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Short communication: Protease activity measurement in milk as a diagnostic test for clinical mastitis in dairy cows

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ABSTRACT

Due to the increasing use of automated milking systems, automated detection of clinical mastitis is becoming more important. Various in- or on-line diagnostic tests are in use, but generally suffer from false mastitis alerts. In this study, we explored a new diagnostic approach based on measurement of protease activity using fluorogenic protease substrates, which can be performed on site, at high speed, and at low costs. Samples from cows with clinical mastitis submitted for bacteriological culture at the University Farm Animal Practice were collected during several months and kept at −20°C until protease activity measurement. A reference set of milk samples from clinically healthy cows were collected on 9 different farms and were tested for protease activity directly and after freezing at −20°C to allow for comparison with the samples from clinical cases. The protease activity in mastitic milk samples was significantly higher than in samples from healthy animals. Based on 71 clinical mastitis samples and 180 milk samples from clinically healthy quarters, the area under the receiver-operating characteristic curve was estimated to be between 0.88 and 0.90, and at a threshold of 38 fluorescence per minute the test had a specificity of 0.99 at a sensitivity of 0.58. Protease activity measured in fresh milk from clinically healthy cows was significantly associated with somatic cell count and parity, but not with electrical conductivity, whereas protease activity in milk that had been frozen was statistically significantly associated with all 3 parameters. This study indicates that protease activity measurement as a stand-alone test can be used for detecting mastitis samples, using milk samples that have been frozen. Because protease activity acts in part on a different biological mechanism than somatic cell count or electrical conductivity, this test may increase the accuracy of mastitis diagnosis in combination with currently available in- or on-line tests in automated milking systems.
Key words: mastitis, protease, milk, cow

Short Communication

Because of the growing popularity of automated milking systems (**AMS**) in dairy herds, automated mastitis detection is becoming increasingly important (Hovinen and Pyörälä, 2011). Especially the automated detection of clinical mastitis is crucial when using an AMS, as no inspection of the udder and the foremilk by the farmer is performed anymore. The most frequently used sensors for automated mastitis detection measure electrical conductivity (**EC**; Hovinen and Pyörälä, 2011), but devices for measuring SCC, L-lactate dehydrogenase, haptoglobin, N-acetyl-β-D-glucosaminidase, temperature, and milk color have been developed as well (reviewed by Rutten et al., 2013). Still, the performance of these systems in terms of sensitivity (**Se**) and specificity (**Sp**) is generally too low to be useful for automated diagnosis of clinical mastitis, mainly because of the low prior probability of mastitis at a given point in time. The low prevalence of mastitis combined with an Sp <1 leads to a low positive predictive value, or, in other words, a high rate of false positive alerts, which is problematic as farmers tend to dislike a high rate of false positive alerts more than a high rate of false negatives (Mollenhorst et al., 2012). Combining several diagnostic tests can help to improve the overall performance by using serial or parallel interpretation of these tests. Such combinations are most helpful if the tests are conditionally independent (i.e. if the test result of one test is independent on the test result of the other test) given disease status (Gardner et al., 2000). Tests are more likely to be conditionally independent if they relate to different biological mechanisms. Therefore, diagnosis of clinical mastitis could be improved by developing tests that aim at other pathways than what is measured by EC or SCC.

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The major protease in milk is plasmin (Larsen et al., 2010), but bovine milk contains many proteases (Kelly et al., 2006), some of which originate from leukocytes (Haddadi et al., 2006) or from contaminating microorganisms (Åkerstedt et al., 2012). Several studies have shown that plasmin activity and, more generally, protease activity (**PA**) increases when mastitis occurs (Moussaoui et al., 2004; Larsen et al., 2010; Hinz et al., 2012). This increase is the result of a complex interaction between somatic cells in milk, the pathogen, and the udder epithelium. Loss of membrane integrity causes an increased permeability of the blood-milk barrier leading to direct influx of plasmin in the milk (Politis et al., 1989); also, the increase of plasminogen activators in the milk increases levels of active plasmin (Heegaard et al., 1994). Measurement of PA in milk may therefore be used as a diagnostic test for mastitis. This potentially adds to the spectrum of diagnostic tests that are already available. Activity-based protease profiling using fluorogenic protease substrates makes quantitative measurement of PA possible at high speed, low costs, and on site. Therefore, such techniques are promising for on-line application in AMS. Recently, we have shown in a limited number of samples that plasmin activity in milk as measured by cleavage of plasmin-specific substrates was significantly associated with mastitis status (Bikker et al., 2014). The test characteristics of PA in milk to detect clinical mastitis, however, are largely unknown. Furthermore, the effect on PA of cow-characteristics, such as parity and DIM, and of other factors, such as freezing of the milk, is poorly described. This study aims (1) to define the test characteristics of PA measurement for diagnosis of clinical mastitis, (2) to assess to what degree PA correlates to the other indicators of mastitis, and (3) to determine to what extent parity and DIM of the cow and freezing of the milk sample affect PA levels.

