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Development of a flow cytometric immunoassay for recombinant bovine somatotropin-induced antibodies in serum of dairy cows

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Administration of recombinant bovine somatotropin (rbST) to enhance milk production in dairy cows is banned within the European Union. Therefore, methods for pinpointing rbST abuse are required. Due to the problematic detection of rbST itself in serum, methods are also focused on detecting changes in rbST-related biomarkers. In this study, a fast and easy-to-perform microsphere-based flow cytometric immunoassay (FCIA) for detection of rbST-induced antibodies in serum was developed. Until now, detection of rbST-induced antibodies was also problematic due to non-specific binding of serum proteins resulting in a high rate of false positive results. Therefore, five different sample preparation methods, i.e. dilution, octanoic acid precipitation, filtration, protein G purification, and a previously described generic FCIA sample preparation were critically compared to overcome non-specific binding to the microspheres. Only the generic FCIA sample pretreatment was effective in reducing non-specific binding. As a result, an absolute decision level for detecting rbST antibodies in serum of dairy cows was determined and its applicability was demonstrated. In accordance with biological expectations from literature, rbST antibodies were induced in three out of four rbST-treated dairy cows. These rbST-induced antibodies were successfully detected for up to 4 weeks after the last rbST treatment, whereas no false positive results were obtained for 27 untreated dairy cows. This is the first method, able to overcome the interference of serum proteins and therefore, can be applied with high confidence for screening unknown herds of cattle for rbST antibodies, an important biomarker for pinpointing at rbST abuse in cattle.

Supporting information may be found in the online version of this article.

Keywords: bovine somatotropin; specific antibodies; biomarker; flow cytometry; growth hormone

Introduction

The administration of recombinant bovine somatotropin (rbST) to enhance milk production in livestock[1,2] is banned within the European Union (EU).[3,4] However, due to the widespread application in several other countries, for example, the USA, Brazil, and South Africa, the illegal use within the EU cannot be excluded. Therefore, methods are required for the detection of rbST abuse. Direct detection of rbST, however, is problematic due to its short half-life in blood, the similarity with the endogenous hormone (bST, also called growth hormone), the low concentrations of bST and rbST in serum, and strong fluctuations therein. Preliminary results regarding the development of a confirmation method for rbST detection based on liquid chromatography-mass spectrometry (LC-MS) show potential.[5] But that method is rather laborious, time-consuming, and requires highly trained personnel and expensive equipment. Moreover, the time window for rbST detection is inherently short due to its short half-life. Research is, therefore, also focused on the development of relatively fast and easy-to-perform screening assays for detection of changes in concentration of rbST-dependent biomarkers having a longer half-life. The following biomarkers are considered as being indicative for (illegal) administration of rbST: rbST-induced antibodies, insulin-like growth factor-1 (IGF-1) and its binding proteins IGFBP2 and 3 and several markers of bone and collagen turnover.[6–9] So far, none of these individual biomarkers have been found reliable for predicting rbST abuse on its own. However, a combination of biomarkers has been suggested for cost-effective screening of dairy herds, similar to the detection of biomarkers for steroids abuse.[10–13] For detection of anti-rbST antibodies in serum, immunoassays on different platforms like radio immunoassay (RIA),[14] ELISA,[15,16] and Western blot[17] have been described.

The aim of this study was to develop an flow cytometric immunoassay to screen herds of dairy cattle for the presence of rbST-induced antibodies. Antibodies are important biomarkers due to their long half-life and specificity.[15,18–20] Although the formation of antibodies to a virtually endogenous protein – only one out of 191 amino acids on the N-terminal end is replaced[21–23] – seems surprising at first, it was observed in approximately 70 out of 90

