Identification of polymorphisms in the bovine collagenous lectins and their association with infectious diseases in cattle

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Abstract

Infectious diseases are a significant issue in animal production systems, including both the dairy and beef cattle industries. Understanding and defining the genetics of infectious disease susceptibility in cattle is an important step in the mitigation of their impact. Collagenous lectins are soluble pattern recognition receptors that form an important part of the innate immune system, which serves as the first line of host defense against pathogens. Polymorphisms in the collagenous lectin genes have been shown in previous studies to contribute to infectious disease susceptibility, and in cattle, mutations in two collagenous lectin genes (MBL1 and MBL2) are associated with mastitis. To further characterize the contribution of variation in the bovine collagenous lectins to infectious disease susceptibility, we used a pooled NGS approach to identify short nucleotide variants (SNVs) in the collagenous lectins (and regulatory DNA) of cattle with (n = 80) and without (n = 40) infectious disease. Allele frequency analysis identified 74 variants that were significantly (p < 5 × 10^{-6}) associated with infectious disease, the majority of which were clustered in a 29-kb segment upstream of the collectin locus on chromosome 28. In silico analysis of the functional effects of all the variants predicted 11 SNVs with a deleterious effect on protein structure and/or function, 148 SNVs that occurred within potential transcription factor binding sites, and 31 SNVs occurring within potential miRNA binding elements. This study provides a detailed look at the genetic variation of the bovine collagenous lectins and identifies potential genetic markers for infectious disease susceptibility.

Keywords Collagenous lectins · Infectious disease · Cattle · Pooled next-generation sequencing · Genetic variants

Introduction

Infectious diseases are a major source of morbidity, mortality, and economic loss to the cattle industry. Infectious respiratory diseases alone account for close to $0.5–1 billion USD annually in North America (Miles 2009), and while estimates for other common infectious diseases, such as mastitis and gastrointestinal disease, are difficult to obtain, they undoubtedly add significantly to the economic impact of infectious disease (Schepers and Dijkhuizen 1991; Halasa et al. 2007; Heikkinä et al. 2012). Infectious diseases also represent a large source of agricultural antimicrobial use, which contributes to the development of antimicrobial resistance (Prescott et al. 2012). The approach to managing the impact of infectious disease has traditionally been to control the pathogen, largely ignoring the potential contributions of an immunologically deficient host (Miles 2009). Recently, however, there has been a broadening in focus to include host factors that contribute to infectious disease susceptibility.

The innate immune system represents the first line of defense against infectious diseases. Pattern recognition receptors (PRRs), a key part of the innate immune system, recognize conserved motifs on pathogens called pattern associated molecular patterns (Janeway 1989). The collagenous lectins are a subset of membrane-bound and/or soluble, circulating C-type lectins that function as PRRs, recognizing carbohydrate residues on the surfaces of bacteria, viruses, and fungi. The collagenous lectin family includes the collectins and ficolins, which share structural and functional similarities. Eleven collagenous lectin genes have been identified in cattle, including the genes encoding mannose-binding lectins A and C (MBL1...
and MBL2); surfactant proteins A and D (SFTPA1 and SFTPD); collectins (CL) 10, 11, 12, 43, and 46; conglutinin (CGN1); and ficolin-1 (FCN1). CL43, CL46, and CGN1 are found in cattle and a few select herbivores, and structural similarities between these collagenous lectins and SFTPD suggest that they are evolutionarily related (Hansen and Holmskov 2002; Gjerstorff et al. 2004a).

Certain collagenous lectins can activate the lectin pathway of complement and can agglutinate or opsonize pathogens (Fujita 2002, 2004). The lectin pathway of complement is activated in part by four MBL-associated serine proteases (MASPs), encoded by two MASP genes, MASP1 and MASP2. The MASP proteins are structurally and functionally similar to C1r and C1s of the classical complement pathway (Thiel et al. 1997; Matsushita et al. 2013) and bind in proenzyme form to the collagen-like domain of the MBLs, FCN-1, and CL-11 (Matsushita and Fujita 1992; Matsushita et al. 2001; Hansen et al. 2010). Following ligand recognition, the MASP proteins lead to the cleavage of complement components C2 and C4, resulting in the activation of complement.

Short nucleotide variants (SNVs) in the collagenous lectin genes are associated with infectious disease susceptibility in a variety of species. A dominant negative missense mutation in human MBL2 leads to an opsonic defect and is a cause of recurring infections in children (Sumiya et al. 1991) and adults (Summerfield et al. 1995). Deficiencies of MBL-C resulting from MBL2 polymorphisms in humans are also associated with HIV, hepatitis B and C, meningococcal disease, and parasitic infections (Eisen and Minchinton 2003). Polymorphisms in the human ficolin genes are associated with leprosy (Boldt et al. 2013; Andrade et al. 2017), pneumonia (van Kempen et al. 2017) and Chagas disease (Luz et al. 2016), while variation in SFTPA2 is associated with different outcomes to influenza A virus infection (Herrera-Ramos et al. 2014). In animals, mutations in the promoter region of porcine MBL2 are associated with decreased expression of MBL-C and are more frequent in animals diagnosed with pneumonia, enteritis, sepsis, or septicemia (Lillie et al. 2007). A missense mutation in porcine MBL1 is associated with decreased serum concentrations of MBL-A (Juul-Madsen et al. 2011). Relatively little is known about genetic variation in bovine collagenous lectins, and the few investigations that have been done have focused solely on mastitis and the MBL genes. Both a missense mutation in exon 2 and a promoter mutation of MBL1 are associated with altered activity of the classical complement pathway as well as with somatic cell score (SCS), a measure of the inflammatory cell content of milk and an indicator of mastitis (Wang et al. 2011; Liu et al. 2011; Yuan et al. 2012). Mutations in the coding region of MBL2 are also associated with SCS and complement activity (Zhao et al. 2012; Wang et al. 2012).

