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N-acetyl -β-D-glucosaminidase activity in cow milk as an indicator of mastitis

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Activity of lysosomal *N*-acetyl-β-D-glucosaminidase (NAGase) in milk has been used as an indicator of bovine mastitis. We studied NAGase activity of 808 milk samples from healthy quarters and quarters of cows with spontaneous subclinical and clinical mastitis. Associations between milk NAGase activity and milk somatic cell count (SCC), mastitis causing pathogen, quarter, parity, days in milk (DIM) and season were studied. In addition, the performance of NAGase activity in detecting clinical and subclinical mastitis and distinguishing infections caused by minor and major bacteria was investigated. Our results indicate that NAGase activity can be used to detect both subclinical and clinical mastitis with a high level of accuracy (0·85 and 0·99). Incomplete correlation between NAGase activity and SCC suggests that a substantial proportion of NAGase activity comes from damaged epithelial cells of the udder in addition to somatic cells. We therefore recommend determination of NAGase activity from quarter foremilk after at least six hours from the last milking using the method described. Samples should be frozen before analysis. NAGase activity should be interpreted according to DIM, at least during the first month of lactation. Based on the results of the present study, a reference value for normal milk NAGase activity of 0·1–1·04 pmoles 4-MU/min/µl for cows with ≥30 DIM (196 samples) could be proposed. We consider milk NAGase activity to be an accurate indicator of subclinical and clinical mastitis.

Keywords: *N*-acetyl -β-D-glucosaminidase, cow milk, indicator of mastitis.

Mastitis is the most economically important disease of dairy cows because of decreased milk quality, involuntary culling of cows and increased workload for the farmers, as well as its effects on bovine welfare (Halasa et al. 2007). Inflammatory markers in the milk are used to detect mastitis and to identify low quality milk that has to be discarded. The most widely used indicator of inflammation in the milk, also used by regulators to monitor bulk milk quality globally, is milk somatic cell count (SCC; IDF, 2013). In automatic milking systems, in addition to SCC, determining milk colour and electrical conductivity, and on-line analysis of L-lactate dehydrogenase incorporated into a decision-support model, are currently used commercially (Chagunda et al. 2006b).

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N-acetyl-β-D-glucosaminidase (NAGase) is a lysosomal glycosidase that is released into milk from epithelial cells of mammary tissue, reflecting destruction of udder tissue (Kitchen et al. 1978, 1980; Fox et al. 1988) and during lysis of inflammatory neutrophils (Kitchen et al. 1978; Kaartinen et al. 1988). NAGase activity in normal milk reported in different studies varies considerably (Table 1.) NAGase activity closely correlates with SCC (Kitchen et al. 1978, 1980; Miller & Paape, 1988; Berning & Shook, 1992). NAGase activity has also been used to distinguish intramammary inflammation and infection (IMI) caused by minor pathogens from those caused by major pathogens (Miller & Paape, 1988; Berning & Shook, 1992; Pyörälä et al. 2011). Due to its epithelial origin, NAGase activity can also separate healthy quarters from those with subclinical (Urech et al. 1999; Bansal et al. 2005; Nielsen et al. 2005) or clinical mastitis (Chagunda et al. 2006a; Larsen et al. 2010). NAGase activity is higher in clinical mastitis

Table 1. Mean milk N-acetyl-β-D-glucosaminidase activity of normal milk. Criteria used for normal milk, fraction of milk sampled, number of samples and temperature and length of incubation of the samples during laboratory analysis in the studies cited is also shown. All studies refer to the original method of Kitchen et al. (1978) with or without modifications

Study	NAGase pmol/min/μl milk†	Incubation at °C/min	Criteria for normal milk	Milk sample	Number of samples
Kitchen et al. (1978)	5·3	37/15	<500 000 cells/ml	Quarter foremilk	15 samples
Fox et al. (1985)	3·2 SE 1·4	37/15	<100 000 cells/ml, culture negative	Quarter foremilk	36 cows 259 samples
Mattila & Sandholm (1985)	Approximately 1	25/15	Culture negative	Quarter foremilk	80 cows 3157 samples
Emanuelson et al. (1987)	5·95 SE 2·06	37/15	Culture negative	Quarter foremilk	19 cows 507 samples
Miller & Paape (1988)	24·05 SE 1·03	50/15	Culture negative	Quarter foremilk	61 cows 896 samples
Berning & Shook (1992)	4·26 SD 1·95	37/15	Culture negative, no treatment	Cow composite milk	179 samples
Bansal et al. (2005)	0·34 SD 0·2	37/15	<100 000 cells/ml, culture negative	Quarter foremilk	37 cows 47 samples
Nielsen et al. (2005)	2·48 (6 h MI) 2·02 (12 h MI)	37/18	CMT 1–2	Quarter foremilk	11 cows 214 and 266 samples
Chagunda et al. (2006a)	39·05 SD 21·93	37/18	≤100 000 cells/ml	Cow composite milk	159 cows 11 799 samples
Piccinini et al. (2007)	32·55 SD 1·94	25/15	<100 000 cells/ml, culture negative	Quarter foremilk	14 quarters 224 samples

