Innate immune gene variation and differential susceptibility to uterine diseases in Holstein cows

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Article history:
Received 14 March 2013
Received in revised form 29 April 2013
Accepted 30 April 2013

Keywords:
Metritis
Endometritis
Toll-like receptors
Candidate gene

Abstract
An immune response is mounted after binding of Toll-like receptors (TLRs) to pathogen-associated molecular patterns. The primary objective of this study was to test for the associations between bovine single-nucleotide polymorphisms (SNPs) and insertion-deletion (indel) mutations occurring in seven bovine TLR genes (TLRs 1, 2, 4, 5, 6, 9, and 10) that are known to recognize bacterial ligands and the most significant uterine diseases in dairy cows, including metritis (MET), clinical endometritis (CE), and cytologic endometritis (CYE). Custom allele-specific genotyping assays derived from multiple bovine TLR sequencing studies were utilized. Genotypes for 110 loci (SNPs and indels) that are known to be variable in domestic cattle were determined, resulting in 46 monomorphic loci, 64 loci with two alleles, and 35 loci that did not meet our inclusion criterion for minor allele frequency (≥0.10). The association between specific TLR genotypes and each of the uterine diseases (MET, CE, CYE) was evaluated by logistic regression with correction for confounding variables. Collectively, seven SNPs produced uncorrected P values ≤0.05 with respect to three different uterine diseases investigated, but none of the SNP associations endured correction for multiple testing (P values >0.05). Several confounding variables, including parity, dystocia, and ketosis before 17 DIM, remained significant after correction for multiple testing. Our analysis of these data suggest that some bovine TLR SNPs (i.e., TLRs 2, 4, 6, 9) may potentially elicit relatively small effects on uterine health in Holstein dairy cows and that some confounding variables are actually more predictive for the incidence of disease than any genetic markers evaluated herein.

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1. Introduction

A key component for bovine reproductive efficiency is the maintenance of adequate uterine health that allows for rapid resumption of reproductive function after calving. However, the challenge of uterine contamination with microorganisms at parturition is unavoidable with bacteria present in the uterus of 80% to 100% of cows within the first 2 weeks after calving [1,2]. Notably, some cows respond to this challenge successfully, but about 20% of the cows will subsequently develop metritis (MET) within 3 weeks postpartum [2,3]. After 3 weeks, a similar proportion of cows will develop clinical endometritis (CE) [4,5], and the prevalence of the subclinical condition will range from 26% to 74% between 40 and 60 DIM [5–7]. Importantly, both metritis and endometritis contribute to increased days to first breeding, decreased conception and pregnancy rate, and increased culling [5–9]. In addition to tangible economic losses consisting of ~$285 per lactation [6], animal welfare is also compromised by uterine diseases; affected cows suffer from loss of appetite, often become dehydrated, and may also show signs of pain [1].
Genetic selection for increased disease resistance in cattle has been proposed [10,11], and a recent study reported that dairy cattle identified as high immune responders (i.e., individuals mounting a significant general antibody and cell-mediated immune response) were at lower risk of developing disorders such as mastitis, metritis, and retained placenta [12]. At the molecular level, genes that represent one of the first lines of host defense by modulating innate immunity responses against a variety of invading pathogens have also been considered putative candidate loci for improving host resistance to disease in agricultural species [13]. Innate immune receptors recognize a variety of molecular ligands derived from diverse microbial species, thereafter eliciting host responses to invading pathogens without requiring prior exposure [14–16]. The totality of this response depends on the action of neutrophils (polymorphonuclear cells (PMNs)), monocytes, and macrophages, with pattern recognition receptors that initiate signaling pathways leading to the release of proinflammatory cytokines [17]. One important group of pattern recognition receptors are the Toll-like receptors (TLRs), which recognize a variety of microbial components (pathogen-associated molecular patterns), and subsequently regulate the activation of both innate and adaptive immunity [14,15,18]. At least 10 members of the TLR gene family are known to exist in mammals, with all members encoding type I transmembrane proteins of the interleukin-1 receptor family that consists of N-terminal leucine-rich repeats involved in ligand recognition, a transmembrane domain, and C-terminal intracellular Toll/IL-1 receptor homologous domain for signal transduction [10,14,15]. Four TLRs have been associated with the recognition of viral constituents (TLRs 3, 7, 8, 9), whereas six (TLRs 1, 2, 4, 5, 6, 9) are known to recognize microbial and/or synthetic ligands [18–20]. Still, the ligand specificity of TLR10 remains elusive [21]. Importantly, previous studies reported that the mammalian TLR genes are primarily expressed by antigen-presenting cells, including macrophages, natural killer cells, and dendritic cells [19,22], with several studies also reporting that some naturally occurring TLR variants increased the risk of severe infections in humans, mice, and domestic cattle [23–25]. Relevant to bovine reproduction, the initial defense of the endometrium against invading microbes is dependent on the innate immune system, including initial recognition by host TLR proteins and secretion of antimicrobial peptides, cytokines, and acute phase proteins [3,26,27]. Moreover, neutrophils provide the initial cellular defense against bacterial colonization within the uterus [26,28–30], and cows diagnosed with puerperal metritis and subclinical endometritis are known to have significantly reduced blood PMN functions during the periparturient period, when compared with cows with normal uterine health [30,31]. Moreover, the ability of neutrophils to perform multiple antimicrobial effector functions as well as the induction of cytokines and chemokines that ultimately serve to recruit other immune cells is largely dependent on recognition and signaling by host TLRs [18]. Therefore, we hypothesized that some naturally occurring variation within the bovine TLR genes may be associated with differential susceptibility to several economically important reproductive diseases in Holstein dairy cattle. Moreover, the primary objective of this study was to test for associations between bovine single-nucleotide polymorphisms (SNPs) and indels within seven bovine TLR genes (TLR 1, 2, 4, 5, 6, 9, and 10) that are known to recognize microbial ligands and the most economically significant uterine diseases affecting dairy cows. Herein, we provide evidence that some bovine TLR variants may potentially elicit small effects related to risk for metritis, clinical endometritis, and cytologic endometritis.

