HHS Public Access

Author manuscript

Sens Int. Author manuscript; available in PMC 2022 May 20.

Published in final edited form as:

Sens Int. 2020 ; 1: . doi:10.1016/j.sintl.2020.100009.

A rapid blood test to monitor immunity shift during pregnancy and potential application for animal health management

Tianyu Zheng[a,1], **Yasmine Moustafa**[b,1], **Caroline Finn**[c], **Sydney Scott**[b], **Christopher J. Haase**[d], **Nathaly A. Carpinelli**[e], **Johan S. Osorio**[e], **Karl K. McKinstry**[c], **Tara M. Strutt**[c,**], **Qun Huo**[b,*]

[a]Nano Discovery Inc., 1060 Woodcock Road Suite 131, Orlando, FL, 32803, USA

[b]Department of Chemistry and NanoScience Technology Center, University of Central Florida, 12424 Research Parkway Suite 400, Orlando, FL, 32826, USA

[c]Burnett School of Biomedical Science, Division of Immunity and Pathogenesis, College of Medicine, University of Central Florida, 6900 Lake Nona Blvd., Orlando, FL, 32827, USA

[d]CJ Haase Veterinary & Immunological Service, 407 Prairie St, Reeseville, WI, 53579, USA

[e]Dairy and Food Science Department, South Dakota State University, Brookings, SD, 57007, USA

Abstract

The immune health of a farm animal can have significant impact on its overall health, welfare and productivity. One of the most vulnerable physiological states for both humans and animals is pregnancy. Many systemic changes correlate with the gravid state, including shifts in the immune system that may impact the ability to respond optimally to pathogen challenge. Because of this, it would be beneficial to be able to monitor the immune health of the pregnant animals closely. Recently, we developed a new nanoparticle-enabled rapid blood test that can detect ongoing immune responses from both laboratory and farm animals. Here, we report that this novel test reveals highly repeatable and acute changes associated with pregnancy and peri-parturition period in laboratory mice and in cattle. We hypothesize that the test score change reflects changes in the immune status of the gravid females related to the humoral immune response. The test is easy to conduct, of low cost, with results obtained in less than 20 min. This rapid test could be potentially used as an onsite test in local farms and small clinics for animal health management.

Keywords

Immune response; Pregnancy; Cattle reproduction; Nanotechnology

**Corresponding author. Tara.Strutt@ucf.edu (T.M. Strutt). *Corresponding author. Qun.Huo@ucf.edu (Q. Huo).

[1]T. Zheng and Y. Moustafa made equal contribution to this study.

Declaration of competing interest

The authors declare the following competing financial interest(s): Q.H. is an owner and officer of Nano Discovery Inc. Nano Discovery Inc. Licensed and commercializes the assay technology reported in the manuscript.

1. Introduction

Pregnancy represents a unique immune state in which humans and animals are more vulnerable to infectious disease. The underlying mechanisms leading to enhanced susceptibility in pregnant females are not entirely clear, but it has been suggested that there is an overall immune suppression [1] that goes beyond the maternal fetal interface to keep inflammation under control. Alternatively, it has been proposed that changes in the Th1/Th2 response potential [2–7] can result in suboptimal immune-mediated attack against invading microbes. Th1 responses are directed by CD4 T cells that are polarized into specialized T helper type 1 lymphocytes by inflammatory factors in the priming environment. Th1 cells mainly stimulates cell-mediated immune response, while Th2-polarized CD4 T cells orchestrate responses characterized by strong antibody-mediated immunity and high antibody titer. Th1 immunity is more protective against intracellular pathogens such as viruses, and Th2 immunity is more important for protection against extracellular pathogens [2]. Pregnancy has been associated with a propensity towards Th2 polarization of naive CD4 T cells [8–12] which may result in compromised immunity against intracellular pathogens and viruses. It is thought the teleological reason behind this state at the maternal fetal interface is that it helps to protect the developing fetus against damaging cell-mediated responses triggered by recognition of allo-antigens that usually trigger strong Th1 activation. Th2-mediated responses that feature more robust antibody production and less cell-mediated attack have been suggested to present a reduced threat to the fetus. Such immune alterations in the periphery could compromise the ability of pregnant females to combat pathogens [13–17]. Additionally, several components of the innate immune system are differentially regulated during the gravid state, including elements of the complement system [18,19].

