to be *Staphylococcus aureus*. Streptococci were differentiated from enterococci based on the absence of growth on MacConkey agar. Identification of streptococci to Lancefield group level was carried out using the Streptococcal Grouping Kit (Oxoid Ltd.). Streptococci that failed to react with antisera to groups A, B, C, or D were tested for aesculin hydrolysis and, if positive, assumed to be *S. uberis*. Quarters were classified as infected with minor (e.g. coagulase negative staphylococci and *Corynebacterium* spp.) or major (e.g. *S. aureus*, *S. uberis*) pathogens based on culture results (Anderson et al., 2010).

Data Analysis

Analysis was performed at quarter level using Statistical Package for Social Sciences (SPSS) software version 22.0 (IBM SPSS, Portsmouth, UK). Variables were analysed using descriptive statistics, and normality was assessed using histograms, normal probability (Q-Q) plots and Shapiro-Wilk tests. Because of the non-normal distribution of the data, differences in median haptoglobin (Hpt) concentration and SCC between postpartum days were assessed by non-parametric tests (Friedman test for overall difference, with post-hoc Dunn-Bonferroni test to determine which groups differ). SCC and Hpt data was natural logarithm converted for data analysis and graphic presentation effects, however distribution was still non-normal. For

each sampling day (0, 3, 5) the relation between milk Hpt concentration and SCC was examined visually and statistically using scatterplots and Spearman's rank correlation test, respectively. Hpt concentrations were compared between culture negative samples and those from infected quarters using boxplots using either lenient or strict definitions of infection. For each sampling day, differences between groups were assessed by Kruskal-Wallis H test and pairwise comparisons using Dunn's procedure with Bonferroni adjustment. Results were considered statistically significant at $P < 0.05$. Receiver operating characteristics (ROC) were analysed to assess test performance (area under the curve, AUC), cut-off values and respective sensitivity (Se) and specificity (Sp) for the diagnosis of infection status, using bacteriological culture as reference method (Dohoo et al., 2011). The selection of the cut-off values was made following the procedure described in Froud and Abel (2014) using Euclidean geometry to find the closest point of the ROC curve to the top-left corner of the ROC space, assuming that sensitivity and specificity should be valued equally.

Results and Discussion

On large dairy farms, the udder health history and status of heifers that enter the lactating herd is often unknown, particularly when heifers are raised in rearing units that are separate from the main farm. Acute phase proteins may have value as biomarkers of udder health and the method for quantification of Hpt lends itself to pen-side test formats (Thomas et al., 2016a). Therefore, we investigated the value of Hpt concentrations in milk from dairy heifers in the first week post-partum as indicator of their udder health status.

Haptoglobin was present in moderate concentrations in colostrum samples (Table 1), in agreement with results reported by others (Thomas et al., 2016b). Thomas' study (2016) did not provide information on the relationship between Hpt and infection status in the first week post calving, but our results show variable Hpt levels in samples from culture negative as well as infected quarters (Table 2). The presence of Hpt in colostrum from healthy mammary glands may be due to the antibacterial properties of APP and their role in passive immunity and protection of the newborn calf (Eckersall et al., 2006; Thomas et al., 2016b). Stress at parturition may also contribute to Hpt production by mammary tissue (Thomas et al., 2016b). The highest median Hpt concentration (5.5 µg/mL, IQR: 3.1 to 9 µg/mL) was observed on day 3 of lactation, and 44% of quarters showed a peak in Hpt at day 3. A similar pattern was previously observed in several cows, although median Hpt concentration decreased from day 1 to day 10 post-partum in multiparous animals (Thomas et al., 2016b).

Geometric mean SCC was higher at calving than at day 3 and 5 of lactation (Table 1), in agreement with other studies (Barkema et al., 1999; Sargeant et al., 2001). Haptoglobin concentration was significantly correlated with SCC overall ($r_s = 0.35$; $P < 0.001$; $n = 286$), at calving ($r_s = 0.21$; $P < 0.05$; $n = 94$; Figure 1) and on day 3 of lactation ($r_s = 0.68$; $P < 0.001$; $n = 96$), but not on day 5, $r_s = 0.18$; $P > 0.05$; $n = 96$ (Supplementary Figure 1). A positive correlation between Hpt and SCC has been described before, and neutrophils are a recognized source of extra-hepatic Hpt in milk (Åkerstedt et al., 2007; Lai et al., 2009). Despite the positive correlation between Hpt and SCC overall, median Hpt concentration and geometric

mean SCC peaked on different days. The correlation between Hpt concentration and SCC was strongest on day 3, when all but one samples tested positive for Hpt (Table 1).