2. Materials and methods

2.1. Study population, general management, and disease monitoring

The study was conducted for 1 year, spanning from October 2010 to October 2011 within the University of Florida Dairy Unit (Gainesville, FL, USA). The study cohort consisted of 358 Holstein cows (164 primiparous and 194 multiparous) that were genotyped. Diagnosis of CE and CYE was not available for a total of 6 and 25 cows, respectively. The research herd consisted of 550 lactating cows, with a yearly rolling herd average for milk production of 9910 kg. The herd was milked twice daily and was a member of the Dairy Herd Improvement Association (Raleigh, NC, USA), with an on-farm computer-based record system (AfIFarm, SAE Afikim Kibbutz Afikim, Israel). Prepartum transition cows that were within 2 weeks of calving were maintained in a maternity barn, fed a low DCAD diet, and monitored for signs of calving by farm employees trained to assist with parturition. Calving events, such as dystocia, twins, and retained fetal membranes (RFMs), were recorded by the farm personnel. Cows that did not expel the fetal membranes within 24 hours after calving were considered to have RFM. After parturition, cows were sent for 2 days to an open hospital facility with shade and a sand covered floor. At 3 days postpartum, healthy cows were moved to the lactating herd and kept in a barn consisting on freestall facilities with a concrete floor. Cows were fed a totally mixed ration formulated to meet or exceed the requirements of lactating dairy cows weighing ~680 kg and producing 45 kg of 3.5% FCM, as recommended by the National Research Council [32]. All cows went through a routine postpartum health monitoring protocol that consisted of an evaluation on Days 4, 7, and 12 after calving, as performed by trained farm personnel or veterinarians from the University of Florida. The protocol included the assessment of attitude, rectal temperature, rectal palpation, and examination of vaginal discharge, udder inspection, assessment of urine ketone bodies (Ketostix, Bayer Corporation, Elkhart, IN, USA) and investigation of abomasum displacement. In addition, automatic health reports were created for every milking event based on individual milk production and milk component levels provided by the AfIMilk meters (SAE Afikim Kibbutz Afikim, Israel). Cows with deviations from pre-established ranges on at least two parameters (milk and milk components) within two consecutive milkings were automatically sorted for a complete health check.
2.2. Enrollment, classification, and diagnostic procedures