Despite the significance of the Th1/Th2 immunity balance in the general health and productivity of pregnant and non-pregnant agricultural animals, there is no convenient diagnostic test in the market that can be used for rapid assessment of the immune health of agricultural animals in farm and veterinary clinic settings. Techniques and methods that are commonly used in the laboratory to evaluate the immune status and functions, such as flow cytometry to analyze immune-related cells and immunoassays to quantify the level of antibodies and cytokines, are complicated, require expensive instruments, and take hours to days to obtain the results. These techniques and tests are thus not suitable for rapid testing and screening of farm animal immune health, especially when dealing with larger numbers of animals.

Recently, we have reported a rapid blood test, D2Dx (from Diameter to Diagnostics), that can effectively detect antibody-mediated immune response from laboratory mouse models and cattle [20,21]. This test uses a gold nanoparticle (AuNP) as a pathogen substitute to probe changes in constituents in the blood related to levels of different antibody isotypes and factors of the complement system from human or animal blood (Fig. 1). Upon mixing the AuNP reagent with a blood serum, proteins from the humoral immune system including IgG, IgM, and complement proteins, will interact with the gold nanoparticles in a way similar to what happens *in vivo* [20]. This interaction is measured and quantified by monitoring the average nanoparticle size change of the assay solution using a relatively low-cost instrument called dynamic light scattering (DLS) [22–26]. The test result, expressed

as a test score (Fig. 1), is obtained within 20 min after blood serum is collected. A portable DLS device developed by Nano Discovery Inc., D2Dx-R, can be operated in small veterinary clinics or on farm sites to perform the test with minimum facility and personal training. Using this test, we were able to detect acute changes in the serum that are associated with infection and more longer-term changes associated with aging [20]. The assay reveals similar results in inbred laboratory mice housed in specific pathogen-free conditions and in outbred cattle populations. We also identified that IgG and IgM antibody isotypes as well as complement proteins in the serum as key molecules that impact the test scores reported in our assay [20].

Indeed, given the potential for the rapid onset of serious health issues in pregnant cattle it is vital to develop diagnostics that can identify high risk animals quickly, at a low cost, and ideally in the field. Our newly developed assay may represent such a platform as it requires only a few drops of blood and can be completed within a few minutes using a mobile device. We show here proof-of-principle that our assay can not only detect changes in the serum associated with different stages of pregnancy, but can help to identify at risk individuals including gravid cattle suffering from infection. These studies thus have direct relevance to improving the management and health of livestock.

2. Material and methods

2.1. Murine models, breeding, and blood collection

All animals were housed at the University of Central Florida at Lake Nona Vivarium in specific pathogen free conditions. All experimental animal procedures were approved and conducted in accordance with the University of Central Florida's Animal Care and Use Committee guidelines. BALB/c and C57BL/6 mice were bred at the University of Central Florida at Lake Nona Vivarium.

C57BL/6 breeding pairs were introduced at 7 weeks of age. BALB/c breeding pairs were introduced at 5 weeks of age. Additional female mice that were not placed in breeding but with matching ages were used to collect control blood samples. Blood samples were collected from each mouse every 7 days until the study ended. Peripheral blood was obtained from mice by submandibular bleeding. Blood samples were collected into 2mL microcentrifuge tubes. Immediately after obtaining the blood sample, the tubes were placed in an upright position for 1 h to allow complete blood clotting. The tubes were centrifuged using an Eppendorf Minispin for 5 min at 10,000 g. The serum was removed to a clean microtube and used immediately for testing.

2.2. Bovine blood collection and processing

Bovine blood samples used for the study were obtained from two sources. For data presented in Fig. 4, bovine blood samples were collected by Dr. CJ Haase from CJ Haase Veterinary & Immunological Service (Reeseville, WI). Holstein cows were housed at an individual private sand free stall dairy farm in south central Wisconsin. The herd consists of 900 total animals. Study procedures were reviewed and approved by the farm owners and overseeing veterinary staff. All animals were found to be in good health based on physical exam by

overseeing Veterinarian and lack of concurrent treatments found on Dairy Comp 305 (Valley Agricultural Software, Tulare, CA). Blood was collected from the coccygeal vein at time of herd health. For data presented in Fig. 5: Blood was sampled from the coccygeal vein before morning feeding during the transition period at −30, −15, −7, 5, 10, and 30 days relative to parturition. Samples were collected into evacuated serum tubes (BD Vacutainer, Becton Dickinson and Co., Franklin Lakes, NJ) containing clot activator for serum. After blood collection, tubes were kept at 21 °C until centrifugation (~30 min). Serum was obtained by centrifugation at 1900×*g* for 15 min at 4 °C. Aliquots of serum were frozen (−20 °C) until further analysis.

