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Research paper

Toll-like receptors TLR1, TLR2 and TLR4 gene mutations and natural resistance to *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle

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ABSTRACT

Toll like receptors (TLRs) are a class of pattern recognition receptors belonging to the innate immune system. Mutations in the protein coding region of TLRs are associated with altered responsiveness to pathogen-associated molecular patterns (PAMPs). A search was performed for novel mutations in bovine TLR1, TLR2 and TLR4 genes associated with the *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection. The work was also focused on the assessment of linkage between well known mutations in TLR genes (TLR2: Arg677Trp, Pro681His and Arg753Gln; TLR4: Asp299Gly and Thr399Ile), and the susceptibility of cattle to MAP infection. Detection of MAP infection in cattle population (n = 711) was based on *IS900* PCR, which revealed 22.50% (n = 160) MAP positivity. Known mutations in TLR2 and TLR4 genes were not found in cattle population. A novel mutation Val220Met was associated (Odd's ratio, OR-3.459) with increased susceptibility to MAP infection. Toll/interleukin-1 receptor (TIR) domain of TLR2 was screened for the presence of mutations, wherein a novel Ile680Val mutation was linked with MAP infection. *In silico* analysis of the bovine TLR4 ectodomain (ECD) revealed the polymorphic nature of the central ECD and irregularities in the central LRR motifs. LRR11 of the TLR4 showed five missense mutations possibly linked with the increased susceptibility to MAP infection. The most critical position that may alter the pathogen recognition of TLR molecule was 4th residue downstream to LRR domain. Two such missense mutations in TLR4 (Asp299Asn downstream to LRR11, and Gly389Ser downstream to LRR15) were associated with MAP infection. Briefly, the work describes novel mutations in the bovine TLRs and presents their association with the MAP infection.

1. Introduction

Gastrointestinal epithelial cells are constantly exposed to antigens from normal microflora, food and pathogens (Furrie et al., 2005). Epithelial cells recognize molecular patterns of bacteria, fungi, virus and parasites with the help of toll like receptors (TLRs) (Takeda et al., 2003). TLRs are a class of pattern recognition molecules with a unique function in the innate immune system and which evolved as the immediate host defense in response to foreign antigens (Cristofaro and Opal, 2006). At least 13 TLRs which recognize molecular patterns from all major classes of pathogens have been identified in mammals (Oshiumi et al., 2003; Tabeta et al., 2004). TLR1, TLR2 and TLR4 recognize bacterial cell

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components. TLR2 and TLR4 are critical in the immune response to Gram positive and negative bacteria (Underhill et al., 1999). TLR1 and TLR6, in association with TLR2 (TLR1-TLR2 and TLR1-TLR6 heteromers), recognize a wide variety of bacterial cell wall components like lipopolysaccharides, teichoic acid and lipoproteins (Buwitt-Beckmann et al., 2006; Kirschning and Schumann, 2002) and induce NFkB signaling pathway (Akira et al., 2006). Nuclear translocation of NFkB induces transcription of proinflammatory cytokine genes and thus innate immune response (Ma et al., 2007). TLR2 has been shown to mediate the immune response to ligands derived from *Mycoplasma*, *Borrelia*, *Treponema*, *Chlamydia*, yeasts and parasites (Ben-Ali et al., 2004; Heldwein et al., 2003; Janeway and Medzhitov, 2002; Lien et al., 1999; Morre et al., 2003; Underhill and Ozinsky, 2002). Similarly, TLR1-TLR2/TLR6 heteromers and TLR4 recognize mycobacterial PAMPs by activated macrophages and dendritic cells (Brightbill et al., 1999; Chang et al., 2006; Drennan et al., 2004; Hawn et al., 2007; Means et al., 1999; Omueti et al., 2007; Roura-Mir et al., 2005; Schumann and Tapping, 2007; Uehori et al., 2005; Underhill et al., 1999).

Mycobacterium avium subspecies *paratuberculosis* (MAP) causes paratuberculosis in cattle, sheep, deer and other ruminants (Moravkova et al., 2007). It is a chronic progressive infection of the gastrointestinal tract and the pathogen is commonly shed by the feces and milk of infected animals (Ayele et al., 2005; Grant et al., 2002). Among various MAP detection methods, cultivation is time consuming, while serological tests (like ELISA) are less sensitive in latently infected cattle (Kohler et al., 2008). Early disease detection with high specificity can be achieved by *IS900* based PCR (Bhide et al., 2006; Bull et al., 2003; Ikonomopoulos et al., 2004; Vansnick et al., 2004).