Individual quarter milk samples collected by the farmer at calving were culture negative for 41% of quarters ($n = 39$), culture positive with fewer than 1000 CFU/mL for 36% ($n = 35$), and culture positive with 1000 CFU/mL or more for 20% of quarters ($n = 19$). Only 3% of samples ($n = 3$) were contaminated, as indicated by growth of 3 or more bacterial morphotypes. This bodes well for the feasibility of on-farm or cow-side collection of high-quality milk samples for diagnostic testing.

On each sampling day, median milk Hpt concentration was greater for infected quarters than for culture-negative quarters, regardless of the definition of infection but differences were not significant for the lenient definition of infection ($n = 54$ or 56% of quarters). For the stringent definition of infection ($n = 19$ or 20% of quarters), the difference with culture negative quarters was significant on day 3 and 5 ($P < 0.05$ and $P < 0.01$, respectively; Supplementary Figure 2). In the absence of a gold standard for IMI, it is difficult to know whether these results imply that Hpt does not accurately detect IMI with low cfu counts (false negative Hpt result), or whether low cfu counts are not truly indicative of IMI (false positive culture result).

Coagulase-negative staphylococci (CNS) were the predominant group of bacteria isolated from culture-positive quarters (89%, $n = 24$), of which 6 presented with 2 bacterial morphotypes (Table 2). A predominance of CNS in samples from dairy heifers collected in the early postpartum period has been described before (Piepers et al., 2010). Haptoglobin results for different culture results are presented in Table 2. There were no statistically significant differences between Hpt concentration in pure and mixed cultures of CNS. Statistical tests to compare Hpt concentration between culture-positive quarters with minor and major pathogens were not conducted because of the small number of major pathogens isolated (*S. uberis* and *S. aureus* in one quarter each). The *S. aureus* positive quarter presented with low concentration of Hpt, similar to quarters infected with CNS in our study, regardless

of day post-calving (Table 2). The presence of *S. aureus* without evidence of inflammation has been described before, and the ability to elicit an inflammatory response is strain dependent (Zadoks et al., 2000). A high Hpt concentration was observed in the quarter that was infected with *S. uberis*, particularly on day 3 post calving. After experimental challenge with *S. uberis*, Hpt levels take at least 36 hours to increase (Thomas et al., 2016a). Thus, the low Hpt level at calving may indicate that the *S. uberis* infection had been acquired very recently. Considering that only two quarters were identified with major pathogens, a larger scale study would be needed to gain better knowledge of the relationship between such infections and Hpt levels in heifers post-partum.

The area under the ROC curve (AUC) of milk Hpt, 0.60 and 0.65 respectively for definition the lenient and strict definition (Figure 3) for both definitions of IMI indicated a low accuracy of Hpt as a diagnostic biomarker (Swets, 1988). The cut-off value established for gland status based on lenient and strict definitions was 4.46 µg/ml or 4.17 µg/ml, respectively (sensitivity = 57%, specificity = 61%; or sensitivity = 60%, specificity = 63%, respectively; Figure 3). The low sensitivity and specificity of the Hpt threshold values confirm the poor performance of Hpt as diagnostic test in heifers at calving, despite the existence of significant associations with SCC and IMI status. These results may be related with the bacterial type predominantly isolated in our samples, i.e. minor pathogens, which generally induce only mild inflammation only (Pyörälä and Taponen, 2009).

Conclusions

The data shows limited potential of milk haptoglobin as diagnostic tests for CNS intramammary infection in heifers during the first week postpartum.

Conflict of interest statement

The authors declare no conflicts of interest. None of the authors of this article has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Acknowledgements

The authors thank the herdsman for his help during the study; Funmilola Clara Thomas and André Santana for help with the ELISA procedures; and Libby Graham, Manuel Fuentes and Kathleen Reynolds for laboratory and logistic support. Financial support from the School of Veterinary Medicine – University of Glasgow is gratefully acknowledged.