2.3. D2Dx test

Gold nanoparticles (AuNPs) used for this study were provided by Nano Discovery Inc. (Orlando, FL). The chemical composition of the AuNP is proprietary. The AuNP has an average diameter of 90 nm. The D2Dx blood test was performed using a D2Dx-R dynamic light scattering reader from Nano Discovery Inc. (Orlando, FL). All size measurements were conducted at an ambient temperature of 25 °C [20].

To perform the blood test, 3 μL of blood serum was mixed with 60 μL of AuNP solution. The mixture was vortexed for about 10 s and then incubated at room temperature. The particle size of the assay solution was measured using D2Dx-R after 20 min of incubation at room temperature (D_2). The particle size of the original pure AuNP was measured by D2Dx-R, and denoted as D_1 (90 nm). The ratio of D_2/D_1 was calculated and reported here as the D2Dx test score.

2.4. Statistical analyses

P values as presented in the figures were determined by either two-tailed unpaired Student's t-test. P values < 0.05 were considered as significant difference. The numbers of asterisks indicate significance levels of P values, for example, the symbols of *, **, ***, and **** represent P values of ≤0.05, ≤0.01, ≤0.001, and ≤0.0001, respectively. If there is no significant difference (P > 0.05) between the groups, the results are presented as "ns", namely, not significant. The correlation coefficient labeled in Fig. 4B was calculated using the CORREL function in Excel.

3. Results

3.1. Murine model study

Two laboratory mouse strains were used in this study: C57BL/6 and BALB/c mice are representative of mouse strains with more Th1- or Th2-dominant immunity, respectively [4,27]. Prior to studies on pregnancy, we conducted D2Dx tests on non-breeding C57BL/6 and BBALB/c mice housed in the same facility to see if the test may be able to detect the Th1/Th2 immunity balance difference between these two mouse strains. In Fig. 2A, the D2Dx test results of these two mice strains are presented across comparable ages. The number of individual mice across the three age groups was 7, 3, 8 for C56BL/6 mice, and 4, 20, 6 for BALB/c mice. At all three age groups, the BALB/c mice exhibited higher test scores than C57BL/6 mice. Using another set of mice (6 C57BL/6 mice and 4 BALB/c

mice), the exact same trend was observed: at the same age, the D2Dx test score of BALB/c mice was always higher than C57BL/6 mice (Fig. 2B). These results demonstrate that the D2Dx test reveals similar trends in mice with distinct genetic backgrounds known to favor Th1 or Th2 response phenotypes in at least certain situations.

We then repeated this analysis in breeding pairs of C57BL/6 and BALB/c mice through several rounds of pregnancy. For C57BL/6 mice, three breeding pairs were monitored weekly from 7 to 17 weeks of age. Blood samples were collected weekly from each breeding mice from 7 to 17 week. Additionally, three female littermates of the breeding females, but not involved in breeding, were used as negative controls to compare to gravid females and collected from 10 to 13 week. For BALB/c mice study, two breeding pairs and two female controls were monitored. All breeding pairs were 5 week-old when breeding began. Blood samples from breeding pair were collected and tested from 5 to 14 week, while the blood samples from female control mice were collected and tested from 7 to 14 week.

Fig. 3A and B presents the D2Dx test scores over the whole study period. The test score of each female breeding mouse is shown individually. The test scores of male breeding mice and female control mice (non-gravid) were averaged separately and presented over time. For both mice strains, we found the D2Dx test score of all pregnant mice increased sharply approximately 7–10 days before parturition. Following the dropping of litters, the test scores generally decreased, although not to the same level prior to pregnancy. The breeder cages were involved in a continuous breeding scheme, meaning that additional pregnancies were expected to occur in the female mice over time. Indeed, when the mice became pregnant again, the test score increased again. Thus, each spike in the curves of the female breeding mice over time represents roughly one cycle of pregnancy and parturition. In contrast, the test scores of male partners and female non-breeding controls all remain rather steady or increased slightly over time. This slight increase is in line with what we have observed previously from a longer-term age-dependent study of C57BL/6 and BALB/c mice [20]. The typical gestation period of mice is 18–21 days. We hypothesize that the D2Dx test can detect significant shifts in blood constituents that might be related to humoral capacity of the gravid mice from middle to late pregnancy, i.e., 7–10 days before parturition.