Mutations in TLRs are the prime cause of reduced pathogen recognition ability and hampered innate immune activation in general (Franchimont et al., 2004; Hawn et al., 2007; Henckaerts et al., 2007; Johnson et al., 2007; Morre et al., 2003; Schroder and Schumann, 2005). Mutations in TLR1 and TLR4 genes cause weak immune response to lipopeptides (Hawn et al., 2007), lipopolysaccharide (Schwartz, 2002), microbial cell wall components (Omueti et al., 2007), and increase susceptibility to invasive aspergillosis (Kesh et al., 2005). TLR2 and TLR4 gene mutations increase risk of infections like tuberculosis (Ben-Ali et al., 2004; Thuong et al., 2007), leprosy (Bochud et al., 2003; Bochud et al., 2008), pneumococcemia or malaria (Khor et al., 2007; Malley et al., 2003; Schroder and Schumann, 2005), urinary tract infections (Tabel et al., 2007) and disease conditions like periodontitis (Fukusaki et al., 2007), acute rheumatic fever (Berdeli et al., 2005) and Crohn's disease (Hong et al., 2007; Lakatos et al., 2005).

This study focuses on the detection of mutations in protein coding region of TLR1, TLR2 and TLR4 and their possible association with MAP susceptibility in cattle.

2. Materials and methods

2.1. Animals and DNA isolation

711 cows from 3 farms located in eastern Slovakia were included in the study. The farms were located in the same geographic area, the distance between two farms being no more than ~80 km. In all the farms autochthonous cattle breeds along with Holstein or Simmental were maintained. For example in the first farm Holstein cattle were simultaneously maintained with Pinzgauer; in the second, Simmentals and dark brown Carpathian cattle were mixed; while in the third farm pure-bred Slovak spotted cattle were raised with Polish red and cross-bred Holstein cattle. These farms were chosen for the present study because of the high incidence (7–10%) of MAP recorded during paratuberculosis surveillance in the years 2004–2006 (unpublished data). Animals with weight loss and/or chronic diarrhea formed a cohort suspected of paratuberculosis. At least 4–5 apparently healthy animals that had close contact with suspected animals were also included in the study. In this way we assured the equal probability of MAP infection on the studied animals. All animals included in this study were of similar age (5–6 years). 3–4 ml of blood from each animal was collected in sterile tubes containing 2 ml of 1.5% EDTA. Buffy coat was separated as described previously and used for total DNA extraction (Bhide et al., 2006).

2.2. Nested PCR for MAP detection

Nested PCR for MAP detection was designed as described previously (Bhide et al., 2006). The primers were designed to amplify *IS900* MAP specific sequence only. The sensitivity and specificity of *IS900* based PCR and its correlation with ELISA is discussed in detail in our previous work (Bhide et al., 2006). On the basis of PCR results, animals were grouped into MAP positive and negative cohorts, and cohorts were subjected to mutation detection in TLR genes.

2.3. SNP detection in TLR genes

Ectodomain (ECD) of the TLR1 was targeted for mutation detection. Primers were designed (DNASTAR) to amplify a gene fragment covering leucine rich repeats (LRR) 8–11. The primers (TLR1/F 5′-GGAGATACTTATGGGGAAAGAGAA and TLR1/R 5′-GTGTATAGACAAGGCCTTCAGTGA) amplified 402 bp of the gene segment. Conditions for PCR were initial denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 1.0 min, 52 °C for 1 min 20 s, 72 °C for 1.0 min with final extension at 72 °C for 10 min.

