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Analysis of sequence variability and protein domain architectures for bovine peptidoglycan recognition protein 1 and Toll-like receptors 2 and 6

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ABSTRACT

The mammalian Toll-like receptors (TLRs) recognize invading pathogens, thereafter provoking innate immune responses, whereas peptidoglycan recognition protein 1 (PGLYRP1) is directly microbicidal. The primary objective of this study was to characterize single-nucleotide polymorphisms (SNPs) and insertion-deletion polymorphisms (indels) within bovine TLR2, TLR6, and PGLYRP1, thereby facilitating future TLR signaling, association, and PGLYRP1 microbicidal assays relevant to bovine innate immunity. Comparative sequence analysis for 10 bovine breeds revealed 83 polymorphisms (82 SNPs, 1 indel), with 15 nonsynonymous SNPs located within predicted functional domains. Of the 83 polymorphisms detected, 72 (87%) are reported here for the first time. Several predicted amino acid replacements encoded by bovine TLR2 and TLR6, but not PGLYRP1, resulted in the confident prediction of protein domain alterations. Prediction and comparison of protein domain architectures for TLR2 and TLR6 revealed six regions of leucine-rich-repeat patterning that was conserved among multiple species. Collectively, differences in the patterns and frequencies of polymorphism were noted between bovine TLRs that predominantly recognize viral ligands (TLRs 3, 7, 8, 9) and those that recognize microbial and/or unknown ligands (TLRs 1, 2, 5, 6, 10).

Advances in agricultural genomics have often been fueled by the supposition that improving host genetics through selective breeding may ultimately aid in the suppression of economically important diseases in food-animal populations. Because the innate immune system in mammals provides host defense against a variety of pathogens without requiring prior exposure [1,2], genes involved in innate immunity have been considered putative candidate loci for potentially improving host genetics in agricultural species. Notably, the field of innate immunity was strongly impelled by the discovery that Toll, a *Drosophila* protein governing developmental polarity, also elicited an effective antifungal immune response in adult *Drosophila* [3–6]. Since the initial description of Toll, nine members of the Toll gene family have been identified within the *Drosophila* genome, whereas mammals are generally considered to possess 10 or 12 functional Toll-like receptor (TLR) genes (TLRs 1–10 in human, TLRs 1–9 and 11–13 in mouse) [2,5–7]. The mammalian TLRs are type I transmembrane proteins of the interleukin-1 receptor (IL-1R) family that possess N-terminal leucine-rich repeats (LRR) involved in ligand recognition, a transmembrane domain, and a C-terminal intracellular Toll/IL-1R homologous domain for signal transduction [1,2,8]. To date, ligand specificities for most mammalian TLRs have been elucidated, with six gene family members (TLRs 1, 2, 4, 5, 6, 9) known to recognize microbial and/or synthetic ligands, and five gene family members (3, 4, 7, 8, 9) known to recognize viral components [for reviews see 1,7,8].

Expression of bovine and ovine TLRs 1–10 was recently identified in selected tissues [9], and all 10 bovine TLRs have been mapped using radiation hybrid technology [10–12]. Detailed polymorphism studies have also emerged for bovine TLRs 1, 3, 4, 5, 7, 8, 9, and 10 [13–15]. However, few detailed reports currently exist regarding the frequency and distribution of genetic polymorphisms within bovine TLRs 2 and 6 [9,10,16–18]. Importantly, bovine TLR2 has been implicated in host responses to mycobacteria [19–21], including *Mycobacterium avium* subsp. *paratuberculosis*, the causative agent of Johne's disease in ruminants [22]. Additionally, TLR2 has been demonstrated to recognize a variety of components derived from gram-positive bacteria, gram-negative bacteria, yeast, spirochetes, and fungi [23–31]. Given the diverse array of microbial pathogen-associated molecular patterns (PAMPs) that TLR2 recognizes, some authors consider it to be the most promiscuous TLR [32]. Interestingly, the promiscuity of TLR2 has primarily been attributed to its ability to heterodimerize with both TLR1 and TLR6, thereby enabling the resulting protein complexes to recognize a variety of microbial PAMPs [31,32]. Recent studies also emphasize the importance of defining TLR polymorphisms by illustrating how certain naturally occurring genetic variants may enhance the risk of severe infections in humans and mice [for reviews see [33,34]. Therefore, we consider TLR2 and TLR6 to be potentially important candidate genes for resistance to microbial diseases of both humans and cattle.

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Unlike the TLR proteins, several of the mammalian peptidoglycan recognition proteins (PGRPs) modulate innate immunity via pattern recognition of invading microbes followed by bactericidal or bacteriostatic activity [35,36]. The peptidoglycan recognition proteins were originally classified into four categories based on transcript length (short, PGRP-S; long, PGRP-L; intermediate, PGRP-Iα, PGRP-Iβ), but have since been formally named peptidoglycan recognition proteins 1, 2, 3, and 4 (PGLYRP1, PGLYRP2, PGLYRP3, PGLYRP4), respectively, by the Human Genome Organization Gene Nomenclature Committee [35,36]. Specifically, mammalian PGLYRP2 hydrolyzes bacterial peptidoglycan, with the remaining three PGLYRPs functioning as bactericidal or bacteriostatic proteins [for reviews see [36–38]. A recent study indicates that bovine PGRP-S (PGLYRP1) has multiple functions and microbial affinities [35]. Interestingly, bovine PGLYRP1 binds to a range of microbial components and kills diverse microorganisms [35]. Therefore, bovine PGRPs may be generalists in both antimicrobial affinity and activity [35]. For these reasons, we consider PGLYRP1 to be a potentially important candidate gene for resistance to microbial diseases of both cattle and humans.

In this study we report a comparative sequence analysis for bovine TLR2, TLR6, and PGLYRP1 using a DNA panel consisting of nine breeds derived from *Bos taurus, Bos indicus,* and crosses thereof (*B. taurus*—Angus, Charolais, Holstein, Limousin; *B. indicus*—Brahman, Nelore; crossbred—Braford, Piedmontese, Romagnola). For each comparative analysis, the Hereford reference sequence from the Bovine Genome Project (http://www.hgsc.bcm.tmc.edu/projects/bovine/) was also included. Detailed information regarding the origins of the breeds surveyed may be accessed at the Oklahoma State University Web site (http://www.ansi.okstate.edu/breeds/cattle/). The results of this study will facilitate future association and TLR signaling studies relevant to bovine innate immunity. Moreover, we also describe how nonsynonymous SNPs (nsSNPs) within bovine TLR2, TLR6, and PGLYRP1 influence protein domain prediction while also comparatively defining conserved domain architectures among cow, human, mouse, rat, dog, and chicken.

Results

Bovine polymorphisms detected

Collectively, 7798 bp corresponding to bovine TLR2, TLR6, and PGLYRP1 were interrogated for nine domestic cattle breeds and compared to the equivalent Hereford reference sequences from the Bovine Genome Project. Overall, 82 SNPs were identified, resulting in an average density of 1 SNP for every 95 bp sequenced. Additionally, one insertion-deletion (indel) polymorphism was detected, with all possible genotypic classes (+/+,+/−, −/−) observed among the 10 cattle breeds surveyed. Of the 82 SNPs identified, 70% (n=57) were transitions and 30% (n=25) were transversions. A search of the published literature on bovine TLR2, TLR6, and PGLYRP1 revealed that the majority of the polymorphisms detected (72 of 83; 87%) are formally reported here for the first time. Notably, predicted amino acid replacements encoded by bovine TLR2 and TLR6, but not PGLYRP1, resulted in the prediction of protein domain alterations using the simple modular architecture research tool (SMART) [39] online (http://smart.embl-heidelberg.de/).

Tlr2

Bovine TLR2 was previously mapped to BTA17 using several radiation hybrid approaches [10,12]. According to the reference sequence provided by the National Center for Biotechnology Information (NCBI; Accession Nos. NM_174197 and NC_007315), the bovine TLR2 mRNA comprises two exons spanning 3513 bp, including both 5′ and 3′ untranslated regions, and encodes a protein consisting of 784 amino acids. Comparative sequence analysis of TLR2 (3224 bp comprising the coding and flanking sequences) for 10 bovine breeds yielded 43 SNPs (Table 1). Of the 29 SNPs detected within the coding region, 15 (52%) were nonsynonymous. Overall, 60% of the SNPs encountered were transitions, with transitions also accounting for the majority (59%) of the genetic variation observed within the coding region of bovine TLR2. The average density of polymorphism observed for bovine TLR2 was 1 SNP per 75 bp sequenced. All bovine TLR2 SNPs and predicted amino acid replacements are described in Table 1. The genomic positions of all bovine TLR2 SNPs, corresponding major and minor allele frequencies, SNP genotypes, amino acid positions, and relevant GenBank accession numbers are also depicted in Table 1.

Two repetitive sequences (short interspersed nuclear elements or SINEs) were detected within the targeted region of bovine TLR2 (NC_007315) using RepeatMasker online (http://www.repeatmasker.org/). Specifically, a SINE classified as ART2A was identified (NC_007315 positions 9214–9594). Single-nucleotide polymorphisms (Table 1) and one indel polymorphism (NC_007315 positions 9214–9215: TG,+/+,+/−, −/−; ss104796296) were detected within the ART2A repeat, with all indel genotypic classes observed within the bovine DNA panel (genotypes not shown). Additionally, a second smaller SINE classified as CHR-2 was also detected (NC_007315 positions 9631–9796). Two bovine SNPs (Table 1) and no indels were observed within the CHR-2 repeat. Two overlapping CpG islands were independently predicted by CpG Plot (156 bp; http://www.ebi.ac.uk/emboss/cpgplot/) and CpGProD (865 bp; http://pbil.univ-lyon1.fr/software/cpgprod_query.html) online and were located within the coding region of bovine TLR2. Moreover, a single plus-strand promoter was predicted for bovine TLR2 using CpGProD (NC_007315, positions 11250–12114) online.

Comparative prediction of TLR2 protein domain architectures using SMART [39] for *B. taurus, B. indicus, Homo sapiens, Mus musculus, Rattus norvegicus, Canis familiaris,* and *Gallus gallus* revealed three clusters of LRR patterning that were conserved among all species investigated (Fig. 1). Predicted amino acid replacements detected (Table 1) for *B. taurus* and *B. indicus* did not result in the SMART prediction of novel protein domain architectures. However, the presence of either isoleucine (Ile) or valine (Val) at amino acid position 211 (Table 1) for *B. taurus, B. indicus,* and crossbred cattle was determined to modulate SMART prediction of a low-complexity region (Fig. 1). The relative locations of predicted amino acid replacements encoded by bovine TLR2 nsSNPs are depicted in Fig. 1.