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rbST-treated cows using an enzyme-linked immunosorbent assay (ELISA).[14,15] During human growth hormone treatment, no antibody formation was observed in adults;[24] however in children, antibody formation was observed in up to 68% of the target group.[25] But the anti-rbST antibody titers were low.[15,19] To be able to use the anti-rbST antibodies as a biomarker in a screening assay, the low antibody concentrations must generate responses that significantly differ from background responses of untreated dairy cows. Until now, high variations in responses in sera of untreated dairy cows as a result of non-specific binding of serum proteins were observed, resulting in a high number of false positive results.[14–17] Moreover, high variations in responses of untreated cows lead to a high decision level for discriminating between treated and untreated cows and consequently also to a high number of false-negative results. So far, in literature, anti-rbST antibodies were monitored by Zwickl *et al.*, comparing the median response of a treated group with a group of untreated cows to avoid the false positive rate of 9%, as 1 out of 11 cows displayed a false positive response.[15] Eppard *et al.* compared a serum sample before and after treatment for each individual cow, thereby only presenting relative responses to treatment to deduct high inter-individual differences between cows.[14] Pinel *et al.* pointed out that because of the high risk of false positive results in their immunoassay, monitoring anti-rbST antibodies should be coupled to a confirmatory technique.[23] Surprisingly, Rochereau-Roulet *et al.* claimed a low false positive rate of 5.5%; however, according to the results presented, 1 out of 9 cows displayed a false positive result yielding a false positive rate of 11.1%.[16] De facto, a method that can be used to screen unknown herds of dairy cattle for the presence of anti-rbST antibodies in real practice is not yet available. To improve specificity and sensitivity of immunoassays an important step is the removal of interfering proteins from the matrix. This can be achieved by different techniques, for example, precipitation,[14,18,26] size exclusion,[27] and affinity purification.[28] Specificity and sensitivity can also be improved by disrupting non-specific binding using detergents and a pH change.[29] In this study, all five different sample preparation methods were critically compared for their ability to reduce non-specific binding. Moreover, we developed a flow cytometric method having multiplex capability – using the multi-analyte profiling (xMAP™) technology[30] – with an effective reduction of non-specific binding for the detection of anti-rbST antibodies in cow serum. Its applicability for screening unknown herds of dairy cattle for rbST antibodies is demonstrated.

Experimental

Materials and instruments

Monsanto rbST standard was obtained from the National Hormone & Peptide Program (NHPP) of Dr Parlow (Torrance, CA, USA). Posilac® 500 mg single-dose syringes and syringes with only the slow-release formulae were purchased from Monsanto company (St Louis, MO, USA). Octanoic acid, hydrochloric acid, potassium phosphate, sodium azide, sodium chloride, sodium hydroxide, sodium phosphate, Tween-20 and the ultrasonic cleaner were purchased from VWR International (Amsterdam, the Netherlands) and glycine was from Duchefa (Haarlem, the Netherlands). N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) was supplied by Fluka (Steinheim, Switzerland) and sodium dodecyl sulfate (SDS) by Serva (Heidelberg, Germany). Bovine serum albumine (BSA), tris(hydroxymethyl)aminomethane, 2-(N-morpholino)ethanesulfonic acid (MES hydrate) and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich Chemie (Zwijndrecht, the Netherlands). R-Phycoerythrin (PE)-labelled goat anti-bovine immunoglobulins (GAB-PE) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). MultiScreen HTS filterplates and microcon centrifugal filter units were purchased from Millipore (Amsterdam, the Netherlands). Protein Lobind Tubes (1.5 ml) and a table centrifuge model 5810R were supplied by Eppendorf (Hamburg, Germany). Protein HP SpinTrap columns were purchased from GE Healthcare (Buckinghamshire, UK). The Luminex 100 IS 2.2 system consisting of a Luminex 100 analyzer and a Luminex XY Platform, which was programmed to analyze a 96-well plate, was purchased from Applied Cytometry Systems (ACS, Dinnington, Sheffield, South Yorkshire, UK). SeroMAP microspheres (bead set 050) and sheath fluid were purchased from Luminex (Austin, TX, USA). The Snijder test tube rotator was purchased from Omnilabo International (Breda, the Netherlands). The microtiter vari-shaker was purchased from Dynatech (Guernsey, UK).

Sample materials

Eight 5-year-old Holstein dairy cows (a-h) were divided into two groups. After two weeks' adaptation, treatment consisted of subcutaneous injections of 500 mg rbST from Posilac® in a slow-release formulae for the first group (e*-h*) and the slow-release formulae only for the second group (a*-d*). The cows were injected with a two-week interval four times and subsequently twice with a one-week interval. During the two-week adaptation period, blood samples were collected weekly. During the treatment period, blood samples were collected shortly before injection, a day after and a week after injection and after the last injection blood samples were collected weekly for four more weeks. The experimental procedure was authorized by the ethical committee of the Faculty of Veterinary Medicine, Ghent University.