Primers for TLR2 were constructed to amplify gene fragments covering earlier reported Pro681His, Arg677Trp and Arg753Gln mutations (Ben-Ali et al., 2004; Berdeli et al., 2005) located in toll/interleukin-1 receptor (TIR) domain. Primers designed for TLR4 spanned both previously described major polymorphism sites, Asp299Gly and Thr399Ile (Garza-Gonzalez et al., 2007) located in ECD (LRRs 11–16). Nucleotide sequences of TLR2 primers were TLR2/F 5′-CAGGAGCTGGAGCACTTCAACC and TLR2/R 5′-GTCTCATCCACGGGCCACTCCA, while, TLR4 oligo sequences were TLR4/F 5′-GGGACTGTGCAACCTGACCA and TLR4/R 5′-GCTCTAAGCCCATGAAGTTTGAA. PCR conditions for TLR2 gene were initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 60 s, 56 °C for 45 s, and 72 °C for 60 s with final extension at 72 °C for 10 min. The cycling conditions for TLR4 were similar to TLR2 except annealing temperature (57 °C).

Table 1
Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* in different cattle breeds.

Breed	Number of animals tested	Number of MAP infected animals (%)	Prevalence of MAP infected animals
Slovak spotted cattle	10	1	10
Slovak spotted cattle × Holstein	16	0	0
Polish red	64	5	3.13
Holstein	136	27	16.45
Pinzgauer	247	0	0
Slovakian Simmental	206	103	64.56
Dark brown Carpathians	32	24	15
Total	711	160	22.50

2.4. Single-strand conformational polymorphism analysis (SSCP)

Briefly, 5 μl of amplified product was mixed with equal amount of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene–cyanol), subjected to denaturation at 95 °C for 10 min and then cooled rapidly on ice. Denatured single-stranded amplimers were loaded onto 6% acrylamide/bisacrylamide (37.5:1, v/v; Bio-Rad) gels. Electrophoresis was performed using 200 V at 8 °C in 0.5% TBE buffer for 20 h in the electrophoresis chamber (Ingeny, The Netherlands). Gels were silver-stained. Samples were grouped based on SSCP profiles by using Gel-Scan software (BioSciTec, Germany).

2.5. DNA sequencing

Representative samples from each SSCP genotype were sequenced on an Avant3100 sequencer (Applied Biosystem). The sequences were aligned, then checked for mutations and validated using SeqScape v.2.1 software (Applied Biosystem). Sequences were submitted to the GeneBank (USA) under the accession numbers: TLR1 (EU532011 and EU532014), TLR2 (AY972156, EU546166 to EU546169) and TLR4 (DQ058897, EU546170).

2.6. In silico bovine LRR motif analysis

Bovine TLR nucleotide sequences obtained in this study were aligned by the ClustalW multiple alignment method (DNASTAR software), translated into putative amino acids and consensus sequences were obtained (BioEdit software). LRR motifs were outlined according to the method described earlier (Matsushima et al., 2007) using PFAM and SSpro4.0 servers (Cheng et al., 2005).

2.7. Statistical analysis

Possible linkage between mutation in TLR genes and increased MAP infection in cattle was calculated by Odd's ratio (OR) (Win episcope software).

3. Results

3.1. MAP prevalence in the population

160 (22.50%) animals were found infected with MAP. The highest percentage (64.56% of 206 animals) of MAP positivity was found in the autochthonous Slovakian Simmental cattle. Prevalence of MAP infection in cattle population is presented in Table 1. Healthy ($n = 551$) and MAP infected ($n = 160$) animals were studied further for the presence of TLR mutations.

3.2. TLR1, TLR2 and TLR4 genotypes and their frequencies in the population

With the help of PCR-SSCP analysis, two TLR1 (TLR1_I and TLR1_II), five TLR2 (TLR2_I–TLR2_V) and two TLR4 (TLR4_I and TLR4_II) genotypes were found. The majority of the animals (95%) displayed TLR1_I genotype (EU532011). TLR2 gene was the most polymorphic and breed-dependent genotype distribution was evident. 58% of Holstein cattle had TLR2_I genotype (AY972156), while 40% of animals showed TLR2_IV (EU546166). Interestingly, in our previous study the high frequency (95%) of TLR2_I genotype was observed in Sahiwal breed (unpublished data). In Slovakian Simmental population, three TLR2 genotypes viz. TLR2_II (11%), TLR2_III (23%) and TLR2_IV (66%) were found. Genotype TLR2_IV occurred most frequently (100%) in Slovak spotted cattle, Pinzgauer, Simmental and dark brown Carpathian breeds. The genotype TLR2_V (EU546169) was observed only in Holsteins. Almost all cattle had TLR4_I genotype (DQ058897; 98%), while the other TLR4 genotype (EU546170) was observed only in pure-breed Holstein.

