Immunogenetics (2011) 63:115–119
DOI 10.1007/s00251-010-0484-6

BRIEF COMMUNICATION

Association of toll-like receptor four single nucleotide polymorphisms with incidence of infectious bovine keratoconjunctivitis (IBK) in cattle

Ranjit S. Kataria · Richard G. Tait Jr · Dinesh Kumar · Manuel A. Ortega · Jose Rodiguez · James M. Reecy

Received: 27 May 2010 / Accepted: 23 September 2010 / Published online: 12 October 2010
© Springer-Verlag 2010

Abstract Toll-like receptor 4 (TLR4) is a receptor protein that binds pathogen ligands, which are mainly associated with Gram-negative bacteria. The objective of this study was to investigate the association of nucleotide polymorphisms in TLR4 with infectious bovine keratoconjunctivitis (IBK), or pinkeye, incidence in American Angus cattle. Animals with previously calculated breeding values for IBK susceptibility were used to identify two SNPs in TLR4; Int1 (A/G) in intron1 (−26 Ex2 position) and Ex3 (C/T) in exon3 (1,678 position). To investigate the possible role of these SNPs in IBK susceptibility, the disease incidence information was collected on 370 calves raised in Iowa at two time points—June or August (disease season) and October (at weaning) and genotyped using PCR-RFLP protocols. In statistical models including year, pasture management group, and SNP, the Int1 SNP had a significant effect on IBK infection rates both in-season ($P<0.05$) and at weaning ($P<0.01$), whereas the Ex3 SNP was not significant ($P>0.79$) at either time point. Furthermore, the Int1 SNP alone could account for 2.1% of phenotypic variation in IBK infection during the disease season and 3.0% of phenotypic variation in IBK infection at the time of weaning. These data indicate that there is a relationship between Int1 genotype and the rate of IBK infection in American Angus cattle.

Keywords Toll-like receptor 4 · Single nucleotide polymorphisms · Pinkeye · Cattle

R. G. Tait Jr · J. Rodiguez · J. M. Reecy (✉)
Department of Animal Science, Iowa State University,
Ames, IA, USA
e-mail: jreecy@iastate.edu

R. S. Kataria · D. Kumar
DNA Fingerprinting Unit,
National Bureau of Animal Genetic Resources,
Karnal, India

M. A. Ortega
Industrial Biotechnology, University of Puerto Rico-Mayaguez,
Mayaguez, Puerto Rico

Being a critical first line of defense and evolutionarily conserved, the innate immune response plays a critical role in the fight against bacterial, viral, and fungal pathogens. Innate immunity relies on recognition of antigens by a small number of weakly specific receptors designated pattern-recognition receptors (PRRs). Toll-like receptors (TLRs) belong to a family of trans-membrane PRR proteins and in mammalians at least 13 TLRs are reported that recognize a variety of ligands from pathogens to trigger immunological responses (Roach et al. 2005). They are vital for microbial sensing and execute two major functions as part of the immune system. First, they recognize lipid, carbohydrate, peptide, and nucleic acid structures associated with pathogens, and second, they activate the immune response (Trinchieri and Sher 2007).

In cattle, 10 TLR genes have been identified, which recognize a variety of microbial ligands and initiate the host defense mechanism. With genomic organization of the bovine TLRs being similar to that of humans and mice, the ten recognized TLR genes have been mapped in cattle (McGuire et al. 2006). Among these identified TLRs, TLR4 is able to recognize lipopolysaccharides cell wall component of Gram-negative bacteria, which are involved in the pathogenesis of many diseases in cattle (Donovan et al. 2005; Mucha et al. 2009). Located on chromosome eight in *Bos taurus*, TLR4 consists of three exons and two introns. Cattle TLR4 mRNA is 3,739 nucleotide long with an open reading frame of 2,526 nucleotides that encodes for 841 amino acids (aa) with a 470

nucleotide long 5′ UTR and 743 nucleotide long 3′ UTR. Tissue specific expression profiling revealed that TLR4 is expressed in the mammary gland, liver, muscle, duodenum, fats, uterus, kidney, heart, lung, pancreas, and ovary in cattle, which indicates that this molecule could be involved in a variety of biological functions (Wang et al. 2009).