Furthermore, blood samples were taken from 20 calves younger than 26 weeks of age and 20 healthy, lactating cows (1–20) varying in the age of 2–5 years old, in different stages of their lactating cycle, to reflect a normal population of untreated animals. Based on the origin of the calves and cows the assumption of being untreated with rbST was justified.

After blood collection, all blood samples were placed at room temperature for 4 h to coagulate. After coagulation, samples were centrifuged for 10 min at 3 000 g, and sera were collected and stored at −80 °C until further use.

Pretreatment of serum samples

Five different techniques were used for sample pretreatment, i.e. dilution, octanoic acid precipitation, protein G affinity purification, filtration with 100 kDa cut-off, and a generic FCIA sample preparation procedure. Octanoic acid precipitation was performed according to Bailly-Chouriberry *et al.*[18] Prior to FCIA samples were diluted 50 times in PBST. For protein G affinity purification, sera were diluted 20 times in PBS and treated according to the manufacturer's protocol. After concentration by affinity purification, samples were diluted in PBST to a 70-time final dilution. For filtration with a 100 kDa filter, samples were diluted 100 times in PBST and treated according to the manufacturer's protocol. The retentate was collected and volumes were adjusted to the initial 100 times dilution. For the generic FCIA sample preparation procedure,[31] serum samples were pretreated by adding 25 µl glycine solution (27.5 mM glycine pH 0.5 (pH adjusted by addition

of HCl)) to 25 μl of serum sample or standard solution in a polypropylene tube under constant vortexing. Samples were then incubated at room temperature for 60 min. After incubation, 50 μl glycine-SDS solution (400 mM Glycine, 0.3% m/v SDS, pH 10 (pH adjusted by addition of NaOH)) was added under constant vortexing. Samples were further diluted to a final dilution of 80 times with 0.1% BSA in PBST.

Bead preparation for the Flow Cytometric Immuno Assay (FCIA)

rbST of Monsanto was coupled to seroMAP bead set 050 according to Bremer *et al.*[31] Briefly, 2.5 x 10^6 beads were coupled using 500 μl of a 100 μg ml^{-1} rbST protein solution in MES buffer. After coupling, the beads were stored in blocking buffer (PBS, 0.1 % BSA, 0.02 % Tween-20 and 0.05 % NaN_3) at 2–8 °C in the dark until use. Under these storing conditions, beads were stable for more than one year.

Direct FCIA

From the diluted crude and pretreated serum samples, 100 μl was added to a filter bottom microtiterplate. Then, beads (10 μl diluted suspension containing about 1250 beads) were added to each well and incubated for 1 h on a microtiterplate shaker. After incubation, the plate was centrifuged (1 min 130 g) and the beads were washed with 200 μl PBST. After washing, 125 μl of a 100 times diluted GAB-PE solution was added and incubated for 30 min on a microtiterplate shaker. After this second incubation step, the plate was centrifuged and 125 μl of PBST was added per well. Then, the beads were detected in the flow cytometer (1 μl s^{-1} was measured until 100 events were reached with a maximum of 50 μl $well^{-1}$).

Inhibition FCIA

The inhibition FCIA was similar to the direct FCIA with the exception that after 100 μl of the pretreated serum sample was added to a filter bottom microtiterplate, 10 μl of PBST or rbST diluted in PBST was added. This mixture was then pre-incubated for 15 min on a microtiterplate shaker and beads were added. Further handling is similar to the direct FCIA.

Statistics

For decision level determination, sera from 27 untreated dairy cows (20 untreated animals and 7 from the animal experiment during their adaption period) were measured in the direct FCIA. The decision level was defined as the average response of these sera, plus two times standard deviation ($p < 0.05$). Accordingly, results were reported with a statistical significance of 95% confidence, suitable for screening assays. Responses from FCIA analyses carried out within the same day were compared with this absolute decision level. However, due to daily variations in assay performance and technical performance of the Luminex 100 IS 2.2 system, such a comparison couldn't be made between days. Therefore, a normalized in between day decision level was determined using the ratio of serum f* to the absolute decision level. No other validation parameters were tested because of the lack of a pure standard of rbST specific antibodies.