3.3. Point mutations in TLR1, TLR2 and TLR4

The missense mutations found in TLR1 gene were Asp119Glu, Ala120Leu, Glu140Lys, Ile144Asn, Gly150Ser, Pro170Ser, Phe201Ile and Val220Met. Silent mutations found in TLR1 genes are depicted in Fig. 1. *In silico* analysis of TLR1 ECD revealed a conserved nature of LRR motifs except LRR10 (634–666 nucleotides), wherein valine was changed to methionine at 220 amino acid position. All other TLR1 mutations were located in extra LRR motifs (Fig. 1).

Seven missense mutations were found in TLR2: Phe679Leu, Ile680Val, His697Arg, Asn706Ser, Lys709Arg, Ile745Val and Lys759Arg (Fig. 2). Well known mutations in TLR2 gene, Arg677Trp, Pro681His and Arg753Gln were not found in the present study. However, 10.9% subjects showed a silent mutation in the codon 677 (A2031G).

LRR motifs in the TLR4 gene were highly heterogeneous. Among six central LRR motifs (LRR11–16), LRR11 was the

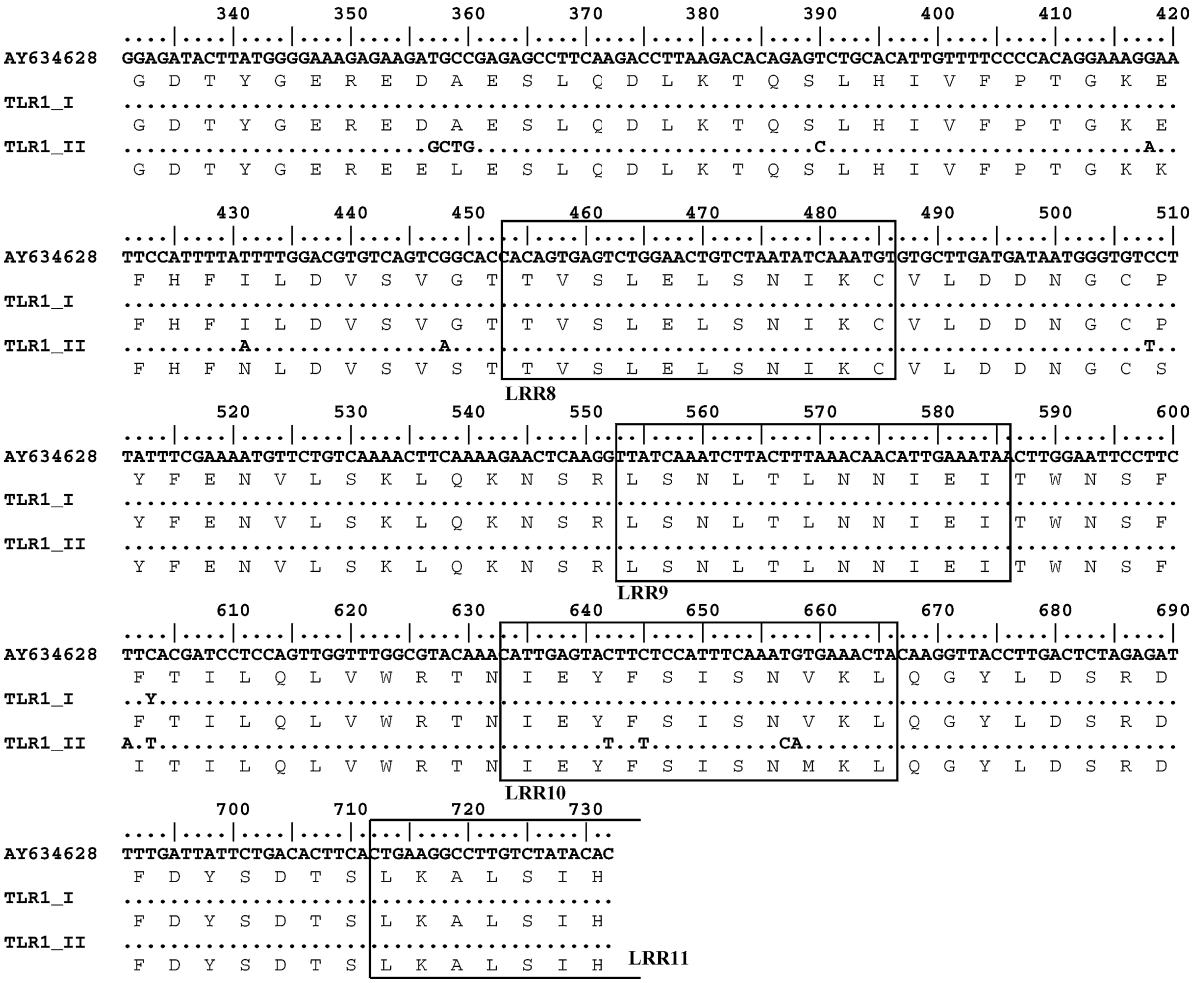

Fig. 1. Mutations in the central part of bovine TLR1 ectodomain. Alignment of nucleotide and amino acid sequences of TLR1 ECD (LRR8–LRR11) is presented. LRR motifs are bracketed (LRR8-nucleotides, nts: 454–486, LRR9-nts: 553–585, LRR10-nts: 634–666 and LRR11-nts: 712–744). Note that LRR11 is incomplete.

most non-conserved motif with four missense mutations: Ile285Arg, Gln287Thr, Phe288Ile and Asp294Asn. Ile337-Met and Asp363Gly mutations were found in LRR13 and LRR14 motifs, whereas LRR12, LRR15 and LRR16 had no amino acid change. Missense mutations in the extra LRR region of the TLR4 were Asp299Asn, Ser351Arg, Ile364Ala, Thr366Ser and Gly389Ser (Fig. 3).