Infectious bovine keratoconjunctivitis (IBK) or pinkeye has a multiple etiology, *Moraxella bovis* a Gram-negative bacteria being the major causative agent. The disease symptoms in cattle range from excessive tear formation, conjunctivitis, and in the worst scenario total blindness. Although not a fatal disease, it is very contagious and results in heavy economic losses to the cattle industry due to indirect effects on animal growth and production. Loss of about 150 million dollars due to pinkeye alone has been reported with the disease affecting more than ten million calves annually in USA. IBK has been found to be responsible for reduction in the weaning weights of calves (Snowder et al. 2005). Putative QTLs reported to be associated with IBK susceptibility have been mapped to chromosome 1 and 20 in cattle (Casas and Stone 2006).

Resistance to several infectious diseases is reported to be affected by variations in innate immune response genes. Because of its importance in immunity against a large number of diseases, particularly to bovine respiratory diseases and Johne's disease, TLR4 is a candidate gene for polymorphism analysis and association studies. Haplotypic variations in bovine TLR4 gene of 11 cattle breeds have already been documented, showing bovine TLR4 gene to be highly polymorphic (White et al. 2003). Nucleotide variations in the promoter and coding regions of the TLR4 gene have been associated with somatic cell score and lactation persistency in cattle (Sharma et al. 2006; Wang et al. 2007). Toll-like receptors have also been implicated in ocular immunity and inflammatory eye disease in human beings (Chang et al. 2006). Thus, the previous reports prompted us to explore the possible relationship between bovine TLR4 variants and pinkeye disease in American Angus cattle.

This study utilized 370 Angus cattle born in the spring of 2003 (n=320) or 2004 (n=50) from the Iowa State University Angus breeding project. Phenotype collection was carried out during the active pinkeye season in August (2003 born) or June (2004 born; in-season), these periods were when active infections were observed in the respective year, and then again in October (at weaning) of each year. October measures represent a management time when producers typically assess cattle for performance measures and could routinely have pinkeye evidence evaluated on an individual basis. At the time of phenotype collection, white blood cell (WBC) samples were collected and stored for future DNA extraction. Severity of pinkeye was measured by scoring the levels of opacity in each eye on a scale of 0 to 4, where score 0 represents no apparent infection, score 1 represents less than 1/3 opacity of the cornea, score 2 represents 1/3 to 2/3 opacity of the cornea, score 3 represents more than 2/3 opacity of the cornea, and a score of four represents a ruptured cornea. Pinkeye severity scores were used to define two traits for analysis: (1) pinkeye infection and (2) blockage of vision. Pinkeye infection was characterized by developing an arbitrary scale of: uninfected animals=100 (zero severity score in both eyes) and infected animals=200 (any score other than 0 in either eye). The blockage of vision trait was calculated by first recoding the severity score 4 to a severity score of three then summing the severity scores for both eyes. Therefore, the range for blockage of vision was 0 to 6, which represents how many sixths of the vision was blocked by pinkeye infection.

DNA was extracted from the buffy coats, using a modified version of the standard SDS-ProteinaseK method (Dracopoli et al. 2000). The WBC pellet was suspended in 3 ml TE buffer followed by addition of 500 μl of 10× TENS (TrisHCl–EDTA–NaCl) buffer, 300 μl of 10% SDS, and 20 μl of RNase A at 20 mg/ml, to each sample and after 2 h of incubation at 37°C in a water bath, 75 μL ProteinaseK at 20 mg/ml was added and incubated overnight in a 55°C water bath. DNA was precipitated by adding 400 μL of 5 M NaCl and two times the volume of 100% chilled ethanol. After washing with 70% ethyl alcohol, the DNA pellets were air-dried briefly and dissolved in TE buffer. DNA quality and quantity was checked by measuring UV absorbance at 260 and 280 nm.

To identify SNPs within the TLR4 gene, 12 animals were selected, six from each group having extreme estimated breeding values for susceptibility or resistance to pinkeye infection (Rodriguez 2006). PCR products that encompassed the entire coding region and some of the adjoining intronic sequences generated from genomic DNA of these 12 animals were first screened for the identification of SNPs. Primers were designed from previous reported sequence of cattle (Acc. No. AY297043) or described by White et al. (2003) to amplify 500–600 bp fragments of exonic regions of TLR4 gene along with adjacent intronic sequence.

