A Rapid Blood Test to Monitor the Immune Status Change of Dairy Cows and to Evaluate their Disease Risk during the Periparturient Period

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Abstract

Background

Dairy cows are at the highest risk of developing clinical and subclinical diseases and disorders in the first few weeks following parturition. During the periparturient period, from approximately 30 days before calving to 30 days postpartum, the immune system of the dairy cows undergoes a multitude of changes to prepare for parturition, colostrum production and lactation. One such change is the transfer of a large amount of immunoglobulin G proteins, especially subclass IgG1 to colostrum, leading to a reduction of IgG1 in the blood. As IgG1 and IgG2 need to maintain a balance to protect animals from intracellular and extracellular pathogens, a disruption of this balance compromises the immune protection of the animals. Rapid tests that can detect these immunological changes may be potentially useful for predicting the risk of dairy cows developing infectious diseases and other adverse health conditions following parturition.

Results

We report here a new rapid test to detect certain immune status changes in the blood serum of dairy cows. This test uses a nanoparticle probe to evaluate the relative quantity of IgG1 and IgG2 in a sample. The nanoparticle probes are aggregated together upon interaction with bovine IgG2, while bovine IgG1 inhibits such interactions. The nanoparticle aggregates are detected by monitoring the color change of the assay solution using a handheld device. We tested the serum samples from 230 dairy cows collected during periparturient period, from 14-7 days before calving to 7-14 days postpartum. Results show that the test clearly detected an immune status change associated with IgG1/IgG2 relative quantity change around the time of parturition. Data analysis using mixed liner model in SAS (Statistical Analysis System) revealed a significant difference (P value = 0.042) in their test responses between healthy cows and cows with mastitis and/or lameness.

Conclusion

The new rapid test we report here can be used to detect and monitor certain immune status change in dairy cows during the periparturient period. The test results may be potentially used to evaluate and predict the health risk of the dairy cows following parturition.

Background

Dairy cows are at the highest risk of developing clinical and subclinical diseases and disorders in the first few weeks postpartum [1-4]. It is believed that more than 50% cows suffer from at least one or more disorders during this period. Mastitis, lameness, retained placenta, metritis, ketosis, and hypocalcemia are among the most common diseases in dairy cattle. The median prevalence of clinical mastitis is around 20-25 cases per 100 cows per year [5]. In some countries and regions, the overall mastitis at cow level can be as high as 60% or more [6]. Milk production loss in combination with treatment costs and culling related to mastitis constitute approximately $116-$325 /case [7]. Lameness is a condition associated

with lesions of hind limb, which is caused by pathogens invading the hooves. Research shows that lameness in dairy cows adversely affect milk production, reproductive performance, longevity, and the general well-being of the animal. The prevalence of lameness among most dairy herds varies from 20-40% [8, 9]. Lameness is the third largest cause of economic loss in the dairy cattle industry, after mastitis, and reproductive disorders.

Around the time of calving, the body of a dairy cow makes many biological and physiological changes and adjustments to prepare for calving, colostrum production and lactation [2, 10, 11]. The immune system of the dairy cow, in particular, undergoes numerous changes in order to help the cow successfully deliver and protect the cow from infectious challenges postpartum. One such change is the transfer of immunoglobulin G proteins, especially subclass IgG1, to colostrum, leading to a reduction of IgG1 in the maternal blood [12-14]. The transfer of IgG1 to colostrum and the feeding of newborn calves with colostrum provides the calves passive immune protection for their first few weeks of life following their birth. As the level of IgG1 and IgG2 should maintain an optimal balance to protect animals from intracellular and extracellular pathogens, a disruption of this balance compromises the immune protection of the periparturient dairy cows.

Another significant change related to the immune system is the elevated inflammatory responses during the periparturient period [15]. Inflammation is the first immune response of an organism when facing a microbial infection or a tissue injury. Studies have shown that a certain degree of inflammation is necessary to initiate calving, assist placental expulsion and protect the dams from postpartum microbial invasion [16]. However, excessive and persistent postpartum pro-inflammatory response has been linked to increased disease risk and decreased milk production [17, 18]. Metabolic changes accompanied with a pro-inflammatory state alter the homeostasis of the immune system, including IgG1/IgG2 balance, exposing dairy cows to increased disease risk in early lactation [19].

