A screening method using Milk Amyloid A measurement in cow milk to significantly reduce the use of intramammary antibiotics at drying off

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Abstract

Milk Amyloid A (MAA) has been suggested in several studies as a biomarker of both clinical and subclinical mastitis. We conducted a study to evaluate the efficiency of the measurement of MAA by ELISA when used to make selective antimicrobial treatment decisions at mammary quarter level on cows at drying off. Mammary quarter milk samples from 112 cows, originating from low bulk tank somatic cell count (SCC) (<250,000 cells / mL) dairy herds and registered for drying off, were collected between two to seven days prior to drying off and once a week after calving, starting from two weeks until six weeks. All milk samples were cultured for bacterial detection and were analyzed for MAA concentration and for somatic cell count (SCC). Cow data and health events history were recorded. We performed a selective dry cow therapy at quarter level based on MAA results. The mammary quarters from cows with an MAA concentration ≥ 1 µg / mL (n=257) were treated with an intramammary antibiotic therapy and were infused with a teat sealant. The other quarters (MAA < 1 µg / mL) were only infused with a teat sealant and were not treated with an antibiotic therapy (n=94) or they were treated by antibiotic therapy and infused with a teat sealant (n=92). We developed an algorithm to identify an intramammary infection (IMI) at mammary quarter level at the end of lactation based on MAA and SCC results. The test characteristics of our screening method, called the MAA-Biotecklait method, were calculated and its negative (NPV) and positive (PPV) predictive values were estimated using bacterial culture as a gold standard. Our method was compared with the somatic cell count recording prior to drying off. The sensitivity and specificity of the MAA-Biotecklait method were both high, respectively 94.5% and 93.0% and the PPV and NPV when estimated in our study population were respectively 96.3% and 89.9%. The predictive values were both above 90% in populations where the proportion of udder quarters with an IMI at drying off ranged from 40% to 65%. By contrast, the sensitivity and the NPV of the SCC were low, respectively 68.7% and 62.8%. We concluded that the use of the MAA-Biotecklait screening method in a selective dry cow therapy programme at the quarter level would allow a significant reduction in the use of intramammary antibiotics at drying off with a low risk of missing infected udder quarters. In our study, its application would have reduced the use of antibiotic treatments by 29% and also would achieve the quantitative objectives of reducing antibiotic use in veterinary medicine by 25%, advocated by the French "ecoantibio 2017 plan". Moreover, we didn't observe a clinical mastitis six weeks after calving for quarters with a negative MAA-Biotecklait test that were not treated with antibiotics and were only infused with a teat sealant. These quarters have achieved success in the treatment and prevention of IMI over the dry period.

Keywords: Milk Amyloid A; Selective dry cow therapy; Reduction of antibiotic use; Algorithm; Intramammary infection.

Introduction

Worldwide, mastitis remains the most common disease of dairy cattle and the most economically important disease in bovine dairy production, with subclinical mastitis accounting for almost two-thirds of the economic loss (Seegers *et al*., 2003; Halasa *et al*., 2007). As part of mastitis control, most dairy producers in North America (USDA, 2008; Dufour *et al*., 2012) and in many European countries (Smith, 2014) treat all quarters of all cows with an intramammary antibiotic therapy at the end of lactation. This practice aims at reducing the prevalence of IMI, both by clearing up any existing IMI already present at drying off and by preventing new IMI from occurring during the dry period (Bradley and Green, 2001).

Antibiotic use creates a selective pressure on bacterial populations and contributes to the development of antimicrobial resistance (Tacconelli, 2009; Landers *et al*., 2012). In this context, organizations such as The Food and Drug Administration, recommend reduction in the use of antibiotics. In France, the "ecoantibio 2017 plan" advocates a cautious and rational antibiotic use. This plan is hinged around quantitative objectives of reducing antibiotic use in veterinary medicine by 25% in 5 years. To meet these recommendations and to achieve these quantitative objectives in dairy production, an alternative approach to treatment of all mammary quarters would be to target antimicrobial treatment only at infected mammary quarters at drying off. Therefore, udder quarters that are not suspected of having an IMI at drying off would enter the dry period without having received intramammary antibiotics. Only an internal teat sealant is administered to them.