In order to address this gap in knowledge, we designed a targeted, next-generation sequencing study that captured the bovine collagenous lectin and related MASP genes as well as surrounding regulatory DNA. We sequenced our target regions in 120 cattle, 80 of which were diagnosed with infectious disease, and 40 of which lacked any evidence of infectious disease. We provide a comprehensive look at the variation in the collagenous lectin genes of these cattle, including in silico predictions of functional effects of identified variants. We also performed association analysis and identified 74 variants significantly associated with infectious disease in cattle.

Materials and methods

Sample selection and library preparation

Samples of liver or lung were collected from cattle presenting to the postmortem service at the Ontario Veterinary College or the Animal Health Laboratory at the University of Guelph. Cattle underwent a complete autopsy under the supervision of a veterinary pathologist certified by the American College of Veterinary Pathologists. Ancillary testing (e.g., bacterial culture, viral PCR, etc.) was performed as necessary to confirm the presence of pathogens. Cattle were broadly divided into two major populations: those with and without evidence of infectious disease (referred to as infectious and noninfectious in this article). They were then subdivided into pools of five animals each based on the similarity of their diagnosis at autopsy (Table 1). The study population consisted predominantly of female Holstein-Friesians (66.7%), with fewer intact and castrated male Holstein-Friesians (8.3%). The remainder (25.0%) was composed of a variety of other breeds and crosses (Table 2).

Tissue samples were stored at −20 °C until processed. DNA was extracted using a commercial column based DNA extraction kit (QIAGEN DNeasy Blood and Tissue kit, Mississauga, ON, Canada), and sample concentration was evaluated via fluorometry (Qubit 2.0, Thermo Fisher Scientific, Mississauga, ON, Canada). Equimolar amounts of DNA from cattle in each group were pooled to obtain a final concentration of 1 μg of DNA in 50 μl of low-EDTA buffer TE. Each pool of DNA was acoustically sheared to a target range of 600 bp (Covaris M220, Woburn, MA, USA).

Following acoustic shearing, each pool of DNA underwent end repair, A-tailing, and adapter ligation (including a unique index) using a KAPA Library Preparation Kit for Illumina Platforms (KAPA Biosystems, Wilmington, MA, USA) as per the manufacturer’s instructions, with the following exception: a single cleanup step was performed following adapter ligation. The cleanup was performed by adding 0.6X PEG/NaCl SPRI solution and only the DNA bound to the magnets was retained. The pools were then combined in equimolar amounts to create a single sequencing library.

Target enrichment was performed using a SeqCap EZ Developer Enrichment Kit as per the SeqCap EZ Library SR User’s Guide v.4.2. Target regions consisted of the
collagenous lectins and related MASP genes and were based on coordinates obtained from the UMD3.1.1 (bosTau8) genome (The Bovine Genome Sequencing and Analysis Consortium et al. 2009) hosted by the University of Santa Cruz, CA (Karolchik et al. 2004) (Table 3). Up to 50 kb upstream and 3 kb downstream of each gene was targeted for sequencing, in an attempt to capture a portion of regulatory DNA. Due to some inconsistencies in the annotation of the bovine collagenous lectins, annotations from NCBI and Ensembl were compared and reviewed, and the most appropriate annotation for each gene was chosen. For example, the MBL1 gene is not annotated in Ensembl or UCSC, while the SFTP A1 gene, located nearby on the same chromosome, has four annotated transcript variants in Ensembl. The NCBI RefSeq accession for MBL1, NM_001010994.3, is identical to the Ensembl SFTP A1 transcript ENSBTAT00000001165, leading to some uncertainty surrounding the true identity of these transcripts. Alignment of the four bovine SFTP A1 transcripts to the coding and protein sequences SFTP A1 and MBL1 from other species using Clustal Omega (Goujon et al. 2010; Sievers et al. 2011) showed that ENSBTAT00000031298 and ENSBTAT0000001165 had the highest percent identity matrices to SFTP A1 and MBL1, respectively. Thus, in contradiction to the annotation found in Ensembl, ENSBTAT00000001165 was considered to represent the bovine MBL1 gene, while only the ENSBTAT00000031298 transcript was considered to represent the SFTP A1 gene. Similarly, we believe the gene annotated as FCNB in Ensembl and FCN1 in NCBI more closely resembles FCN1, and is referred to as such in this study. Coordinates for COLEC10 and MASP2 were adjusted slightly based on sequence homology to other species in order to ensure they contained start codons.