Tlr6

Bovine TLR6 was previously mapped to BTA6 [10] and is part of the 50-kb TLR6-TLR1-TLR10 gene cluster previously described [17]. The bovine TLR6 mRNA comprises four exons totaling 3095 bp, which includes 5′ as well as 3′ untranslated regions, and encodes a protein consisting of 793 amino acids (NCBI Accession No. NC_007304) [17]. Notably, the coding region of TLR6 is distributed among two exons, the latter of which is shared by TLR1 according to the most recent NCBI reference sequences for bovine TLR6 and TLR1 (NC_007304; build 3.1). In this study we interrogated 2609 bp of bovine TLR6. The targeted region included the entire coding sequence as well as 5′ and 3′ flanking sequences. Comparative sequence analysis of 2609 bp for 10 bovine breeds yielded 28 SNPs and no indels (Table 2). Of the 25 SNPs detected within the coding region of bovine TLR6, 10 were nonsynonymous (Table 2). Overall, nearly 86% of the SNPs observed were transitions. Moreover, transitions accounted for 88% of the genetic variation detected within the coding region of bovine TLR6. The average density of polymorphism observed for bovine TLR6 was 1 SNP per 93 bp sequenced. All bovine TLR6 SNPs and predicted amino acid replacements are presented in Table 2. The genomic positions of all SNPs, major and minor allele frequencies, SNP genotypes, amino acid positions, and relevant GenBank accession numbers are also provided in Table 2.

Table 1
Single-nucleotide polymorphisms (SNPs) detected in bovine TLR2 by comparative sequence analysis of 10 domestic cattle breeds[a] and the corresponding GenBank accession numbers for the TLR2 sequences generated for each breed

Allele[b]	Genomic[c]	Observed frequency[d]	Amino acid position[e]	Amino acid[f]	First report[g]	(SNP genotype): bovine breed[h]	dbSNP ID
T/G	9,128	0.90/0.10	NA	NA	Y	(G): P	ss104796293
A/G	9,163	0.90/0.10	NA	NA	Y	(G): N	ss104796294
T/C	9,166	0.80/0.20	NA	NA	Y	(C): N; (Y): Bd, Bn	ss104796295
A/T	9,273	0.80/0.20	NA	NA	Y	(T): N; (W): Bd, Bn	ss104796297
A/C	9,400	0.80/0.20	NA	NA	Y	(C): N; (M): Bd, Bn	ss104796298
G/A	9,431	0.95/0.05	NA	NA	Y	(R): Bn	ss104796299
G/A	9,463	0.80/0.20	NA	NA	Y	(A): N; (R): Bd, Bn	ss104796300
G/A	9,474	0.95/0.05	NA	NA	Y	(R): Bd	ss104796301
T/C	9,510	0.80/0.20	NA	NA	Y	(C): N; (Y): Bd, Bn	ss104796302
C/T	9,564	0.95/0.05	NA	NA	Y	(Y): An	ss104796303
G/A	9,579	0.95/0.05	NA	NA	Y	(R): Bd	ss104796304
A/C	9,589	0.80/0.20	NA	NA	Y	(C): N; (M): Bd, Bn	ss104796305
G/T	9,675	0.85/0.15	NA	NA	Y	(T): N; (K): Bd	ss104796306
C/T	9,708	0.80/0.20	NA	NA	Y	(T): N; (Y): Bd, Bn	ss104796307
T/C	10,095	0.80/0.20	62	Asn/Asn	Y	(C): N; (Y): Bd, Bn	ss104796308
G/T	10,098	0.55/0.45	63	**Glu/Asp**	N[i,j]	(T): An, Hn (K): Bd, Bn, Ch, L, P	ss104796309
G/A	10,111	0.90/0.10	68	**Gly/Ser**	Y	(A): N	ss104796310
G/A	10,364	0.95/0.05	152	**Arg/Gln**	Y	(R): Ro	ss104796311
A/G	10,540	0.55/0.45	211	**Ile/Val**	N[j]	(G): N, Ro (R): Bd, Bn, Ch, L, P	ss104796312
T/A	10,590	0.90/0.10	227	**Phe/Leu**	Y	(A): N	ss104796313
T/G	10,779	0.90/0.10	290	**Asp/Glu**	Y	(G): L	ss104796314
G/T	10,854	0.95/0.05	315	Arg/Arg	Y	(K): Bd	ss104796315
T/A	10,887	0.80/0.20	326	**His/Gln**	Y	(A): N; (W): Bd, Bn	ss104796316
G/A	10,919	0.80/0.20	337	**Arg/Lys**	Y	(A): N; (R): Bd, Bn	ss104796317
A/C	10,938	0.95/0.05	343	Ile/Ile	Y	(M): Bn	ss104796318
C/T	11,123	0.95/0.05	405	**Thr/Met**	Y	(Y): N	ss104796319
A/G	11,159	0.80/0.20	417	**Asn/Ser**	Y	(G): N; (R): Bd, Bn	ss104796320
A/C	11,217	0.80/0.20	436	Gly/Gly	Y	(C): N; (M): Bd, Bn	ss104796321
T/G	11,413	0.95/0.05	502	**Ser/Ala**	Y	(K): N	ss104796322
C/**T**	11,541	0.90/0.10	544	Phe/Phe	Y	(C): An, Bd, Bn, Ch, Hn, L, P, N, Ro	ss104796323
G/A	11,597	0.80/0.20	563	**Arg/His**	Y	(A): N; (R): Bd, Bn	ss104796324
T/C	11,616	0.75/0.25	569	His/His	Y	(C): N; (Y): Bd, Bn, Ro	ss104796325
A/C	11,688	0.80/0.20	593	Ala/Ala	Y	(C): N; (M): Bd, Bn	ss104796326
G/T	11,691	0.80/0.20	594	Ala/Ala	Y	(T): N; (K): Bd, Bn	ss104796327
C/T	11,723	0.90/0.10	605	**Thr/Met**	Y	(T): N	ss104796328
C/T	11,748	0.95/0.05	613	His/His	Y	(Y): Bn	ss104796329
C/G	11,904	0.80/0.20	665	**His/Gln**	Y	(G): N; (S): Bd, Bn	ss104796330
T/C	11,934	0.80/0.20	675	His/His	Y	(C): N; (Y): Bd, Bn	ss104796331
T/C	11,964	0.85/0.15	685	Ile/Ile	Y	(C): N; (Y): Bd	ss104796332
G/C	12,033	0.95/0.05	708	Val/Val	Y	(S): Bd	ss104796333
G/A	12,123	0.80/0.20	738	Glu/Glu	Y	(A): N; (R): Bd, Bn	ss104796334
C/T	12,204	0.80/0.20	765	Pro/Pro	Y	(T): N; (Y): Bd, Bn	ss104796335
G/A	12,257	0.90/0.10	783	**Arg/Lys**	Y	(A): L	ss104796336

Breed	GenBank	Breed	GenBank	Breed	GenBank
Angus	EU746464	Charolais	EU746465	Nelore	EU746461
Braford	EU746457	Holstein	EU746459	Piedmontese	EU746462
Brahman	EU746458	Limousin	EU746460	Romagnola	EU746463

[a] An, Angus; Bd, Braford; Bn, Brahman; Ch, Charolais; Hn, Holstein; L, Limousin; N, Nelore; P, Piedmontese; Ro, Romagnola. All bovine TLR2 sequences are compared to the Hereford TLR2 sequence (GenBank Accession No. NC_007315).
[b] Alleles are depicted as major allele/minor allele, with the Hereford (NC_007315) allele depicted in bold.
[c] Genomic position based on GenBank Accession No. NC_007315.
[d] Observed frequencies for major and minor alleles, respectively.
[e] Amino acid position based on GenBank Accession No. NC_007315 (Hereford).
[f] Amino acid(s) encoded by the major and minor alleles, respectively, with predicted amino acid replacements illustrated in bold.
[g] Novelty of the polymorphism: Y, first known report of the polymorphism; N, polymorphism has previously been reported.
[h] Breed reference and annotation of TLR2 SNPs deviating from GenBank Accession No. NC_007315 (Hereford). Heterozygous SNP genotypes are depicted using the IUPAC codes for heterozygosity; homozygous genotypes are indicated by a standard, single nucleotide (A, C, T, G).
[i] McGuire et al. [10].
[j] Opsal et al. [18].

No repetitive sequences were detected within the targeted regions of bovine TLR6 (NC_007304) using RepeatMasker online (http://www.repeatmasker.org/). However, it should be noted that the current library for bovine repeats may not be complete, thereby rendering some repeats unrecognizable by RepeatMasker. No putative CpG islands were identified by either CpG Plot (http://www.ebi.ac.uk/emboss/cpgplot/) or CpGProD online (http://pbil.univ-lyon1.fr/software/cpgprod_query.html). Moreover, no putative promoters were predicted by CpGProD online.

Comparative prediction of TLR6 protein domain architectures via SMART [39] for *B. taurus, B. indicus, H. sapiens, M. musculus,* and *R. norvegicus* revealed three clusters of LRR patterning that were conserved among all species investigated (Fig. 2). Both *C. familiaris* and *G. gallus* were excluded from comparative protein domain analyses because no complete TLR6 amino acid sequence was available for *C. familiaris* (http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9615) and no orthologous TLR6 amino acid sequence could be clearly defined for *G. gallus* [40,41] (http://www.ncbi.nlm.nih.gov/mapview/map_search.

