**Plasmin measurement as a new diagnostic test for mastitis in dairy cattle**

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**Abstract**

**Mastitis is a major economic issue in the farming of dairy cattle. It causes high economic losses due to veterinary costs, milk which can’t be used for consumption and due to decreased production and increased culling rates. Current tests like somatic cell count, electrical conduction and CMT-test are not always reliable. There are regularly false negative and false positive results so that there is need for new diagnostic tests. The objective of the present study was to determine test characteristics of plasmin substrates as a diagnostic test for clinical mastitis in dairy cattle. One quarter of 180 different cows was sampled by hand milking. The somatic cell count and electrical conductivity were determined for each sample. Then buffer, sampled milk coming from one cow and substrate are put together. This mix is incubated and after that the mingling is placed in the Fluostar for one hour. The plasmin, a protease, begins to cut the substrate what leads to the release of electrons what can be measured. A significant difference in increased fluorescence was measured between cows without and cow with mastitis. This was done by compare the increased fluorescence from samples of milk from apparently healthy cows with those with mastitis. The aera under the curve was 0,760. When looking at the threshold-value 14,86, a sensitivity of 81% and specificity of 61% are found. It was also seen that there is a correlation between plasmin concentration and somatic cell count (SCC) and a negative correlation between plasmin concentration and electric conductivy (EC).**

**Introduction**

Economic losses due to mastitis are high. It includes veterinary costs, decreased production and fertility, milk that must be discarded and sometimes death [1; 11]. There are currently several methods to detect mastitis amongst which electrical conductivity and somatic cell count are the most frequently used. However, these methods aren’t totally reliable. The specificity (SP) and sensitivity (SE) of these tests to detect clinical mastitis are very different using another time window. As longer the measurements are done, the more cases of mastitis are detected [12]. The SP and SE are also dependent of the chosen threshold. As higher the threshold, as high the SP and as lower the SE. This means that sometimes cows are declared negative while there are positive and vice versa. This is much more work for the farmer ti control the cows for which the alert was given. False negative and false positive results are problematic. Results should always be interpreted critically. The perfect test has a high sensitivity and high specificity, is easy to use, cheap and quick. Actually, it is important to detect mastitis in early stadia for example when using milking-robots. That’s something which works badly today with current tests and farmers still lose lots of money due to mastitis. So, a new or better detection method is wanted. It is known that during the inflammatory process of mastitis the blood milk barrier leak and is passable for some proteases. One of these proteases is plasmin which is involved during the fibrinolysis process. F.J. Bikker suggested that tailor made fluorogenic plasmin substrates could be used to diagnose mastitis in milk samples [4]. The reaction between the enzyme and substrate leads to a certain degree of fluorescence. On the basis of this fluorescence a distinction could be made between healthy milk samples and mastitis milk samples [4].

When there is mastitis in cows, the number of somatic cells (neutrophils and mononuclear cells) is much higher in milk [3; 8; 9]. Epithelial cells decrease in number and activity in the udder because of damage. This leads to a decreased milk production [10]. There are several methods to detect it, all with different values for sensitivity and specificity [12].

The CMT-test and gives only an indication on how high the somatic cell count in the milk is but not what the really amount. It is possible to detect a higher number of somatic cells in the milk by forming of a clump. As clinical mastitis could be seen at the aspect of the milk, this test should be used to confirm it or by subclinical mastitis. Therefore a few milliliters are needed. A bacteriological examination is then essential to know what type of bacteria is present to use an adequate antibiotic.

Milk recording is done in 80% of the farms in the Netherlands [3]. The somatic cell count is given for the farm but also for each cow. The SCC of a healthy quarters is, in general, lower than 50 000 cells/mL [3]. The DCC of DeLaval is an example of a device that may be used to detect clinical mastitis. This test gives help to detect a high quantity of leukocytes in the milk. Another plus point is that the farmer could buy it so he could detect quickly clinical mastitis. The costs to examine a sample at home with the DCC are about 5euro [3]. The disadvantage of this test is that it isn’t known where the elevated quantity of leukocytes comes from. A higher number of those cells isn’t always due to mastitis, but can be related for example to trauma. At only mildly elevated SCC value, the quarter which has a higher SCC can’t be found with the CMT-test.