3.2. Bovine study

Given the findings above, and that we previously found the D2Dx test score to reveal similar patterns in mice housed in controlled specific-pathogen free conditions and in cattle in the field, we next tested bovine blood samples collected from two different locations. In one study, blood samples were collected from 79 pregnant dairy cows from a dairy farm in Wisconsin. These cows were at different stages of pregnancy. We divided the cows into several groups according to their DCC value (days carrying calf), with each group covering an approximately 50 day span of DCC. Fig. 4 shows the average test score of each DCC group. As a general trend, the D2Dx test score increased steadily as pregnancy progressed (Fig. 4A), similar to what was observed from breeding mice. This test score increase over DCC can be roughly fit into a linear curve, with a coefficient of determination $R^2 = 0.95$ (Fig. 4B).

In another study, we analyzed blood samples from 20 Holstein cows during the peripartal period, at −30, −15, −7, 5, 10, and 30 days relative to parturition. Among the 20 cows, 8 were healthy, 8 were subsequently found to have developed ketosis, and 4 were diagnosed with mastitis after calving. Data presented in Fig. 5A are the average test score of each group of cows (healthy, ketosis, and mastitis) during the study period. Fig. 5B is the same plot as Fig. 5A, without standard deviation, but with the average test score of each cow group combined for each sampling day. Fig. 5C is the average test score of healthy, ketosis and mastitis group on day −30 and day −15 combined. From Fig. 5A and B, even though the sample size is relatively small, a clear trend was observed: roughly the test score was the highest just before calving (day −30 and −15). Close to calving and after calving, the test score decreased gradually. Furthermore, we observed that the test score of the healthy group appeared to be able to return to or close to non-pregnant value more quickly than the ketosis and mastitis group. On day 30 after calving, the healthy group maintained a low-test score, while the test score of ketosis and mastitis group increased again to a substantially higher level. The post-calving test scores of the healthy group on day 10 and day 30, roughly at a value between 3.0 and 4.0, are very close to the average test scores of cows in very early pregnancy (as shown in Fig. 4).

Additionally, if we combine the test scores of each cow before calving on day −30 and −15, one can see that the average test scores of the ketosis and mastitis group are higher than the healthy group (Fig. 5C, p value for normal-ketosis group is 0.02; and p value for normal-ketosis/mastitis combined group is 0.01). These data suggest that the D2Dx test is sensitive enough to determine differences in components of humoral immunity regulated by the gravid state from those changes that are induced in gravid (or postpartum) animals by concurrent infection. It is acknowledged here that the number of cows used in this observational study is quite limited. More studies need to be conducted to confirm the difference between healthy and high-risk cows. However, the pattern of increasing test score with progressing pregnancy in general is clear.

4. Discussion

Overall, our study demonstrates that D2Dx test can effectively detect acute changes in systemic components related to immunity balance change during pregnancy and parturition. Our previously reported study established the use of D2Dx test to detect antibody-dependent immune responses in blood that also involve activation of the complement system [20,21]. Both our study in laboratory mice as well as our studies in farm cattle revealed significant and reproducible test score increases during pregnancy. The score reached the highest level just close to parturition and then started to decline around and immediately after parturition. Furthermore, our limited data observed from 20 transition cows suggests that unusually high D2Dx test scores may be linked to increased health risk of transition cows following calving. The exact basis of the increasing test scores in the gravid mice and cattle reported here are as yet unclear, but we hypothesize that they relate to changes in the absolute levels of specific antibody isotypes and/or complement components present in the blood. Whether or not the changes in test score related to pregnancy depend on the same changes in constituents of humoral immunity that we have defined during infection in our previous work requires future study.