Results and discussion

Detection of antibodies specific for rbST in crude serum

To assess the presence of rbST-specific antibodies, first a decision level based on non-treated animals needs to be established. As a start, crude serum samples of 20 untreated calves and 27 untreated dairy cows (20 untreated animals and 7 from the animal experiment during their adaption period) were measured with the direct FCIA. An average response of 194 ± 99 mean fluorescence intensity (MFI) was calculated for 50 times in PBST diluted calves sera. For the adult dairy cow sera an average response of 1150 ± 792 MFI was calculated even after a 100 times dilution in PBST. In other words, although adult dairy cow sera were diluted a factor of two more, the average response determined was six times higher than for calves. This result clearly shows the influence of (pre)puberty on the analysis results and therefore a realistic control group for assessing the presence of rbST-specific antibodies is a prerequisite. In Figure 1, the results for the 27 untreated control adult dairy cows are displayed in more detail.

The individual dairy cows clearly show large differences in responses, as the coefficient of variation is 69% in MFIs. To distinguish treated from untreated dairy cows a decision level, defined as the average response of the crude sera from untreated dairy cows

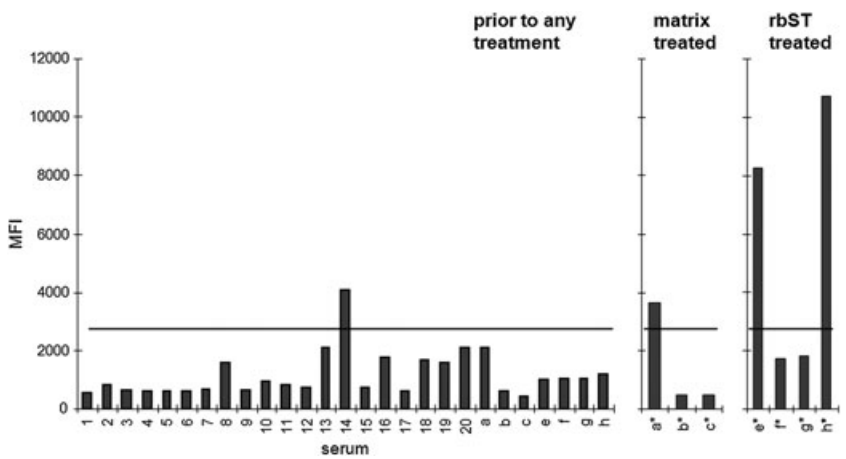

Figure 1. FCIA responses (bars) of 100 times PBST diluted serum samples of 27 untreated adult dairy cows (1–20; a-h), serum samples collected a week after the treatment period of 3 matrix only (a*, b*, c*) treated; and 4 rbST (e*,f*,g*,h*) treated cows and their corresponding decision level (line).

plus two times standard deviation (2SD) was set to 2734 MFI. Although this decision level was over two times the average response, serum 14 showed a response higher than the decision level and therefore was depicted as a false positive sample as shown in Figure 1.

To assess the suitability of this decision level, sera collected one week after the treatment period, from four dairy cows (e*, f*, g*, h*) treated with rbST and three control dairy cows (a*, b*, c*) treated with matrix only were analyzed (unfortunately, one control animal, denoted d, died during the experiment). The obtained responses were compared with the decision level as shown in Figure 1. Only two out of the four rbST-treated animals displayed responses over the decision level, implying presence of rbST-induced antibodies. In other words, 50% of the treated dairy cows were incorrectly classified as negative. Furthermore, the response of one control dairy cow resulted in a second false positive classification. These results clearly show that discriminating between treated and untreated dairy cows using just a dilution of crude serum without further sample preparation is unreliable due to non-specific binding.