We collected samples from animals with clinical mastitis and a set of control samples from clinically nonmastitic animals. Samples from animals with clinical mastitis were collected at the University Farm Animal Practice (ULP, Harmelen, the Netherlands). In total, 71 milk samples were submitted to the practice's laboratory for bacteriological culture with explicit notion that they were collected from a quarter with clinical mastitis. These samples were collected during 4 mo from the end of June through October 2013. Samples were collected aseptically by farmers, which had received training in collection of sterile milk samples for bacteriological culture by their veterinarian. Bacteriological culture was performed according to National Mastitis Council guidelines (Hogan et al., 1999), and the remainder of the sample was stored at −20°C for 5 to 20 wk until further examination.

For the control samples from clinically healthy quarters, a convenience sample of 9 herds was selected from the same veterinary practice based on willingness of the farmers to participate and on having a conventional (nonautomatic) milking system. On each farm, 20 cows were selected by sampling all clinically healthy animals on one side of the parlor until the number of 20 was reached. From each cow, 1 quarter was sampled, sequentially collecting a sample from the right front, right hind, left front, and left hind quarter. After standard teat cleaning by the farmer, sampling was performed by discarding the first 3 squirts of milk and then collecting about 10 mL of milk in a sterile plastic vial. Animals that showed any signs of clinical mastitis, such as abnormal milk or abnormalities of the udder, were not sampled. For all animals sampled, parity and DIM were recorded.

The samples from clinically healthy cows were cooled with ice packs and transported to the laboratory for SCC measurement with a DCC DeLaval cell counter (DeLaval International AB, Tumba, Sweden) and for EC measurement with a hand-held conductivity meter (Cyanamid Animal Health, Etten-Leur, the Netherlands). After this, the milk samples were split into 2 aliquots. Protease activity was determined on 1 of the 2 aliquots immediately and on the other after it had been frozen at −20°C for at least 1 wk.

A protease substrate with broad protease specificity, Fitc-Ahx-NleKKKKVLPIQLNAATDK-KDbcyl (Cummings et al., 2002), was synthesized by in house (Department of Oral Biochemistry, Academic Centre for Dentistry Amsterdam) peptide chemistry and purified to >95% purity. The identity of the substrate was confirmed by in house mass spectroscopy (Veerman et al., 2007). Protease activity was analyzed as described earlier (Kaman et al., 2013; Bikker et al., 2014). In short, milk samples were centrifuged at 1,000 × g for 1 h at 4°C, after which the skim milk was transferred to a new vial, leaving fat, cells, and debris behind. The skim milk was directly used for analysis or stored at −20°C. Skim milk was mixed 1:1 vol/vol with 50 mM Tris, 1 mM Ca^{2+}, pH 9.0, and 16 μM substrate. Plates were read for 60 min with 2-min intervals at 37°C on a fluorescence microplate reader (Fluostar Galaxy, BMG Laboratories, Offenburg, Germany) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Samples were analyzed in duplicate and the average of the 2 measurements was used for further analyses. Protease activity was defined in fluorescence per minute (**F/min**) calculated over a read-out period of 10 min (**PA_10**), 30 min (**PA_30**), or 60 min (**PA_60**).