Electrical conductivity (EC)

Ions and among others lactose determine the osmolarity of milk. When there is an inflammation, the blood milk barrier is damaged. The permeability of the capillaries is increased, the tight junctions are damaged and there is a decreased active transport. The concentration of ions in milk changes also: Na+ and Cl- come massively into the milk while K+ goes to the extracellular fluid. These changes cause an increase in conductivity but no change in osmolarity. The disadvantage of this method to detect mastitis is the variation in factors affecting the EC. Temperature and a longer interval between milking give an increasing in EC. A higher fat percentage is associated with a lower EC. There are also other factors such as estrus that influences EC. Tests to measure the EC aren’t good enough since there are many false positive alerts in automatic milking systems [12].Electrical conductivity has a sensitivity (SE) of 77,0% and a specificity (SP) of 69,0% with a time window of 1 day (Nielen et al., 1995 cited in [12]). When using a larger time window, the SE and SP increase considerably, respectively 100% and 99,8 when using a time window of 7 days [De Mol and Woldt, 2001 cited in [12]).

Plasmin is the principal protease in milk [5] and is also present in normal milk. The activity of this protease is higher in mastitic than in normal milk (Grieve & Kitchen, 1985; Saeman *et al.,* 1988 cited in [6]). It is firstly secreted as plasminogen in the blood. Then it is transported as plasminogen from blood into milk [5] where it’s activated during storage (Driessen & van der Waals, 1978; Alichanidis *et al.,* 1986 cited in [6]) or before milking when milk is held in the mammary lumen (Donnelly & Barry, 1983; Schaar, 1985 cited in [6]). It plays a role in fibrinolysis as dissolution of blood clots [6]. The increase in plasmin is associated with the migration of polymorphonuclear neutrophils from blood into the milk. This leads to the release of proteolytic enzymes which damage the blood-milk barrier [2].

There are several parameters that influence the plasmin concentration in the milk. The most important is to have a healthy cow. When compared with a cow suffering of mastitis, a significant difference in plasmin and plasminogen can be noticed. Plasmin and plasminogen concentrations increased respectively from 0,18 to 0,37mg/L and from 0,85 to 1,48mg/L when the somatic cell count increased from less than 250 000 tot more than 1 000 000 (Politis et al, 1989 cited in [6]. Other factors like stage of lactation, lactation number and breed also have an effect on concentrations of plasmin [4].

The tailor made substrate that is used has the following composition:

FITC-Ahx–*Amino acid 1–Amino acid* 2-KDbc, with lysine- serine or arginine-arginine as amino acids. Before use Pek054, other substrates were examined so that the best substrate could be used to test the samples.

The goal of this study is to look if plasmin could be used as parameter to detect clinical mastitis in dairy cattle.

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Materials and Methods

The bacteriological cultures were made by vets working at the Laboratory of University Farm Animal Practice. The cultures were done on sheep-blood agar-plate and on a MacConkey plate (to detect Gram negative bacteria). 24hour and 48hour later, the plates are examinated and a colonia is taken to have a pure culture of the pathogen.

Apparently healthy cows

Milk samples were collected at nine farms who voluntarily participated with the project. At all 9 farms, a milk sample from 20 apparently healthy cows was collected by hand milking during the morning milking (8 farms) and during evening milking (one farm). The choice was made to sample all the cows standing at one side of the milking parlour. A quarter from each cow was sampled according to the following scheme: right front, right hind, left front and left hind. The quarter was first cleaned by the farmer with a cloth. Then two or three squirts of milk were discarded and the about 10 mL of milk was collected in a sterile plastic vial. Gloves were used for a good hygiene, but no aseptic measures were taken.