DNA was amplified in a 15 μl PCR reaction that contained 1 μl (50 ng) genomic DNA, 2.5 pmol of each forward and reverse primers, 5.5 μl of distilled water, and 7.5 μl of *GoTaq* Green Master Mix (Promega, Madison, WI). Amplification was carried out using PCR cycles of 2 min at 94°C, followed by 35 cycles of denaturation at 94°C, respective primer's annealing temperature and extension at 72°C for 30 s each, and a final 5 min extension at 72°C. All PCR products were checked on 1.5% agarose gel. PCR products were treated with Exo-SAP (USB) enzymes and sent to the DNA Facility, Iowa State University for sequencing.

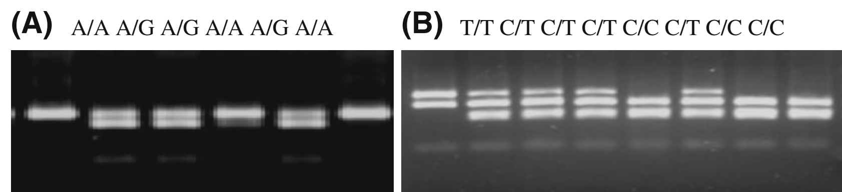

Fig. 1 RFLP genotyping of Int1 (−26Ex2) SNP using AciI (**a**) and Ex3 (1,678) SNP using ApeKI (**b**) restriction enzymes

Sequences were aligned and SNPs were detected manually across the exonic and adjoining intronic regions of TLR4.

After preliminary screening of SNPs in the extreme pinkeye EBV animals, two SNPs were identified for genotype analysis: one in intron1 at Int1 (−26 Ex2 position i.e. AciI enzyme RFLP site; rs8193046) with nucleotides A/G and another synonymous SNP in exon3 at position 1,678 (ApeKI enzyme RFLP site; rs8193060) with nucleotides C/T, both of these SNPs have been previously reported by White et al. (2003). The test population of 370 calves was then genotyped for each of these two SNPs by PCR-RFLP. The primers used for PCR amplification were: exon2 and adjacent introns: forward 5′-TCTTTGCTCGTCCCAGTAGC-3′ and reverse 5′-AAGTGAATGAAAAGGAGACCTCA-3′ and exon3: forward 5′-AGGTAGCCCAGACAGCATTTCACTCC-3′, and reverse 5′-GCTCTCGCCCCTGCCATACTTTT-3′. Each sample was analyzed by digesting 4 µl of PCR product, at 37°C overnight with AciI and for the RFLP of ApeKI, samples were incubated at 75°C overnight as recommended by the manufacturer (New England BioLabs). Each RFLP product was checked on a 3% NuSieve agarose gel for size and genotypes were recorded (Fig. 1).

Genotype frequencies of the animals in this study (n=370) were analyzed with PROC FREQ of SAS (v. 9.1, SAS Institute, Cary, NC). Int1 allele frequencies were determined to be A=0.265; G=0.735 and genotype frequencies were observed to be: A/A=0.051, A/G=0.427, G/G=0.522. Ex3 SNP allele frequencies were C=0.628; T=0.372 and genotype frequencies were observed to be: C/C=0.378, C/T=0.500, T/T=0.122. Each SNP was tested to see if it was in Hardy–Weinberg equilibrium (Falconer and Mackay 1996) within the test population. While both SNPs had an excess of heterozygotes compared to the Hardy–Weinberg expectation, there was no evidence (P>0.05) to reject the null hypothesis that each SNP was in Hardy–Weinberg equilibrium. There was a small amount of linkage disequilibrium between these two SNPs (R^2=0.138).

Two traits (pinkeye infection and blockage of vision) were evaluated at both time points (in-season and at weaning) using the GLM Procedure of SAS in a series of four analyses. All P values reported are nominal P values. Fixed effects initially tested for their significance in all four analyses included class effects of: year, pasture management group (2003, n=2; 2004, n=1), age of dam in years (2003, 2 to 7; 2004, 2 to 8; 26 dams had progeny in both years), gender of calf, Int1 genotype, Ex3 genotype, Int1×Ex3 genotype interaction, and covariate of age of calf, in days, at time of measurement. Stepwise removal of non-genetic effects which were not significant (P>0.05) removed age of dam, gender of calf, and age of calf for all analyses except blockage of vision evaluated at weaning which still indicated age of dam was significant (P<0.01).