Considering the critical role of immune functions in the overall health, production, and reproduction performance of dairy cows, simple and convenient tests that can allow rapid evaluation of the immune status and conditions of dairy cows will provide a valuable tool for dairy farm management. Although laboratory tools and tests are available to measure and evaluate different cellular and molecular components of the immune system, these are typically not suitable for on-site farm testing. Here we report the use of a newly developed, D2Dx™ immunity test, to monitor the immune status change in dairy cows during the transition period [20, 21]. This is a single-step test requiring only the mixing of a testing reagent with a small volume of un-diluted and un-treated blood serum or plasma providing a test result within 30 seconds. This quick test can be conveniently performed on-site or in house at small local veterinary clinics thereby avoiding having to send samples out for offsite processing and waiting for test results to be returned.

The principle of the test is illustrated in Figure 1. This test uses a gold nanoparticle probe to evaluate the relative quantity of IgG1 and IgG2 in a blood sample. The nanoparticle is designed to interact with bovine IgG1 and IgG2 through two different modes: upon interacting with IgG2, the nanoparticle forms large

aggregates; with IgG1, the protein will bind to the nanoparticle surface to form a protein layer, however, will not cause nanoparticle aggregate formation. The nanoparticle aggregates are detected by monitoring the color change of the assay solution using a handheld reader device, CT-100 from Nano Discovery Inc. The color change is due to the surface plasmon resonance shift in aggregated gold nanoparticles, a phenomenon that has been well studied and established for biosensing and diagnostic test applications [22-25]. In the presence of both IgG1 and IgG2, as in the case of bovine blood samples, the two proteins will compete to interact with the nanoparticles through the two different binding modes. The degree of color change is reflective of the relative quantities of IgG1 and IgG2 in a sample.

In this study, we tested blood serum samples from 230 dairy cows collected during their periparturient period, 14-7 days before and after calving. The 115 of the cows had mastitis and/or lameness, whereas the other 115 of the cows had no observed clinical problems.

Materials And Methods

Bovine blood sample source

This study was approved by the University of Idaho Animal Care and Use Committee (# 2017-52). Between March 2018 to May 2019, blood samples were collected from dairy cows in several dairy farms located in the Pacific Northwest region of the United States. A total of 230 cows were enrolled. Blood samples were collected from each cow on day − 14, -7, + 1, +7 and + 14, relative to parturition using BD Vacutainer® red top serum collection tubes (Franklin Lakes, NJ, U.S.A.). All samples were processed on the same day upon collection and the serum samples were isolated, aliquoted, and stored immediately at -80^{o}C. The status of mastitis was determined by the California Mastitis Test (CMT) in the milking parlor. Lameness was determined by visualizing during walking or examining the hooves. All of the health data were collected 90 days before to 90 days after calving.

For the current study, we tested the serial draw blood samples (day − 14, -7, + 1, +7, + 14, relative to parturition) from 230 cows randomly selected from a study pool of total 1203 cows. Among 230 cows, half of them (115) had no documented clinical diseases (healthy group), while the other half (115) had documented mastitis and/or lameness conditions (diseased group). Within the mastitis/lameness group, more than 78% of cows had mastitis. No other criteria were applied for sample selection from the study pool.

D2dx™ Immunity Test

D2Dx™ immunity test kits (catalog D2Dx-BV-500, lot number bv05212020) were received from Nano Discovery Inc. (Orlando, Florida). Each kit contains the reagent and cuvettes for 500 tests. A handheld reader device, CT-100 from Nano Discovery Inc. was used to read the test result. To perform the test, 50 µL of testing reagent solution was first placed into the cuvette using a micropipette. Then 10 µL of an undiluted bovine blood serum sample was added. After mixing the assay solution for 5 seconds using a

mini-vortex mixer, the cuvette was placed in CT-100, and the result was read automatically in 30 seconds. The response of the test was reported directly as the absorbance change of the assay solution over 30 seconds of reaction time.