†SD standard deviation, SE standard error of the mean, MI milking interval.

than in subclinical mastitis (Pyörälä et al. 2011). It is affected by days in milk (DIM) (Mattila et al. 1986; Miller & Paape, 1988; Berning & Shook, 1992; Chagunda et al. 2006a; Piccinini et al. 2007), parity (Mattila et al. 1986; Chagunda et al. 2006a; Piccinini et al. 2007) and phase of milking (Bansal et al. 2005; Nielsen et al. 2005). Variation in milk NAGase activity between milkings in healthy udder quarters is low (Åkerstedt et al. 2011).

A fluorometric method is mostly used in NAGase activity detection and it has been proven to be effective, cheap and easy (Kitchen et al. 1978). The milk NAGase releases 4-MU from the substrate 4-MUAG in an acidic environment. The 4-MU fluoresces in an alkaline environment, which can be measured to indicate milk NAGase activity. The advantage of determining NAGase activity is that milk storage, e.g. by freezing, does not disturb the analysis (Mattila & Sandholm, 1985). Currently NAGase analysis requires laboratory facilities, although an automated on-line method with an optical biosensor-based immunoassay was suggested (Welbeck et al. 2011).

The aim of this study was to establish a reference value for normal milk NAGase activity, and to find optimal conditions for the analysis. An additional aim was to study the performance of NAGase activity in detecting clinical and subclinical mastitis and in differentiating intramammary infection caused by minor pathogens and major pathogens. The effects on milk NAGase activity of milk SCC, mastitis causing pathogen and quarter (fore/hind), parity and DIM of the cow and season were determined.

Material and methods

Number of samples

Milk from healthy quarters of cows with spontaneous subclinical or clinical mastitis was collected from 64 dairy herds (1–114 samples per herd, a total of 808 samples) in southern Finland or Estonia by veterinarians or trained personnel during 2000–2010. Table 2 shows the number of herds and samples in each udder health category. Only one milk sample per cow was used for each category. Cows had calved on average 2·4 times (1–8).

Sampling scheme and categorisation of samples

To determine local signs of mastitis, milk colour and consistency were assessed as normal or changed (flakes, clots and discoloration). Udders were palpated and checked for any abnormal signs, such as heat, oedema, swelling, chronic changes, wounds and teat tramps. If there were local signs, rectal temperature was measured and appetite and general attitude of the cow were gauged. A California Mastitis Test (CMT) was performed for each cow using the Scandinavian scoring system of 1 to 5 (Klastrup & Schmidt-Madsen, 1974). Milk samples were taken for SCC, determination of NAGase activity, and bacterial culture from one healthy quarter of a healthy cow or the quarter with the highest CMT score of a cow with subclinical or clinical mastitis. Quarter, DIM, parity, season and the time from last milking were recorded. Strip milk

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Table 2. Number and origin of herds and samples used, days in milk (DIM) and parity of cows and categorisation of samples in nine different udder health categories

Category SCC mean (range)	*N*	Herds Samples/herd	DIM mean range	Parity mean	Dataset†
Healthy total, SCC < 100 000/ml	220	9 2–49	139 5–397	1·8	1, 2
Healthy, DIM ≥ 30 d 25 560 cells/ml (1000–94 000 cells/ml)	196	9 2–42	154 30–397	1·8	1, 2
Healthy, DIM < 30 d, 27 750 cells/ml (1000–91 000 cells/ml)	24	7 1–7	17 5–29	1,9	1, 2
Subclinical mastitis total, SCC 100 000–10 000 000/ml	207	19 1–57	188 3–477	2·2	1, 2
Subclinical mastitis, SCC 100 000–199 000/ml 141 890 cells/ml (100 000–197 000 cells/ml)	47	9 1–15	171 3–414	2·0	1, 2
Subclinical mastitis, SCC 200 000–299 000/ml 244 080 cells/ml (200 000–295 000 cells/ml)	25	7 1–8	181 12–311	2·4	1, 2
Subclinical mastitis, SCC 300 000–399 000/ml 346 670 cells/ml (301 000–396 000 cells/ml)	24	7 1–6	218 46–429	2·1	1, 2
Subclinical mastitis, SCC 400 000–999 000/ml 622 330 cells/ml (404 000–985 000 cells/ml)	45	7 1–11	188 12–477	2·2	1, 2
Subclinical mastitis, SCC ≥ 1 000 000/ml 2 792 420 cells/ml (1 018 000–10 000 000 cells/ml)	66	9 1–17	191 4–469	2·5	1, 2
Clinical mastitis total	381	57 1–114	103 1–395	2·8	3, 4, 5
Clinical mastitis with local signs	293	27 1–111	103 1–395	2·8	3, 4, 5
Clinical mastitis with systemic signs	87	51 1–10	102 1–377	2·7	3, 4, 5