For consistency of models, all four analyses were reduced to include class effects of year and pasture management group as non-genetic effects and Int1, Ex3, and Int1×Ex3 interaction as genetic effects. These analyses indicated there was no significant interaction (P>0.30) between Int1 and Ex3 in any of the four analyses. It is important to recognize that due to the low minor allele frequencies there were some combinations of genotypes between Int1 and Ex3 that were not observed in our population. Therefore, analyses including only year, pasture management group, and single SNP genotype (Int1 or Ex3) were conducted. Each SNP was also fit as a class effect as the only term in the model to identify what proportion of the phenotypic variation that a SNP could account for.

The Int1 and Ex3 genotypes were also redefined as covariates to fit an additive allele substitution genetic effect in the model. Genotypes for Int1 were recoded as A/A=0, A/G=1, G/G=2. Where Ex3 genotypes were recoded as C/C=0, C/T=1, and T/T=2. The allele substitution model included fixed effects of year and pasture management group, with Int1 or Ex3 allele substitution variables fit as a covariate.

Animals involved in this study exhibited a pinkeye infection rate of 38.7% in-season and 46.8% at weaning, with 32.7% of animals identified as infected at both time-points (Table 1). Average blockage of vision values was 0.85 in-season and 0.75 at weaning. Model R^2, significance of fixed effects, and significance of Int1 for pinkeye

Table 1 Incidence of IBK recorded in 370 animals at two time points-in-season (June or August) and at wean (October)

Evaluation time-point	Non-infected		Infected	
	Frequency	Percentage	Frequency	Percentage
In-season	227	61.35	143	38.65
Weaning	197	53.24	173	46.76
Both time-points	175	47.3	121	32.7

Table 2 Significance analysis of non-genetic and Int1 genetic (fit as class) effects on pinkeye incidence and blocked vision at two evaluation time points

Trait	Evaluation time-point	R^2	Year (*p* value)	Pasture group (*p* value)	Int1 (*p* value)
Pinkeye infection	In-season	0.2225	< 0.0001	< 0.0001	0.0404
Blocked vision	In-season	0.1754	0.0003	< 0.0001	0.0587
Pinkeye infection	Weaning	0.0389	0.5645	0.1187	0.0045
Blocked vision	Weaning	0.0201	0.0462	0.9500	0.1624

Table 3 Significance analysis of non-genetic and Int1 genetic (fit as allele substitution covariate) effects on pinkeye incidence and blocked vision at two evaluation time points

Trait	Evaluation time-point	R^2	Year (*p* value)	Pasture group (*p* value)	Int1 (*p* value)
Pinkeye infection	In-season	0.2187	< 0.0001	< 0.0001	0.0311
Blocked vision	In-season	0.1723	0.0002	< 0.0001	0.0379
Pinkeye infection	Weaning	0.0348	0.5307	0.1006	0.0023
Blocked vision	Weaning	0.0157	0.0521	0.9842	0.1581

infection and blocked vision at both evaluation time-points are reported in Table 2. Int1 was significantly ($P<0.05$) associated with pinkeye infection both in-season and at weaning. In a similar model, the Ex3 SNP was not associated ($P>0.37$) with pinkeye infection or blocked vision at either time-point (data not shown). Least square means of different genotypes of the two SNPs from these models are shown in Fig. 2, which demonstrates that the A allele of Int1 SNP had higher rates of infection; whereas, not much variation in the infection rates existed among Ex3 genotypes.

Table 3 shows results for model R^2, significance of fixed effects, and significance of G SNP allele substitution effects at Int1 for pinkeye infection and blocked vision at both evaluation time-points. Addition of a G allele at Int1 was significant for pinkeye infection in-season, with each allele reducing pinkeye infection by 8.2%. Furthermore, addition of a G allele at Int1 was also significant for pinkeye infection at weaning, with each G allele reducing pinkeye infection by 13.3%. The fixed effects and Ex3 genetic allele substitution model indicated that Ex3 was not significant ($P>0.16$) for pinkeye infection or blocked vision at either time-point (data not shown).