D2Dx™ immunity test of bovine IgG1 and IgG2 protein in pure buffer solution

To illustrate and confirm the effectiveness of D2Dx™ immunity test kit (catalog D2Dx-BV-500) to detect the relative quantity and different ratios of bovine IgG1/IgG2, we conducted the testing of purified bovine IgG1, IgG2 and their mixture solutions using the D2Dx™ immunity test kit. Purified bovine IgG1 and IgG2 were purchased from Biorad (IgG1, catalog pep003, 1 mg/mL; IgG2, catalog pep004, 1 mg/mL, Hercules, California). IgG1/IgG2 mixtures were made at the following volume ratios 5/1, 2/1, 1/1, 1/2, 1/5 and 1/10 (IgG1/IgG2, v/v). The testing of these purified IgG1, IgG2, and IgG1/IgG2 mixture solutions was conducted using the same protocol as used for bovine serum testing as stated above. The test result was reported directly as the absorbance change of the assay solution over 30 seconds of reaction time.

ELISA assay of bovine IgG1 and IgG2 in bovine blood serum samples

The level of bovine IgG1 and IgG2 in 39 selected bovine serum samples was determined using ELISA kits from Bethyl Laboratories (Montgomery, Texas), catalog E11-116 for bovine IgG1 (lot E11-116-190912) and catalog E11-117 for bovine IgG2 (lot E11-117-190814). A SpectroStar Nano plate reader from BMG LabTech (Cary, North Carolina) was used to read the ELISA results.

Statistical analysis

The data obtained from days relative to parturition (period) were analyzed as a repeated measures linear mixed model in SAS (V. 9.4, SAS Inst. Inc., Cary, NC, U.S.A.) with disease presence/absence and day and their interaction as fixed effects and cows as random effect. A repeated measures association between days was additionally accounted for through a compound symmetry correlation structure. The statistical model was $Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk}$ where the Y_{ijk} = the response of IgG concentration, μ = overall mean, α_i = fixed effect of disease (*i* = control, disease (mastitis or lameness)), β_j = fixed effect of time, $(\alpha\beta)_{ij}$ = disease-time interaction, and e_{ijk} = residual error term assumed to be $N(0, \sigma^2)$. In addition, the data obtained from prepartum, calving and postpartum were analyzed by Proc TTEST in SAS to compare prepartum vs. calving, prepartum vs postpartum and calving vs. postpartum. The statistical model was: $Y_{ij} = \mu + R_i + e_{ij}$ where the μ = overall mean, Ri = fixed effect of diseases (*i* = healthy, diseased), and e_{ij} = residual error term assumed to be $N(0, \sigma^2)$. The significance was declared at $P < 0.05$.

Results

Detection of bovine IgG1/IgG2 relative quantity using the D2Dx™ immunity test

To illustrate and confirm the effectiveness of the D2Dx™ immunity test reagent in detecting different bovine IgG1/IgG2 ratios, pure bovine IgG1, IgG2 and their mixtures at a quantity ratio of 5/1, 2/1, 1/1, 1/2, 1/5 and 1/10 (IgG1/IgG2, w/w) were reacted with the immunity test reagent. The responses detected as the absorbance change of the assay solutions are presented in Fig. 2. Upon reaction with pure bovine IgG2, there is a clear color change of the assay solution, while upon mixing with bovine IgG1, there is no color change. When IgG1 and IgG2 are mixed together, the color change of the assay solution increases with an increasing amount of IgG2, that is, a decreasing IgG1/IgG2 ratio.