In order to be successful, a selective dry cow therapy requires an easy, cost-effective and rapid method to identify cows with an IMI at the time of drying off (Sanford *et al*., 2006). The measurement of MAA by ELISA seems to be adapted for this purpose. MAA is suggested as a reliable biomarker of both clinical (Molenaar *et al*. 2009; Kovác *et al*., 2011; Pyörälä *et al*., 2011) and subclinical (Eckersall *et al*., 2006; Gerardi *et al*., 2009; Safi *et al*., 2009; Pyörälä *et al*., 2011) bovine mastitis. A specific ELISA kit for the measurement of MAA is commercially available. Its reliability when used in the diagnosis of subclinical mastitis in dairy cows was tested and was proved (Gerardi *et al*., 2009).

The objective of the study was to evaluate the efficiency of the measurement of MAA by ELISA when used to make selective antimicrobial treatment decisions at drying off at quarter level on cows originating from low bulk tank SCC. We developed a screening test (MAA-Biotecklait method) based on an algorithm to identify an IMI at quarter level at the end of lactation. Our algorithm has been developed based on MAA and SCC assay results carried out prior to drying off. The test characteristics and predictive values were determined using bacteriological culture as a reference method. Our method was compared with the SCC recorded just prior to drying off.

Materials and methods

Cows

Cows were selected from low bulk tank SCC (<250,000 cells / mL) dairy herds in the year prior to the trial. They were required to present with an apparent good health status, with no evidence of clinical mastitis, no intramammary anti-biotherapy and no systemic anti-inflammatory treatment within the last three weeks prior to drying off. Cows were also required to be pregnant in order to calve once again. A total of 112 Prim 'Holstein cows from 6 herds in 4 different locations in France were selected for the testing and were enrolled at drying off. All cow data and health events history of each cow were recorded.

Milk analysis and selective antibiotic treatment at drying off

Individual quarter milk samples of each cow selected for the testing were collected between two to seven days prior to drying off and once a week after calving, starting from two weeks until six weeks. The milk samples were collected aseptically in duplicate according to the procedures recommended by the Laboratory Handbook on Bovine Mastitis (National Mastitis Council, 1999). Immediately after sampling, one replicate of

each quarter milk sample was frozen to -20°C before submission to the laboratory for standard bacteriological culture and species identification. The other quarter milk sample replicates were stored at 4°C and were sent to a lab for SCC and MAA analyses. Milk analysis for MAA and for SCC was performed by a dairy laboratory that contributes to a French DHI programme (Oxygen Laboratoires d'Analyses, Maroeuil, France). The concentration of MAA was determined using a commercial ELISA kit (Milk Amyloid A-MAA Assay Kit, cat. no. TP-807; Tridelta Development Ltd, Maynooth, Ireland) in accordance with the manufacturer's recommendations. SCC was assessed by fluoro-opto-electronic cell counting (Somacount FCM; Bentley Instruments, Maroeuil, France). The mammary quarters were treated at drying off according to their MAA concentration results. A MAA cut-off value of 1 µg / mL was used. This value is in agreement with the study of Åkerstedt *et al*. 2011 in which MAA was measured below 1 µg / mL in the clinically healthy quarters. Earlier studies suggested cut-off values between 0·8 and 1·4 µg / mL to indicate an inflammatory response in quarters and in cow composite milk samples (Grönlund *et al*. 2003; Jacobsen *et al*. 2005; O´Mahony *et al*. 2006). The quarters with an MAA concentration ≥ 1 µg / mL (n=257) were treated with an antibiotic therapy and were infused with a teat sealant. The other quarters (MAA < 1 µg / mL) were only infused with a teat sealant and were not treated with an antibiotic therapy (n=94) or they were treated with an antibiotic therapy and infused with a teat sealant (n=92).

Bacteriological culture and IMI definitions

Bacteriological culture and species identification were performed by a COFRAC accredited laboratory (LABÉO Manche, Saint-Lô, France). The milk samples were cultured using standardized protocols based on the NMC guidelines (Hogan *et al*., 1999). A mammary quarter was defined as having an IMI if ≥ 100 cfu / mL of milk of any pathogen was cultured as either pure or mixed growth, except coagulase negative staphylococci (CNS). For CNS, a definition ≥ 200 cfu / mL was used. These definitions are in accordance with the recent publication of characterization of IMI based on single sample bacteriological testing (Dohoo *et al*., 2011). A sample from which three or more different species were cultured was classified as contaminated.