Following enrichment, the library was sequenced using MiSeq Reagent Kit v3 (600-cycle) sequencing chemistry on an Illumina MiSeq (San Diego, CA, USA). In order to achieve adequate depth of sequencing, the same library was sequenced twice. All statistical analyses were performed in R unless otherwise specified (R Core Team 2017).

### Bioinformatic analysis of NGS data

The sequencing data was processed in two stages (Fig. 1). In the first stage, data from each run was processed separately, while in the second stage, data from the same pool from different runs was merged and then further processed. The data from both sequencing runs was first trimmed using Trimmomatic v.

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**Table 1** Cattle were placed into groups of five animals each based on the similarity of the diagnosis determined at autopsy.

| Status   | Group | Diagnosis                                      |
|----------|-------|------------------------------------------------|
| Noninfectious | Group 1 | Normal (no lesions)                           |
|          | Group 2 | Fractures or trauma                            |
|          | Group 3 | Dental malocclusion, peripheral neuropathy     |
|          | Group 4 | Intestinal accident or musculoskeletal trauma |
|          | Group 5 | Neoplasia                                      |
|          | Group 6 | Metabolic disease                              |
|          | Group 7 | Congenital malformations                        |
|          | Group 8 | Organ torsion or rupture                        |
| Infectious | Group 9 | Endocarditis                                    |
|          | Group 10 | Meningitis                                     |
|          | Group 11 | Bronchopneumonia 1                             |
|          | Group 12 | Bronchopneumonia 2                             |
|          | Group 13 | Pneumonia (M. haemolytica)                     |
|          | Group 14 | Mycoplasma arthritis, osteomyelitis, or pneumonia |
|          | Group 15 | Pneumonia (T. pyogenes)                        |
|          | Group 16 | Sepsis                                         |
|          | Group 17 | Omphalophlebitis                                |
|          | Group 18 | Foot abscess or ulcer                           |
|          | Group 19 | Infectious arthritis                           |
|          | Group 20 | Abortion or perinatal death of infectious cause |
|          | Group 21 | Diarrhea                                        |
|          | Group 22 | Mastitis                                        |
|          | Group 23 | Multifocal abscesses                            |
|          | Group 24 | Metritis or endocarditis                        |

**Table 2** Breakdown of the study population by breed and gender

| Breed                  | F   | M | MN | Total |
|------------------------|-----|---|----|-------|
| Noninfectious          |     |   |    |       |
| Holstein-Friesian      | 30  | 1 | 2  | 33    |
| Limousin               | 1   | 1 |    | 2     |
| Hereford-Limousin      | 1   | 1 |    | 1     |
| Limousin cross         | 1   | 1 |    | 1     |
| Not available          | 1   |   | 1  | 1     |
| Angus                  | 1   |   | 1  | 1     |
| Jersey                 | 1   |   | 1  | 1     |
| Subtotal               | 34  | 4 | 2  | 40    |
| Infectious             |     |   |    |       |
| Holstein-Friesian      | 50  | 7 |    | 57    |
| Limousin               | 2   | 2 | 1  | 5     |
| Shorthorn              | 1   | 1 |    | 2     |
| Red Angus              | 1   |   | 1  | 1     |
| Wagyu                  | 1   |   | 1  | 1     |
| Black Angus            | 1   | 3 | 2  | 6     |
| Simmental cross        | 1   |   | 1  | 1     |
| Unspecified beef breed | 2   |   |    | 2     |
| Angus                  | 1   | 1 |    | 2     |
| Angus-Simmental        | 1   |   | 1  | 1     |
| Charolais cross        | 1   |   | 1  | 1     |
| Jersey                 | 1   |   | 1  | 1     |
| Subtotal               | 59  | 16| 5  | 80    |
| Total                  | 93  | 20| 7  | 120   |

MN male neutered (steer)
0.36 (Bolger et al. 2014) based on the following criteria: (a) leading and trailing bases with a quality score of less than 20 were trimmed, (b) reads were trimmed if quality dropped below an average score of 20 over a 5 bp sliding window, and (c) reads were dropped if they were less than 75 bp in length. Reads were then mapped to the bovine genome UMD3.1.1 using BWA-MEM algorithm of BWA v. 0.75 (Li and Durbin 2009). PCR and optical duplicates were removed with Picard v. 1.127 (http://broadinstitute.github.io/picard/, accessed 2018-01-12). The Genome Analysis Toolkit (GATK) Best Practices Guidelines (DePristo et al. 2011; Van der Auwera et al. 2013) were followed for in/del realignment and base quality score recalibration (BQSR) using GATK v. 3.6 (McKenna et al. 2010). At this point, BAM files for each pool generated during the different runs were merged. Each merged BAM file was reprocessed for PCR duplicates and in/del realignment. Variant calling was performed on merged BAM files using the joint genotyping protocol outlined in the GATK Best Practices guidelines. Variants were filtered using separate hard filters for SNVs and in/dels. Multiallelic variants and spanning deletions were excluded, and known variants were obtained from dbSNP v. 150 (Sherry et al. 2001). Evaluation of target capture between the noninfectious and infectious populations was performed by comparing the overall mean of the mean of each pool within each population using a two-way ANOVA and a least-square means post-hoc test.