†1: samples collected for this study during herd visits during 2000–2002.
2: samples collected for this study during herd visits in during 2009–2010.
3: samples collected for this study from cows with mastitis in during 2000–2002.
4: samples collected during a treatment trial on clinical *E. coli* mastitis during 2003–2006 (Suojala et al. 2010).
5: samples collected in a mastitis treatment trial in Estonia during 2007–2009 (Kalmus et al. 2013).

samples for NAGase activity analysis were collected from 33 healthy quarters after milking.

To determine values for normal milk, milk from clinically healthy cows with ≥30 DIM and milk SCC of the quarter <100 000 cells/ml was used. To determine values for milk from early lactation, normal milk from clinically healthy cows in their first 5–29 DIM with no visual oedema was used. Milk samples were taken at least six hours after the last milking. To determine values for subclinical mastitis, quarters with no local or systemic clinical signs were selected. A quarter with subclinical mastitis was defined as a quarter with milk SCC ≥100 000 cells/ml independent of bacterial status (IDF, 2011, 2013). For clinical mastitis samples, quarters of cows with clinical mastitis signs were selected. Cows were further categorised as having mild to moderate (local) or severe (systemic) mastitis (IDF, 2011).

Sampling technique

Foremilk samples were collected for CMT scoring, evaluation of the milk appearance and determination of milk SCC. An additional 10 ml was collected aseptically for milk bacteriology and determination of NAGase activity. Samples were cooled immediately and transported to the laboratory of the Department of Production Animal Medicine for SCC and bacterial culturing. Samples were stored at −20 °C for subsequent NAGase activity analysis.

Sample analyses

Milk SCC was determined using either an electronic cell-counter DCC (DeLaval International AB, Hamra, Sweden) at the laboratory of the Department of Production Animal Medicine or a fluoro-optic cell-counter Fossomatic (Foss Electric, Hillerød, Denmark) at Valio's laboratory. The higher quantification limit was set to 10 000 000 cells/ml milk. Conventional methods were used for bacteriological culturing (Hogan et al. 1999). Overall 386 milk samples from 588 quarters with subclinical or clinical mastitis were bacteriologically positive.

NAGase activity was measured after each sampling using a fluorometric method using micro templates, modified from that of Kitchen et al. (1978) and Mattila & Sandholm (1985). Control milk samples with a known 4-MU concentration were from a cow with mastitis (control 1, high NAGase activity) and from a clinically healthy cow (control 2, low NAGase activity). Control samples were used to determine NAGase activity of the sample milk (high control) and to

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follow degradation of the substrate (low control). Control samples were frozen at −70 °C.

Frozen milk samples were thawed to 20 °C and inserted into a black template. Ten microliters of sample, control milk and distilled water were pipetted in the wells in duplicate. In each well, 40 µl of substrate (2·25 mM 4-MUAG, M-2133 Sigma-Aldrich®) in citrate buffer (0·25 M, pH 4·6) was added.

The template was placed in a dark box on an automatic shaker for one minute. After mixing, a 14-minute incubation at 20 °C was used to allow NAGase to release 4-MU from the substrate in an acidic environment. To stop the reaction, 150 µl of glycine buffer (0·2 M, pH 11) was added into each well and mixed for one minute. The fluorescence of the resultant 4-MU was measured with a Fluoroscan Ascent Type 374, (Labsystems, Espoo, Finland) using a 355 nm excitation filter and a 460 nm emission filter. The means of the relative fluorescence units of the duplicate samples were calculated and reduced by the mean of the blanks. NAGase activity was calculated according to the equation: NAGase activity of the sample = fluorescence of (sample–blank) × NAGase activity of control 1/fluorescence of (control 1–blank). NAGase activity of the control 1 was determined from the standard curve (see below). NAGase activity was expressed in pmoles of 4-MU/min/µl milk at 20 °C.