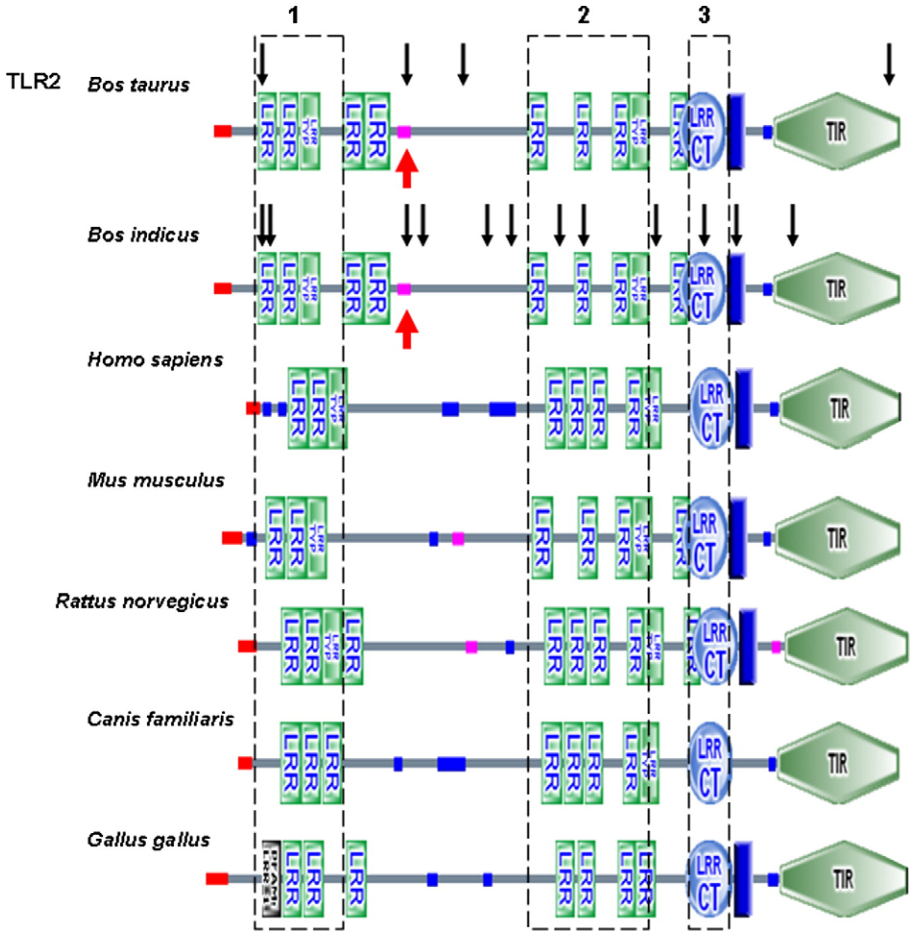

Fig. 1. Comparative evaluation of TLR2 protein domain architectures for *B. taurus, B. indicus, H. sapiens, M. musculus, R. norvegicus, C. familiaris,* and *G. gallus.* Domain diagrams were generated using SMART [39] and are not precisely scaled. Only confidently predicted and nonoverlapping domains are depicted. Nucleotide and/or amino acid sequences for all species except *B. indicus* were retrieved from GenBank (*B. taurus* NC_007315, *H. sapiens* NP_003255, *M. musculus* NP_036035, *R. norvegicus* NP_942064, *C. familiaris* NP_001005264, *G. gallus* NP_989609). The *B. indicus* TLR2 sequence was derived from this study. Patterns of leucine-rich repeats conserved across all taxa are indicated by a dashed black box. SMART-predicted protein domains and regions are as follows: signal peptides are indicated by small red boxes; low-complexity regions are indicated in pink; regions of intrinsic disorder are indicated by small, blue, horizontal boxes; leucine-rich repeats are depicted as LRR, LRR TYP, PFAM LRR, and LRRCT; vertical blue rectangles indicate the transmembrane domain; TIR indicates Toll-interleukin 1-resistance homologous domain. Black arrows indicate the locations of predicted amino acid replacements encoded by bovine TLR2 nsSNPs (63 Glu/Asp, 68 Gly/Ser, 211 Ile/Val, 227 Phe/Leu, 290 Asp/Glu, 326 His/Gln, 337 Arg/Lys, 405 Thr/Met, 417Asn/Ser, 502 Ser/Ala, 563 Arg/His, 605 Thr/Met, 665 His/Gln, 783 Arg/Lys; Table 1). Note that 152 Arg/Gln (Table 1) is not illustrated because the origin of the polymorphism (*B. taurus, B. indicus*) was not apparent. Predicted amino acid replacements (excluding 152 Arg/Gln) encoded by bovine TLR2 did not result in the prediction of novel protein domain architectures for either species of *Bos*. One predicted amino acid replacement (211 Ile/Val; Table 1) was determined to modulate either confident prediction (211 Val) or ablation (211 Ile) of a low-complexity protein domain for *B. taurus, B. indicus,* and crossbred cattle using SMART [39], as indicated by large red arrows.

cgi?taxid=9031). Interestingly, two predicted amino acid replacements (214Asp → Asn, 494Phe → Ile; Table 2), both independently and/or collectively, were determined to modulate SMART prediction of two LRR domains (Pfam-LRR1) for *B. taurus* (Fig. 2). Specifically, two Pfam-LRR1 domains were confidently predicted via SMART for the *B. taurus* TLR6 reference sequence (NP_001001159 from NC_007304), and two predicted amino acid replacements (214Asp → Asn, 494Phe → Ile) were subsequently determined to ablate the confident prediction of these domains. The two Pfam-LRR1 domains were not confidently predicted for *B. indicus* TLR6 using SMART (Fig. 2). In addition to domain alterations predicted by amino acid replacements, a 5′ and a 3′ transmembrane domain were also predicted for the bovine TLR6 reference sequence (*B. taurus;* NC_007304, NP_001001159; Fig. 2). The relative locations of all predicted amino acid polymorphisms encoded by bovine TLR6 nsSNPs (Table 2) are depicted in Fig. 2.

Pglyrp1

Bovine PGLYRP1 has been placed on BTA18, according to build 3.1 of the bovine genome (http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9913). The bovine PGLYRP1 mRNA comprises three exons totaling 688 bp, which includes 5′ as well as 3′ untranslated regions, and encodes a protein consisting of 190 amino acids, according to the reference sequence provided by NCBI (NCBI Accession No. NC_007316). Comparative sequence analysis of 1965 bp spanning the coding and contiguous flanking regions of bovine PGLYRP1 for a panel of 10 cattle breeds revealed 11 SNPs and no indels (Table 3). Of the 3 SNPs detected within the coding region of bovine PGLYRP1, 2 were nonsynonymous. Overall, nearly 64% of the SNPs observed were transitions. However, transversions accounted for the majority (66%) of the genetic variation detected within the coding region. The average density of polymorphism observed for bovine PGLYRP1 was 1 SNP per 179 bp sequenced. All bovine PGLYRP1 SNPs and predicted amino acid replacements are presented in Table 3. Additionally, the genomic positions of all bovine PGLYRP1 SNPs, corresponding major and minor allele frequencies, SNP genotypes, amino acid positions, and relevant GenBank accession numbers are also provided in Table 3.

Analysis of bovine PGLYRP1 (2064 bp; NC_007316) via RepeatMasker online (http://www.repeatmasker.org/) revealed five repetitive

Table 2
Single-nucleotide polymorphisms (SNPs) detected in bovine TLR6 by comparative sequence analysis of 10 domestic cattle breeds[a] and the corresponding GenBank accession numbers for the TLR6 sequences generated for each breed

Allele[b]	Genomic[c]	Observed frequency[d]	Amino acid position[e]	Amino acid[f]	First report[g]	(SNP genotype): bovine breed[h]	dbSNP ID
A/T	13,851	0.90/0.10	NA	NA	Y	(T): An	ss104796337
T/G	14,066	0.90/0.10	43	**Leu/Arg**	Y	(G): Bn	ss104796338
A/G	14,121	0.80/0.20	61	Gln/Gln	Y	(G): Bn, N	ss104796339
A/G	14,197	0.85/0.15	87	**Arg/Gly**	Y	(G): Bn; (R): N	ss104796340
G/A[i]	14,578	0.50/0.50	214	**Asp/Asn**	N[j]	(A): Bd, Bn, N (R): An, Ch, Hn, L	ss104796341
G/A	14,589	0.80/0.20	217	Ala/Ala	Y	(A): Bn, N	ss104796342
C/T	15,060	0.90/0.10	374	Asp/Asp	N[k]	(Y): Ch, Ro	ss104796343
A/G	15,121	0.80/0.20	395	**Thr/Ala**	Y	(G): Bn, N	ss104796344
T/C	15,213	0.80/0.20	425	Ser/Ser	Y	(C): Bn, N	ss104796345
C/T	15,312	0.80/0.20	458	His/His	Y	(T): Bn; (Y): N	ss104796346
T/A	15,418	0.95/0.05	494	**Phe/Ile**	Y	(W): An	ss104796347
T/C	15,453	0.85/0.15	505	Asn/Asn	Y	(C): Bn; (Y): N	ss104796348
C/T	15,492	0.95/0.05	518	Cys/Cys	Y	(Y): L	ss104796349
C/**T**	15,515	0.55/0.45	526	**Ala/Val**	N[k,l]	(C): Bd, Bn, N (Y): An, Ch, Hn, L, Ro	ss104796350
G/A	15,516	0.70/0.30	526	**Val/Ala**[m]	N[l]	(A): Bd (R): An, Ch, Hn, L	ss104796351
C/G	15,555	0.80/0.20	539	**Asp/Glu**	Y	(G): Bn, N	ss104796352
G/A	15,568	0.85/0.15	544	**Val/Ile**	Y	(A): Bn; (R): N	ss104796353
G/A	15,703	0.55/0.45	589	**Val/Ile**	N[k,l]	(A): Bd, Ch, L (R): An, Hn, Ro	ss104796354
C/T	15,753	0.70/0.30	605	Leu/Leu	N[l]	(T): Bd (Y): An, Ch, Hn, L	ss104796355
A/G	15,943	0.80/0.20	669	**Ile/Val**	Y	(G): Bn, N	ss104796356
C/T	15,960	0.70/0.30	674	His/His	N[l]	(T): Bd (Y): An, Ch, Hn, L	ss104796357
A/G	15,966	0.80/0.20	676	Arg/Arg	Y	(G): Bn, N	ss104796358
T/C	15,990	0.80/0.20	684	Ile/Ile	Y	(C): Bn, N	ss104796359
T/C	16,038	0.80/0.20	700	Phe/Phe	Y	(C): Bn, N	ss104796360
T/**C**	16,041	0.65/0.35	NA	NA	Y	(T): Bd, Bn, Ch, L, N (Y): An, Hn, Ro	ss104796361
G/**A**	16,068	0.65/0.35	NA	NA	Y	(G): Bd, Bn, Ch, L, N (R): An, Hn, Ro	ss104796362
C/**T**	33,094	0.60/0.40	701	Val/Val	N[k]	(C): An, Ch, N, P, Ro (Y): Hn, L	ss73689418
C/T	33,118	0.95/0.05	709	Ser/Ser	N[n]	(Y): N	ss73689419