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Table 1

Quarter-level haptoglobin concentration, somatic cell count and microbiology results of 24 dairy heifers ($n=96$ quarters)

	Day in milk		
	1 (at calving)	3	5
Haptoglobin ($\mu\text{g/mL}$)			
Positive samples ^b	76 (79%)	95 (99%)	72 (75%)
Mean ^a	7.7 ± 1.2	8.2 ± 1	5.6 ± 0.8
Geometric mean	3.4	5.8	2.7
Q1	1.9	3.1	1.1
Median	3.5	5.5**	3**
Q3	7.1	9	7
IQR	5.2	5.8	5.9
Somatic cell count (x1000 cells/mL)			
LnSCC mean ^a	6 ± 0.3	5.5 ± 0.1	4.8 ± 0.1
Geometric mean ^a	381	255	124
Q1	112	90	58
Median	721	200*	85**‡
Q3	1706	558	197
IQR	1594	468	139
Microbiology ^b			

Culture-negative	39 (41%)
<1000 CFU/mL	35 (36%)
≥1000 CFU/mL	19 (20%)
Contaminated	3 (3%)

Q1 – first quartile; Q3 – third quartile; IQR – interquartile range

^a Values are mean ± standard error of mean

^b number of quarters (% of quarters) with detectable levels of haptoglobin

Significant differences with day of calving (Friedman test, post hoc Dunn-Bonferroni test) * $P < 0.01$; ** $P < 0.001$;
with day 3 on milk † $P < 0.001$.

Table 2Milk haptoglobin concentration ($\mu\text{g/mL}$) in relation to culture results ($n=93$ quarters)

	Day in milk		
	1 (at calving)	3	5
Culture-negative ($n = 39$) ^{c,a}	30 (77%)	39 (100%)	27 (69%)
	3 (1.9 to 5.8)	4.6 (3.2 to 6.8)	2.7 (0.4 to 5.2)
Culture-positive ($n = 54$) ^c	43 (78%)	53 (98%)	42 (78%)
Infected			
Lenient definition ($n = 54$) ^a	5.2 (1.9 to 11.1)	6.7 ** (3 to 10.3)	4.1 † (2.1 to 8.4)
Strict definition ($n = 19$) ^a	2.3 (1.1 to 21.9)	8.1 * (4.5 to 14.2)	6.3 (3.6 to 8.6)
Pure culture			
Minor pathogens			
CNS ($n = 18$) ^a	4.8 (0.4 to 15.4)	7.5 (4 to 10.3)	5.4 (2.1 to 13.8)
<i>Corynebacterium</i> sp. ($n = 1$) ^{a,b}	10	4.5	9.3
Major pathogens			
<i>Streptococcus uberis</i> ($n = 1$) ^{a,b}	2.3	49.3	8.4
<i>Staphylococcus aureus</i> ($n = 1$) ^{a,b}	5.4	6.1	4.3
Mixed culture			
CNS ($n = 6$) ^a	12.4 (1.9 to 32.8)	7.3 (4.3 to 12.5)	8.6 (4.2 to 35.8)

^a Median $\mu\text{g/mL}$ (Q1 to Q3); IQR – interquartile range; Q1 – first quartile; Q3 – third quartile

^b Haptoglobin value

^c Number of quarters (% of quarters) with detectable haptoglobin

Significant differences with day of calving (Friedman test, post hoc Dunn-Bonferroni test) * $P < 0.05$; ** $P < 0.01$;
with day 3 on milk † $P < 0.01$.

Figure legends

Figure 1 Correlation between haptoglobin concentration and somatic cell count in milk from heifers on the day of calving ($n = 94$ quarters of 24 heifers). Linear regression equation and R^2 (coefficient of determination) value are shown.