This was an observational cohort study with cows enrolled within 3 days after parturition and followed until 35 ± 3 days after parturition. Cows that had cesarean section or fetotomy were excluded from the study. The farm was visited twice per week and cows were assigned a body condition score at calving, at enrollment, and at 35 DIM using a scale of 1 to 5 according to Ferguson et al. [33]. Diagnosis of MET was performed during routine health evaluations at Days 4, 7, and 12 after calving; cows with deviations on milk and milk components were also submitted for complete health check. Cows were examined at 35 ± 3 DIM for CE and CYE. Diagnosis of CE consisted in the inspection of vaginal discharge using the Metrichcheck device (Simcro, Waikato, New Zealand) and a score on a scale of 0 (no material) to 5 (grossly purulent and with an odor) [34]. Examination procedures for CYE diagnosis included the collection of endometrial cytology samples using the cyobrush technique [6] and the preparation of glass microscopic slides for cell count under the microscope to determine the proportions of PMNs in the sample [6].

Three reproductive disorders mainly caused by bacteria [1,5,8] were the primary outcomes of interest, and thus, considered as individual binary dependent variables as follows: (1) MET, defined as cows that had an abnormally enlarged uterus, reddish-brown fetid uterine discharge with or without a fever within 21 days postpartum [1]; (2) CE, defined as the presence of purulent (>50% pus) or mucopurulent (≈50% pus, 50% mucus) uterine discharge detectable in the vagina at 35 ± 3 days postpartum [4]; and (3) CYE, defined as the presence of ≥10% of PMNs in a cytological sample taken from cows at 35 ± 3 days postpartum [35]. For detection of PMN, 200 cells were counted from each slide, and the results were expressed as a percentage of total cells (excluding erythrocytes). Data from the routine postpartum examinations and information related to variables potentially affecting uterine disease (i.e., calving, production, and health events) were collected from on-farm software (AffiFarm farm information software) for potential inclusion in the logistic regression models.

2.3. DNA isolation procedures and TLR genotyping

Whole blood (10 mL per cow) was collected from the coccygeal vein into vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) with EDTA, and DNA was isolated using a commercial kit, according the manufacturer’s recommendations (MoBio UltraClean Tissue and Cells DNA Isolation Kit, Carlsbad, CA, USA). Purified DNA was assessed for quality and concentration by standard spectrophotometric methods. Bovine TLR sequencing, validation of variable sites, and haplotype studies have previously been reported for many beef and dairy cattle breeds (n = 37, including Holsteins), with validated SNPs and indels incorporated within an allele-specific genotyping system [10,16,20,36]. For this study, we selected validated SNPs and indels (n = 110) within the 5′-upstream regions, introns, and those encoding nonsynonymous or putative nonsense mutations in bovine TLR genes recognizing bacterial ligands (TLRs 1, 2, 4, 5, 6, 9, and 10; for review see [10,13–20]). Genotyping assays were performed using the KASPar allele-specific fluorescent genotyping system (Kbiosciences, Hertfordshire, UK), as previously described [10,16]. Thermal cycling parameters and reaction concentrations followed the manufacturer’s recommendations, with some modifications to the MgCl2 concentrations. Primer sequences and MgCl2 concentrations are available on request. Genotype clustering and calling were performed using KlusterCaller and/or SNPviewer software (Kbiosciences). Genotype quality was assessed by manually inspecting the cluster data for every marker across all research cows. Failed assays (i.e., reaction failure, no data) and/or individual data points (genotypes), which did not reside within the high-quality genotype clusters, were subsequently rerun, and a follow-up quality control analysis was performed by manual inspection. Assay reruns that provided high-quality genotypes were included in the final data set.