D2dx™ Immunity Test Of Bovine Blood Samples

Figures 3A and B are the immunity test results of 230 cows on day − 14, -7, + 1, +7 and + 14 relative to parturition (A-healthy group; B-diseased group). The model based marginal means of the two cow groups at different days are shown in Fig. 3C. Overall, we observed a significant general trend of time ($P = 0.001$): from pre-calving to calving, there is an increase in the test response; and from calving to postpartum, the test responses declines from a peak value to levels seen in pre-calving period. There is not a significant between health status and time interaction ($P = 0.73$). All days were significantly different from adjacent times for the healthy group ($P \leq 0.01$). The diseased group had a similar pattern of significance except for day 7 vs. 14 ($P = 0.30$). In addition, the mixed model considers the interaction between the health status effect and time, and the P value of the interaction is 0.73, confirming that both healthy cows and diseased cows show similar time-dependent test response changes during the periparturient period. This trend of change appears to mirror the expected IgG1/IgG2 quantity change during the periparturition period, where a decrease of IgG1 level in blood plasma during pre-calving period leads to a decreased IgG1/IgG2 ratio; therefore, an increased D2Dx™ immunity test response; and then a return of plasma IgG1 level following parturition leads to an increased IgG1/IgG2 ratio and, subsequently, a decreased immunity test response.

During the study period, we also observed a significant difference between the control and diseased groups. The diseased cow group has a higher average test response than the control cow group ($P = 0.04$). This observation indicates that diseased cows may have lower IgG1/IgG2 ratio than the control cows during the entire periparturition period.

ELISA analysis of IgG1 and IgG2 level in selected cows during the study period

To further confirm that the changes detected by the D2Dx™ immunity test are results of or associated with bovine IgG1/IgG2 ratio change in cows around the time of parturition, we conducted ELISA analysis of bovine IgG1 and IgG2 in selected blood samples. Total 39 serum samples from 13 randomly chosen cows that represent pre-calving (-14 and − 7), calving (day 1), and post-calving (day 7 and 14) were analyzed. Figures 4A and B are the results (absorbance at 450 nm) of bovine IgG1 and IgG2 analysis, respectively. From pre-calving to calving, there is a clear drop of IgG1 level in the blood, and the level is recovered or partially recovered following parturition. The difference between pre-calving to calving, and between calving to post-calving, is statistically significant, as confirmed by the P values (< 0.0001,

student T test). These data confirmed what has already been reported by other studies [12–14]. On the other hand, the level of IgG2 remained almost unchanged from pre-calving, to calving, and post calving. As a result, the ratio of IgG1/IgG2 decreased from pre-calving to calving, and then returned to pre-calving status following parturition. A lower IgG1/IgG2 ratio gives a higher D2Dx™ immunity test response. The D2Dx™ immunity test response increase observed from pre-calving to calving, and then the decline from calving to postpartum reflect this IgG1/IgG2 ratio change in the periparturient cows.

Discussions

D2Dx™ immunity test is a simple, single-step test that may be performed on-site at the farm or in local veterinary clinics. The test is also rapid; taking less than 1 minute to perform and to obtain results. As confirmed by the testing of pure bovine IgG1, IgG2, and their mixture solutions, the D2Dx™ immunity test responds to IgG1/IgG2 ratio change in pure buffer solution.

In bovine blood, the concentration of IgG1 and IgG2 is typically around 10–20 mg/mL, and the two IgG subclasses are maintained approximately at a 1/1 ratio [26]. Several weeks before calving, IgG1 is selectively secreted to the colostrum. The concentration of IgG1 in colostrum is enhanced by 10 fold and can often reach more than 50 mg/mL [27, 28]. This selective secretion of IgG1 to colostrum leads to a decline of IgG1 concentration in blood, for example, according to the study by Sasaki et al., from 14 to ~ 5 mg/mL [14]. At the same time, most studies do not find significant change of IgG2 level in the blood around peri-parturition period. This means the IgG1/IgG2 ratio in the blood decreases during colostrogenesis. As the D2Dx™ immunity test can detect IgG1/IgG2 ratio variations, we hypothesized that this test may be able to detect the relative IgG1/IgG2 quantity change in dairy cows during the periparturient period.

From the testing of close to 1000 serum samples collected from 230 dairy cows, we clearly detected an immune status change in the periparturient cows around prepartum period and this change is associated with the IgG1/IgG2 relatively quantity change during this time. Except for a few cases, almost all cows, regardless of their health conditions, follow the same trend: their immunity test responses increased from 1–2 weeks before calving to the day of calving; and then started to decline back following parturition. This trend is an exact reflection of immune homeostasis disruption known to occur in dairy cows and other reproductive animals during parturition [2, 29, 30].