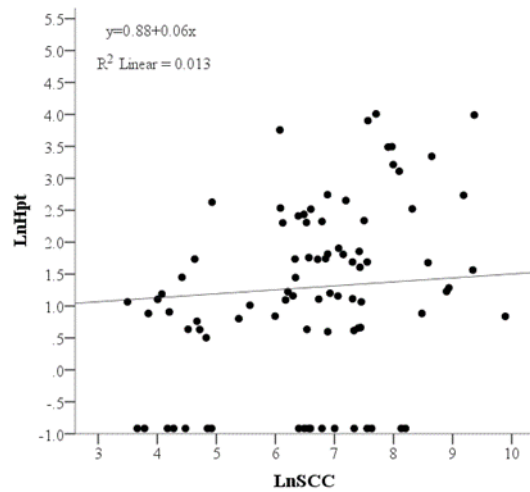


Figure 2 Concentration of haptoglobin (natural logarithm transformed - LnHpt) in milk from culture-negative and culture-positive at the calving day. Quarters culture-negative ($n = 39$), with low CFU count (<1000 CFU/mL; $n = 35$) and high CFU count (≥ 1000 CFU/mL; $n = 19$). The plots show the median (line within box), 1st and 3rd quartiles (box), range (whiskers) and outliers (dots).

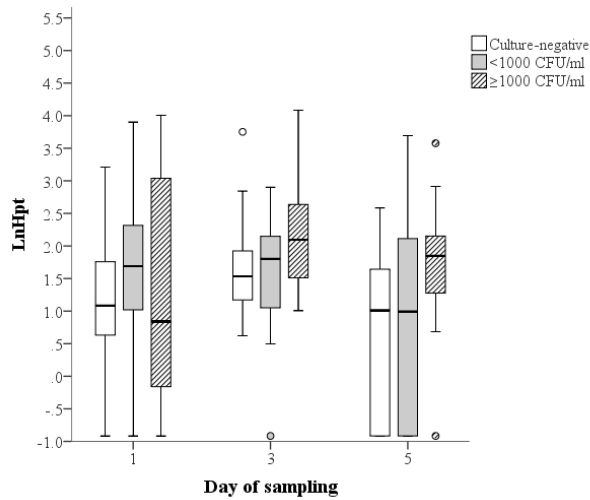
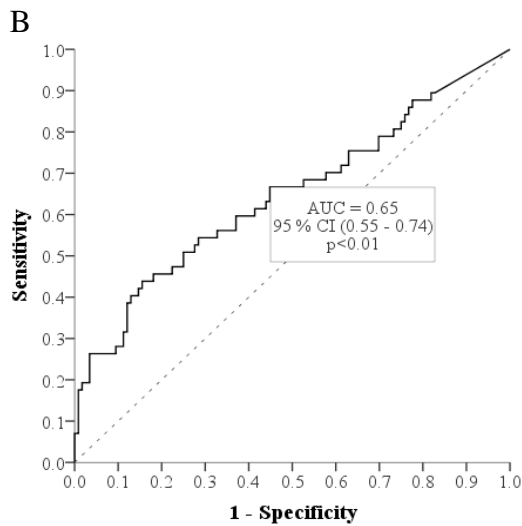
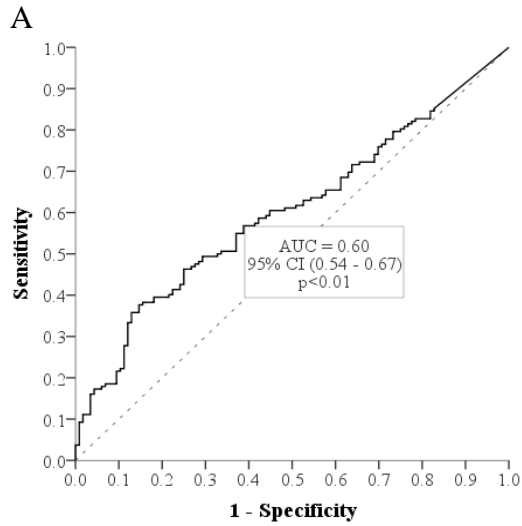


Figure 3 Receiver operating characteristic (ROC) curves of milk haptoglobin (Hpt) for detection of intramammary infection (IMI) in dairy heifers. A; gland status based on lenient definition of IMI (culture-positive in a non-contaminated milk sample). B; gland status based on strict definition of IMI (≥ 1000 CFU/mL). AUC – area under the ROC curve; 95% CI – 95% confidence interval.



Highlights

Pilot study into milk haptoglobin as an indicator of udder health in heifers after calving

- Specialization in dairy farming demands use of udder health screening tools in heifers
- Haptoglobin level is significantly and positively correlated with SCC in post-partum heifers
- Haptoglobin level and culture of CNS are in poor agreement as markers of udder health