Receiver operating characteristic (**ROC**) curves were plotted to show the diagnostic value of PA in differenti-

ating samples from clinically healthy and clinically diseased animals. Curves were plotted for PA_10, PA_30, and PA_60 using only data from samples that had been frozen to make samples from healthy and diseased quarters comparable. In samples from clinically healthy animals, the difference between PA_60 in fresh and in frozen samples was tested using a paired *t*-test and Pearson correlation coefficient was calculated between 10log-transformed-SCC, EC, and PA_60 (on frozen samples and on fresh samples). Associations of parity, DIM, udder quarter, and farm with PA_60 (on frozen and on fresh samples) were tested using regression models. The effect of farm was modeled as a random effect to correct for potential clustering. Parity was recoded into a 5-class categorical variable (parity 1, 2, 3, 4, and >4). The fit of the model and the assumptions of homoscedasticity and normality were checked by visual inspection of the plots of standardized residuals against predicted values and histograms and quantile-quantile plots of standardized residuals. The analyses were performed in R (version 3.0.2; R Foundation for Statistical Computing, Vienna, Austria). The ROC curves were made using the pROC library (Robin et al., 2011); for the mixed model, the LME function from the NLME library was used (Pinheiro et al., 2009).

The main goal of our study was to estimate the value of PA measurement as a diagnostic test to identify cases of clinical mastitis (i.e. to differentiate between milk samples from clinically healthy, including subclinically infected, and clinically mastitic quarters). Table 1 shows that PA in milk from clinically mastitic quarters is significantly higher than PA in milk from clinically healthy quarters, resulting in area under the ROC curve values of up to 0.90 (Figure 1). This indicates that PA measurement is of value in diagnosing clinical mastitis. At a threshold of 38 F/min, PA_60 had an Sp of 0.99 (95% CI = 0.97–1.00) and an Se of 0.58 (95% CI = 0.46–0.69). The finding that PA is higher in mastitic milk is in line with previous findings in experimental studies (Larsen et al., 2010; Hinz et al., 2012; Bikker et al., 2014), but to our knowledge, the current study is the first estimating diagnostic test characteristics of PA measurement. Rutten et al. (2013) summarized the diagnostic performance in terms of Se and Sp of other sensors for automated detection of mastitis that can be used in- or on-line. In comparison to these studies, PA measurement in milk samples that had been frozen performs worse than some sensors, but better than others. However, such comparisons are hampered by the fact that designs and analyses of diagnostic studies vary (Hogeveen et al., 2010). Moreover, many of these studies used multiple tests in combination or employed algorithms to increase test performance (e.g., Chagunda et al., 2006; Mollenhorst et al., 2010), complicating a direct comparison with the test performance of PA measurement. Comparison to EC and SCC within the current study would have allowed for comparing the test performance of PA measurement with EC and SCC, but this could not be performed because the clinical milk samples had been frozen, which affects SCC test results (Barkema et al., 1997) and likely also EC.

The majority of samples from clinical mastitis in our study yielded either *Streptococcus uberis* (34%) or *Escherichia coli* (21%), but a range of other pathogens were identified at lower frequencies. In 14% of the clinical cases, no pathogen was cultured. The samples from clinical mastitis were collected based on submission to the laboratory and were not a random selection from the population. Possibly, the clinical mastitis cases in our study may have been more severe than average cases in the population, and, thus, the difference between samples from healthy and diseased animals in our data

Table 1. Descriptive statistics for protease activity (fluorescence/min), SCC (cells/mL or $\log_{10}$ cells/mL), electrical conductivity (EC; mS/cm) measurements, and parity and DIM of quarter samples from clinically healthy and clinically mastitic cows used in this study[1]

	Clinically healthy quarter samples						Clinically mastitic quarter samples					
Item	n	Mean	SD	Median	Minimum	Maximum	n	Mean	SD	Median	Minimum	Maximum
PA_10 (frozen)	180	−4.88	23.87	−5.76	−78.78	68.21	71	98.88	115.05	61.46	−88.79	517.34
PA_10 (fresh)	180	−8.87	80.72	−9.33	−385.09	395.63						
PA_30 (frozen)	180	−2.13	15.28	−2.42	−45.15	64.74	71	72.81	73.02	50.48	−23.76	404.94
PA_30 (fresh)	180	0.57	63.14	−3.02	−156.7	405.60						
PA_60 (frozen)	180	2.10	11.77	2.26	−27.23	53.53	71	52.3	50.55	42.17	−12.13	351.82
PA_60 (fresh)	180	18.14	51.24	6.44	−95.45	37.86						
SCC (×1,000)	180	206	497	31	1	3,153						
$\log_{10}$ SCC	180	4.59	0.79	4.48	3.00	6.50						
EC	177	7.31	1.18	7.30	5.00	12.40						
Parity	169	2.64	1.70	2.00	1.00	9.00						
DIM	168	183	130	155	5	560						

[1]Protease activity (PA) was calculated over a read-out period of 10 min (PA_10), 30 min (PA_30), and 60 min (PA_60) in samples that were either processed immediately (fresh) or after having been frozen at −20°C (frozen). Comparisons of PA between clinically healthy and clinically mastitic cows using a student's t-test were significant in all 3 cases ($P < 0.001$).