While alterations in immune response potential are likely unavoidable and necessary for a successful pregnancy, an unbalanced or suppressed response may expose the post-partum animals to an increased risk of diseases and other health problems. As found from the study of 20 transition cows, cows that developed mastitis and ketosis after calving exhibited exceptionally high-test scores (>10), significantly higher than the healthy transition cows. It may be possible to perform the D2Dx test on pregnant cows 30 to 15 days before calving, and use the test scores to predict the potential health risk level of the cows during transition period. For high risk cows, preventive treatment may be applied, while the low risk cows may be spared from excessive treatment, especially antibiotic treatment.

5. Conclusions

During pregnancy many physiological changes occur including shifts in immune response potential in animals and humans. While necessary for maintaining a successful pregnancy, suboptimal immune-mediated attack against certain invading microbes may result. It is thus important to be able to monitor the immune health of the pregnant animals closely. Here we demonstrate that our previously reported nanoparticle-enabled blood test, D2Dx, may be used to detect the changes in the immune status of both laboratory and farm animals. The test is easy to conduct, of low cost, with results obtained in less than 20 min. This rapid test could be potentially used as an onsite test in local farms and small clinics for cattle health management.

Acknowledgments

Funding information

TMS is supported by funds from the Department of Health and Human Services Eunice Kennedy Shriver National Institute of Child Health & Human Development grant award R21HD093948.

References

[1]. Erlebacher A, Why isn’t the fetus rejected? Curr. Opin. Immunol. 13 (2001) 590–593. [PubMed: 11544009]

[2]. Spellberg B, Edwards JE Jr., Type 1/Type 2 immunity in infectious diseases, Clin. Infect. Dis. 32 (2001) 76–102. [PubMed: 11118387]

[3]. Bretscher PA, On the mechanism determining the Th1/Th2 phenotype of an immune response, and its pertinence to strategies for the prevention, and treatment, of certain infectious diseases, Scand. J. Immunol. 79 (2014) 361–376. [PubMed: 24684592]

[4]. Reiner SL, Locksley RM, The regulation of immunity to Leishmania Major, Annu. Rev. Immunol. 13 (1995) 151–177. [PubMed: 7612219]

[5]. Kidd P, Th1/Th2 balance: the hypothesis, its limitations, and implications for health and diseases, Alternative Med. Rev. 8 (2003) 223–246.

[6]. Romagnani S, T-cell subsets (Th1 versus Th2), ann. Allergy, asthma, Immunol. 85 (2000) 9–18, 21. [PubMed: 10923599]

[7]. Mosmann TR, Sad S, The expanding universe of T-cell subsets: Th1, Th2 and more, Immunol, Today Off. 17 (1996) 138–146.

[8]. Reinhard G, Noll A, Schlebrusch H, Mallmann P, Ruecker AV, Shifts in the Th1/Th2 balance during human pregnancy correlate with apoptotic changes, Biochem. Biophys. Res. Commun. 245 (1998) 933–938. [PubMed: 9588218]

[9]. Morelli S, Mandal M, Goldsmith LT, Kashani BN, Ponzio NM, The maternal immune system during pregnancy and its influence on fetal development, Res. Rep. Biol. 6 (2015) 171–189.

[10]. Sykes L, MacIntyre DA, Yap XJ, Teoh TG, Bennett PR, The Th1/Th2 dichotomy of pregnancy and preterm labor, Mediat. Inflamm. (2012), 2012: 967629.

[11]. Makhseed M, Raghupathy R, Azizieh F, Omu A, Al-Shamali E, Ashkanani L, Th1 and Th2 cytokine profiles in recurrent aborters with successful pregnancy and with subsequent abortions, Hum. Reprod. 16 (2001) 2219–2226. [PubMed: 11574519]

[12]. Yang L, Wang Y, Ma X, Wang S, Zhang L, Changes in expression of Th1 and Th2 cytokines in bovine peripheral blood mononuclear cells during early pregnancy, Indian J. Anim. Res. 50 (2016) 466–470.

[13]. Aleri JW, Hine BC, Pyman MF, Mansell PD, Wales WJ, Mallard B, Fisher AD, Periparturient immunosuppression and strategies to improve dairy cow health during the periparturient period, Res. Vet. Sci. 108 (2016) 8–17. [PubMed: 27663364]

[14]. Sordillo LM, Nutritional strategies to optimize the dairy cattle immunity, J. Dairy Sci. 99 (2015) 4967–4982.

[15]. Trevisi E, Jahan N, Bertoni G, Ferrari A, Minuti A, Pro-inflammatory cytokine profile in dairy cows: consequences for new lactation, Ital. J. Anim. Sci. 14 (2015) 285–292.