Breed	GenBank	Breed	GenBank	Breed	GenBank
Angus	EU746466	Charolais	EU746469	Nelore	EU746474
Braford	EU746467	Holstein	EU746470	Piedmontese	EU746472
Brahman	EU746468	Limousin	EU746471	Romagnola	EU746473

[a] An, Angus; Bd, Braford; Bn, Brahman; Ch, Charolais; Hn, Holstein; L, Limousin; N, Nelore; P, Piedmontese; Ro, Romagnola. All bovine TLR6 sequences are compared to the Hereford TLR6 sequence (GenBank Accession No. NC_007304).
[b] Alleles are depicted as major allele/minor allele, with the Hereford (NC_007304) allele depicted in bold.
[c] Genomic position based on GenBank Accession No. NC_007304 (Hereford).
[d] Observed frequencies for major and minor alleles, respectively.
[e] Amino acid position based on GenBank Accession No. NC_007304 (Hereford).
[f] Predicted amino acid(s) encoded by the major and minor alleles, respectively, with predicted amino acid replacements depicted in bold.
[g] Novelty of the polymorphism: Y, first known report of the polymorphism; N, polymorphism has previously been reported.
[h] Breed reference and annotation of TLR6 SNPs deviating from GenBank Accession No. NC_007304 (Hereford). Heterozygous SNP genotypes are depicted using the IUPAC codes for heterozygosity; homozygous genotypes are indicated by a standard, single nucleotide (A, C, T, G).
[i] Both alleles are equally represented in the sample.
[j] Opsal et al. [17].
[k] McGuire et al. [10].
[l] Menzies and Ingham [9].
[m] Ala or Val at amino acid position 526 is modulated by variation encoded by genomic position 15,515.
[n] Seabury et al. [15].

sequences. Altogether, the repetitive sequences totaled 480 bp and contained the following five repeats: SINE/MIR (594–687), SINE/MIR (745–785), SINE/MIR (1221–1359), SINE/BovA (1393–1504), SINE/MIR (1615–1708). Six SNPs detected for bovine PGLYRP1 occur within four of the repetitive sequences described above (see Table 3 for SNP positions). All of the repetitive sequences identified were located in noncoding regions, according to the bovine PGLYRP1 reference sequence provided by NCBI online (NC_007316). Two putative CpG islands (NC_007316; 48–431 and 1832–2007) were predicted using CpG Plot (http://www.ebi.ac.uk/emboss/cpgplot/) online. One overlapping CpG island and putative promoter (NC_007316; 1–840) was also predicted via CpGProD (http://pbil.univ-lyon1.fr/software/cpgprod_query.html) online.

Comparative prediction of PGLYRP1 protein domain architectures using SMART [39] for *B. taurus, B. indicus, H. sapiens, M. musculus, R. norvegicus,* and *C. familiaris* revealed three domains that were conserved among all taxa as follows: signal peptide, animal peptidoglycan recognition protein homologous to bacteriophage T3 lysozyme (PGRP), and a zinc amidase/*N*-acetylmuramoyl-L-alanine amidase activity (Ami_2) domain that overlapped with PGRP (Fig. 3). Predicted amino acid replacements encoded by bovine PGLYRP1 nsSNPs (Table 3) did not result in the prediction of novel protein domain architectures or domain alterations using SMART [39]. The domain locations and distribution of all bovine PGLYRP1 predicted amino acid replacements for *B. taurus* and *B. indicus* are comparatively

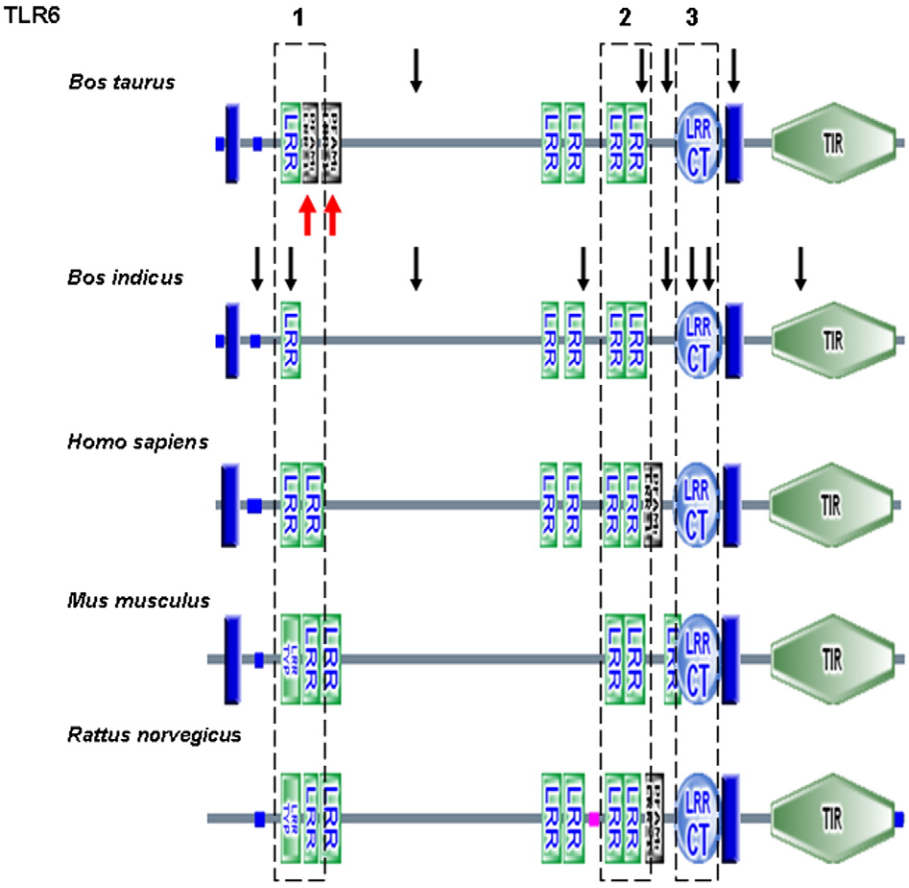

Fig. 2. Comparative evaluation of TLR6 protein domain architectures for *B. taurus, B. indicus, H. sapiens, M. musculus,* and *R. norvegicus.* Domain diagrams were generated using SMART [39] and are not precisely scaled. Only confidently predicted and nonoverlapping domains are depicted. Nucleotide and/or amino acid sequences for all species except *B. indicus* were retrieved from GenBank (*B. taurus* NC_007304, NP_001001159; *H. sapiens* NP_006059; *M. musculus* NP_035734; *R. norvegicus* NP_997487). The *B. indicus* TLR6 sequence was derived from this study. Patterns of LRRs conserved across all taxa are indicated by dashed, black boxes. SMART-predicted protein domains and regions are as follows: regions of low complexity are indicated in pink; regions of intrinsic disorder are indicated by small, blue horizontal boxes; leucine-rich repeats are depicted as LRR, LRR TYP, PFAM LRR, and LRRCT; vertical blue rectangles indicate the transmembrane domain; TIR indicates Toll-interleukin 1-resistance homologous domain. Black arrows indicate the locations of predicted amino acid polymorphisms encoded by bovine TLR6 nsSNPs (43 Leu/Arg, 87 Arg/Gly, 214 Asp/Asn, 395 Thr/Ala, 494 Phe/Ile, 526 Ala/Val, 539 Asp/Glu, 544 Val/Ile, 589 Val/Ile, 669 Ile/Val; see Table 2). Two predicted amino acid replacements (214Asp → Asn; 494Phe → Ile; Table 2), either independently or collaboratively, ablate confident prediction of the two PFAM LRR domains indicated by large red arrows.

illustrated in Fig. 3. Additionally, a consensus PGLYRP1 protein domain architecture representing *H. sapiens, M. musculus, R. norvegicus,* and *C. familiaris* is depicted in Fig. 3. *Gallus gallus* was excluded from the comparative protein domain analysis because no orthologous PGLYRP1 (PGRP-S) amino acid sequence could be definitively identified using the Chicken Genome Resources online (http://www.ncbi.nlm.nih.gov/genome/guide/chicken/).

Discussion

Domain architecture and nsSNPs

Previous studies provide evidence that the LRR-containing ecto-domains of the TLR proteins are likely to facilitate detection of invading PAMPs [8,33,42–44]. Comparative prediction of TLR2 protein domain architectures for multiple mammalian lineages and one outgroup avian lineage revealed at least three conserved regions of LRR patterning among all species investigated (Fig. 1). Notably, comparative protein domain analysis for TLR2 revealed that *C. familiaris* (GenBank NP_001005264) was the only species for which a transmembrane domain was not confidently predicted by SMART [39]. Signal peptides and Toll-interleukin-1 resistance (TIR) domains, as defined by SMART [39], were predicted for TLR2 of all species investigated (Fig. 1). Nonsynonymous SNPs were detected within most major protein domains predicted for bovine TLR2 (Fig. 1), with predicted amino acid replacements observed within the LRR, transmembrane, and TIR domains (Fig. 1). The majority of all predicted amino acid replacements detected for bovine TLR2 were observed for breeds influenced and/or derived from *B. indicus* (Table 1; Fig. 1). Interestingly, one predicted amino acid replacement (211Ile/Val) encoded by a nsSNP at TLR2 genomic position 10,540 (Table 1) was determined to modulate the presence or absence of a low-complexity protein domain for *B. taurus, B. indicus,* and crossbred cattle during sequential SMART [39] analyses (Fig. 1). Notably, the low-complexity region was not predicted for the bovine TLR2 reference sequence (*B. taurus;* NC_007315, NP_776622), which encoded Ile at amino acid position 211. With the exception of *C. familiaris* lacking a predicted transmembrane domain, the predicted protein domain architectures for TLR2 were similar for all species investigated, indicating a detectable level of conservation across mammal and bird lineages.