Determination of the standard curve

NAGase activity analysis was based on a standard curve by measuring the fluorescence of 12 different known 4-MU concentrations (1–450 µM 4-MU in glycine-buffer) (methylumbelliferone, M-1381 Sigma-Aldrich®). Using the curve, NAGase activity of the control milk 1 and the upper and the lower quantification limits for the analysis were determined from the linear section of the curve, above the value of the blank. The fluorescence of control 1–blank was divided by the slope, which produced the respective molarity of control 1. The upper quantification limit for NAGase activity was 24·49 pmol of 4-MU/min/µl milk at 20 °C and the lower 0·10 pmol of 4-MU/min/µl milk at 20 °C.

Repeatability of the analysis

Intra-assay CV (coefficient of variation) was determined with 45 pairs of samples of control milk 1 in one plate, resulting in an intra-assay CV of 3·9%. The inter-assay CV was determined using 7 pairs of samples of control milk 1 in 7 different plates, 3 different days and intervals of more than 26 d over 3 months, resulting in a value of 4·9%.

Kinetics of the reaction

End-point kinetics with known control milk and a 15-min incubation time were used. To study the linearity of the reaction, we studied control milk 1 (4 pairs/plate) and control milk 2 (2 pairs/plate) on six different plates, with incubation times of 1, 2, 5, 10, 15 and 20 min. The reaction was linear for 20 min, although slight non-linearity was apparent after 10 min.

Statistical methods

Reference values for normal milk were calculated as mean ± 1·96 × SD for cows with ≥30 DIM (196 samples). NAGase activity and SCC were transformed to logarithmic (ln) values to normalise the data. The associations between NAGase activity of normal milk and different predictors were studied using a linear mixed model with herd as a random effect. The linear assumption was confirmed with graphical assessment of linearity between SCC and NAGase activity. DIM was categorised as <30, 30–239 and ≥240 d and parity as first, second and ≥3. The quarter was used as a dichotomous variable (fore/hind quarter). Season was classified as Dec to Feb, March to April, June to Aug, Sep to Nov. The dataset was either 1, 2, 3, 4 or 5. Variables were first tested individually and subsequently the variables of the model were chosen using stepwise selection (forward-backward). The random effects Tobit regression model for censored data was used to investigate associations between milk NAGase activity and predictors with mastitis samples. The Tobit regression model was chosen because 27% of the NAGase activity results were over the maximum working limit of the assay (24·5 pmol of 4-MU/min/µl) with mastitis samples, which violate the regression model's assumptions. In the Tobit regression, all cases falling above (or below) a specified threshold value are censored, although these cases remain in the analysis (Long, 1997). Logarithmic (ln) transformation of milk NAGase activities was used to achieve a normal distribution of uncensored data; 153 samples from 573 were censored at the level of 24·5 pmol of 4-MU/min/µl. All other model building strategies and predictors in this model were as described for the model of normal milk samples above. Additionally, different bacterial species and severity of mastitis (clinical/subclinical) were included. Suitability of the model was tested by examining distribution and homogeneity of variances of residuals.

Pearson correlation was used to study the association between NAGase activity (ln) and SCC (ln). Paired sample *t*-tests were used to compare NAGase activity between fore and strip milk and independent sample *t*-tests for comparison of quarters infected with minor or major bacteria. ANOVA was used to compare NAGase activity in different health categories and a Bonferroni adjustment was used to make pairwise comparisons among the categories. Sensitivity (SE) and specificity (SP) of NAGase activity in differentiating among udder health categories was determined using ROC-analysis. Statistical software Stata 11.0 (StataCorp, Texas, USA) was used.

Results

Milk NAGase activity and correlation between it and SCC

Mean, median, range, and SD of NAGase activity in different health categories are presented in Table 3, in addition to

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Table 3. Mean, median, range and SD of NAGase activity (pmol of 4-MU/min/µl milk at 20 °C) and Pearson correlation coefficients between NAGase activity and SCC of quarters from healthy cows or cows with subclinical or clinical mastitis