[16]. Velasova M, Damaso A, Prakashbabu BC, Gibbons J, Wheelhouse N, Longbottom D, Van Winden S, Green M, Guitian J, Herd-level prevalence of selected endemic infectious diseases of dairy cows in Great Britain, J. Dairy Sci. 100 (2017) 9215–9233. [PubMed: 28843682]

[17]. McConnel CS, Lombard JE, Wagner AE, Garry FB, Evaluation of factors associated with increased dairy cow mortality on United States dairy operations, J. Dairy Sci. 91 (2008) 1423–1432. [PubMed: 18349234]

[18]. Regal JF, Gilbert JS, Burwick RM, The complement system and adverse pregnancy outcome, Mol. Immonol. 67 (2015) 56–70.

[19]. Denny KJ, Woodruff TM, Taylor SM, Callaway LK, Complement in pregnancy: a delicate balance, Am. J. Reprod. Immunol. 69 (2013) 3–11. [PubMed: 22925193]

[20]. Zheng T, Crews J, McGill JL, Dhume K, Finn C, Strutt T, McKinstry KK, Huo Q, A single-step gold nanoparticle–blood serum interaction assay reveals humoral immunity development and immune status of animals from neonates to adults, ACS Infect. Dis. 5 (2019) 228–238. [PubMed: 30521752]

[21]. Zheng T, Finn C, Parret CJ, Dhume K, Hwang JH, Sidhom D, Strutt TM, Li Sip YY, McKinstry KK, Huo Q, A rapid blood test to determine the active status and duration of acute viral infection, ACS Infect. Dis. 3 (2017) 866–873. [PubMed: 28918638]

[22]. Zheng T, Bott S, Huo Q, Techniques for accurate sizing of gold nanoparticles using dynamic light scattering with particular application to chemical and biological sensing based on aggregate formation, ACS Appl. Mater. Interfaces 8 (2016) 21585–21594. [PubMed: 27472008]

[23]. Huo Q, Litherland SA, Sullivan S, Hallquist H, Decker DA, Rivera-Ramirez I, Developing a nanoparticle test for prostate cancer scoring, J. Transl. Med. 10 (2012) 44. [PubMed: 22404986]

[24]. Huo Q, Cordero A, Bogdanovic J, Colon J, Baker CH, Goodison S, Pensky M, A facile nanoparticle immunoassay for cancer biomarker discovery, J. Nanobiotechnol. 9 (2011) 20.

[25]. Pecora R, Dynamic light scattering measurement of nanometer particles in liquids, J. Nanoparticle Res. 2 (2000) 123–131.

[26]. Berne BJ, Pecora R, Dynamic Light Scattering: with Applications to Chemistry, Biology, and Physics, John Wiley & Sons, New York, 1976.

[27]. Watanabe H, Numata K, Ito T, Takaqi K, Matsukawa A, Innate immune response in Th1- and Th2-dominant mouse strains, Shock 22 (2004) 460–466. [PubMed: 15489639]

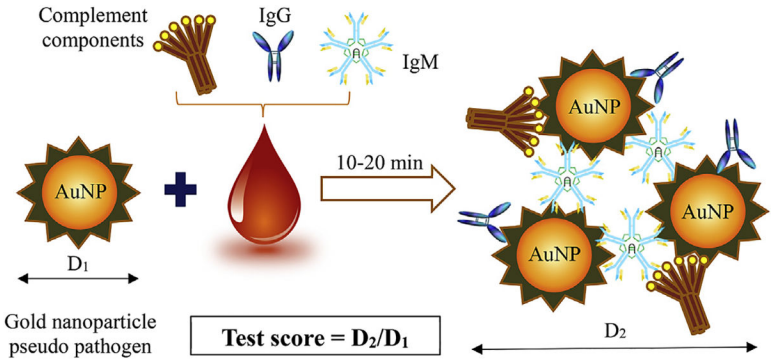

Fig. 1.