Comparative prediction of TLR6 protein domain architectures for five mammalian lineages revealed at least three conserved regions of LRR patterning across all taxa investigated (Fig. 2). The majority of all nsSNPs and predicted amino acid replacements detected for bovine TLR6 were observed for breeds derived from *B. indicus.* No signal peptides were predicted for the five mammalian TLR6 amino acid sequences evaluated using SMART [39]. Interestingly, a 5′ and a 3′ transmembrane domain were predicted for the bovine TLR6 reference

Table 3
Single-nucleotide polymorphisms (SNPs) detected in bovine PGLYRP1 by comparative sequence analysis of 10 domestic cattle breeds[a] and the corresponding GenBank accession numbers for the PGLYRP1 sequences generated for each breed

Allele[b]	Genomic[c]	Observed frequency[d]	Amino acid position[e]	Amino acid[f]	First report[g]	(SNP genotype): bovine breed[h]	dbSNP ID
G/C	130	0.55/0.45	34	Gly/Gly	Y	(C): Hn, L, P, Ro (S): Ch	ss104796363
T/C	254	0.80/0.20	76	**Tyr/His**	Y	(C): Bn, N	ss104796364
C/T	661	0.95/0.05	NA	NA	Y	(Y): N	ss104796365
C/G	677	0.90/0.10	NA	NA	Y	(G): An	ss104796366
G/A	972	0.75/0.25	NA	NA	Y	(A): Bn; (R): Bd, N, P	ss104796367
G/A	979	0.95/0.05	NA	NA	Y	(R): Bd	ss104796368
G/C	1395	0.95/0.05	NA	NA	Y	(S): N	ss104796369
C/T	1411	0.95/0.05	NA	NA	Y	(Y): Ro	ss104796370
C/T	1457	0.90/0.10	NA	NA	Y	(T): Ro	ss104796371
T/C	1686	0.65/0.35	NA	NA	Y	(Y): Bd, Hn, P	ss104796372
T/G	1954	0.90/0.10	184	**Trp/Gly**	Y	(G): An	ss104796373

Breed	GenBank	Breed	GenBank	Breed	GenBank
Angus	EU746455	Charolais	EU746450	Nelore	EU746453
Braford	EU746448	Holstein	EU746451	Piedmontese	EU746454
Brahman	EU746449	Limousin	EU746452	Romagnola	EU746456

[a] An, Angus; Bd, Braford; Bn, Brahman; Ch, Charolais; Hn, Holstein; L, Limousin; N, Nelore; P, Piedmontese; Ro, Romagnola. All bovine PGLYRP1 sequences are compared to the Hereford PGLYRP1 sequence (GenBank Accession No. NC_007316).
[b] Alleles are depicted as major allele/minor allele, with the Hereford (NC_007316) allele depicted in bold.
[c] Genomic position based on GenBank Accession No. NC_007316 (Hereford).
[d] Observed frequencies for major and minor alleles, respectively.
[e] Amino acid position based on GenBank Accession No. NC_007316 (Hereford).
[f] Amino acid(s) encoded by the major and minor alleles, respectively, with predicted amino acid replacements illustrated in bold.
[g] Novelty of the polymorphism: Y, first known report of the polymorphism depicted; N, polymorphism has been previously reported.
[h] Breed reference and annotation of PGLYRP1 SNPs deviating from GenBank Accession No. NC_007316 (Hereford). Heterozygous SNP genotypes are depicted using the IUPAC codes for heterozygosity; homozygous genotypes are indicated by a standard, single nucleotide (A, C, T, G).

sequence (*B. taurus;* NC_007304, NP_001001159). Comparative alignment of the TLR6 protein domain architectures predicted by SMART [39] revealed two transmembrane domains for all species investigated except *R. norvegicus* (Fig. 2). To date, no other mammalian TLR loci have been reported or predicted to contain two transmembrane domains. The biological significance of this predicted protein domain architecture is currently unknown. Predicted bovine amino acid replacements encoded by nsSNPs were determined to modulate protein domain alterations during SMART [39] analyses (Fig. 2). Specifically, two amino acid replacements (214Asp → Asn, 494Phe → Ile), both independently and/or collaboratively, were determined to abolish confident prediction of two distinct LRR domains (Pfam-LRR1; amino acid positions 101–119 and 123–146) using SMART [39] (see Fig. 2). Representatives of *B. indicus* (Brahman, Nelore) investigated herein were monomorphic at TLR6 amino acid positions 214 and 494 (encoding 214Asn, 494Phe; Table 2). Therefore, the two LRR domains (Pfam-LRR1) spanning amino acid positions 101–119 and 123–146 were not confidently predicted for *B. indicus* TLR6 using SMART [39] (Fig. 2). In contrast, nsSNPs corresponding to TLR6 amino acid positions 214 and/or 494 (Table 2) were observed for bovine breeds representing *B. taurus* (Angus, Charolais, Holstein, Limousin), with relevant variation (Asp214Asn; Phe494Ile) modulating either confident prediction or ablation of the two bovine TLR6 LRR (Pfam-LRR1) domains as described above (see Table 2; Fig. 2). Collectively, the distribution of nsSNPs detected for bovine TLR6 included most major protein domains predicted by SMART [39], with predicted amino acid replacements noted within the LRR, transmembrane, and TIR domains (Fig. 2).

Among the loci interrogated in this study, comparative sequence analysis of PGLYRP1 for 10 domestic cattle breeds revealed the fewest nsSNPs. However, it should be noted that only three variable nucleotide sites were observed within the PGLYRP1 coding sequence, two of which were nonsynonymous (Table 3). The two nsSNPs were observed within regions encoding the overlapping PGRP/Ami_2 domains (*B. indicus*) and a 3′ unstructured region of bovine PGLYRP1 (*B. taurus*), with no nsSNPs detected within the predicted signal peptides or 5′ unstructured regions (Table 3; Fig. 3). Interestingly, only representatives of *B. indicus* (Brahman, Nelore) were observed to encode nsSNPs within the major protein domains (PGRP/Ami_2) confidently predicted by SMART [39] (Table 3; Fig. 3). Unlike bovine TLR2 and TLR6, nsSNPs detected for bovine PGLYRP1 did not manifest as confidently predicted protein domain alterations during sequential SMART [39] analyses. Comparative analysis of the predicted protein domain architectures for PGLYRP1 of six mammalian lineages revealed a single consensus configuration that was conserved among all species evaluated (Fig. 3).

Bovine quantitative trait locus (QTL) alignment with TLR2, TLR6, and PGLYRP1

Currently, three online databases that provide public access to bovine QTLs are available for fine mapping and genomic orientation toward candidate genes through QTL alignment with the bovine genome. Each database is hosted by an independent academic institution, with one sponsored by Texas A and M University (Bovine QTL Viewer: http://bovineqtlv2.tamu.edu/index.html) and the other two hosted by Iowa State University (http://www.animalgenome.org/QTLdb/cattle.html) and the University of Sydney, Australia (http://www.vetsci.usyd.edu.au/reprogen/QTL_Map/). As previously reported [15], a search of the three databases for health-related QTL identified on BTA6 revealed two bovine QTLs overlapping the TLR10-TLR1-TLR6 gene cluster. Specifically, a genome-wide suggestive QTL affecting bovine spongiform encephalopathy was determined to overlap with the TLR10-TLR1-TLR6 gene cluster on BTA6 [45]. A second QTL of genome-wide significance for clinical mastitis [46] also overlapped with the chromosomal region harboring the bovine TLR10-TLR1-TLR6 gene cluster. Similar queries of all the available bovine databases did not reveal any health-related QTL that overlapped with the chromosomal position of TLR2 or PGLYRP1, located on BTA17 and BTA18, respectively. However, a microsatellite marker on BTA18 (BM2078) was significantly associated with *M. avium paratuberculosis* infection in cattle during a recent genome scan [47]. The physical distance between BM2078 and PGLYRP1 is approximately 7 Mb based on build 3.1 of the *B. taurus* genome (http://www.ncbi.nlm.nih.gov/mapview/

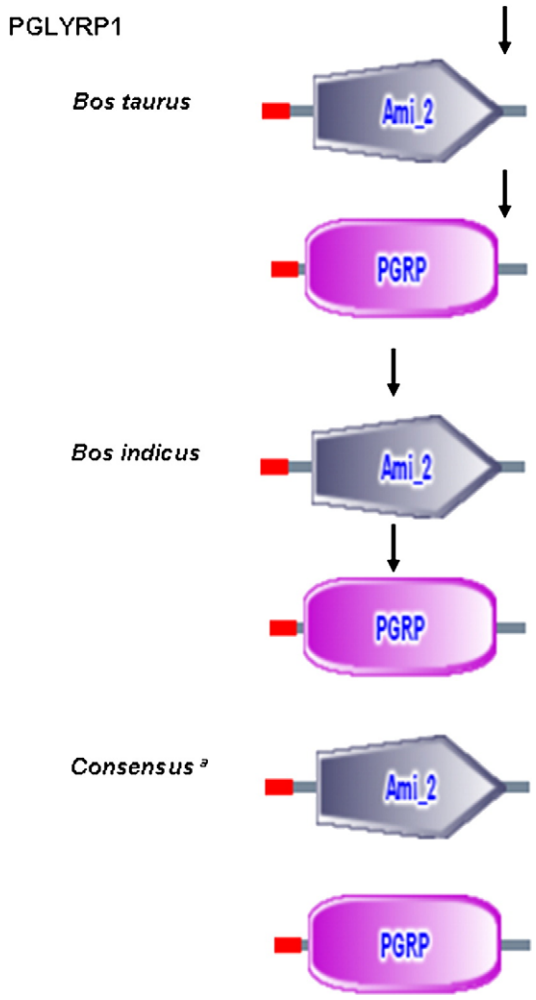

Fig. 3. Comparative evaluation of PGLYRP1 protein domain architectures for *B. taurus*, *B. indicus*, *H. sapiens*[a], *M. musculus*[a], *R. norvegicus*[a], and *C. familiaris*[a]. Domain diagrams were generated using SMART [39] and are not precisely scaled. Only confidently predicted domains are depicted. Nucleotide and/or amino acid sequences for all species except *B. indicus* were retrieved from GenBank (*B. taurus* NC_007316, NP_776998; *H. sapiens* NP_005082; *M. musculus* NP_033428; *R. norvegicus* NP_445825; *C. familiaris* XP_855038). The *B. indicus* PGLYRP1 sequence was derived from this study. SMART-predicted domains and protein regions are indicated as follows: signal peptides are indicated by small red boxes; PGRP indicates animal peptidoglycan recognition protein homologous to bacteriophage T3 lysozyme; Ami_2 indicates zinc amidase/*N*-acetylmuramoyl-L-alanine amidase activity. PGRP and Ami_2 overlap. Black arrows illustrate the locations of predicted amino acid polymorphisms encoded by bovine PGLYRP1 nsSNPs (76 Tyr/His, 184 Trp/Gly; Table 3). Predicted amino acid replacements encoded by bovine PGLYRP1 did not result in the prediction of novel protein domain architectures or protein domain alterations for either species of *Bos*.

map_search.cgi?taxid=9913). Subsequent interval mapping failed to provide further statistical support for a *M. avium paratuberculosis* infection QTL on BTA18 [47].