Statistical analysis

An algorithm has been developed to identify an IMI at quarter level at the end of lactation. It is based on MAA and SCC assay results of the quarter milk samples collected from the 112 cows selected in this study (MAA-Biotecklait Method). Further details are not given for reasons of commercial sensitivity. The characteristics of the MAA-Biotecklait method and of the SCC method were calculated using 2 × 2 contingency tables and by using bacteriological culture as a reference method. Sensitivity was defined as the proportion of quarters with an IMI that were classified as positive with a screening method. Conversely, specificity was defined as the proportion of mammary quarters without an IMI that were classified as negative with a screening test. The PPV was the proportion of udder quarters with positive screening test results that truly had an IMI. The NPV was the proportion of quarters with negative screening test results that did not have an IMI. A threshold of 100,000 cells / mL for primiparous cows or of 150,000 cells / mL for multiparous cows was used for the calculation of sensitivity and specificity of the SCC method. The performance of the MAA-Biotecklait method in different cow populations with varying degrees of infected mammary quarters was examined. The predictive values were calculated for prevalence estimated ranging from 1 to 100% and using the formulas based on Bayes' theorem.

Results

Bacteriological culture

The distribution of pathogens isolated in quarter milk samples is presented in table 1. The environmental streptococci were grouped together and encompassed *Aerococcus viridans* (n=10), *Enterococcus faecalis* (n=3), *Lactococcus gravieae* (n=4), *Micrococcus* sp. (n=1), *Streptococcus dysgalactiae* (n=2) and *Streptococcus uberis* (n=3). Gram-negative bacteria were also amalgamated into a grouping, and included *Acinetobacter* sp. and *Acinetobacter baumannii*. The most frequently isolated pathogens at the end of lactation were minor pathogens, in descending order, CNS, followed by environmental Gram-positive pathogens *Corynebacterium* spp. and environmental streptococci, predominantly *Aerococcus viridans*. Two bacteria species were isolated in some udder quarter samples (12%), with CNS constantly present. The percentage of contaminated samples and of samples lost was respectively 29.3% and 14.5%. According to our extrapolated bacteriological culture results, the prevalence of an infected quarter prior to drying off in our cow population was 65.5%.

Table 1. Distribution of pathogens isolated in quarter milk samples collected between two to seven days prior to drying off. Results are presented as the number quarter and as a percentage of total quarter infected or not with an organism. The number of analyzed bacteriological culture results was n=249.

Pathogens	Number of quarters	Percentage of total quarters
No growth	86	34.5
Coagulase negative staphylococci (CNS)	78	31.4
Corynebacterium spp. (C. spp.)	27	10.9
Streptococci[a] (Strep.)	23	9.2
CNS + C. spp.	20	8.0
CNS + Strep[b]	10	4.0
Coliform	0	0
Gram-negatives[c]	2	0.8
Strep. agalactiae	3	1.2
Contaminated	130	-
No sample	64	-

[a]Streptococci include: *Aerococcus viridans, Enterococcus faecalis, Lactococcus gravieae, Micrococcus sp., Streptococcus dysgalactiae and Streptococcus uberis.*
[b]Strep. encompass *Aerococcus viridans, Streptococcus uberis and Lactococcus gravieae.*
[c]*Acinetobacter sp.* and *Acinetobacter baumannii*

Test characteristics and predictive values of the screening methods

The characteristics of the MAA-Biotecklait and SCC screening methods to identify an IMI at mammary quarter level were calculated by using bacteriological culture as a reference method. The predictive values of the methods were estimated considering only prevalence of IMI in our studied population (65.5%) (Figure 1).

The sensitivity and the specificity of the MAA-Biotecklait method were both high respectively 94.5% and 93.0%. The estimation of the PPV was 96.3% and that of the NPV was 89.9%. By contrast, a high specificity (97.7%) was obtained using SCC method whereas the sensitivity was low (68.7%). The calculated PPV and NPV for this method were respectively 98.2% and 62.8%.