2.4. Statistical analysis

Bovine TLR variable sites (SNPs, indels) that were monomorphic across all investigated cows were excluded from further investigation. The minimum minor allele frequency (MAF) required for inclusion of an SNP or indel in all single-marker tests of association was 0.10, as previously described [10]. Data for variables that may potentially influence the manifestation of uterine disease [8,37,38] were noted, scored, and assembled for further investigation including season of calving; parity; body condition score (BCS) at calving (BCSCv), at enrollment (BCSEn), and at 35 DIM (BCS35); dystocia (scored from 0 which = no assistance to 5 which = C-section/fetotomy required); ketosis before and after 17 DIM; RFM; hypocalcemia; twins; calf dead on arrival; abortion; sire; dam; and maternal grand sire. Notably, only cases of ketosis occurring before the onset of a uterine disease event were considered in this study. Additionally, some variables were further categorized as follows: parity as 1 (primiparae) or ≥2 (multiparae); season of calving: winter (January to March), spring (April to June), summer (July to September), and fall (October to December); dystocia: no = score < 3, yes = score ≥ 3. Variables with individual count data >5 and ≤10% missing data that exhibited confounding effects (P ≤ 0.10) via logistic regression with respect to uterine disease (MET, CE, CYE) were subsequently used as covariates to correct the TLR single-marker logistic regression models. Specifically, we used two regression models referred to as the reduced model and the full model, with the significance of the genetic marker estimated after removing the effects of confounding variables by performing a full versus reduced model analysis. For the “full versus reduced” logistic regression model, logistic regression equations are obtained for both the full and reduced models. The reduced model includes only the dependent variable (MET, CE, CYE) and any confounding covariates as defined above. The full model includes all of the variables, including the dependent variable, all confounding covariates, and each genetic marker. A likelihood ratio statistic is calculated to find the
significance of including the full model regressors versus not including them. The restricted likelihood of the reduced model is represented by \( L_0 \), and \( L_1 \) is the restricted likelihood of the full model. Both \( L_0 \) and \( L_1 \) are computed as below:

\[
l_0 = \log L_0 = \sum_{i=1}^{n} y_i \log \left( \frac{1}{1 + e^{-\beta y_i + \gamma_i}} \right) + (1 - y_i) \log \left( \frac{1}{1 + e^{-\beta y_i + \gamma_i}} \right),
\]

\[
l_1 = \log L_1 = \sum_{i=1}^{n} y_i \log \left( \frac{1}{1 + e^{-\beta y_i}} \right) + (1 - y_i) \log \left( \frac{1}{1 + e^{-\beta y_i}} \right),
\]

and \( P = P(X > -2(l_0 - l_1)), \) where \( X \sim \chi^2(m - k) \), where \( m \) are the degrees of freedom of the full model and \( k \) are the degrees of freedom of the reduced model. Here, \( \beta \) is the reduced model vector of slope coefficients and \( \hat{\beta} \) is the full model vector of slope coefficients. All logistic regression analyses were performed for genotypes that were numerically recoded for the evaluation of additive, dominant, and recessive inheritance models using SVS7 (version 7.7.2; Golden Helix, Bozeman Montana), with all relevant formulae and coding procedures for inheritance models described in detail online (http://doc.goldenhelix.com/SVS/latest/formulas_theories.html#ftlogreg). For all numerically recoded marker genotypes that were based on genetic inheritance models (i.e., additive, dominant, recessive) and the number of major or minor alleles present, we excluded markers from the regression analyses that possessed numerical categories that occurred \( \leq 5 \) times regardless of the MAF. The reason for exclusion is that sparse data in certain categories may lead to \( P \) values \( < 0.05 \), but with very broad confidence intervals for the odds ratios, which can lead to spurious conclusions about the true economic or biomedical importance of the marker in question. All single-marker \( P \) values were corrected for multiple testing by applying the FDR correction (http://sdpproject.com/utilities/?show=FDR) [39] to the raw \( P \) values derived from association tests against each dependent variable (MET, CE, CYE).