**Variant analysis**

In silico analysis of the variants was performed for coding region variants, downstream 3 kb, and the upstream 5 kb of potential regulatory DNA. Variant density was evaluated both in terms of target regions and functional genomic regions using a one-way ANOVA and Kruskal-Wallis post hoc test. Correlations between variant density and GC content as well as indel number were assessed using a Pearson’s correlation coefficient. Missense coding variants were analyzed using Polyphen2 (Adzhubei et al. 2010) and the SIFT algorithm (Sim et al. 2012) run by the Variant Effect Predictor (VEP) hosted by Ensembl (McLaren et al. 2010). For Polyphen2, batch submission was used and a FASTA file containing the protein sequences was submitted. For VEP, options were left at their default settings.

Transcription factor (TF) binding site (TFBS) analysis was performed on the 5 kb upstream to the start codon for each target gene using CIS-BP v. 1.02 (Weirauch et al. 2014). The 3 kb downstream from the stop codon of the targeted genes was analyzed for potential miRNA recognition elements (MREs). Although the 3′ UTR was annotated within Ensembl for all genes with the exception of COLEC10 and SFTPD, we opted to analyze the entire 3 kb for each gene to maintain consistency. Multiple MRE discovery algorithms were used to maximize the accuracy of the predictions (Riffo-Campos et al. 2016). miRanda v. 3.3a (Enright et al. 2003) and Targetscan v. 7.0 (Agarwal et al. 2015) were used to identify MREs binding mature miR sequences from cattle accessed from miRbase 21 (Kozomara and Griffiths-Jones 2013). The energy threshold parameter of miRanda was set to −20 kcal/mol, with other parameters for both programs left at their default settings. The intersection of the seed region of predicted MREs from both programs was obtained using BEDtools v. 2.25.0 (Quinlan and Hall 2010). BEDtools was then used to identify variants in our dataset that intersected with the seed sequence of MREs predicted by both algorithms.

### Table 3

The regions targeted for resequencing are given for each gene included in the study. Genes in close proximity to each other were sequenced as a single unit.

| Name   | Ensembl ID         | Chr | Target start | Target end | Total bp |
|--------|--------------------|-----|--------------|------------|----------|
| MASP1  | ENSBTAG00000012467 | 1   | 80,546,924   | 80,652,367 | 105,443  |
| COLEC11| ENSBTAG00000016225 | 8   | 112,860,707  | 112,896,491| 35,784   |
| FCN1   | ENSBTAG00000048155 | 11  | 106,773,026  | 106,834,643| 61,617   |
| COLEC10| ENSBTAG00000017343 | 14  | 47,260,662   | 47,359,061 | 98,399   |
| MASP2  | ENSBTAG00000012808 | 16  | 43,449,518   | 43,481,362 | 31,844   |
| COLEC12| ENSBTAG00000007705 | 24  | 35,627,928   | 35,866,269 | 238,341  |
| MBL2   | ENSBTAG00000007049 | 26  | 6,294,785    | 6,351,912  | 57,127   |
| CGN1   | ENSBTAG00000006536 | 28  | 35,541,900   | 35,726,104 | 184,204  |
| CL46   | ENSBTAG00000048082 | 28  |              |            |          |
| CL43   | ENSBTAG00000047317 | 28  |              |            |          |
| SFTPD  | ENSBTAG00000046421 | 28  | 35,764,587   | 35,870,565 | 105,978  |
| MBL1   | ENSBTAT0000001165a| 28  |              |            |          |
| SFTPA1 | ENSBTAT00000031298a| 28  |              |            |          |

*Both of these transcript IDs are from the gene ENSBTAG00000023032 (SFTPA1). The transcript given for MBL1 is identical to the NCBI RefSeq accession for MBL1 (NM_001010994.3), and percent identity matrices comparing the coding and protein sequences of these accessions to MBL1 and SFTPA1 in other species supports their identities as we have determined them based on in silico analysis.

0.36 (Bolger et al. 2014) based on the following criteria: (a) leading and trailing bases with a quality score of less than 20 were trimmed, (b) reads were trimmed if quality dropped below an average score of 20 over a 5 bp sliding window, and (c) reads were dropped if they were less than 75 bp in length. Reads were then mapped to the bovine genome UMD3.1.1 using BWA-MEM algorithm of BWA v. 0.75 (Li and Durbin 2009). PCR and optical duplicates were removed with Picard v. 1.127 (http://broadinstitute.github.io/picard/, accessed 2018-01-12). The Genome Analysis Toolkit (GATK) Best Practices Guidelines (DePristo et al. 2011; Van der Auwera et al. 2013) were followed for in/del realignment and base quality score recalibration (BQSR) using GATK v. 3.6 (McKenna et al. 2010). At this point, BAM files for each pool generated during the different runs were merged. Each merged BAM file was reprocessed for PCR duplicates and in/del realignment. Variant calling was performed on merged BAM files using the joint genotyping protocol outlined in the GATK Best Practices guidelines. Variants were filtered using separate hard filters for SNVs and in/dels. Multiallelic variants and spanning deletions were excluded, and known variants were obtained from dbSNP v. 150 (Sherry et al. 2001). Evaluation of target capture between the noninfectious and infectious populations was performed by comparing the overall mean of the mean of each pool within each population using a two-way ANOVA and a least-square means post-hoc test.