Notably, TLR2 recognizes a variety of microbial products and generally functions as a heterodimer with either TLR1 or TLR6 [8,31]. The TLR2/TLR1 heterodimer is known to recognize a variety of lipoproteins, including those derived from mycobacteria and meningococci [8,26,48], whereas the TLR2/TLR6 heterodimer recognizes mycoplasma lipoproteins, peptidoglycan, and components derived from both protozoa and fungi [8,49]. Importantly, TLR2 may also recognize ligands such as the gram-positive cell wall component lipoteichoic acid, mycobacterial-derived lipoarabinomannan, and atypical lipopolysaccharide as either homodimers and/or heterodimers with other non-TLR proteins [for specific review see 8]. Recent studies indicate that TLR2/6 heterodimers also recognize *Bacillus anthracis*, the causative agent of anthrax [50], and that bovine TLR2 mRNA abundance significantly increases during mastitis infection [11]. For these reasons, TLR2, TLR6, and TLR1 should not be discounted as potentially important candidate genes for bovine genetic improvement, vaccine development [51], or innate immunologicals [52] aimed at suppressing economically important diseases in domestic cattle.

Peptidoglycan (PGN) is a major constituent of bacterial cell walls and one of the main microbial components detected by the mammalian innate immune system [53]. The recognition of PGN in mammals is modulated by several gene families, including the TLRs, nucleotide-binding oligomerization domain-containing proteins, and the PGRPs [for review see 53]. Bovine PGLYRP1 is found within neutrophils as well as eosinophils and exhibits the same immune staining pattern as β-defensin [35,54]. To date, relatively few studies exist with respect to the bovine PGRPs [35,55]. Importantly, previous studies indicate that bovine PGLYRP1 is capable of killing microorganisms in which PGN is exposed (*Staphylococcus aureus, Listeria monocytogenes*), buried (*Salmonella typhimurium*), or even absent (*Cryptococcus neoformans*), thereby providing evidence for a diverse role in bovine innate immunity [35,55]. For this reason, we consider bovine PGLYRP1 to be a potentially important candidate gene underlying differential susceptibility to gram-positive bacteria, gram-negative bacteria, and fungi [35,55].

Comparison of polymorphisms among the bovine TLRs

Using the same bovine DNA panel, recent studies provide detailed descriptions of 222 SNPs and 15 indels within 33,022 bp of the bovine TLRs that recognize viral (TLRs 3, 7, 8, and 9) PAMPs [14], as well as microbial (TLRs 1, 5) and/or unspecified (TLR10) ligands [15]. Notably, because TLR10 has been shown to form functional heterodimers with both TLR1 and TLR2, it is possible that TLR10 may collaboratively aid in the recognition of a variety of microbial PAMPs [56]. Including polymorphism data reported in this study, comparative sequence analysis for 10 bovine breeds revealed a total of 293 SNPs and 16 indels localized within 38,855 bp spanning bovine TLRs 1, 2, 3, 5, 6, 7, 8, 9, and 10 ([14,15], this study). Notably, bovine TLR

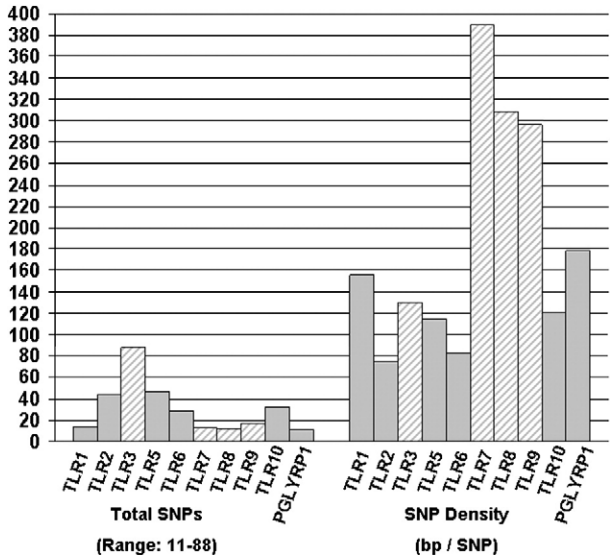

Fig. 4. Comparative evaluation of the total observed SNPs and average SNP density (bp/SNP) for bovine PGLYRP1 as well as TLRs 1, 2, 3, 5, 6, 7, 8, 9, and 10. Total observed SNPs and average SNP densities for bovine PGLYRP1, TLR2, and TLR6 were derived from this study. Total observed SNPs and average SNP densities for all other TLR loci were derived from previous studies [14,15]. Collectively, bovine TLRs predominantly recognizing viral ligands (TLRs 3, 7, 8, 9) revealed a significantly lower average SNP density ($p=0.0001$; two-tailed, Fisher's exact test) than those recognizing microbial and/or unknown ligands (TLRs 1, 2, 5, 6, 10).

Table 4
Oligonucleotide primers for PCR amplification and sequencing of bovine PGLYRP1, TLR2, and TLR6

	5′ to 3′
PGLYRP1[a]	
PGLYRP1_1 (666 bp)[b]	F: ATCTCCGCGTGTCCTTTC
	R: TGACCCTGAAGCTGAGAGAG
PGLYRP1_2 (694 bp)[b]	F: TGGATTCGCTTGGTAAAGTC
	R: AGAGGTTGTAGGTGGCAGAG
PGLYRP1_3 (965 bp)[b]	F: AGAAGATGGGCTCGTGTATG
	R: GTGTGAGAAGACGGACAGG
TLR2[c]	
TLR2_1 (816 bp)[b]	F: TCCTGCTCCATATTCCTACG
	R: TGACTGTGTTTGACATCATGG
TLR2_2 (668 bp)[b]	F: CTCATTCATTTATGGCTGGC
	R: GACCTGAACCAGGAGGATG
TLR2_3 (681 bp)[b]	F: AGATCACCTATGTCGGCAAC
	R: CATGGGTACAGTCATCAAACTC
TLR2_4 (774 bp)[b]	F: AGCATCCATCAGTGAAATGAG
	R: GGTAAGAAGGAGGCATCTGG
TLR2_5 (730 bp)[b]	F: AGTTTAACCCAGTGCCTTCC
	R: TGGAGTCAATGATGTTGTCG
TLR2_6 (436 bp)[b]	F: CCTACTGGGTGGAGAACCTC
	R: ACCACCAGACCAAGACTGAC
TLR6[d,e]	
TLR6_1 (876 bp)[b]	F: ATTGAGAGTAATCAGCCAAT
	R: GTAAGGTTGGTCCTCCAGTG
TLR6_2 (805 bp)[b]	F: ACTACCCATTGCTCACTTGC
	R: CTATACTCCCAACCCAAGAGC
TLR6_3 (845 bp)[b]	F: GACACACGCTTTATACACATGC
	R: CACTGACACACCATCCTGAG
TLR6_4 (604 bp)[b]	F: GCCAAGTATCCAGTGACGTG
	R: AATGGTGTTCTGTGGAATGG

[a] Contig accession number used for PGLYRP1 primer design: NC_007316.
[b] Expected amplicon sizes based on NC_007316, NC_007315, NC_007304.
[c] Contig accession number used for TLR2 primer design: NC_007315.
[d] Contig accession number used for TLR6 primer design: NC_007304.
[e] TLR6_3F and TLR6_4R were used for PCR amplification to ensure specificity for bovine TLR6, with TLR6_3F, TLR6_3R, TLR6_4F, and TLR6_4R used for direct sequencing.

SNP and indel polymorphisms were detected using the same bovine DNA panel in all three studies. A comparative evaluation of total observed SNPs and average SNP density for bovine TLRs 1, 2, 3, 5, 6, 7, 8, 9, and 10, as well as bovine PGLYRP1, is depicted in Fig. 4. Overall, bovine TLRs recognizing viral PAMPs (3, 7, 8, 9) revealed a significantly lower average SNP density ($p=0.0001$; two-tailed Fisher's exact test) than those recognizing microbial and/or unknown ligands (TLRs 1, 2, 5, 6, 10; see Fig. 4). We previously reported a similar result [15]. However, the addition of TLR2 and TLR6 SNP data enhances the significance of the difference previously noted [15]. Further comparison between TLRs primarily recognizing viral PAMPs [14] and those recognizing microbial and/or unknown ligands ([15], this study) also revealed a more than threefold difference in the total number of SNPs detected within the coding regions (viral PAMPs, 33; microbial/unknown, 101). However, no significant difference (two-tailed Fisher's exact test) was noted for a comparison of the distribution of nonsynonymous (viral PAMPs, 16; microbial/unknown, 48) and synonymous (viral PAMPs, 17; microbial/unknown, 53) SNPs between the two TLR groups ([14,15], this study). Therefore, it is currently unclear whether bovine TLRs that primarily recognize nonviral ligands are subject to different functional and/or selective constraints, as compared to those primarily recognizing viral ligands, or whether significant differences noted in average SNP densities may somehow be related to the sampling scheme in all three studies. The answers to these questions currently remain unknown.

As we previously reported [15], no nsSNPs were detected within the predicted transmembrane or TIR domains of bovine TLRs 3, 7, 8, and 9 [14], whereas a total of 10 nsSNPs were detected within the predicted transmembrane and/or TIR domains of bovine TLRs 1, 2, 5, 6, and 10 ([15], Figs. 1 and 2 of this study). This trend remains one of the most remarkable disparities noted during comparative sequence analyses of the bovine TLRs [14,15]. As previously hypothesized [15], the biological significance of this disparity might be related to the fact that the TLR transmembrane domain is required for determining cellular localization (intracellular versus cell surface) [for review see [57]. Specifically, TLRs 3, 7, 8, and 9 reside in acidified intracellular compartments that are conducive to the degradation, release, and subsequent recognition of foreign viral or bacterial (TLR9, bacterial CpG DNA) nucleic acids [for review see [57]. For this reason, any alterations in the normal cellular localization patterns may be potentially deleterious to bovine innate immunity.