3.4. Linkage between TLR mutations and susceptibility to MAP infection

Linkage between TLR mutations and susceptibility to MAP infection was observed in this study. 85.7% (n = 18; OR-3.459) of the population carrying Val220Met mutation was MAP infected. 20.39% (n = 32) of the total MAP infected individuals possessed missense mutations (His697Arg, Asn706Ser, Lys709Arg, Ile745Val and Lys759Arg) in TLR2 gene. Interestingly, 14 individuals showing apparent clinical symptoms carried one missense mutation (Ile680-Val) in TLR2 gene and 4 in TLR4 gene (Gly298[Arg,Trp], Asp299Asn, Gly389Ser, Gly389Ser). This indicates the most probable linkage between TLR mutations and increased susceptibility to MAP infection (Table 2).

4. Discussion

A linkage between TLR gene mutations and increased susceptibility to bacterial infections or disease conditions was reported earlier (Ben-Ali et al., 2004; Berdeli et al., 2005; Bochud et al., 2003, 2008; Fukusaki et al., 2007; Hong et al., 2007; Khor et al., 2007; Lakatos et al., 2005; Malley et al., 2003; Schroder and Schumann, 2005; Tabel et al., 2007; Thuong et al., 2007).

The ectodomain of the TLR1 consists of 20 LRRs which take part in the mycobacterial cell wall peptidoglycan recognition (Ozinsky et al., 2000). Mutations in TLR ectodomains may alter the PAMP recognition ability (Bell et al., 2003; Hamann et al., 2004). A central part of TLR1 ectodomain (LRR10) is prone to missense mutations and more irregular than other LRRs (Matsushima et al., 2007). A regular LRR motif comprised of 11 amino acids: L*xx*L*x*L*xx*N*x*L, wherein '*x*' is any amino acid, 'L' is Leu, Ile,