As parturition leads to a disruption of homeostasis in the body of the reproductive animals, naturally it only makes sense that the animal body will attempt to recover the original balance after parturition. If the body fails to restore this balance on time and/or fully, the animal may be exposed to an increased risk of infectious diseases and other health problems. Studies have found that a certain degree of inflammation is necessary to induce birth and to protect the cows from bacterial infection in the first few days postpartum [16]. Cows that can resolve this inflammation more quickly are less susceptible to diseases, whereas cows that sustain high inflammatory responses are more likely to experience transition diseases such as metritis and mastitis [15]. The D2Dx™ immunity test results obtained in the present study

supports this hypothesis. Overall, we found cows with mastitis and/or lameness to have more elevated test response than cows without clinical diseases in average during the four weeks of periparturient period. More notably, the postpartum test response of diseased cows remained at elevated levels more frequently than the healthy cows, suggesting that the diseased cows may have had more difficulty to recover their homeostasis following parturition.

The $D2D_X$™ is not a traditional bioassay that measures the absolute quantity of a specific target protein. Even though the test was designed to detect bovine IgG1/IgG2 relative quantity change, the test result is not an absolute quantity measurement of the IgG1/IgG2 ratio. In our previously published study [21], we explained in details the mechanism of this test. Briefly, the test uses a nanoparticle “pseudo” pathogen to probe the humoral immune status of a blood sample. When the nanoparticle probe is mixed with a blood sample, IgG (including bovine IgG1 and IgG2) and IgM proteins will bind with the nanoparticle. Once IgG and IgM are bound to the nanoparticle, the entire structure will be recognized by the complement system as an antibody-coated pathogen particle, and a cascade of reactions and interactions will follow between the blood proteins and the IgG or IgM-bound nanoparticle. As we demonstrated in our previous studies [21], the D2Dx™ immunity test response is determined by the collective effect of IgG, IgM, complements, and possibly other proteins in the blood. In our current study, we further confirmed that a change of IgG1/IgG2 ratio in the blood will lead to a change of the D2Dx™ immunity test response. To use the D2Dx™ immunity test for decision making purpose, and to evaluate the immune status and predict the risk level of individual cows, a clinical cutoff value should be established from testing of a sufficiently large number of healthy and diseased animals. The clinical sensitivity and specificity of the test for specific applications shall be determined from such studies.

Limitations of the current study must be considered. First, this study was conducted on frozen serum samples. This test remains to be validated using fresh blood samples if it were to be used as an on-farm test. Second, this study did not record any subclinical disorders or diseases. It is believed that in the first few weeks of lactation, more than 50% of cows may suffer from at least one subclinical disease or disorder [15]. This means that a significant number of cows classified as “healthy” cows may actually have had some sub-clinical diseases or disorders. If we consider this factor, the test response difference between the real healthy cows and diseased cows could be even larger and more significant than what we have observed. More extensive studies over a longer period should be conducted to make further evaluations. If the D2Dx™ immunity test can detect cows with subclinical diseases and disorders, the test can add major benefit to dairy cow health monitoring and management.

Conclusion

The D2Dx™ immunity test can detect IgG1/IgG2-associated immune status change in dairy cows around the periparturient period. The test shows strong potential for monitoring the immune health and evaluating the disease risk of dairy cows following calving.

Declarations

Ethics approval and consent to participate

This study was approved by the University of Idaho Animal Care and Use Committee (# 2017-52).

Consent for publication

Not applicable

Availability of data and materials

The datasets during and/or analysed during the current study are available from the corresponding author on reasonable request

Competing interests

QH is an owner and a co-founder of Nano Discovery Inc. that produces D2Dx™ immunity test. All other authors declare no competing interest.

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Authors' contributions

CYT, HCH, TW were involved in bovine blood sample collection and health data recording; RH and QH conducted immunity test and data analysis; CYT and WJP conducted statistical analysis and participated in manuscript writing; PR and QH oversaw the study, conducted data analysis, result interpretation, and participated in manuscript writing.