Comparison of sample pretreatment for reducing non-specific binding

High inter-individual differences in anti-rbST response and false positive results when analyzing crude sera were described before[14,15,17] and different explanations were put forward. First, it was suggested that the control animals used in the experiments could have been treated with rbST in the past and therefore revealed an immunological memory effect.[17] Second, highly abundant proteins in sera might lead to strong non-specific binding and third, specific proteins, such as, for sample, growth hormone binding protein (GHBP), might generate specific but undesired binding.[17] In this study all these possible explanations were investigated. Based on the origin of the dairy cows the assumption of being untreated with rbST was justified. Non-specific binding can be reduced by lowering the protein content of serum samples. For this several methods are described; for example, octanoic acid precipitation[18] and protein G affinity purification.[28] Furthermore, to ensure GHBP (a 70 kDa protein) removal, serum can be passed over a filter with a 100 kDa cut-off to retain the 150 kDa anti-rbST in the retentate. Besides the above mentioned methods, also a recently developed generic FCIA sample preparation procedure[31] which disrupts non-specific binding was tested. For testing these different sample pretreatments, six serum samples were selected based on their responses obtained with the crude sera in the FCIA; two serum samples from rbST-treated dairy cows, one positive (e*) and one false-negative (f*), two blank serum samples (3, 4) and two blank serum samples with high responses including one false positive sample (14, 18). After the different sample pretreatments, i.e. octanoic acid precipitation, protein G purification, filtration, and the generic FCIA sample preparation, responses were determined in the FCIA. Although dilution differences were minor, large differences in absolute response were measured, respectively 3236, 711, 886, and 180 MFI for the different sample pretreatments of serum 18. Therefore, to simplify the visual comparison of the methods, the responses were normalized to serum 3 for each sample pretreatment method, and the results are shown in Figure 2. Discrimination between positive and negative serum samples was not possible using dilution of crude serum, octanoic acid precipitation, protein G purification, and filtration. For all these methods, one or both of the high-responsive negative serum samples (14, 18), still gave responses higher than the positive serum sample f*.

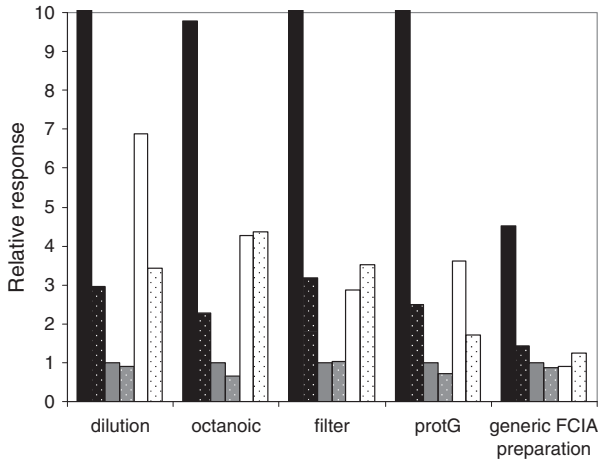

Figure 2. Effect of different sample treatments, PBST dilution, octanoic acid precipitation, 100 kD filtration, protein G purification and generic FCIA sample preparation on cow sera collected one week after the rbST treatment period e* ( ) and f* ( ), untreated cows with low responses 3( ), 4( ), and untreated cows with high responses 14( ) and 18( ); all responses are normalized to serum 3.

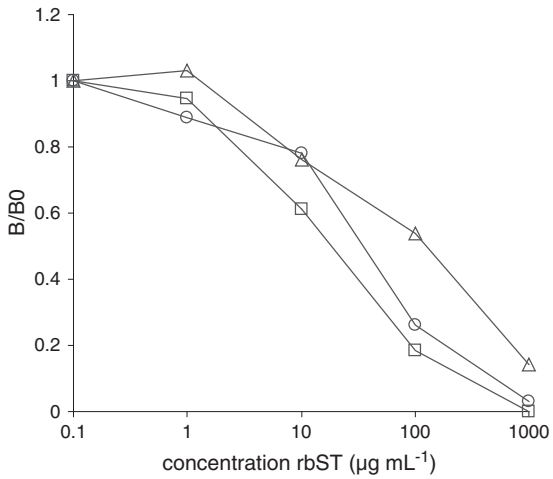

Figure 3. Inhibition curves obtained with rbST in the inhibition FCIA using serum samples of rbST treated cows tested positive for rbST induced antibodies e* (○), g* (Δ) and h* (□) after the generic FCIA sample preparation and average background response correction.