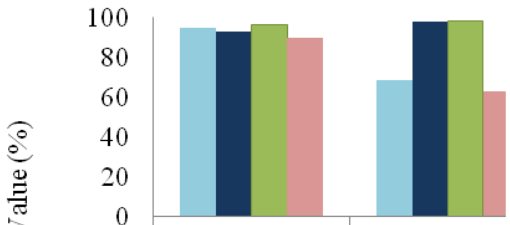
The performance of the MAA-Biotecklait method in different cow populations, with varying degrees of infected quarters, was more specifically examined. The predictive values were calculated for prevalence estimation ranging from 1 to 100% (Figure 2).

The PPV and NPV were both high, above 90%, in populations where the proportion of udder quarters with an IMI at drying off ranged from 40% to 65%. These were also maintained at high enough levels (> 85%) in populations where the prevalence at a quarter level of an IMI ranged from 30% to 75%.

Discussion

The aim of a selective treatment at a mammary quarter level is to reserve intramammary antibiotic therapy for quarters that are suspected of having an IMI at drying off. No antibiotic is administrated to quarters presumed not to have a subclinical mastitis at the end of lactation. An internal teat sealant (ITS) is only applied to protect them against an infection during the non-lactating period. This practice would contribute to reduce antibiotic use in dairy production. Therefore, such a treatment programme requires a screening method to identify the health status of udder quarters at drying off. An ideal method would have maximum sensitivity and specificity to minimize the proportion of false-negative and of false positive results (Dingwell *et al.*, 2003; Midleton *et al.*, 2004; Sanford *et al.*, 2006). Therefore, the treatment of infected quarters and reduction of antibiotic use for healthy quarters would be magnified. Our results indicate that the screening test to detect an IMI at drying off using SCC at a threshold value of 100,000 cells / mL for primiparous cows or of 150,000 cells / mL has low sensitivity. The use of this method as part of a selective programme at a mammary quarter level at the end of lactation thus seems unsuitable. In our cow population, more than 30% of infected quarters would be missed and not treated. In contrast, our results show that when used to detect an infected quarter at drying off, the MAA-Biotecklait screening method had high sensitivity (94.5%) and high specificity (93.0%). As a result, this method could be used to make selective dry cow treatment decisions with the confidence that very few infected udder quarters would be missed and that high proportion of the healthy mammary quarters would be not treated with antibiotic therapy. They would be only infused with a teat sealant.

In our study, the most frequently isolated pathogens in mammary quarters were coagulase negative staphylococci. The prevalence of this group of species in our total mammary quarter population was 43.4%. Similar results of prevalence were reported in earlier studies. In a German study, 35% of quarters with subclinical mastitis harbored CNS (Tenhagen *et al.*, 2006). The highest prevalence of intramammary infections with CNS was reported in Finland, where CNS were isolated from 50% of the quarters positive for bacterial growth in a nationwide survey (Pitkälä *et al.*, 2004). The effect on somatic cell



	MAA-Biotecklait Test	SCC Test
Sensitivity	94,5	68,7
Specificity	93,0	97,7
PPV	96,3	98,2
NPV	89,9	62,8

Figure 1. Test characteristics, positive (PPV) and negative (NPV) predictive values of MAA-Biotecklait and SCC methods to identify an IMI at mammary quarter level at the end of lactation in cow population originated from herds with a low bulk tank SCC (<250,000 cells/mL). A threshold of 100,000 cells/mL for primiparous cows or of 150,000 cells/mL for multiparous cows was used for SCC method.