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Not applicable

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Figures

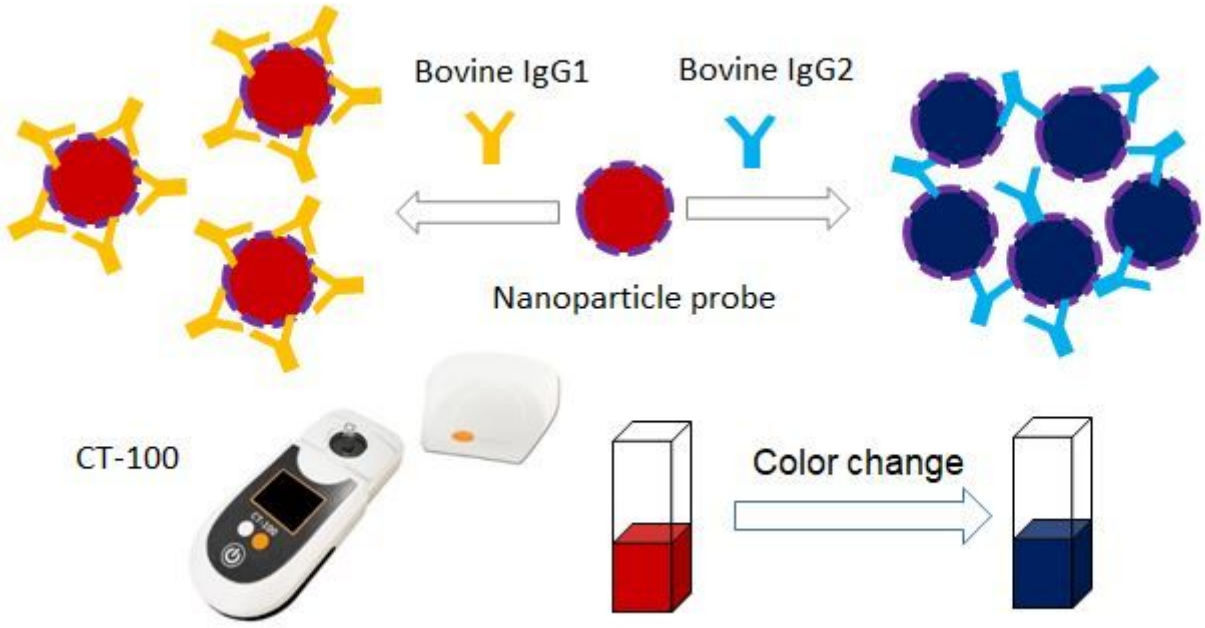

Figure 1

Illustration of the principle behind the D2Dx™ immunity test. The interaction of bovine IgG2 with the nanoparticle probe causes a nanoparticle aggregate formation and a color change from red to blue, while the interaction of bovine IgG1 with the nanoparticle probe leads to adsorption of the protein on the nanoparticle surface, but not aggregate formation, and no color change. In the presence of both IgG1 and IgG2, IgG1 and IgG2 will compete to bind with the nanoparticle probe through the two different binding modes. The color change of the assay solution from red to blue is detected using a handheld reader device, CT-100.

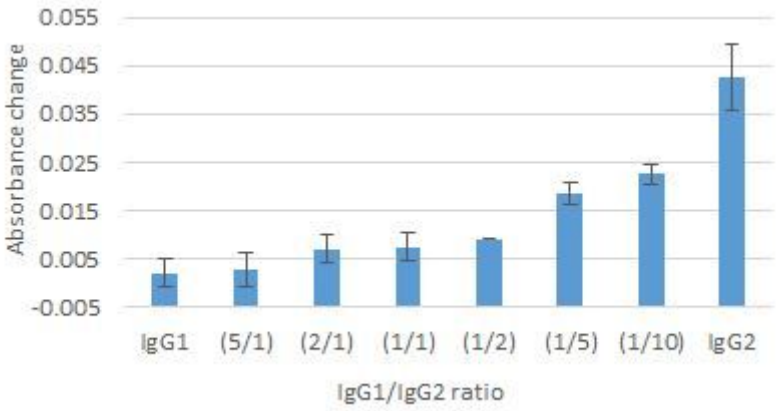

Figure 2

D2Dx™ test response of bovine IgG1, IgG2 and their mixtures. Both IgG1 and IgG2 have the same concentration of 1.0 mg/mL. The IgG1/IgG2 ratios indicated in the graph represent both v/v and w/w ratios. Error bars represent the standard deviation from at least two measurements.