Furthermore, comparing the four negative serum samples (3, 4, 14, and 18) extreme inter-individual differences in responses were displayed, resulting in a high SD and decision level and therefore complicating the distinction between treated and untreated dairy cows. However, for the generic FCIA sample preparation, responses of all negative sera were lower than the responses of both positive sera (e*, f*) and the inter-individual differences in responses were greatly reduced. Therefore, in the following experiments, the generic FCIA sample preparation was used. Moreover, applicability of the generic sample preparation method can also be advantageous to in the earlier mentioned RIA,[14] ELISA,[15,16] and Western blot.[17]

Although the research was not focused on determining the origin of the non-specific interaction, some characteristics of the interfering proteins can be deduced from the previous experiments. Most probably it is an over 100 kDa protein with affinity for protein G columns, pointing into the direction of an IgG (like) protein, as suggested by Pinel *et al.*[17] The specificity of the

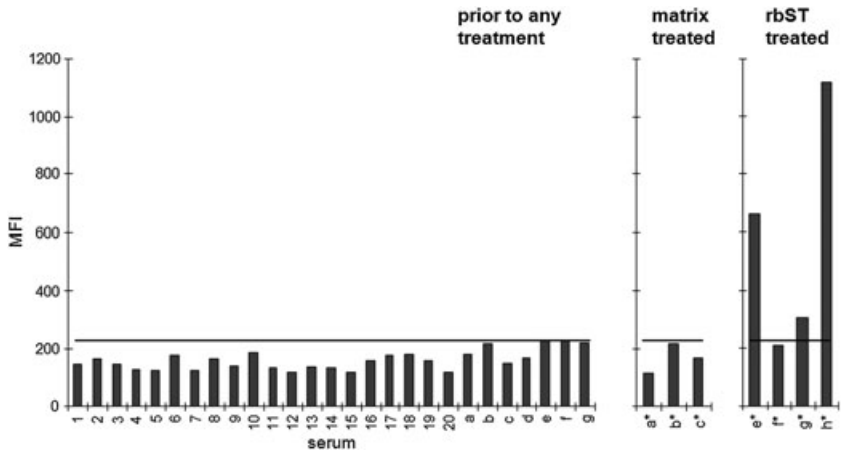

Figure 4. FCIA responses (bars) of 27 untreated cows (1–20; a-h), serum samples collected a week after the treatment period of 3 matrix only (a*, b*, c*) treated and 4 rbST (e*,f*,g*,h*) treated cows and their corresponding decision level (line) using generic FCIA sample preparation.

induced antibodies for rbST was proven in this study by the ability to bind rbST in solution and therefore inhibit binding to the beads as shown in Figure 3.

FCIA applicability to real samples

As it was possible to eliminate the majority of the non-specific responses by the generic FCIA sample preparation, a new decision level for discriminating between treated and untreated dairy cows could be determined. For this, the sera of 27 untreated dairy cows were pretreated using the generic FCIA sample preparation before analysis in the FCIA. An average response of 160 ± 34 MFI was found, resulting in a decision level of 228 MFI (Figure 4). Now only relatively small inter-individual differences were observed for the sera of the untreated dairy cows: a standard deviation of roughly 20% was obtained, much lower than the 90% SD observed by Rochereau-Roulet *et al.*[16] Furthermore, in our case no false positive results were obtained at all, which favourably compares with previous studies.[14–17]

For evaluating the applicability of the assay, further sera of the animal experiment (four rbST-treated and three matrix-treated dairy cows) were analyzed and responses were compared to the decision level (Figure 4). Three out of the four rbST-treated dairy cows gave responses above the decision level indicating the presence of antibodies specific for rbST. These results are in agreement with literature,[14,15] where in 70–80 % of rbST-treated cows antibodies specific for rbST could be found. To assess the presence of antibodies specific for rbST in time, responses of weekly collected serum samples of the animal experiment were determined in FCIA and compared with the in-between day decision level. For the rbST treated dairy cows an increase in response, with a peak after the second rbST treatment was detected, however, no unambiguous dose-response relation could be established. Then treatment-induced antibody responses decreased, but remained above decision level for all responding animals (Figure 5). Even four weeks after the last rbST treatment, the endpoint of the animal experiment, antibodies specific for rbST were still detectable in the serum samples. When the weekly serum samples of the animal experiment were analyzed, matrix treated cow a* and c* were below decision level over the whole time period, while cow b* was correctly classified negative, except for 2 out of 15 time points (Figure 5). Since