Illustration of the principle of D2Dx immunity test. In this test, a gold nanoparticle (AuNP) pseudo pathogen is mixed with a blood serum sample. Proteins that are major part of the humoral immune system, including IgG, IgM and complement proteins, will interact with the nanoparticles in a way similar to what happens *in vivo*. This interaction will lead to gold nanoparticle aggregate formation and the nanoparticle aggregates are detected by measuring the average particle size change of the assay solution using a particle sizing technique called dynamic light scattering. The test result is expressed as a test score, defined as the ratio of the average particle size of the assay solution product (D_2) versus the average particle size of the original AuNP solution (D_1). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

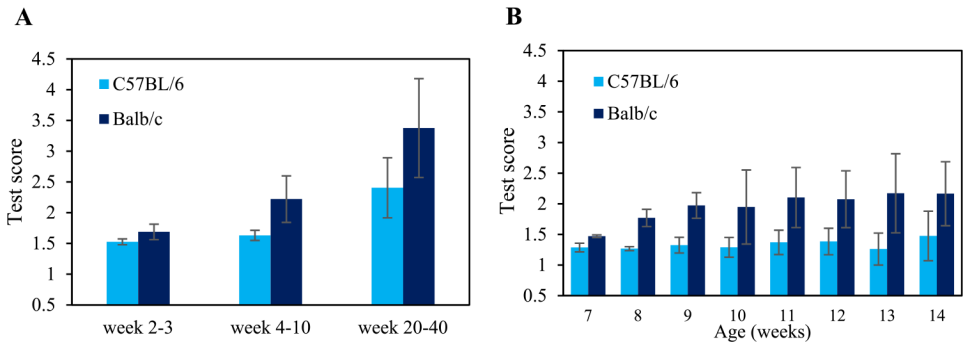

Fig. 2.

D2Dx immunity test score of two mouse model strains, C57BL/6 and BALB/c, from two studies. (A) The number of mice in the three age groups is 7, 3, 8 for C56BL/6 mice, and 4, 20, 6 for BALB/c mice, respectively. (B) The ages of C57BL/6 and BALB/c mice are matched exactly. The test was conducted weekly for seven weeks, starting from week 7. The number of mice used in this study is 6 for C57BL/6 and 4 for BALB/c mice, respectively. Error bars represent the standard deviation of each mouse strain group.

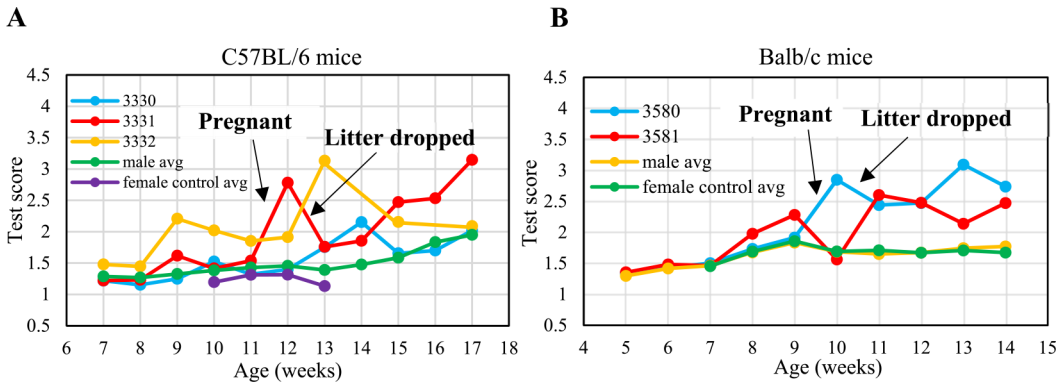

Fig. 3.

D2Dx immunity test score change of C57BL/6 (A) and BALB/c (B) mice during pregnancy and parturition. For C57BL/6 group (A), 3330, 3331, and 3332 are three female breeding mice. For BALB/c group (B), 3380 and 3381 are the female breeding mice. Male average is the average test score of corresponding male mice partners to the female breeding mice (3 for C57BL/6 group, and 2 for BALB/c group). Female controls are not placed for breeding. 3 non-breeding female C57BL/6 and 2 non-breeding female BALB/c mice were used as negative controls. Breeding began when the C57BL/6 mice were 7 weeks old and the BALB/c mice were 5 weeks old. Blood samples were collected every 7 days from each study mouse.