	n	Mean/Median	Range	SD	Correlation between lnNAGase and lnSCC
Healthy	220	0·62/0·59	0·14–2·59	0·33	0·04
DIM ≥ 30	196	0·56[a]/0·56†	0·14–1·55	0·24	0·04
DIM < 30	24	1·04[ab]/0·98	0·31–2·59	0·54	0·06
DIM ≥ 240	31	0·61/0·59	0·21–1·44	0·27	0·12
Subclinical mastitis	207	2·42/1·35	0·30–21·67	3·07	0·73***‡
SCC 100 000–199 999	47	0·99[a]/0·88	0·30–4·25	0·64	0·25
SCC 200 000–299 999	25	1·02[ab]/1·02	0·57–1·68	0·31	0·10
SCC 300 000–399 999	24	1·20[ab]/0·96	0·48–5·65	1·01	0·18
SCC 400 000–999 999	45	1·90[ab]/1·43	0·51–6·38	1·33	0·35*
SCC>1 000 000	66	4·78[b]/2·79	1·01–21·67	4·40	0·43***
Clinical mastitis	381	16·65/19·4	0·48–24·49	8·28	Not measured
With local signs	294	16·19[c]/18·11	0·48–24·49	8·48	Not measured
With systemic signs	87	18·19[c]/22·45	1·32–24·49	7·39	Not measured

†Different letters indicate statistically significant differences between mean NAGase activities ($P < 0·05$) between different health categories.
‡$P < 0·05$, *** $P < 0·001$.

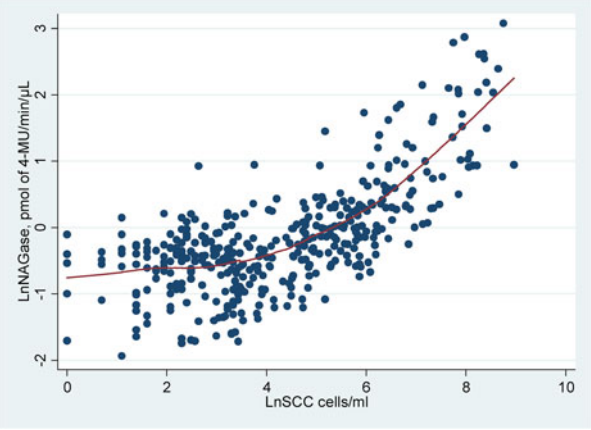

Fig. 1. Milk lnSCC and lnNAGase activity and smooth loess trendline in healthy quarters and in quarters with subclinical mastitis.

correlation between SCC and NAGase activity. Figure 1 shows the association between lnNAGase activity and lnSCC for all normal and subclinical quarters ($n = 427$; coefficient of correlation 0·75; $P < 0·001$). Strip milk NAGase activity was 0·97 (SD 0·44) pmoles 4-MU/min/µl, significantly different from foremilk NAGase activity at 0·71 (SD 0·19) pmoles of 4-MU/min/µl ($P < 0·001$). Based on these results we found a reference value for normal milk of healthy cows of 0·1–1·04 pmoles 4-MU/min/µl milk for cows with ≥30 DIM.

Performance of NAGase activity in differentiating among health categories

According to the ROC-analysis, a cut-off of 0·76 pmoles 4-MU/min/µl milk differentiated healthy quarters from those with subclinical mastitis at a SE of 85%, SP of 84% and accuracy of 0·85. The cut-off point of 1·19 pmoles 4-MU/min/µl milk differentiated healthy quarters from those with clinical mastitis with an SE of 99% and an SP of 98% leading to 0·99 accuracy. The cut-off point 5·3 pmoles 4-MU/min/µl milk differentiates quarters with subclinical mastitis from those with clinical mastitis with an SE of 86% and an SP of 90% at 0·88 accuracy.

Effect of the bacterial species isolated in the milk on NAGase activity and discrimination performance

NAGase activity and SCC in mastitis caused by different pathogens are shown in Table 4. Milk from quarters with growth of minor pathogens (coagulase-negative staphylococci (CNS) or *Corynebacterium* sp.) had a mean NAGase activity of 3·19 pmol/min/µl milk at 20 °C (SD 5·24), and milk from quarters with major pathogens (*Staphylococcus aureus, Streptococcus uberis, Streptococcus dysgalactiae, Streptococcus agalactiae. Escherichia coli or Trueperella pyogenes*) had a mean NAGase activity of 15·96 pmol/min/µl milk at 20 °C (SD 8·81) ($P < 0·0001$). A cut-off of 2·63 pmol/min/µl milk at 20 °C had a SE of 91%, a SP of 85%, and an accuracy of 0·88 in differentiating major pathogens from minor pathogens.