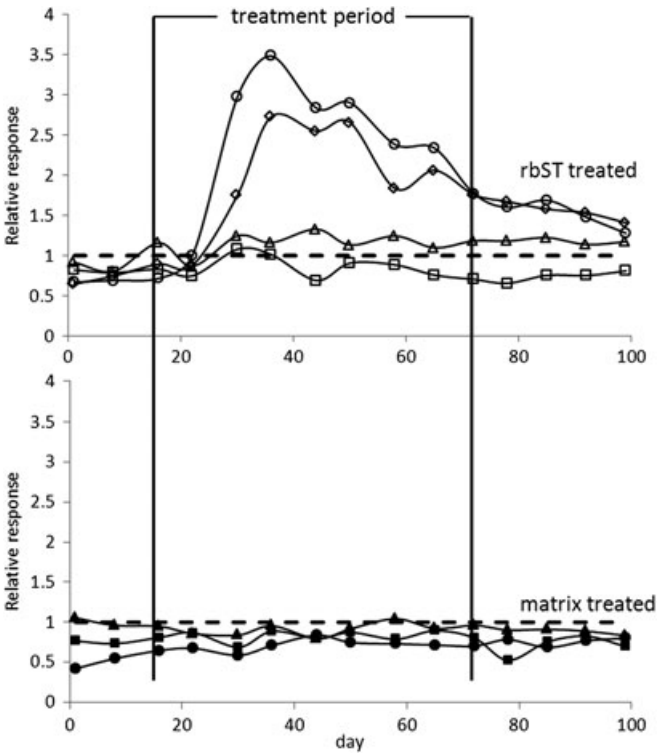

Figure 5. Effect of matrix only- and rbST treatment on serum of dairy cows in time. Responses of weekly collected serum samples of matrix treatment only, a* (■), b* (▲), c* (●) and rbST e* (◊), f* (□), g* (Δ) and h* (○) treated dairy cows. Treatment started at the left vertical line and ended at the right vertical line; all responses are compared with the in between day decision level.

matrix treated cow b* is considered false positive, this would still lead to a low maximal false positive rate of 3.7% only.

As expected from literature only three out of the four treated animals produce anti-rbST antibodies. This is not problematic however, since multiple cows from a herd might be sampled and then at least one cow will display a positive result. Hence, this requires development of a rule on the number of cows to be sampled based on the ability of finding positive results in rbST-treated cows. Eventually, samples might be pre-screened by the method presented and additional certainties obtained by applying a multiplex biomarker approach in a laboratory. Such

a multiplex FCIA can provide a detailed biomarker profile in serum, ultimately pinpointing with the highest confidence to rbST and related protein abuse in cattle.

Conclusions

The generic FCIA sample preparation method reduced non-specific binding of serum proteins to rbST-coated beads. Consequently, inter-individual variation in signals between non-treated dairy cows was low, resulting in a relatively low decision level. Using this FCIA sample preparation the signal from the developed anti-rbST antibodies in rbST-treated dairy cows differed significantly from the background responses in non-treated cows. Hence, an absolute decision level could be determined and the applicability of our method for screening herds of dairy cattle was demonstrated. Antibodies against rbST were induced in three out of the four treated dairy cows, a realistic percentage. They could be detected up to at least four weeks after the last rbST treatment and hardly any false positive results were observed in 27 untreated dairy cows. In conclusion, a unique anti-rbST antibody screening assay was presented which can successfully discriminate between treated and untreated dairy cows within unknown herds of cattle.

Due to the multiplex capability of the developed assay, the confidence level of the assay can be increased by combination with assays for different biomarkers, ultimately pinpointing to rbST abuse in single cows.

Supporting information

Supporting information may be found in the online version of this article.

Acknowledgements

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