Conclusions and future studies

Including the results reported herein, detailed polymorphism studies have now emerged for all 10 of the bovine TLR loci [13–15]. Moreover, we also provide the first detailed study of genetic variation within bovine PGLYRP1. The primary objective of this study was to characterize SNPs and indels that will enable future association and TLR signaling studies relevant to bovine innate immunity. In addition, we also sought to understand how nsSNPs within bovine PGLYRP1, TLR2, and TLR6 influence the prediction of protein domain architecture, while also comparatively defining conserved domain patterning among mammalian and nonmammalian taxa. Collectively, 83 polymorphisms were identified within the three genes investigated, with at least 15 nsSNPs located within predicted domains considered to be functionally relevant. Comparative analysis of predicted protein domain architectures for TLR2 and TLR6 revealed six regions of LRR patterning that was conserved among all species investigated. Future work will entail SNP validation and haplotype inference for larger sample sets to enable association studies as well as QTL fine mapping in cattle. Additionally, the bovine TLR SNPs described here, as well as those previously described [13–15], will facilitate future ligand recognition studies as well as allele-specific PGLYRP1 microbicidal assays that may help elucidate molecular mechanisms modulating differential susceptibility to important diseases affecting domestic cattle.

Materials and methods

Primers and PCR amplification

All PCR primers were designed using Primer3 online (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), with a 58 °C optimal annealing temperature. PCR primers for amplification and direct sequencing of TLRs 2 and 6 and PGLYRP1 are presented in Table 4. In total, three primer pairs were designed for PGLYRP1, six primer pairs were designed for TLR2, and four primer pairs were designed for TLR6. Working stocks were diluted to 10 μM for PCR amplification and direct sequencing.

A single step-down thermocycling procedure was used to generate amplicons for bovine TLR2, TLR6, and PGLYRP1 amplicon 3 (PGLYRP1-3F and PGLYRP1-3R; Table 4) using 25-μl PCRs and thermal conditions as previously described [15]. Thermal cycling parameters and 25-μl PCRs utilized to generate the two remaining 5′ amplicons for bovine PGLYRP1 (Table 4; PGLYRP1-1, PGLYRP1-2) were also identical to those previously described [15], with the following exception: initial annealing temperature of 60 °C followed by a final annealing temperature of 57 °C. To ensure PCR specificity for TLR6, we generated amplicons for all cattle breeds investigated using the primer pair TLR6_3F and TLR6_4R (expected size ~1059 bp; Table 4), with 25-μl PCRs and thermal parameters as previously described [15]. All resulting PCR products were visualized via agarose gel electrophoresis and subsequently purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations.

Sequencing

Direct sequencing of all PCR products for bovine PGLYRP1, TLR2, and TLR6 was initially performed on a control sample to verify sequence identities prior to the polymorphism screen. Thereafter, all purified bovine TLR amplicons were sequenced directly in both directions using Big Dye Terminator Cycle Sequencing technology in conjunction with GeneAmp 9700 PCR Systems (Applied Biosystems, Foster City, CA, USA) in 10-μl reaction volumes. Sequencing reaction volumes, constituents, concentrations, and thermal cycling parameters followed Seabury et al. [15]. All sequencing reactions were purified using G-50 Sephadex columns (Biomax, Odenton, MD, USA) according to the manufacturer's recommended protocol. Purified sequencing reactions were dried using a Speed Vac and stored at −20 °C. Each sample was rehydrated with 15 μl of ABI HiDi formamide (Applied Biosystems) and resolved on an ABI 3130 automated sequencer (Applied Biosystems).

DNA samples

DNA from an Angus bull (J.E.W.38) [15] was utilized for primer optimization and verification of amplicon sequence identities via BLAST (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=9913) and/or CLUSTALW (http://clustalw.ddbj.nig.ac.jp/top-e.html) alignment with the relevant Hereford reference sequence from the Bovine Genome Project. Bovine DNA samples representing multiple cattle breeds were also available in a local repository [15,58]. In addition to the Angus sample used for optimization, one bovine DNA sample was also selected from the following breeds: Braford, Brahman, Charolais, Holstein, Limousin, Nelore, Piedmontese, and Romagnola. The source of the DNA was commercially available semen [58]. Bovine DNA samples were specifically chosen with the intent of surveying a diverse spectrum of germplasm representing both *B. taurus* and *B. indicus* [14,15]. All sequences generated in this study were compared to the publicly available Hereford sequence from the Bovine Genome Project (http://www.hgsc.bcm.tmc.edu/projects/bovine/).

Comparative sequence analysis

All bovine sequences were assembled and analyzed via Sequencher 4.7 (Gene Codes Corp., Ann Arbor, MI, USA) using the more rigorous default assembly parameters. Sequence quality was verified by electropherogram inspection and confirmed by Sequencher 4.7 quality analysis score. Heterozygous nucleotides were flagged manually during electropherogram inspection and confirmed by Sequencher 4.7 analysis of overlapping sequences. Several amplicons were evaluated by a secondary round of PCR and sequencing to validate observed polymorphisms. Heterozygous nucleotides were annotated with the appropriate IUPAC-IUB code for heterozygosity within Sequencher 4.7, and the final consensus sequences were exported for further analysis. Electropherograms displaying evidence of indel polymorphism were imported into Mutation Surveyor 3.00 (SoftGenetics, State College, PA, USA) for resolution of heterozygous indels by deconvoluting the sequence traces into two separate sequences. Both alleles from the Mutation Surveyor heterozygous indel prediction were also observed as homozygous indel polymorphisms within the sample of 10 bovine breeds, with all possible genotypic classes (+/+,+/−, −/−) observed within the sample. Forward and reverse sequences flanking a heterozygous indel were also used to manually identify the position and sequence of the indel. Mutation Surveyor heterozygous indel prediction and manual methods were fully congruent as previously described [15].

The online utility SMART [39] (http://smart.embl-heidelberg.de/) was used for comparative prediction of TLR and PGLYRP1 protein domain architectures. For all protein domain searches and predictions the normal mode of SMART was used with the following settings: include PFAM domains, signal peptides, internal repeats, and intrinsic protein disorder. Using the settings described above, SMART was also employed to investigate how predicted amino acid replacements influence the prediction of protein domain architectures for bovine PGLYRP1, TLR2, and TLR6. Predicted amino acid replacements were sequentially evaluated in SMART according to how they were observed in the sample (genotypically).

Statistical analysis

Two-tailed Fisher's exact tests were performed using the freeware STAT-SAK (G.E. Dallal). Two-by-two tables constructed for comparisons are available upon request. A $p < 0.05$ was considered statistically significant.

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References

[1] T. Vasselon, P.A. Detmers, Toll receptors: a central element in innate immune responses, Infect. Immun. 70 (2002) 1033–1041.
[2] T. Kaisho, S. Akira, Toll-like receptor function and signaling, J. Allergy Clin. Immunol. 117 (2006) 979–987.
[3] D. Stein, S. Roth, E. Vogelsang, C. Nusselein-Volhard, The polarity of the dorsoventral axis in the *Drosophila* embryo is defined by an extracellular signal, Cell 65 (1991) 725–735.
[4] B. Lemaitre, E. Nicolas, L. Michaut, J.M. Reichhart, J.A. Hoffman, The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults, Cell 86 (1996) 973–983.
[5] S. Tauszig, E. Jouanguy, J.A. Hoffman, J.-L. Imler, Toll-related receptors and the control of antimicrobial peptide expression in *Drosophila*, Proc. Natl. Acad. Sci. USA 97 (2000) 10520–10525.
[6] B. Beutler, Inferences, questions, and possibilities in Toll-like receptor signaling, Nature 430 (2004) 257–263.
[7] S. Akira, K. Takeda, Toll-like receptor signaling, Nat. Rev. Immunol. 4 (2004) 499–511.
[8] A.P. West, A.A. Koblansky, S. Ghosh, Recognition and signaling by Toll-like receptors, Annu. Rev. Cell Dev. Biol. 22 (2006) 409–437.
[9] M. Menzies, A. Ingham, Identification and expression of Toll-like receptors 1–10 in selected bovine and ovine tissues, Vet. Immunol. Immunopathol. 109 (2006) 23–30.
[10] K. McGuire, M. Jones, D. Werling, J.L. Williams, E.J. Glass, O. Jann, Radiation hybrid mapping of all 10 characterized bovine Toll-like receptors, Anim. Genet. 37 (2005) 47—50.
[11] T. Goldammer, H. Zerbe, A. Molenaar, H.J. Schuberth, R.M. Brunner, S.R. Kata, H.M. Seyfert, Mastitis increases mammary mRNA abundance of beta-defensin 5, toll-like-receptor 2 (TLR2), and TLR4 but not TLR9 in cattle, Clin. Diagn. Lab. Immunol. 11 (2004) 174–185.
[12] S.N. White, S.R. Kata, J.E. Womack, Comparative fine maps of bovine toll-like receptor 4 and toll-like receptor 2 regions, Mamm. Genome 14 (2003) 149–155.
[13] S.N. White, K.H. Taylor, C.A. Abbey, C.A. Gill, J.E. Womack, Haplotype variation in bovine Toll-like receptor 4 and computational prediction of a positively selected ligand-binding domain, Proc. Natl. Acad. Sci. USA 100 (2003) 10364–10369.
[14] E.J. Cargill, J.E. Womack, Detection of polymorphisms in bovine Toll-like receptors 3, 7, 8, and 9, Genomics 89 (2007) 745–755.
[15] C.M. Seabury, E.J. Cargill, J.E. Womack, Sequence variability and protein domain architectures for bovine Toll-like receptors 1, 5, and 10, Genomics 90 (2007) 502–515.
[16] D. Werling, J. Piercy, T.J. Coffey, Expression of TOLL-like receptors (TLR) by bovine antigen-presenting cells—potential role in pathogen discrimination? Vet. Immunol. Immunopathol. 112 (2006) 2–11.
[17] M.A. Opsal, D.I. Vage, B. Hays, I. Berget, S. Lien, Genomic organization and transcript profiling of the bovine toll-like receptor gene cluster *TLR6-TLR1-TLR10*, Gene 384 (2006) 45–50.
[18] M.A. Opsal, S. Lien, S. Brenna-Hansen, H.G. Olsen, D.I. Vage, Association analysis of the constructed linkage maps covering TLR2 and TLR4 with clinical mastitis in Norwegian red cattle, J. Anim. Breed. Genet. 125 (2008) 110–118.
[19] K.G. Meade, E. Gormley, M.B. Doyle, T. Fitzsimons, C. O'Farrelly, E. Costello, J. Keane, Y. Zhao, D.E. MacHugh, Innate gene repression associated with Mycobacterium bovis infection in cattle: toward a gene signature of disease, BMC Genomics 8 (2007) 400 doi:10.1186/1471-2164-8-400.