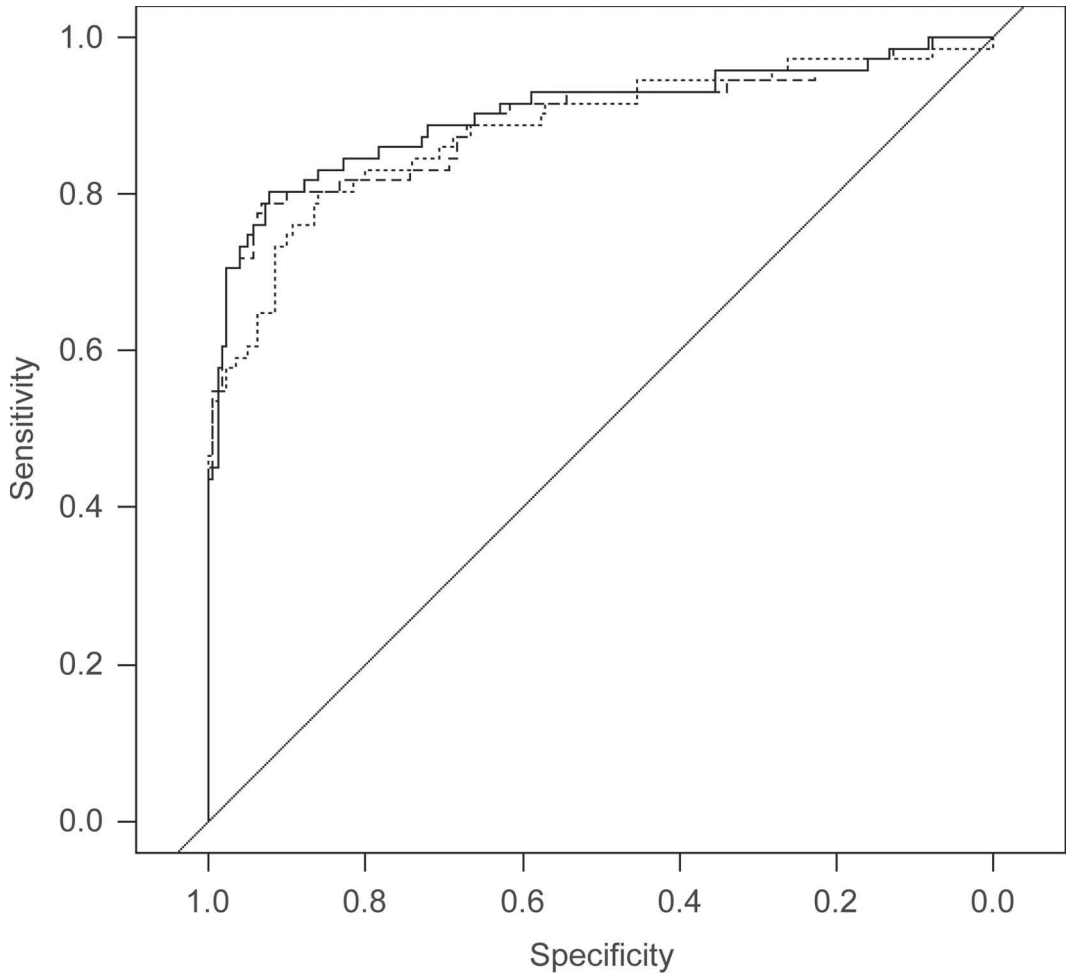

Figure 1. Receiver operating characteristic (ROC) curves showing the diagnostic value of PA in differentiating between samples from clinically healthy and clinically mastitic animals. Protease activity (fluorescence per minute) was calculated over a read-out period of 10 min (dotted line), 30 min (dashed line), and 60 min (solid line), corresponding to area under the ROC curve (95% CI) of 0.88 (0.83 to 0.94), 0.89 (0.84 to 0.95), and 0.90 (0.85 to 0.95), respectively.