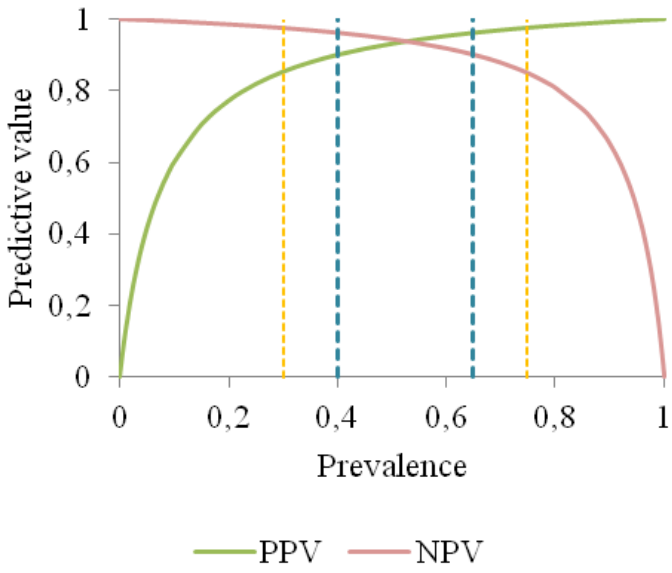


Figure 2. Positive predictive values (PPV) and negative predictive values (NPV) of the MAA-Biotecklait screening method when used to diagnose an IMI at mammary quarter level at drying off in cows from herds with low bulk tank SCC (<250,000 cells/mL) and for prevalence of IMI ranging from 0 to 100%. The vertical blue dashed lines indicate the prevalence range (40-65%) for which the PPV and the NPV both are above 90%. The yellow lines indicate the prevalence range (30-75%) for which the PPV and the NPV both are above 85%.

count is accepted to be generally limited or nonexistent for CNS as a group (Vanderhaeghen *et al.*, 2014). This nonexistent effect would explain the low sensitivity obtained in our study for the SCC method. In contrast, our results indicate that the sensitivity of the MAA-Biotecklait method was high. This method is based on an algorithm developed from SCC level and from the MAA concentration in milk. Therefore, taking into account the MAA concentration in milk seems to enable us to detect mammary quarters infected by CNS at the end of lactation. The possible use of MAA concentration levels in milk samples to detect mammary quarters infected by coagulase-negative staphylococci has been previously described (Pyörälä *et al.*, 2011).

The predictive values of a positive or negative MAA-Biotecklait test across cow populations with varying levels of infected udder quarters at drying off were calculated from the test characteristics of the MAA-Biotecklait method. As illustrated in Figure 2, for a prevalence of infected quarters ranging from 40% to 65%, the PPV and the NPV were both high, above 90%. Then, the proportion of udder quarters truly infected with a negative test (1 - NPV < 10%) that would not receive an intramammary antibiotic treatment would be small in herds where the prevalence is ranged from 40% to 65%. Moreover, in herds with this prevalence range, few of udder quarters truly healthy with a positive test sentence (1 - PPV < 10%) would be treated with an antibiotic therapy. Similar but more moderated results were obtained for a prevalence ranging from 30% to 75%. Consequently, the use of the MAA-Biotecklait method to make selective treatment decisions at udder quarter level at drying off would allow the significant reduction of antibiotic use on dairy farms, with a low risk of missing infected udder quarters. In our study, its application would have reduced the use of antibiotic treatments by 29%. The use of the MAA-Biotecklait method at drying off will allow us to achieve the quantitative objectives of reducing antibiotics in veterinary medicine by 25% advocated by the French "ecoantibio 2017 plan". In parallel, the dairy producer will obtain cost savings regarding the management of herds.

Conclusions and perspectives

The MAA-Biotecklait screening method, based on an algorithm developed from SCC and MAA assay results of quarter milk samples has high sensitivity and specificity, high positive and negative predictive values, when used to diagnose an infected cow mammary quarter at drying off. Its application in a selective dry cow therapy programme at the mammary quarter level would allow a significant reduction in the use of intramammary antibiotics at drying off with a low risk of missing infected udder quarters. Moreover, our first results at calving show no clinical mastitis six weeks after calving for quarters with a negative MAA-Biotecklait test that were not treated with antibiotics and were only infused with a teat sealant. These quarters have achieved success in the treatment and prevention of IMI over the dry period.

We plan to reinforce our algorithm by the addition of thousands of MAA and SCC assays to the database that is used to support it. We are launching a cost-effective service at drying off for dairy farmers based on our MAA-Biotecklait method from September 2015. We use a simple and high throughput laboratory ELISA assay. The MAA assay and the access to our algorithm are available in France from our group Biotecklait and worldwide from Tridelta Development Ltd. We also intend to launch other services for the dairy farmers based on our algorithm.

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