When the Int1 SNP was fit as the only effect in the model (Table 4), it accounted for 2.1% of phenotypic variation in pinkeye infection during the pinkeye season and 3.0% of phenotypic variation in pinkeye infection at weaning. In contrast, when the Ex3 SNP was fit as the only effect in the model, it was not significant ($P>0.79$) for pinkeye infection, and accounted for less than 0.15% of phenotypic variation of pinkeye infection in-season or at weaning. This further indicates and confirms the association of G allele at Int1 with the resistance to pinkeye infection.

Toll-like receptor four is the TLR that recognizes the lipopolysaccharides cell-wall component of Gram-negative bacteria. The fact that *Morexella bovis*, or recently isolated *Moraxella bovoculi* sp. *nov.* (Angelos et al. 2007) are Gram-negative bacteria that are mainly associated with IBK/pinkeye in cattle, prompted the investigation of the possibility that genetic variations in TLR4 are associated with resistance to IBK, a major disease problem among cattle in USA and other countries like Australia. The

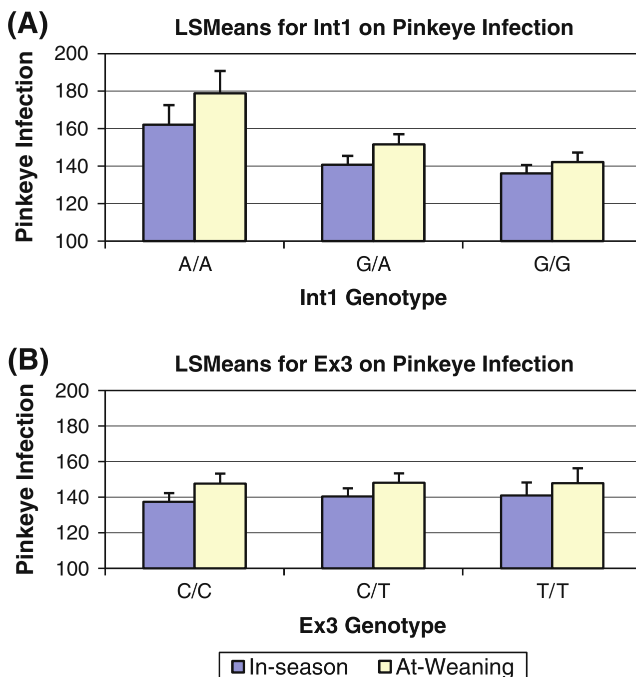

Fig. 2 Least square means showing Int1 (**a**) and Ex3 (**b**) genotypes effects on the pinkeye infection (100 as non-infected and 200 as infected) in season and at weaning time points

Table 4 Significance analysis of Int1 (fit as class) on pinkeye incidence and blocked vision at two evaluation time points

Trait	Evaluation time-point	R^2	Int1 (*p* value)
Pinkeye infection	In-season	0.0210	0.0202
Blocked vision	In-season	0.0187	0.0315
Pinkeye infection	Weaning	0.0303	0.0035
Blocked vision	Weaning	0.0088	0.1984

polymorphism within TLR4 gene has been associated with varied immune response as well as inflammation in several diseases in mouse models and also in human beings (Miller et al. 2005). Recently, Mucha et al. (2009) have described the polymorphisms within the TLR4 gene to be associated with resistance to *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle. Two of the mis-sense mutations, Asp299Asn within the LRR (leucine rich repeats) 11 and Gly389Ser within LRR15 were found to be associated with varied susceptibility to the disease. SNPs in the intronic regions of different genes have also been associated with various disease conditions (Tokuhiro et al. 2003; Qu et al. 2007). Earlier studies have identified a maternal effect related to the incidence of IBK. In addition, it has been reported and also that the crossbred calves sired by tropically adapted breeds having a lower incidence of IBK compared with purebred and crossbred *Bos taurus* types (Snowder et al. 2005). These results indicate that intron1 SNP at Ex2(−26) position of TLR4 gene is associated with infectious bovine kerato-conjunctivitis or the pinkeye incidence in American Angus, which can be used for future work trying to understand the interaction between the disease and host defense. Also, it will be interesting to investigate these SNPs in the indicus cattle which are reported to be relatively resistant to IBK.