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Transcription factor (TF) binding site (TFBS) analysis was performed on the 5 kb upstream to the start codon for each target gene using CIS-BP v. 1.02 (Weirauch et al. 2014). The
species was set as *Bos taurus*, and a motif model of “PWM - LogOdds” with a minimum threshold of 8 was selected; only cis acting TFBSs were considered. Variants falling within predicted TFBSs were identified using BEDtools. Many of the TFBSs shared identical sequences and bound TFs belonging to the same family, and were thus collapsed into a single result with results reported by TF family. Putative TFBSs were further refined by identifying conserved 50-bp-long DNA sequence motifs within the 5 kb upstream from the start codon for each gene across eight different species, cattle (UMD3.1.1), pig (Sscrofa10.2), horse (EquCab2), rat (Rnor_6.0), mouse (GRCm38), gorilla (gorGor3.1), chicken (Galgal4), and human (GRCh38), using MEME v 4.10.1 (Bailey and Elkan 1994). The bovine specific genes *CGN, CL43, and CL46* were aligned to the sequences of SFTP D from other species, as they are believed to be evolutionarily related (Hansen and Holmskov 2002; Gjerstorff et al. 2004b). A minimum E-value of 0.05 was used to consider a motif conserved. The conserved motifs were then examined for the presence of TFBSs predicted by CIS-BP and containing variants.

### Allelic association

The estimated frequency of variant alleles was compared between the noninfectious and infectious populations using Popoolation2 (Kofler et al. 2011). Processed BAM files for each pool were combined using Samtools into a single BAM file for each population, and an mpileup file was generated using minimum mapping and base qualities of 20 (Li et al. 2009). Popoolation2 was used to transform the mpileup file into a sync file, which was then down-sampled according to the recommendations of Popoolation2 to 400 reads per population using the method “fraction” to mitigate the impact of variable read depths on statistical testing. Fisher’s exact test was used to determine the significance of allele frequency estimates between the two populations. A minimum of 5% of the reads (20) across both populations combined was required for the allele to be considered in the allele frequency estimation. The Benjamini-Hochberg procedure was used to correct for multiple testing (Benjamini and Hochberg 1995) with adjusted p value cutoffs labeled as described in the BADGE system (Manly 2005).

### Results

Evaluation of target capture efficiency showed that the median of the mean target coverage was 172.5; however, there were significant differences (p < 0.05) both in terms of the depth of coverage for each target region, as well as the total depth of coverage between the noninfectious and infectious populations (Fig. 2). The depth of coverage between the two populations at individual target regions was not statistically different.

Joint genotyping identified a total of 5439 unique variants, all of which were present in both the noninfectious and infectious populations. These included 5418 SNVs and 21 in/dels. The majority (5317, 97.7%) of the variants identified were present in dbSNP 150, while 122 were novel discoveries. A further 32,794 variants were present in dbSNP 150 within our target intervals. Of these, 2922 were present in our population but were excluded due to various filtering parameters, 29,696 loci were not variant in our population, and 356 loci were not successfully sequenced.

A total of 83 coding variants (40 synonymous, 43 missense), 2297 intronic variants, 309 variants within
the downstream 3 kb of the stop codon, 414 variants within the 5 kb upstream of the start codon, and 2915 variants in the region between 5 and 50 kb upstream of the start codon were identified. The density of variants was examined both by gene and by region (Fig. 3). Significant differences \( (p < 0.05) \) in variant density were observed between genes, with the highest density of variants found in the \textit{FCN1} gene and surrounding DNA (Fig. 3a). Variant density by region was not significantly different, though the coding region tended to have the lowest density (Fig. 3b).

Among the coding variants, 43 missense variants were identified. In silico predictions of the effects of the mutations using either algorithm identified a total

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\begin{align*}
\text{Fig. 2} & \quad \text{The overall mean of the mean depth of coverage for each pool in the noninfectious and infectious populations by gene target. There were no significant differences between populations in a gene target; however, there was a significant difference in depth of coverage between different gene targets (two-way ANOVA and least-squares means post hoc comparison, } p < 0.05). \text{Genes that share a letter are not significantly different: for example, MASP2 is not significantly different from MASP1 but is significantly different from the remaining gene targets. Solid line represents the median, dashed line is the mean. The hinges represent the 1st and 3rd quantiles, while the whiskers represent } 1.5\times \text{ the interquartile range. Data points beyond this range are illustrated as solid circles.}
\end{align*}
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\begin{align*}
\text{Fig. 3} & \quad \text{a Variant density across the entire study population was significantly different amongst the targeted genes. Variant density in these genes was significantly different than \textit{COLEC12 and MASP1} (dagger). Variant density was significantly different from MASP2, COLEC10, COLEC12, and MASP1 (one-way ANOVA and Kruskal-Wallis post hoc test, } p < 0.05) \text{(double dagger). b Variant density between functional genomic regions was not significantly different. Note that due to the proximity of some genes (e.g., \textit{MBL1 and SFTP1}), some variants occurring downstream, upstream, or within the introns and coding regions of \textit{SFTP1} were also counted as being upstream from \textit{MBL2}, thus, the total of variants by region is different than the total of unique variants discovered. Solid line represents the median, dashed line is the mean. The hinges represent the 1st and 3rd quantiles, while the whiskers represent } 1.5\times \text{ the interquartile range. Data points beyond this range are illustrated as solid circles.}
\end{align*}
\]
of 11 SNVs expected to have damaging or possibly damaging effects on protein structure or function (Table 4), with three of the variants (within SFTPD, MBL2, and CL43) predicted to be damaging by both algorithms.