[20] D.J. Weiss, C.D. Souza, O.A. Evanson, M. Sanders, M. Rutherford, Bovine monocyte TLR2 receptors differentially regulate the intracellular fate of *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. *avium*, J. Leukocyte Biol. 83 (2008) 1–8.
[21] D. Werling, J. Piercy, T.J. Coffey, Expression of TOLL-like receptors (TLR) by bovine antigen-presenting cells—potential role pathogen discrimination? Vet. Immunol. Immunopathol. 112 (2006) 2–11.
[22] C.J. Clark, The pathology and pathogenesis of paratuberculosis in ruminants and other species, J. Comp. Pathol. 116 (1997) 217–261.
[23] A. Yoshimura, E. Lien, R.R. Ingalls, E. Tuomanen, R. Dziarski, D. Golenbock, Recognition of gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2, J. Immunol. 163 (1999) 1–5.
[24] R. Schwandner, R. Dziarski, H. Wesche, M. Rothe, C.J. Kirschning, Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2, J. Biol. Chem. 274 (1999) 17406–17409.
[25] T.K. Means, E. Lien, A. Yoshimura, Y. Wang, D.T. Golenbock, M.J. Fenton, The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors, J. Immunol. 163 (1999) 6748–6755.
[26] O. Takeuchi, S. Sato, T. Horiuchi, K. Hoshino, K. Takeda, Z. Dong, R.L. Modlin, S. Akira, Role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins, J. Immunol. 169 (2002) 10–14.
[27] C. Werts, R.I. Tapping, J.C. Mathison, T.H. Chuang, V. Kravchenko, I. Saint Girons, D.A. Haake, P.J. Godowski, F. Hayashi, A. Ozinsky, D.M. Underhill, C.J. Kirschning, H. Wagner, A. Aderem, P.S. Tobias, R.J. Ulevitch, Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism, Nat. Immunol. 2 (2001) 346–352.
[28] D.M. Underhill, A. Ozinsky, A.M. Hajjar, A. Stevens, C.B. Wilson, M. Bassetti, A. Aderem, The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens, Nature 401 (1999) 811–815.
[29] B.N. Gantner, R.M. Simmons, S.J. Canavera, S. Akira, D.M. Underhill, Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2, J. Exp. Med. 197 (2003) 1107–1117.
[30] H. Heine, E. Lien, Toll-like receptors and their function in innate and adaptive immunity, Int. Arch. Allergy Immunol. 130 (2003) 180–192.
[31] A. Ozinsky, D.M. Underhill, J.D. Fontenot, A.M. Hajjar, K.D. Smith, C.B. Wilson, L. Schroeder, A. Aderem, The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors, Proc. Natl. Acad. Sci. USA 97 (2000) 13766–13771.
[32] M. Triantafilou, F.G.J. Gamper, R.M. Haston, M.A. Mouratis, S. Morath, T. Hartung, K. Triantafilou, Membrane sorting of Toll-like receptor (TLR)-2/6 and TLR2/1 heterodimers at the cell surface determines heterotypic associations with CD36 and intracellular targeting, J. Biol. Chem. 281 (2006) 31002–31011.
[33] S. Merx, W. Zimmer, M. Neumaier, P.A. Nejad, Characterization and functional investigation of single nucleotide polymorphisms (SNPs) in the human TLR5 gene, Hum. Mutat. 880 (2006) doi: 10.1002/humu.9409.
[34] J. Texereau, J.-D. Chiche, W. Taylor, G. Choukroun, B. Comba, J.-P. Mira, The importance of Toll-like receptor 2 polymorphisms in severe infections, Clin. Infect. Dis. 41 Suppl. 7 (2005) S408–S415.
[35] C.C. Tydell, J. Yuan, P. Tran, M.E. Selsted, Bovine peptidoglycan recognition protein-S: antimicrobial activity, localization, secretion, and binding properties, J. Immunol. 176 (2006) 1154–1162.
[36] M. Wang, L.-H. Liu, S. Wang, X. Li, X. Lu, D. Gupta, R. Dziarski, Human peptidoglycan recognition proteins require zinc to kill both gram-positive and gram-negative bacteria and are synergistic with antibacterial peptides, J. Immunol. 178 (2007) 3116–3125.
[37] E. Gelius, C. Persson, J. Karlsson, H. Steiner, A mammalian peptidoglycan recognition protein with N-acetylmuramoyl-L-alanine amidase activity, Biochem. Biophys. Res. Commun. 306 (2003) 988–994.
[38] X. Lu, M. Wang, J. Qi, H. Wang, X. Li, D. Gupta, R. Dziarski, Peptidoglycan recognition proteins are a new class of human bactericidal proteins, J. Biol. Chem. 281 (2006) 5895–5907.
[39] I. Letunic, R.R. Copley, B. Pils, S. Pinkert, J. Schultz, P. Bork, SMART 5: domains in the contexts of genomes and networks, Nucleic Acids Res. 34 (2006) D257–D260.
[40] A. Yilmaz, S. Shen, D.L. Adelson, S. Xavier, J.J. Zhu, Identification and sequence analysis of chicken Toll-like receptors, Immunogenetics 56 (2005) 743–753.
[41] A.M. Keestra, M.R. de Zoete, R. van Aubel, J. van Putten, The central leucine-rich repeat region of chicken TLR16 dictates unique ligand specificity and species-specific interaction with TLR2, J. Immunol. 178 (2007) 7110–7119.
[42] J.K. Bell, G.E.D. Mullen, C.A. Leifer, A. Mazzoni, D.R. Davies, D.M. Segal, Leucine-rich repeats and pathogen recognition in Toll-like receptors, Trends Immunol. 24 (2003) 528–533.
[43] S. Akira, S. Sato, Toll-like receptors and their signaling mechanisms, Scand.J. Infect. Dis. 35 (2003) 555–562.
[44] E. Andersen-Nissen, K.D. Smith, R. Bonneau, R.K. Strong, A. Aderem, A conserved surface on Toll-like receptor 5 recognizes bacterial flagellin, J. Exp. Med. 204 (2007) 393–403.
[45] C. Zhang, D.-J. de Koning, J. Hernandez-Sanchez, C.S. Haley, J.L. Williams, P. Wiener, Mapping of multiple quantitative trait loci affecting bovine spongiform encephalopathy, Genetics 167 (2004) 1863–1872.
[46] H. Klungland, A. Sabry, B. Heringstad, H.G. Olsen, L. Gomez-Raya, D.I. Vage, I. Olsaker, J. Odegard, G. Klemetsdal, N. Schulman, J. Vilkki, J. Ruane, M. Aasland, K. Ronningen, S. Lien, Quantitative trait loci affecting clinical mastitis and somatic cell count in dairy cattle, Mamm. Genome 12 (2001) 837–842.
[47] M.G. Gonda, B.W. Kirkpatrick, G.E. Shook, M.T. Collins, Identification of a QTL on BTA20 affecting susceptibility to *Mycobacterium avium* ssp. *paratuberculosis* infection in US Holsteins, Anim. Genet. 38 (2007) 389–396.
[48] L.M. Wetzler, The role of Toll-like receptor 2 in microbial disease and immunity, Vaccine 21 (Suppl. 2) (2003) S55–60.
[49] O. Takeuchi, T. Kawai, P.F. Muhlradt, M. Morr, J.D. Radolf, A. Zychlinsky, K. Takeda, S. Akira, Discrimination of bacterial lipoproteins by Toll-like receptor 6, Int. Immunol. 13 (2001) 933–940.
[50] M. Triantafilou, A. Uddin, S. Maher, N. Charalambous, T. Hamm, A. Alsumaiti, K. Triantafilou, Anthrax toxin evades Toll-like receptor recognition, whereas its cell wall components trigger activation via TLR2/6 heterodimers, Cell Microbiol. 9 (2007) 2880–2892.
[51] T. Parkinson, The future of toll-like receptor therapeutics, Curr. Opin. Mol. Ther. 10 (2008) 21–31.
[52] K.L. Rosenthal, Tweaking innate immunity: the promise of innate immunologicals as anti-infectives, Can.J. Infect. Dis. Med. Microbiol. 17 (2006) 307–314.
[53] R. Guan, R.A. Mariuzza, Peptidoglycan recognition proteins of the innate immune system, Trends Microbiol. 15 (2007) 127–134.
[54] N.Y. Yount, J. Yuan, A. Tarver, T. Castro, G. Diamond, P.A. Tran, J.N. Levy, C. McCullough, J.S. Cullor, C.L. Bevins, M.E. Selsted, Cloning and expression of bovine neutrophil β-defensins: biosynthetic profile during neutrophilic maturation and localization of mature peptide to novel cytoplasmic dense granules, J. Biol. Chem. 274 (1999) 26249–26258.
[55] C. C Tydell, N. Yount, D. Tran, J. Yuan, M.E. Selsted, Isolation, characterization, and antimicrobial properties of bovine oligosaccharide-binding protein, J. Biol. Chem. 277 (2002) 19658–19664.
[56] U. Hasan, C. Chaffois, C. Gaillard, V. Saulnier, E. Merck, S. Tancredi, C. Guiet, F. Briere, J. Vlach, S. Lebecque, G. Trinchieri, E.E.M. Bates, Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88, J. Immunol. 174 (2005) 2942–2950.
[57] S. Bauer, Toll-erating self DNA, Nat. Immunol. 7 (2006) 13–15.
[58] C.M. Seabury, R.L. Honeycutt, A.P. Rooney, J.N. Derr, Novel prion protein gene (*PRNP*) variants and evidence for strong purifying selection in functionally important regions of bovine exon 3, Proc. Natl. Acad. Sci. USA 101 (2004) 15142–15147.

Web resources

Bovine Genome Project (http://www.hgsc.bcm.tmc.edu/projects/bovine/)
Bovine QTL Viewer, Texas A and M University (http://bovineqtlv2.tamu.edu/index.html)
CattleQTLdb, Iowa State University (http://www.animalgenome.org/QTLdb/cattle.html)
Combined QTL Map of Dairy Cattle Traits, University of Sydney, Australian Dairy CRC (http://www.vetsci.usyd.edu.au/reprogen/QTL_Map/)
CpGProD (http://pbil.univ-lyon1.fr/software/cpgprod_query.html)
CpGPlot (http://www.ebi.ac.uk/emboss/cpgplot/)
MapViewer, NCBI (http://www.ncbi.nlm.nih.gov/mapview/)
National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/)
Oklahoma State University Department of Animal Science, Breeds of Cattle (http://www.ansi.okstate.edu/breeds/cattle/)
Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)
RepeatMasker (http://www.repeatmasker.org/)
Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/)