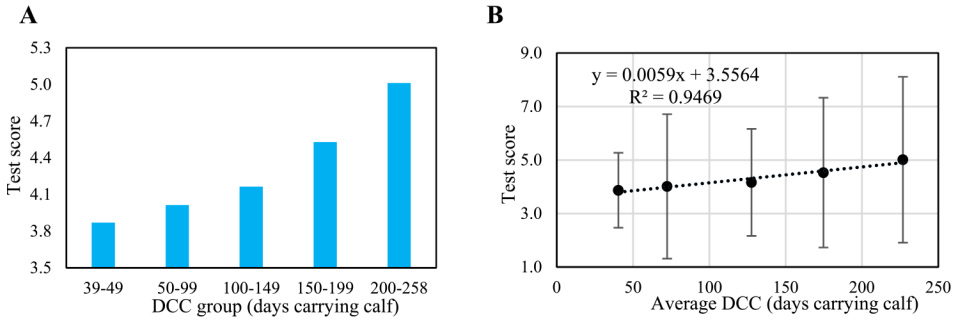

Fig. 4.

D2Dx test score change of pregnant dairy cows with progressing DCC (days carrying calf). Total 79 cows were divided into 5 DCC groups. Each DCC group expands approximately 50 days. (A) is the average test score of each DCC group. N = 5, 17, 14, 17 and 26 for DCC group 39–49 days; 50–99 days; 100–149 days; 150–199 days; and 200–258 days, respectively. (B) is the plot of the average DCC versus the D2Dx test score. The D2Dx-DCC correlation can be fitted into a linear curve, with an R^2 value of 0.95.

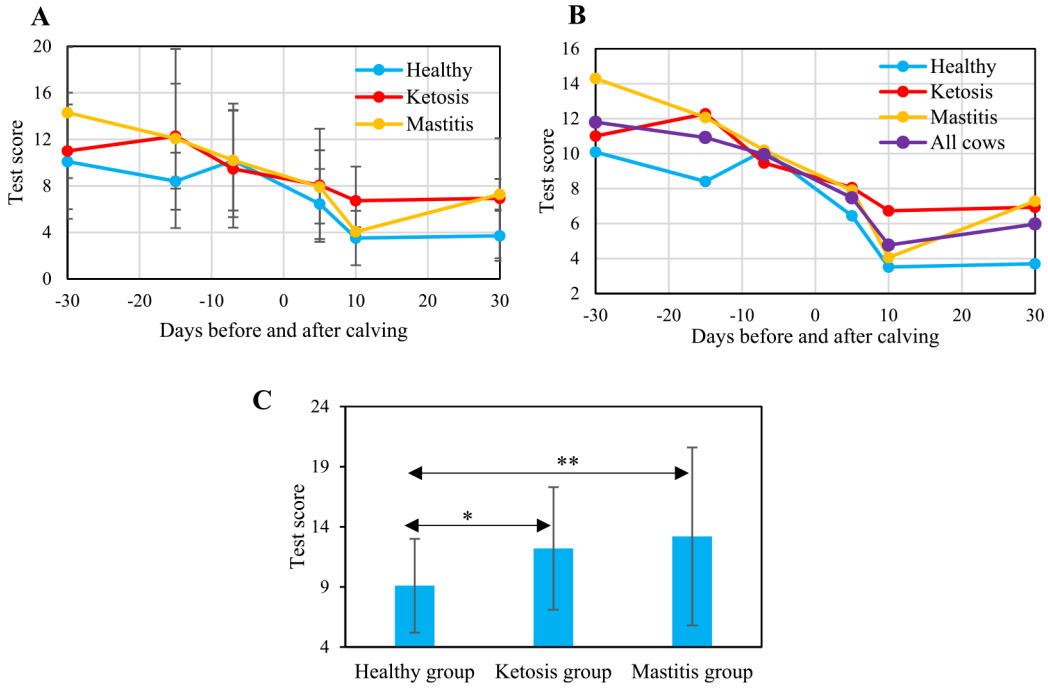

Fig. 5.

D2Dx test score of dairy cows during transition period. Total 20 cows were studied. Among the 20 cows, 8 were observed as healthy, 8 developed ketosis, and 4 developed mastitis following calving. From each cow, 6 blood samples were collected on day −30, −15, −7, 5, 10 and 30 before and after calving. (A) The average test score of each cow group (healthy, ketosis and mastitis), with standard deviation shown in the plot. (B) The same plot as (A), without standard deviation, but with the average test score of all 20 cows added in the plot. (C) is the average test score of healthy, ketosis and mastitis group on day −30 and −15 combined.