set may have been overestimated. The selection of the healthy cows was not entirely random as well. Possibly, by sampling the first cows entering the milking parlor, we have selected for higher ranked and possibly healthier cows, again increasing the difference between healthy and diseased animals and therefore overestimating the test performance. Conversely, using smarter algorithms than just using a threshold for differentiating between healthy and affected quarters may further optimize test characteristics, for instance by comparing PA of quarters within an udder or by comparing PA measurements over time (Hogeveen et al., 2010). In general, studies that combine multiple sensors tend to perform better than studies that look at a single sensor (Hogeveen et al., 2010). Albenzio et al. (2012) showed higher PA levels for infected than for uninfected mammary glands within the same SCC range, suggesting that, although PA is positively associated with SCC, PA is also stimulated directly by udder pathogens. Therefore, PA measurement seems to target a different biological mechanism than SSC or EC, and combination of PA measurement with the existing tests may increase the overall test-performance. This hypothesis should be tested in follow-up studies that ideally also investigate to what extend PA measurement has added value over other mastitis tests, such as L-lactate dehydrogenase, N-acetyl-β-D-glucosaminidase, milk color measurement, and so on.

Associations between the 3 indicators of mastitis (SCC, EC, and PA) in milk samples from healthy quarters were moderately positive and statistically significant ($P < 0.01$), except for the association between PA_60 determined in fresh milk and EC, which was not significantly different from 0 (Table 2). This suggests that high PA is not only indicative of clinical mastitis, but may also have some value in differentiating subclin-

Table 2. Pearson correlation coefficient (*P*-value) between $\log_{10}$-transformed SCC, electrical conductivity (EC), and protease activity determined after a 60-min read-out period (PA_60) in fresh and in frozen milk samples

Item	10log SCC	EC	PA_60 (frozen)	PA_60 (fresh)
$\log_{10}$ SCC	—	0.38 (0.00)	0.31 (0.00)	0.24 (0.00)
EC		—	0.46 (0.00)	−0.04 (0.58)
PA_60 (frozen)			—	0.36 (0.00)
PA_60 (fresh)				—

ically infected from noninfected udders. Higher-parity cows had significantly higher PA levels, suggesting that older cows more often had subclinical mastitis, as older cows had higher SCC on average. Still, even after correcting for the effect of 10log-transformed SCC, the effect of parity on PA was significant (data not shown), which is in line with Bastian and Brown (1996). Associations of DIM and quarter location with PA (in fresh or frozen samples) were not significant ($P > 0.05$).

In our study, samples from clinical mastitis were frozen for logistic reasons. To make the samples from healthy animals comparable to the clinical samples, we froze aliquots of these samples and tested again for PA, allowing us to also study the effect of freezing milk samples on protease levels. Freezing significantly lowered the PA in milk (*P*-value of a paired *t*-test for PA_60 <0.001) and also introduced some bias or random error in the measurements, as can be seen from the fact that the correlation coefficient between PA_60 (fresh) and PA_60 (frozen) was only 0.36. If the effect of freezing the samples would have been limited to bringing the PA down with a fixed amount or a fixed percentage, the correlation coefficient between PA in fresh and in frozen samples would have been high. Our findings, however, suggest that other factors may interact with the effect of freezing, which may have had an effect on the test performance in our study. Although it seems counterintuitive that the error introduced by freezing would improve the test characteristics of the PA measurement, it is still unclear how the test would have performed had the freezing step been omitted. Therefore, future studies should perform PA measurements on fresh milk samples, because that is the kind of milk that will be used in practice. Ideally, longitudinal studies should be performed to gain insight in the development of PA during the onset of clinical mastitis, which will show how early PA measurement can detect a clinical case.

In conclusion, our study suggests that PA measurement may be an addition to the current panel of in- or on-line mastitis tests that can be used in automatic milking systems. Based on measurements in milk samples that were frozen, test characteristics of PA measurement are reasonable, although improvement can probably be made by making combinations with other tests and by the use of algorithms that incorporate other information available. In milk from healthy quarters, PA is positively associated with SCC, EC, and higher parity, but freezing significantly affects PA levels, which makes further study on the value of this test in fresh samples a necessity. Therefore, prospective longitudinal field studies are needed to properly show the value of PA as a diagnostic test for mastitis in fresh milk in combination with other tests available.

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