A total of 20,147 potential cis TFBSs were predicted in the upstream 5 kb of the targeted genes. Of these, 1351 contained a SNV identified in this study. In highly conserved DNA sequence motifs (based on our multispecies comparison of upstream regulatory regions), 148 TFBSs containing a SNV were found across 10 of the targeted genes (Fig. 4a). These TFBSs were members of 30 TF families (Fig. 4b).

Analysis of the 3 kb downstream from the stop codon using both algorithms identified 469 potential miRNA recognition elements (MREs). Within the seed region of the predicted MREs there were 31 SNVs (Fig. 5), two of which impacted two separate miRs. A total of 28 unique miRs were predicted to bind in these regions, 5 of which bound multiple loci. A single MREs intersected with the annotated 3' UTR of SFTPA1.

Evaluation of the frequency of variant alleles identified 25 BADGE class I ($p < 2 \times 10^{-5}$) and 49 class II ($p < 5 \times 10^{-6}$) variants that were significantly associated with either the Non-infectious or Infectious populations (Fig. 6). Seventeen associations were found clustered in intron 2 of MASP1 (Fig. 6b), and a further 48 associations were present in a ~21 kb region ~29 kb upstream from CGN1, the first gene of the bovine collectin locus (Fig. 6c). Four associations were found distributed upstream, downstream, and within intron 8 of FCN1 (Online Resource 2a); four associations were found in the introns 4 and 5 and the downstream region of COLEC11 (Online Resource 2b); and a single association was found in intron 2 of COLEC12 (Online Resource 2c).

### Table 4

| Gene | Chr | Pos  | rsID | Ref  | Alt  | Protein change | Protein domain | Polyphen2 Prediction       | SIFT Prediction | Polyphen2 Score | SIFT Score |
|------|-----|------|------|------|------|----------------|----------------|-------------------------|----------------|----------------|------------|
| FCN1 | 11  | 106,827,968 | rs382216843 | C   | T   | Arg142Cys     | FBG            | Probably damaging       | Jacketed       | 0.986          | Tolerated 0.06 |
| MBL2 | 26  | 6,344,919  | rs21061099 | A   | C   | Pro42Gln      | CLD            | Probably damaging       | Jacketed       | 0.974          | Tolerated 0    |
| SFTPD| 28  | 35,820,078 | rs380240341 | C   | T   | Pro132Ser     | CLD            | Probably damaging       | Jacketed       | 0.958          | Tolerated 0.05 |
| CGN  | 28  | 35,598,640 | rs208842091 | G   | A   | Arg173His     | CLD            | Possibly damaging       | jacketed       | 0.72           | Tolerated 0.16 |
| CL46 | 28  | 35,677,371 | rs383278255 | C   | T   | Pro185Leu     | CLD            | Possibly damaging       | jacketed       | 0.672          | Tolerated 0.32 |
| CL43 | 28  | 35,718,034 | rs42967143  | A   | G   | Thr147Ala     | CLD            | Possibly damaging       | Jacketed       | 0.659          | Tolerated 0.9  |
| CL43 | 28  | 35,718,807 | rs211678602 | G   | T   | Gly185His     | neck region    | Possibly damaging       | Jacketed       | 0.634          | Tolerated 0.01 |
| MASP2| 16  | 43,463,621 | rs207667073 | G   | A   | Gly102Ser     | CUB            | Possibly damaging       | Jacketed       | 0.191          | Tolerated 0.01 |
| FCN1 | 11  | 106,828,710 | rs385211468 | C   | T   | Thr193Met     | FBG            | Benign                  | Jacketed       | 0.042          | Tolerated 0.03 |
| CGN  | 28  | 35,602,463 | rs466869949 | A   | C   | Glu102Asp    | CRD            | Benign                  | Jacketed       | 0.036          | Tolerated 0.04 |
| SFTPD| 28  | 35,824,136 | rs110476851 | C   | G   | Ala288Gly    | CRD            | Benign                  | Jacketed       | 0.002          | Tolerated 0.02 |

FBG fibrinogen-like domain, CLD collagen-like domain, CUB complement C1r/C1s, Uegf, Bmp1, CRD carbohydrate recognition domain.