When the twenty samples were taken, they were put in the cooler with ice packs. From the farms, there were directly driven to the Laboratory of University Farm Animal Practice in Harmelen to measure the SCC with the DCC DeLaval cell counter according to the manufacturer’s instruction. The tubes were swung two times before measurement.

After sampeling the cows on the farm, the further activities took place in Amsterdam at the ACTA (Academisch centrum tandheelkunde Amsterdam). The electrical conductivity was first measured with the milk checker. Then, each sample taken the same morning at the farm was aliquotted in two 1.5 mL Eppendorf tubes. Three frozen samples of cows with clinical mastitis were thawed and then put into two Eppendorf cups. When done, the 46 cups were put into the centrifuge for 60 minutes at 14.000x g. Then the cups were removed out the centrifuge and 100µL skimmed milk was taken in each cup and pipetted into the wells of the 96-well plate. Before that, 100µL buffer (Tris (Tromethamine) + gelatin) was pipetted in the wells. The mix of skimmed milk and buffer was incubated at 37 degrees C for 10 minutes and then 16µL of substrate Pek054 was added rapidly.The plate is immediately placed in the FLUOstar (fabrikant+land van herkomst toevoegen). Plates were read for one hour with 2min interval at 37°C on a fluorescence microplate reader (FLUOstar Galaxy, BMG Laboratories, Offenburg, Germany) with an excitation wavelength of 485nm and an emission wavelength of 530nm. Protease activity was defined in increased fluorescence per minute F/min). The experiments were performed in duplicates [4].

Mastitis samples

The mastitis samples were taken by the vets of the University Farm Animal Practice or brought by the farmer. The milk must be taken sterile. After that, a bacteriological examination of the milk is done to identify the pathogens present in it. Then the tubes with milk were put in the freezer. When these samples were needed, there were taken with ice-packs to bring them to the laboratory in Amsterdam.

The same process was followed as the samples coming from farms. They were defrosting in the original tube. Then two Eppendorf tubes were filled and they were put in the centrifuge for the same time as samples of healthy cows. Then the same mix of buffer, milk and substrate were made and the 96 well plate was put into the FLUOstar.

Statistical analyses

Statistical analyses were made using IBM SPSS Statistics for Windows version 20.0 (IBM Corp, Armonk NY, USA). P values<0,05 were considered statistically significant.

To see if there’s a significant difference between the compagnies in terms of somatic cell count, electrical conductivity and results of the FRET-technique, the Kruskal-Wallis test was used.

To compare the somatic cell count, the electrical conductivity and FRET-results mutually the Mann-Whitney test was used.

Specificity and sensitivity were calculated on the basis of a ROC-curve.

Results

In total, 180 samples of apparently healthy cows were taken in the farms and 123 frozen samples of cows with mastitis were examined. Mastitis of collected monsters was caused by different pathogens. 16 of the 123 samples were culture negative, 2 were caused by yeast and 2 samples were not examined. In the 103 other cases of mastitis, bacteria were cultured. E.*Coli* is one of the most frequent gram-negative bacteria which cause mastitis [1]. Table 1 shows which bacteria was the cause of clinical mastitis in the collected samples.

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| Bacteria | Amount | Percentage |
| Streptococcus spp.:* Strep. *uberis*
* Strep. *agalactiae*
* Strep*. dysgalactiae*
 | 383215 | 30,926,00,84,1 |
| Enterobacter spp.:* E. *coli*
* Klebsiella *pneumonia*
* E. *coli* andklebsiella *pneumoniae*
 | 312731 | 25,222,02,40,8 |
| Staphylococcus *aureus* | 7 | 5,7 |
| Coagulase negative staphylococcus | 11 | 8,9 |
| Truepella *pyogenes* | 1 | 0,8 |
| Enterococcus spp. | 2 | 1,6 |
| Mix of two bacteria:* Streptococcus spp. x staphylococcus spp.
* Enterobacter spp. x streptococcus spp.
* Enterobacter spp. x staphylococcus spp.
* Mixed flora or unknown bacteria
 | 136313 | 10,64,92,40,82,4 |
| Culture negative | 16 | 13,0 |
| yeast | 2 | 1,6 |
| Not examinated | 2 | 1,6 |