Associations between NAGase activity and cow and milk variables in different health categories

Associations between NAGase activity and different variables for normal milk and milk from quarters with subclinical or clinical mastitis are shown in Table 5. According to the model, NAGase activity increased with increasing SCC and was higher in healthy hind quarters than in healthy fore quarters (0·71 *vs* 0·55 pmoles 4-MU/min/µl milk). NAGase activity was also higher during the first 30 d of milking and tended

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Table 4. Proportion of quarters with clinical mastitis (CM) and mean, median and range of NAGase activity and SCC in milk of 573 quarters with subclinical or clinical mastitis classified by bacterial species isolated in the milk

		Milk NAGase pmol/min/µl in 20 °C				SCC*1000 cells/ml milk			
Bacteria	CM	*n*	Mean	Median	Range	*n*	Mean	Median	Range
Coryneform	23%	26	3·35	1·56	0·34–14·26	22	1069	670	115–4253
CNS	10%	58	3·12	1·03	0·48–24·49	52	650	364	117–4530
S. aureus	65%	71	12·35	8·85	0·95–24·49	27	2528	1386	177–10 000
Str. dysgalactiae	72%	25	16·37	20·50	1·42–24·49	9	3727	3103	1162–10 000
Str. uberis	83%	72	16·50	22·6	0·55–24·49	13	1945	895	328–10 000
E. coli	99%	87	17·08	18·1	1·32–24·49				
Str. agalactiae	100%	9	23·34	24·49	17·96–24·49				
T. pyogenes	100%	13	19·22	24·49	1·75–24·49				
Others†	65%	212	10·11	6·66	0·30–24·49	76	1281	417	100–10 000

†No growth (*n* = 146, of which clinical 58%), yeast, *Enterococcus* sp., *Klebsiella* sp., *Coliform* sp., *Lactococcus* sp., *Streptococcus* sp. and samples with two different bacterial species (*n* = 41, of which clinical 95%).

Table 5. Associations between milk lnNAGase activity and cow and milk variables of healthy quarters (SCC < 100 000 cells/ml milk and bacteria negative) and of quarters with subclinical or clinical mastitis (CI = confidence interval)

	Healthy quarters, *n* = 220			Quarters with subclinical or clinical mastitis, *n* = 573		
Variable	Effect	*P*-value	CI of 95%	Effect	*P*-value	CI of 95%
Milk lnSCC	0·10	< 0·0001	0·055–0·14			
Hind *vs* fore quarter	0·11	0·013	0·02–0·20	0·09	0·210	−0·05–0·24
DIM 30–239 *vs* <30	−0·60	<0·0001	−0·73–0·46	−0·30	0·004	−0·51–0·10
DIM ≥ 240 *vs* <30	−0·58	<0·0001	−0·73–0·38	−0·19	0·330	−0·57–0·19
Parity 2 *vs* 1	0·18	0·001	0·07–0·28	0·12	0·290	−0·11–0·35
Parity ≥3 *vs* 1	0·19	0·001	0·08–0·31	0·33	0·003	0·11–0·54
Dataset 1 *vs* 2	−0·70	<0·0001	−0·79–0·61			
Dataset 4 *vs* 1 and 3				−0·35	0·375	−1·13–0·43
Dataset 2 and 5 *vs* 1 and 3				0·18	0·231	−0·11–0·46
Clinical *vs* subclinical				1·86	<0·0001	1·54–2·17
Str. agalactiae vs CNS				1·97	<0·0001	1·70–2·24
E. coli vs CNS				1·27	<0·0001	0·56–1·98
Str. dysgalactiae vs CNS				1·21	<0·0001	0·60–1·82
Str. uberis vs CNS				1·02	<0·0001	0·62–1·41
T. pyogenes vs CNS				0·85	<0·0001	0·47–1·24
S. aureus vs CNS				0·68	0·001	0·29–1·07
Others *vs* CNS				0·35	0·014	0·07–0·62
Corynebacterium sp. *vs* CNS				0·11	0·737	−0·55–0·77
Constant	−0·15	0·106	−0·34–0·03	0·11	0·622	−0·33–0·55

to continue to decrease until 100 d after calving (Fig. 2). NAGase activity was lower in milk from primiparous than older cows. Samples taken and analysed in later years had lower NAGase activity than samples analysed in 2000–2002 (0·45 *vs* 0·77 pmoles 4-MU/min/µl milk in healthy quarters). Season had no effect on milk NAGase activity.