### Discussion

Associating polymorphisms with infectious disease susceptibility has important implications in agricultural economics, animal breeding, and animal health and welfare. Probing the underlying genetics of complex traits like innate immunity is challenging and requires large numbers of animals with well-defined phenotypes (Ron and Weller 2007). The phenotypes of our study samples were determined through complete autopsies supervised by certified veterinary pathologists. This included evaluation of gross tissues, ancillary testing as required, and review of histopathological specimens to define the extent and nature of disease. In order to address the considerable expense required to sequence large numbers of animals, we opted to use a pooled and targeted next-generation sequence approach. Pooled sequencing has been shown to be an accurate and cost-effective method of variant discovery and allele frequency estimation in next-generation sequencing experiments (Bansal et al. 2011; Mullen et al. 2012; Rellstab et al. 2013; Bertelsen et al. 2016), as well as in genome-wide association studies (Keele et al. 2015). While whole genome sequencing would provide a more complete picture of disease associated variants, the cost remains prohibitive, despite decreasing sequencing costs. Instead, targeted resequencing allows specific regions of interest in the genome to be queried, providing much more detail than the available high density SNP array from Illumina: of the 5439 variants found in our study, only 223 are present in the Illumina BovineHD BeadChip (https://support.illumina.com/array/array_kits/bovinehd_dna_analysis_kit/downloads.html, accessed 2018-01-12).

Allele frequency estimation identified 74 significant variants (BADGE class II or higher), after correction for multiple testing, that were associated with infectious disease. Over half of the significant alleles occurred in a 29-kb segment of DNA.
upstream from \textit{CGN1}, and these alleles met the highest level of statistical significance proposed by the BADGE system (Manly 2005). \textit{CGN1} is the first gene in the bovine collectin locus, a 260-kb region on chromosome 28 which includes the \textit{CL46}, \textit{CL43}, \textit{SFTPD}, \textit{MBL1}, and \textit{SFTPA1} genes (Gjerstorff et al. 2004a), and several of these genes have been implicated in infectious disease susceptibility. Plasma concentration of conglutinin is known to be heritable and low levels of plasma conglutinin are associated with increased incidence of respiratory disease in cattle (Holmskov et al. 1998). Whole

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transcriptome sequencing of the abomasum of parasite-susceptible and resistant calves found that resistant animals expressed higher levels of CL46 (Li et al. 2011). In cattle breeds native to China, certain MBL1 haplotypes are associated with somatic cell score, an indirect marker of mastitis (Wang et al. 2011). The block of highly significant associations discovered here could theoretically impact any of the genes within the intron of MASP1 (b) and upstream of the collectin locus (c). The red line indicates the cutoff for class II significance ($p < 5 \times 10^{-6}$). The depth of sequencing (total from all pools) and the regions with probes designed for target capture are shown to illustrate gaps in sequencing and variant discovery.

Two class I and 15 class II associations were found in intron 2 of MASP1. Human MASP1 encodes three protein isoforms with distinct functions, MASP-1, MASP-3, and MAP1 (Beltrame et al. 2015). Only a single transcript is annotated for bovine MASP1, which best corresponds to the MASP-3 isoform, and an additional 4 transcripts are predicted by NCBI. As with human MASP1, the transcript and predicted transcripts that encode the three MASP1 isoforms share the first 8 exons; thus, the cluster of variants noted in intron 2 is also present in intron 2 for all of these predicted transcripts. Genetic mutations resulting in human MASP1 deficiency are associated with infectious disease, and several intronic mutations leading to MASP1 deficiency...
have been identified, though none are identical to the associations found here (Ingels et al. 2013; Beltrame et al. 2015). Again, the relative similarity of the allele frequencies for the different loci suggests significant linkage disequilibrium. It should be noted that mutations that inhibit the function of the MASPs (and complement-activating collagen-like lectins) may also confer benefits: excess activation of complement can contribute to tissue damage or autoimmune disease, and decreased or more moderate levels of complement activity may therefore be beneficial in some scenarios (Beltrame et al. 2015).

A further four associations were found in COLEC11 and two in COLEC12; however, no in silico consequences were predicted for any of the six. There is little known about the relative importance of CL-K1 and CL-P1, the proteins encoded by these two genes, in the innate immune response to infectious disease, and, to the authors knowledge, these are the first reported associations between mutations in these genes and infectious disease of cattle. Recently, CL-P1 was shown to have a soluble form that can activate the alternative pathway of complement (Ma et al. 2015), and CL-K1 is capable of activating the lectin pathway of complement (Ma et al. 2013), presenting possible pathways through which genetic mutations could hamper the immune system.

Although in silico analysis of the disease-associated alleles did not identify any biological effects, several interesting predictions were made regarding other variants present in our dataset. Two missense mutations found in MBL2 and one in MASP2 (Table 3) were previously shown to be associated with SCS in Chinese Holstein cattle (Wang et al. 2012; Wu et al. 2015). The frequency of these three variants was not significantly different in our populations of infectious and noninfectious cattle. Variant rs210611099:c.125C>A was rare, with only eight alleles predicted across both populations (MAF 3.3%). Though rare, this is substantially higher than the reported allele frequency of 0.27% in Chinese Holstein cattle (Wang et al. 2012), and may be the result of different breeds (Chinese Holsteins versus the mixture of breeds common to North America in our study). The mutation occurs in the collagen-like domain (CLD) of MBL-C and is predicted in silico to have a significant impact on protein structure and function. Previously reported mutations in the CLD had an impact on MBL-C driven complement activation (Larsen et al. 2004), as well as on higher-order oligomerization through disruption of the Gly-X-Y collagen-like repeats (Sumiya et al. 1991). Thus, despite its rarity, the role of this variant in infectious disease susceptibility remains of interest. Genotyping of a larger number of animals may be useful in clarifying the discrepancy between studies in allele frequencies and may provide the statistical power required to determine whether this rare variant plays a role in bovine innate immune defense.