### 3. Results

The final study cohort consisted of 358 Holstein cows (164 primiparae and 194 multiparae). Overall, 109/358 (30.4%), 65/352 (18.5%), and 108/333 (32.4%) cows were diagnosed with MET, CE, and CYE, respectively. Genotypes for 110 known TLR variable sites (i.e., bovine SNPs and indels [10]) were determined. Altogether, 46 of these loci were monomorphic in our cohort, and 35 did not meet our MAF inclusion criterion, thus leaving 29 variable loci with MAF \( \geq 0.10 \) for inclusion in the association analyses. Logistic regression models were constructed for each of 29 variable sites to estimate the relative odds of uterine disease (MET, CE, and CYE) based on our defined diagnostic criteria with adjustment for the effects of confounding variables. Collectively, single-marker logistic regression models for seven SNPs produced uncorrected \( P \) values \( \leq 0.05 \) with respect to the three different uterine disease classifications (MET, CE, CYE) of interest. These consisted of four mutations located in bovine TLR9 (MET), two SNPs (TLR4, TLR6) that produced suggestive associations with CE, and one mutation (TLR2) indicative of a potential association with CYE (see Tables 1–3).

Covariates included in the final model for MET comprised calving season (\( P < 0.10 \)), parity (\( P < 0.0012 \)), dystocia (\( P < 0.0000095 \)), BCSEn (\( P < 0.031 \)), and ketosis \( < 17 \) DIM (\( P < 0.000027 \)). Covariates that were included in the final model for CE were calving season (\( P < 0.10 \)), dystocia (\( P < 0.000027 \)), ketosis within 17 DIM (\( P < 0.08 \)), and hypocalcemia (\( P < 0.058 \)). No covariates were retained in the final model for CYE. Following correction for multiple testing, \( P \) values for all TLR markers exceeded the 5% significance level.

### 4. Discussion

Although none of the SNPs reported statistically significant associations after correction for multiple testing, it is interesting to note the specific properties of the TLR markers that were most predictive for the uterine health outcomes of interest (MET, CE, CYE). For example, three of the SNPs located in bovine TLR9 (TLR9_A945G, TLR9_G1187A, and TLR9_C2788T) that produced uncorrected \( P \) values \( \leq 0.05 \) with respect to metritis are non-synonymous mutations that occurred under a recessive model of inheritance, with the minor allele increasing the risk for metritis occurrence (Table 1). This trend is consistent with the potential for diminished receptor

### Table 1

<table>
<thead>
<tr>
<th>Marker</th>
<th>dbSNP ID</th>
<th>Model</th>
<th>ORa</th>
<th>95% CIb</th>
<th>P valuec</th>
<th>Major allele</th>
<th>Risk allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR9_A945G</td>
<td>rs55617138</td>
<td>Recessive</td>
<td>2.00</td>
<td>1.11 3.60</td>
<td>0.020</td>
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<td>G</td>
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<td>TLR9_G1187A</td>
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<td>1.89</td>
<td>1.04 3.40</td>
<td>0.035</td>
<td>G</td>
<td>A</td>
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<td>TLR9_G1401A</td>
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<td>Recessive</td>
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<td>1.00 3.29</td>
<td>0.050</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td>TLR9_C2788T</td>
<td>ss469376155</td>
<td>Recessive</td>
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<td>1.12 3.58</td>
<td>0.019</td>
<td>C</td>
<td>T</td>
</tr>
</tbody>
</table>

Only SNPs with uncorrected \( P \) value \( \leq 0.05 \) are presented.

- \( a \) Odds ratio adjusted for significant covariates.
- \( b \) 95% confidence interval for odds ratio.
- \( c \) \( P \) value not corrected for multiple comparisons.
function as a result of mildly deleterious genetic variation and should be further investigated with respect to specific bacterial pathogens that are known to commonly cause metritis. Moreover, at least one recent human study indicates that minor alleles generally confer risk for disease more often than protection [40], which is also consistent with widespread purifying selection [41]. In contrast, for the two SNPs that produced uncorrected P values <0.05 with respect to CE (TLR4_C9564T, intron, and TLR6_G14578A, missense D214H), the minor allele was associated with a lower risk for disease (Table 2). Finally, the single SNP that was most predictive for risk of CYE (uncorrected P value <0.05) in our analysis (TLR2_C9564T) is located in the intron region of bovine TLR2, and the major allele was associated with increased risk for CYE in both the additive and dominant models (Table 3). Interestingly, variable genetic loci with minor alleles that confer protection, or major alleles that increase risk for disease, could potentially be pleiotropic, whereby the major or minor allele can have opposite effects for different traits; for example, in relation to an unknown fitness trait that drives selection pressure and a uterine health trait (MET, CE, CYE) that we investigated in this study [40,42].

