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Short communication: Relationship between natural antibodies and postpartum uterine health in dairy cows

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ABSTRACT

Postpartum uterine diseases of dairy cows compromise animal welfare and may result in early removal from the herd or impaired reproductive performance. The relationship between poor immune status around calving and uterine diseases is well established; however, that between natural antibodies (NAb) and uterine health has not yet been studied. The objective of this study was to evaluate the association of circulating NAb levels around parturition with puerperal metritis, clinical endometritis, and the intrauterine presence of the *Escherichia coli* virulence factor FimH. One hundred six pregnant heifers were enrolled; NAb in serum samples collected at 30 ± 3 d prepartum and at 2 ± 1 and 35 ± 3 d in milk (DIM) were measured by ELISA. Puerperal metritis was defined as the presence of fetid, watery, red-brown uterine discharge and rectal temperature >39.5°C at 6 ± 1 DIM. Clinical endometritis was defined as presence of pus in the uterine lavage sample collected at 35 ± 3 DIM. The intrauterine presence of the *fimH* gene at 2 ± 1 DIM was evaluated by PCR. The overall optical density (wavelength of 650 nm) of ELISA-detected serum NAb was lower for cows diagnosed with puerperal metritis than for cows that did not have puerperal metritis. Additionally, cows diagnosed with clinical endometritis tended to have lower levels of NAb than did cows without clinical endometritis. Finally, FimH-positive cows had lower overall levels of serum NAb compared with FimH-negative cows. In conclusion, NAb detected in serum around parturition was associated with uterine health of dairy cows.
Key words: natural antibodies, metritis, endometritis, immune system

Short Communication

Postpartum uterine diseases of dairy cows are important for animal welfare and economic reasons because they contribute to cow discomfort, early removal from the herd, and reproductive failure. In North America, metritis affects 10 to 20% of cows (LeBlanc et al., 2011), whereas the incidence of endometritis is approximately 28%, ranging from 5.3 to 52.6% (Dubuc et al., 2010; Cheong et al., 2012). *Escherichia coli*, *Trueperella pyogenes*, *Bacteroides* spp., and *Fusobacterium necrophorum* are the primary bacterial causes of uterine diseases (Dohmen et al., 1995; Miller et al., 2007; Bicalho et al., 2012). The virulence factor FimH, an *E. coli* type 1 pilus adhesive protein that plays a critical role in adhesion and colonization of epithelial surfaces (Mooi and de Graaf, 1985), is highly associated with uterine diseases and impaired reproductive performance in dairy cows (Bicalho et al., 2010, 2012).

The relationship between poor immune status around calving and uterine disease is already well established (Cai et al., 1994; Kimura et al., 2002; Kim et al., 2005; Hammon et al., 2006; Galvão et al., 2010), and recruitment of PMNL to the endometrial surface and the uterine lumen is critical for the immune defense of the uterus (Bondurant, 1999). However, these studies have focused only on the cellular component of the innate immune system.

Natural antibodies (**NAb**) are an important component of the humoral part of the innate immune system (Avrameas, 1991); they are present in the sera of normal, nonimmunized individuals (Sidman et al., 1986; Madi et al., 2009). They are produced by B-1 cells and spontaneously generated without the need for antigenic stimulation (Tarlinton et al., 1995). Natural antibodies can bind to pathogen-associated molecular patterns (Baumgarth et al., 2005), and they may represent the first line of defense by directly neutralizing the pathogen and activating the complement system (Ochsenbein and Zinkernagel, 2000). It has been reported that plasma NAb concentration is decreased during the periparturient period (van Knegsel et al., 2007), and higher levels of NAb tended to be associated with decreased risk of mastitis (Thompson-Crispi et al., 2013). However, to the best of our knowledge, the relationship between NAb and postpartum uterine health has not yet been studied. Therefore, the objective of this study

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was to evaluate the association between circulating NAb levels detected at 30 ± 3 d prepartum and at 2 ± 1 and 35 ± 3 DIM, the incidence of puerperal metritis and clinical endometritis, and the intrauterine presence of the *E. coli* virulence factor FimH.

This study was conducted from June 16 to November 16, 2012, in a commercial dairy farm located near Ithaca, New York, and 106 pregnant heifers were enrolled. Serum samples were collected from each cow 3 times during the study period: at 30 ± 3 d prepartum and at 2 ± 1 and 35 ± 3 DIM. To obtain serum samples, blood was collected from the coccygeal vein or artery using a Vacutainer tube without anticoagulant and a 20-gauge × 2.54-cm Vacutainer needle (Becton Dickinson and Co., Franklin Lakes, NJ). After collection, all blood samples were transported to the laboratory on ice and centrifuged at 2,000 × *g* for 15 min at 4°C, and the serum was harvested and frozen at −80°C.