The second MBL2 missense variant associated with SCS, rs210426415:c.92G>A, leads to the substitution of glutamine for arginine in the N-terminal domain of MBL-C. This domain is believed to utilize conserved cysteine residues to facilitate the functionally critical oligomerization of MBL-C (Wallis and Drickamer 1999). This variant was predicted by both in silico algorithms to have a low impact on protein structure and function, and was not associated with disease in our study, possibly the result of a smaller cohort of animals diagnosed with mastitis. Thus, although genotyping of larger numbers of North American cattle may reveal an association with infectious disease, this study does not provide evidence for further investigation.

The MASP2 variant previously shown to be associated with SCS, rs207667073.G>A, results in an amino acid change in vitro in the innate immune defense against Haemophilus influenza through negative regulation of phagocytosis (Tay et al. 2015). Gram-negative pulmonary pathogens related to H. influenza, notably M. haemolytica, P. multocida, and H. somni, are important causes of respiratory disease in cattle; thus, this predicted MRE affected by a variant may hold relevance for future investigations.

In silico prediction of transcription factor binding sites relies on the observation that the amino acid sequence of the DNA-binding domain of transcription factor proteins largely predicts their DNA-binding specificity, and does so in a highly conserved manner (Kasahara et al. 2006; Jolma et al. 2013; Weirauch et al. 2014). Regulatory DNA is also highly conserved in animals (Nitta et al. 2015). Thus, to reduce the large number of predicted TFBSs identified in the targeted genes, conserved DNA sequences present in the potential promoter region were identified by comparing sequences from up to eight different animals including domestic livestock and more distantly related species (human, gorilla, cattle, horse, pig, rat, mouse, and chicken). This conservative approach narrowed the results to 148 potential TFBSs, some of which were similar to TFBSs predicted or shown in previous studies to be involved in the regulation of collagenous lectins. For example, we identified binding sites for SRF (a member of the MADS box family of transcription factors) in SFTPA1, and an SRF binding site was also identified in human SFTPA2 (Grageda et al. 2014). Initial characterization of the promoter of CL43 included in silico prediction of TFBSs (Hansen et al. 2003), and the same binding sites were predicted in our study for
Myb, ARNT, cEBP, Myc, MyoD, Mzf-1, N-Myc, and USF; however, only Mzf-1 was both present within a conserved motif and contained a SNV. This discrepancy may be the result of more stringent requirements in our study (inclusion of trans binding sites and sites outside of conserved sequences), or potentially due to differing prediction algorithms. HNF3alpha (also known as FOXA1) is known to regulate the expression of human and chicken MBL2 (Naito et al. 1999; Kjærup et al. 2013), and while binding sites were predicted in CL43 and COLEC11, no binding site was predicted for bovine MBL2.

The density of variants varied significantly by gene, but not by gene region (Fig. 3). FCN1 and the two surfactant protein genes, SFTPA1 and SFTPD, had a significantly higher variant density than the bottom two (for FCN1) and four (for SFTPA1 and SFTPD) genes. Both intrinsic and extrinsic factors can affect the degree of sequence variation. Intrinsically, the GC content and the number of indels in a region are predictive of variation (Hodgkinson and Eyre-Walker 2011). The GC content of the FCN1 gene was the second highest of the targeted genes at 55.1%; however, the overall correlation between GC content and the density of variants was not significant ($R = 0.36, p = 0.23$, Online Resource 4). Although the FCN1 target contained the highest number of indels (13/21), there was no correlation between number of indels and variation density ($R = 0.36, p = 0.23$, Online Resource 5). Extrinsically, genes that interact with the environment (such as innate immune response genes) can often be found in “hot spots” in the genome, which show higher levels of variation in response to increased adaptive pressures (Chuang and Li 2004). Interestingly, a recent study on the equine collagenous lectins found that two equine ficolin genes, FCN1 and FCN1-like, also had the highest variant density amongst the equine collagenous lectins (Fraser, unpublished). Five of the eight coding region mutations found in FCN1 were located within the fibrinogen-like domain, and three were missense. Although the relative role of the ficolin genes in the innate immune response of these two species is still under investigation, the increased variation observed in both species might suggest increased evolutionary pressure to adapt to different pathogens.

Variation in innate immune genes can significantly impact the susceptibility of animals to infectious diseases. Here, we have comprehensively documented the variation in a subset of innate immune genes, the collagenous lectins, in cattle with and without infectious diseases. Comparison of mutant alleles in the two populations identified 74 alleles associated with infectious disease. These alleles warrant further investigation, both in terms of population level frequencies, and, if confirmed to be significantly associated with disease susceptibility, in terms of their biological mechanisms of action.

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