Discussion

Reference values for milk NAGase activity of normal milk

We found a reference value for milk NAGase activity of normal milk analysed in conditions defined in the present study, of 0·1–1·04 pmoles 4-MU/min/µl. This value is low compared with values for normal milk found in most other studies. This can partly be explained by the low SCC (<100 000/ml) used here as the criterion for normal milk, compared with other studies using higher SCC limits, or no growth in bacteriological culture as a criterion for normal milk (Kitchen et al. 1978; Emanuelson et al. 1987; Miller & Paape, 1988; Berning & Shook, 1992; Nielsen et al. 2005). We also used a lower incubation temperature (20 °C) compared with most other studies. Reasons for that were practical, as warming of the samples during incubation was not needed and fewer values were above the higher detection limit. We also tested an incubation temperature of

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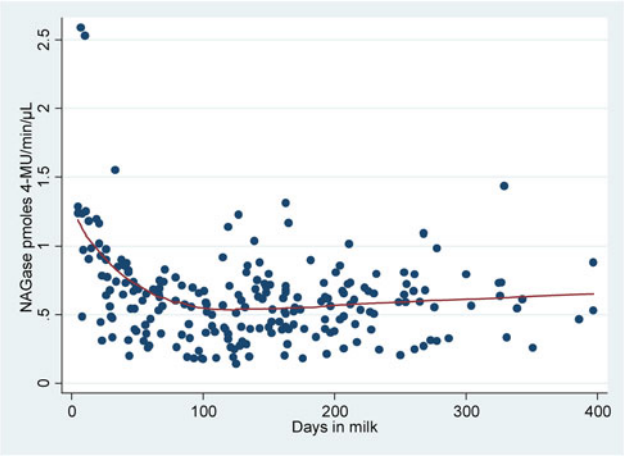

Fig. 2. Milk NAGase activity and smooth loess trend line according to the stage of lactation (days in milk) of the cow in 220 healthy quarters.

37 °C with a few samples of normal milk. Fluorescence values were three times as high as those at a 20 °C incubation temperature used in our study (data not shown). Differences in incubation temperatures could explain higher NAGase activities for normal milk reported in most other studies. The study of Mattila & Sandholm (1985), using a similar incubation temperature as here, and that of Bansal et al. (2005), with similar criteria for normal milk, are the only reports that agree with our findings. We (in contrast to Bansal et al. 2005; Nielsen et al. 2005; Chagunda et al. 2006a) used freezing and thawing of the samples, which increases NAGase activity because NAGase from inside the cells is released (Kaartinen et al. 1988). Some of the other studies also used freezing, but this information was not available for all studies and cannot explain the lower NAGase activities reported in our study.

We used an end-point-method and control milk with known NAGase activity. Incubation time was fixed to 15 min, which has been used in most other studies (Kitchen et al. 1978), except by Nielsen et al. (2005) and Chagunda et al. (2006a), who used 18 min. In our study, the reaction was linear for at least 20 min of incubation. The effect of 15 compared with 20 min incubation time on NAGase activities was minimal for normal milk, but considerable for abnormal milk.

The repeatability of our analysis was comparable with that for other studies (Nielsen et al. 2005; Chagunda et al. 2006a). The inter-assay CV was low, but the time when samples were collected and analysed affected the results of normal milk (according to the model for normal milk), possibly because of different technicians performing the analysis. This highlights the importance of standardisation of methods used for sampling, storage of samples and analysis. As regards mastitic milk, the varying consistency of the sample may present a challenge for the repeatability of the analysis. We tried to overcome this problem with centrifugation of a few clotted samples, with no success.

NAGase activity was higher in strip than in foremilk of healthy quarters. This is consistent with the results by Fox et al. (1988) and Bansal et al. (2005). In the study of Nielsen et al. (2005) and Urech et al. (1999), NAGase activity was high in foremilk and again increased towards the end of milking. Using quarter milk samples is preferred because in composite milk of cows having one or more inflamed quarters, NAGase activity has been found to be almost as low as in milk from totally healthy cows (Nielsen et al. 2005), because of a dilution effect from the healthy quarters.

In conclusion, most other studies have reported higher NAGase activities for normal milk probably because of less stringent criteria for normal milk, higher incubation temperature or longer incubation time. Because of unexplained differences in values for normal milk, exact description of the methods of analysis is needed.

Physiological factors that affect NAGase activity

Healthy quarters and quarters with subclinical or clinical mastitis of primiparous cows had lower NAGase activity than those of older cows, as previously reported in many studies (Mattila & Sandholm, 1985; Mattila et al. 1986; Nyman et al. 2014). Chagunda et al. (2006a) found no effect of parity on milk NAGase activity. Season had no effect on NAGase activity in healthy quarters in this study, but Chagunda et al. (2006a) and Nyman et al. (2014) reported higher milk NAGase activity values during winter months. In our study, NAGase activity was higher in hind than fore quarters of healthy cows.