Puerperal metritis diagnosis was performed at 6 ± 1 DIM by the research team. Puerperal metritis was defined as the presence of fetid, watery, red-brown uterine discharge and rectal temperature >39.5°C (Sheldon et al., 2006). Clinical endometritis was diagnosed at 35 ± 3 DIM by visual inspection of a uterine lavage sample for the presence of purulent secretion, as described previously (Machado et al., 2012); clinical endometritis was defined by the presence of pus in the lavage sample. Body condition scores were determined for all study cows at 30 ± 3 d prepartum and at 2 ± 1 and 35 ± 3 DIM by a single investigator using a 5-point scale with a quarter-point system as previously described (Edmonson et al., 1989).

Cervical swabs were collected at 2 ± 1 DIM for FimH detection by PCR; cows were restrained and the perineum area was cleansed and disinfected with 70% ethanol solution. The swab was manipulated inside the cervix and exposed to uterine secretion. The swabs were kept inside sterile vials at 4°C until processed in the laboratory. In the laboratory, swab samples were immersed in 1 mL of PBS in a 15-mL Falcon tube and vortexed to disperse any mucus, bacteria, or cells. Total DNA was isolated from 400 μL of the suspension by using a QIAmp DNA minikit (Qiagen, Santa Clara, CA) according to the manufacturer's instructions for DNA purification from blood and body fluids. Some convenient modifications, such as the addition of 400 μg of lysozyme and incubation for 12 h at 56°C, were included to maximize bacterial DNA extraction. Total DNA was eluted in 100 μL of sterile DNase- and RNase-free water (Promega, Madison, WI). The concentration and purity of DNA were evaluated by optical density using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE), and the *fimH* gene was amplified by PCR. All reactions were performed in a 25-μL volume using Green GoTaqMasterMix (Promega), 10 pmol of primers (forward: TGCAGAACGGATAAGCCGTGG; reverse: GCAGTCACCTGCCCTCCGGTA), and 1 μL of DNA extract. All thermal cycling protocols were performed in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA); the thermal cycling protocol was 95°C for 12 min, followed by 25 cycles at 94°C for 30 s, 63°C for 30 s, and 68°C for 3 min, followed by a final extension at 72°C for 10 min. Negative controls consisting of the PCR mixture without DNA were included in all PCR runs. Amplification products were separated by electrophoresis through a 1.2% (wt/vol) agarose gel, stained with 0.5 μg/mL ethidium bromide, and visualized with a Kodak Gel Logic 100 Imaging System (GL 100, Scientific Imaging Systems, Eastman Kodak Co., New Haven, CT). Positive results were considered to be amplicons of 508 bp.

Because keyhole limpet hemocyanin (**KLH**) is a metalloprotein found in the hemolymph of the giant keyhole limpet, *Megathura crenulata* (Harris and Markl, 1999), dairy cattle are naive to it. Therefore, it is unlikely that it would be recognized by specific serum immunoglobulins of dairy cattle, which makes KLH a good antigen with which to measure NAb. Hence, NAb in the serum samples were measured by ELISA as follows: ELISA micro-titer plates (Greiner Bio-One, Frickenhausen, Germany) were coated with 0.1 *M* carbonate buffer pH 9.2 containing 1 μg/mL of KLH (Sigma Aldrich, St. Louis, MO). Binding of antigen to microtiter wells was carried out overnight at 4°C; nonspecific binding sites were blocked with PBS containing 3% fish gelatin (Sigma Aldrich) for 3 h at room temperature. Dilutions of bovine serum samples were then added to the ELISA plates; serum samples were diluted in proportions of 1:40 in PBS containing 0.5 *M* NaCl and 0.5% Tween-20. The serotype-specific antibody bound to the ELISA plate was detected with anti-bovine IgG antibody conjugated with horseradish peroxidase, diluted according to the manufacturer's instructions (Sigma Aldrich), followed by addition of the substrate, 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma Aldrich). The optical density (**OD**) of each well was measured after 20 min at 650 nm using an ELISA plate reader (Synergy HT microplate reader, BioTek Instruments, Winooski, VT). The amount of color produced was proportional to the amount of primary antibody bound to the protein on the bottom of the wells. The assays were performed in 3 wells for each sample; in 2 wells, the assays were performed in duplicate, as described above, and the third well was a blank. The blank well was prepared using the same steps performed in the duplicates, except that it was not coated with the antigen. Data were reported as OD at 650 nm ($\mathbf{OD_{650}}$), a result of the average OD of the