*Table 1: causes of clinical mastitis in the collected samples.*

We looked at the correlation between electrical conductivity and somatic cell count, somatic cell count and the values ​​obtained using the FRET technique (fluorescence resonance energy transfer) of the samples of the apparent healthy cows and between conductivity and values ​​of the FRET technique. A correlation between SCC and EC and between SCC and values obtained using the FRET technique was found. Between EC and values of the FRET, a negatieve correlation was found. The correlation coefficients and p-values can be found in table 1.

|  |  |  |
| --- | --- | --- |
|   | Rho  | p-value |
| FRET- electrical conductivity  | -0.236  | 0.002  |
| FRET- SCC  | 0.135  | 0.070  |
| SCC - electrical conductivity  | 0.345  | 0.000  |

*Table 1: correlation coefficients and P-values between FRET-EC, FRET-SCC and SCC-EC*

A significant difference in SCC, EC and FRET-values between companies is found. There is a negative correlation between EC and FRET-values. This is probably due to a farm-effect. That’s to say the farms al have a different somatic cell count quantity and so have different electrical conductivity results what has an effect on the results of the FRET-technique. The p-values can be found in table 2.

|  |  |  |  |
| --- | --- | --- | --- |
|  | SCC  | EC  | FRET  |
| p-value | 0,028  | 0,000  | 0,000  |

*Table 2: p-values of SCC, EC and FRET-values between companies*

The difference in the increased fluorescence of healthy cows and cows with mastitis using the FRET-technique is significant. This means that a cow with high SCC could be detected with this technique. By means of a ROC-curve, it is possible to find what the sensitivity and specificity of this test is. A sensitivity of 81% and specificity of 61% is found by the threshold valie 14,86. The aera under the curve is 0,760.



Discussion:

The goal of this study was to look if plasmin could be used as parameter to detect clinical mastitis in dairy cows. According the results, a significant difference in plasmin concentration has been measured between the group of healthy cows and cows with clinical mastitis.

Now this part of the study is have given results, there should be a follow-up study to look more in detail to some aspects. The effect of thawing on fresh samples of cows with mastitis should be investigated. Unfortunately this did not work in the scheduled time. It should be also useful to investigate whether the SCC correlated better with fresh or thawed samples. It might be an idea to centrifuge the samples and then remove the fat and sediment out of the Eppendorf cups and then to centrifuge again to obtain a pure skimmed milk. That to obtain precise results. Maybe the whole milk without centrifuging should be used to make the measurements.

A longitudinal study should be carried out so that cows can be followed up during a longer period of time. This means that multiple measurements can be done in cows suffering of mastitis en perhaps some healthy cows would develop a mastitis during the study. This gives a better picture of the relationship between SCC and whether or not mastitis and thus the concentration of plasmin.

Using a test designed to detect mastitis is in itself less reliable than to use a combination of several methods. When tests are combined specificity and sensitivity goes up. It might be an idea to use this study to use this test with for example the electrical conductivity which is already used in automatic milking systems. The question is then how reliable is EC? The ultimate goal is to have a test with a high specificity, but also a high sensitivity. This is because you want to detect all the cows that are really suffering of mastitis. A farmer does not want to get every time an alert coming from the automatic milking system while there is nothing going on.

As this is a model study with a unique sample per cow, it just gives an idea of research that could be done in the future. The results still represent a useful contribution to understanding what’s the relation of concentration of plasmin in the milk and presence or no of mastitis. Subclinical mastitis could be detected with this test and this test could be used in milk robots in farms. This study adds information to the knowledge we had in particularly that there is a significant difference in the plasmin concentration between healthy and cows with mastitis. Now a new study should examine and compare the plasmin concentration in fresh mastitis samples with the plasmin concentration in frozen mastitis samples to look if freezing affects or increase the plasmin concentration.

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