Relevant to the purpose of our investigation, genetic studies of fertility-related diseases provide a wide range of estimates of heritability and genetic correlations among traits, with some results that are contradictory [43]. Limited information is currently available on genes associated with reproductive diseases in dairy cattle, and thus a need clearly exists, yet no conclusive results have been reported [44]. Nevertheless, important bovine health-related QTLs have been localized to genomic regions either proximal to or overlapping one or more TLR loci [20,36,45], with one recent study also providing evidence that some bovine TLR variation modulates small effects related to bovine paratuberculosis susceptibility [10]. Herein, we evaluated the association between known polymorphisms in seven bovine TLR genes and the occurrence of uterine diseases. We used an observational cohort design, which is a distinctive feature of this study, where an aggressive approach to disease classification resulted in a low probability of disease misclassification. Similarly, the availability and use of custom genotyping assays derived from both Sanger and Roche 454 pyrosequencing of many distantly related cattle from more than 31 distinct breeds (including Holsteins; [10,16]) provided for a reasonable examination of bovine TLR variation with respect to the incidence of uterine disease.

The incidence of MET, CE, and CYE reported in our study population (30%, 18%, and 32%, respectively) was within the ranges reported elsewhere [4,5,7]. Moreover, multiple factors are likely to affect the incidence of uterine diseases in high-producing dairy cows [46–48]. Consequently, our statistical analyses included a number of confounding variables, with some of these variables actually being more predictive of uterine disease than any individual genetic marker evaluated in this study. The occurrence of metritis in this population was associated with calving in the summer and fall versus winter and spring, primiparity, dystocia, BCSEn, and ketosis <17 DIM, which is largely in agreement with previous reports [9,46,47]. Moreover, calving in the fall, dystocia, ketosis within 17 DIM, and hypocalcemia also influenced the probability of clinical endometritis, as previously described [4,48].

<table>
<thead>
<tr>
<th>Marker</th>
<th>dbSNP ID</th>
<th>Model</th>
<th>OR</th>
<th>95% CI</th>
<th>P value</th>
<th>Major allele</th>
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<td>rs469376065</td>
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<td>0.22</td>
<td>0.91</td>
<td>0.020</td>
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<tr>
<td>TLR6_C14578A</td>
<td>rs43702941</td>
<td>Recessive</td>
<td>0.26</td>
<td>0.06</td>
<td>1.20</td>
<td>0.045</td>
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</table>

Only SNPs with uncorrected P value <0.05 are presented.

4.1. Conclusion

Uterine health is one of many important factors that ultimately influences fertility in dairy cows. In turn, reproductive health itself is influenced by a number of environmental variables, which make it difficult to accurately estimate the precise role of host genetic components. Herein, we demonstrate that known variation within seven bovine TLR genes recognizing pathogen-associated molecular patterns does not modulate large effects on risk for uterine disease, as classified in the study described here. Weak associations were observed between SNPs occurring in four bovine innate immune genes (TLRs 2, 4, 6, 9) and

<table>
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<th>P value</th>
<th>Major allele</th>
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<td>0.38</td>
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</tr>
</tbody>
</table>

Only SNPs with uncorrected P value <0.05 are presented.

a Odds ratio adjusted for significant covariates.

b 95% confidence interval for odds ratio.

c P value not corrected for multiple comparisons.
utero health in Holstein dairy cows, thereby suggesting that variation in bovine innate immune genes may potentially modulate small effects on the incidence of uterine disease. However, classical variables such as dystocia and ketosis (<17 DIM) remain more predictive of MET in this population than any TLR variant investigated. Similarly, the same is true for the predictive value of dystocia on CE for our study population. Future studies employing whole-genome approaches are needed to help elucidate unknown genetic risk factors for uterine diseases in high-producing Holstein dairy cows.

Acknowledgment

This project was supported by the National Research Initiative Competitive grant no. 2009-35205-05058 from the US Department of Agriculture—National Institute of Food and Agriculture to CMS.

References