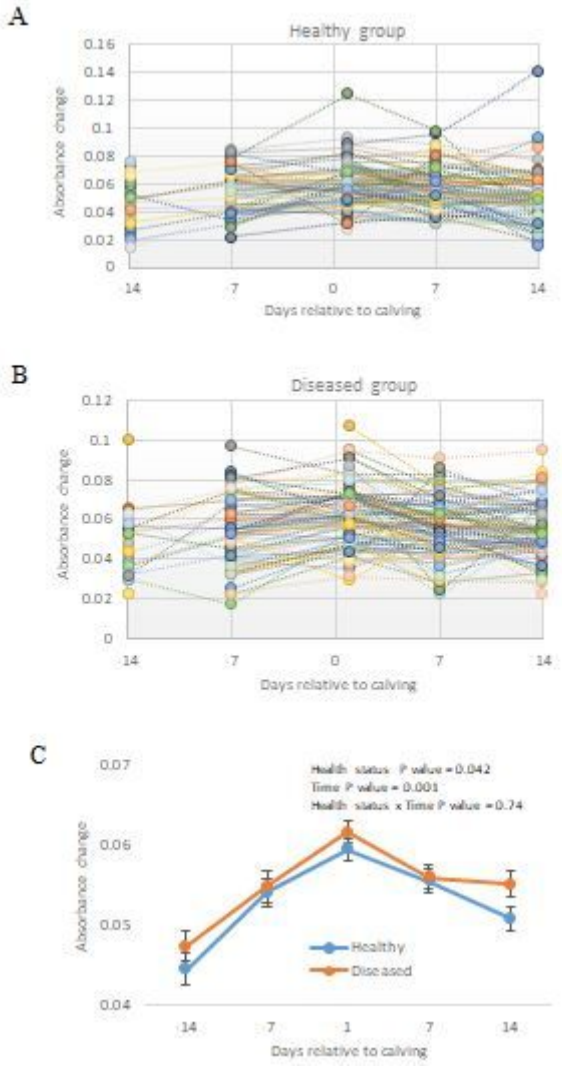

Figure 3

D2Dx™ test response of 230 cows during the periparturient period. A-healthy group (n=115); B-diseased group (n=115); C- least square means of healthy and diseased groups (health status P = 0.042; time P = 0.001).

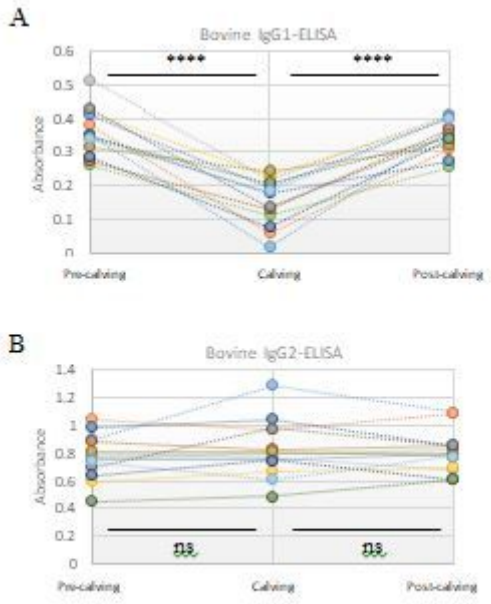

Figure 4

ELISA analysis of bovine IgG1 (A) and IgG2 (B) in 39 blood serum samples. The differences for IgG1 levels between pre-calving and calving; and between calving and post-calving, are both significant ($P < 0.0001$, ****). The differences for IgG2 levels between pre-calving and calving; and between calving and post-calving are both not significant (ns; $P > 0.05$).