Acknowledgements RSK and DK acknowledge the financial support received from Department of Biotechnology, Govt. of India in the form of overseas associateship. Authors recognize Iowa State University Center for Integrated Animal Genomics for financial support of phenotype collections.

References

Angelos JA, Spinks PQ, Ball LM, George LW (2007) *Moraxella bovoculi* sp. nov., isolated from calves with infectious bovine keratoconjunctivitis. Int J Syst Evol Microbiol 57:789–795

Casas E, Stone RT (2006) Putative quantitative trait loci associated with the probability of contracting infectious bovine keratoconjunctivitis. J Anim Sci 84:3180–3184

Chang JH, McCluskey PJ, Wakefield D (2006) Toll-like receptors in ocular immunity and the immunopathogenesis of inflammatory eye disease. Br J Ophthalmol 90:103–108

Donovan DM, Kerr DE, Wall RJ (2005) Engineering disease resistant cattle. Transgenic Res 14:563–567

Dracopoli NC, Haines JL, Korf BR, Morton CC, Seidman CC, Seidman JG, Smith DR, Boyle AL (2000) Current protocols in human genetics, vol. 3. Wiley, New York, A.3B.3, A.3C.2

Falconer DS, Mackay TFC (1996) Introduction to quantitative genetics, 4th edn. Longman, Essex, p 10

McGuire K, Jones M, Werling D, Williams JL, Glass EJ, Jann O (2006) Radiation hybrid mapping of all ten characterized bovine toll-like receptors. Anim Genet 37:47–50

Miller SI, Ernst RK, Bader MW (2005) LPS, TLR4 and infectious disease diversity. Nat Rev Microbiol 3:36–46

Mucha R, Bhide MR, Chakurkar EB, Novak M, Mikula I Sr (2009) Toll-like receptors TLR1, TLR2 and TLR4 gene mutations and natural resistance to *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle. Vet Immunol Immunopathol 28:381–388

Qu H, Montpetit A, Ge B, Hudson TJ, Polychronakos C (2007) Toward further mapping of the association between the *IL2RA* locus and type 1 diabetes. Diabetes 56:1174–1176

Roach JC, Glusman G, Rowen L, Kaur A, Purcell MK, Smith KD, Hood LE, Aderem A (2005) The evolution of vertebrate Toll-like receptors. Proc Natl Acad Sci USA 102:9577–9582

Rodriguez JE (2006) IBK (pinkeye) in Black Angus Cattle. Dissertation, Iowa State University, Ames, IA, USA

Sharma BS, Leyva I, Schenkel F, Karrow NA (2006) Association of toll-like receptor four polymorphisms with somatic cell score and lactation persistency in Holstein bulls. J Dairy Sci 89:3626–3635

Snowder GD, VanVleck LD, Cundiff LV, Bennett GL (2005) Genetic and environmental factors associated with incidence of infectious bovine keratoconjunctivitis in preweaned beef calves. J Anim Sci 83:507–518

Tokuhiro S, Yamada R, Chang X, Suzuki A, Kochi Y, Sawada T, Suzuki M, Nagasaki M et al (2003) An intronic SNP in a RUNX1 binding site of SLC22A4, encoding an organic cation transporter, is associated with rheumatoid arthritis. Nat Genet 35:341–348

Trinchieri G, Sher A (2007) Cooperation of Toll-like receptor signals in innate immune defence. Nat Rev Immunol 7:179–190

Wang X, Xu S, Gao X, Ren H, Chen J (2007) Genetic polymorphism of *TLR4* gene and correlation with mastitis in cattle. J Genet Genom 34:406–412

Wang X, Luoreng Z, Xu S, Gao X, Li J, Ren H, Chen J (2009) The structure and sequence analysis of TLR4 gene in cattle. Agr Sci China 8:632–637

White SN, Taylor KH, Abbey CA, Gill CA, Womack JE (2003) Haplotype variation in bovine toll-like receptor four and computational prediction of a positively selected ligand-binding domain. Proc Natl Acad Sci USA 100:10364–10369