NAGase activity of healthy quarters or quarters with clinical mastitis were affected by DIM, as reported in other studies (Mattila et al. 1986; Miller & Paape, 1988; Berning & Shook, 1992; Chagunda et al. 2006a; Piccinini et al. 2007; Nyman et al. 2014). NAGase activity for early lactation (DIM < 30) of healthy quarters was almost twice as high as that for the remaining lactation. This agrees with earlier studies where NAGase activity was higher from DIM 4 to as far as DIM 40 (Berning & Shook, 1992; Chagunda et al. 2006a; Piccinini et al. 2007). NAGase activities for milk from quarters with mastitis was also higher in late lactation, but not for healthy quarters. Miller & Paape (1988) suggested determination of different reference values for early, middle and late lactation. According to our study, this would be necessary at least for early lactation. No change in milk NAGase activities was recorded after the first 100 d in healthy quarters also in Chagunda et al. (2006a) and Piccinini et al. (2007). In some studies NAGase activity increased towards late lactation (Berning & Shook 1992; Nyman et al. 2014), but in the study by Mattila et al. (1986) the effect was eliminated by taking the decreasing milk yield into account.

No simple correlation existed between NAGase activity and SCC in normal milk, but a mild association was found in the model looking at associations between model predictors and normal milk of healthy quarters. Correlations reported in other studies have been higher,

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0·41 ($n = 11\,799$, composite milk) (Chagunda et al. 2006a) and 0·39 ($n = 485$) in milk from healthy cows (Nyman et al. 2014). We had a high correlation (0·73) between NAGase activity and SCC in milk in quarters with subclinical mastitis. This is expected because part of the NAGase activity comes from milk somatic cells in addition to epithelial cells (Kitchen et al. 1978, 1980; Emanuelson et al. 1987; Miller & Paape, 1988; Berning & Shook, 1992). Changunda et al. (2006a), using composite milk, and Bansal et al. (2005), using quarter milk, reported lower correlation (0·59; $n = 28\,508$ and 0·45; $n = 142$) between SCC and NAGase activity for subclinical mastitis than in our study.

Performance of NAGase-test in detection of subclinical and clinical mastitis

NAGase activity was very low in milk of healthy quarters, increased in subclinical mastitis and was highest in quarters with clinical mastitis, as reported by Pyörälä et al. (2011). With a low cut-off point, NAGase activity differentiated healthy quarters from those with subclinical mastitis very accurately (0·85) compared with other studies (Urech et al. 1999; Bansal et al. 2005; Nielsen et al. 2005).

Clinical mastitis in our study was differentiated from healthy quarters with a fairly low cut-off point for milk NAGase activity, with extremely high (0·99) accuracy. Furthermore, quarters with subclinical mastitis were differentiated from those with clinical mastitis with a high accuracy (0·88), which can be explained by the destruction of the udder epithelium in cows with clinical signs. Compared with the findings of Chagunda et al. (2006a), the performance of milk NAGase activity in our study was better than milk L-lactate dehydrogenase in their study, probably because we used quarter milk and not composite milk.

Performance of NAGase-test in detection of IMI

There was a significant difference in milk NAGase activity of quarters infected with minor or major bacteria, as reported earlier (Miller & Paape, 1988; Berning & Shook, 1992; Pyörälä et al. 2011). The test performed very well in separating quarters infected with major pathogens from those with minor pathogens, in accordance with Mattila & Sandholm (1985).

Differences in NAGase activity in milk from quarters with IMI caused by different bacteria were apparent in the model for clinical and subclinical mastitis, but further investigation of the data (not shown) revealed that this was mainly in quarters with subclinical mastitis and not in those with clinical mastitis. Overall, milk from quarters with clinical mastitis had a much higher NAGase activity than in milk from quarters with subclinical mastitis. Our results agree with those from earlier studies of Pyörälä et al. (2011) where *T. pyogenes* and *E. coli* were associated with the highest NAGase activity and CNS with the lowest.

Milk NAGase activity as an indicator of mastitis

We described here reference values for milk NAGase activity, which can be used in further studies, according to the conditions listed: (1) Determination of NAGase activity from quarter foremilk after at least six hours from the last milking. (2) Samples should be frozen before analysis. (3) NAGase activities should be interpreted taking DIM of the cow into account, at least in the first month of lactation. Agreement between NAGase activity and SCC was not complete, which suggests that a substantial part of milk NAGase comes from damaged epithelial cells of the udder and may be seen partly as a result of epithelial destruction.

We conclude that milk NAGase activity can be used for the detection of subclinical and clinical mastitis with a high accuracy. Unlike milk SCC, a high NAGase activity tells us that epithelial cell destruction in the udder is likely, which helps to assess the prognosis for recovery from mastitis. Milk NAGase activity is a good measure of udder health.

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