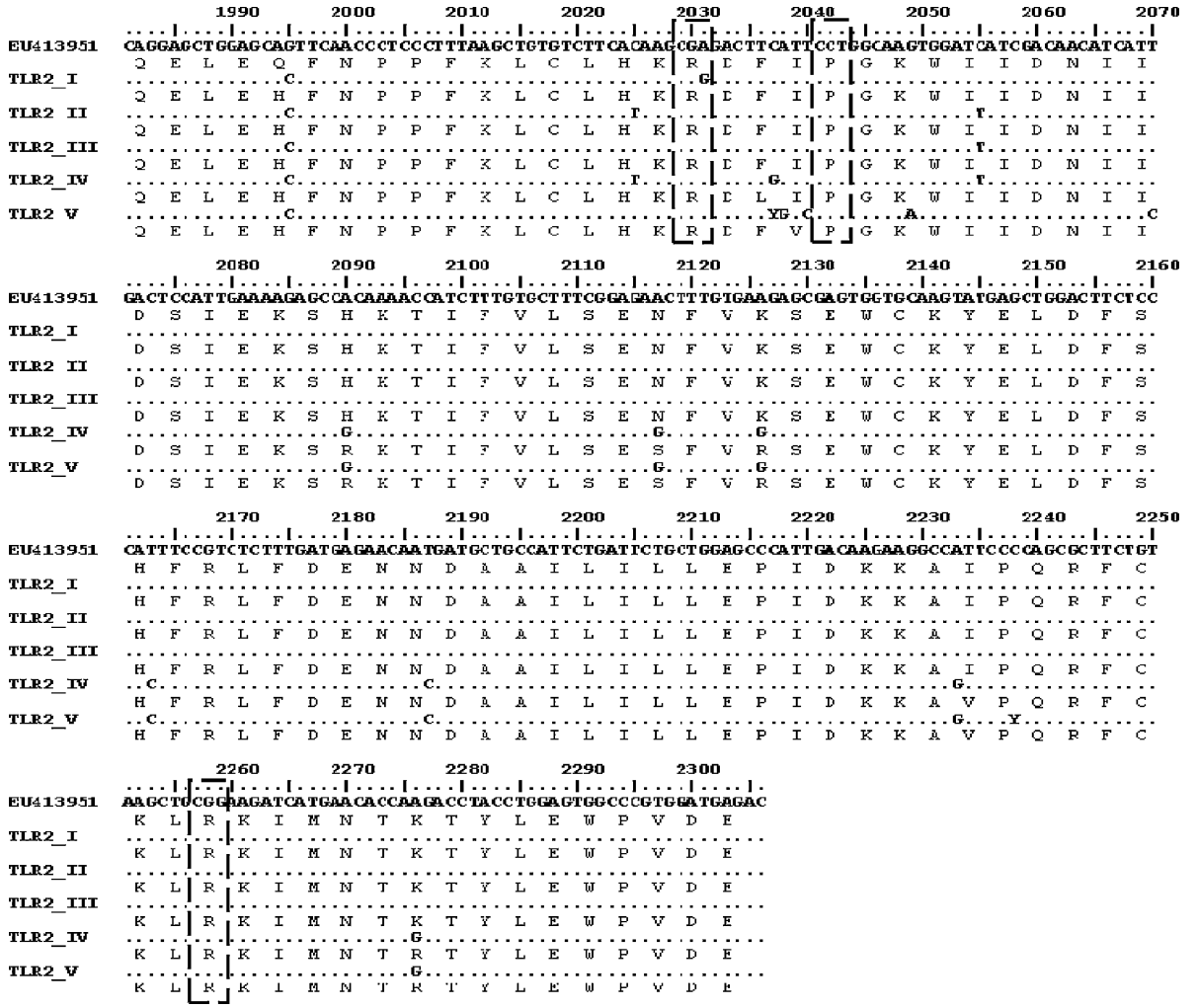

Fig. 2. Mutations in bovine TLR2-TIR ectodomain. Alignment of nucleotide and amino acid sequences of TLR2-TIR domain is presented. Well known mutation sites Arg677 (nts: 2029–2031), Pro681 (nts: 2041–2043) and Arg753 (nts: 2257–2259) are bracketed with dashed lines.

Table 2
Missense mutations in TLR1, TLR2 and TLR4 genes linked with MAP infection.

Gene	Nucleotide substitution[d]	Aminoacid change[d]	Genotype frequency of the mutated amino acid	Allele frequency of the mutated amino acid	OR[a]/number of animals carrying mutation
TLR1	G658A	Val220Met	0.03	0.03	3.459[b]/21
TLR2	A2038G	Ile680Val	0.02	0.02	–[c]/14
TLR4	G892Y	Gly298[Arg,Trp]	0.02	0.01	–/14
	G895A	Asp299Asn	0.02	0.02	–/14
	G1165A	Gly389Ser	0.02	0.02	–/14
	T1167C	Gly389Ser	0.02	0.02	–/14

[a] OR: Odd's ratio.
[b] 18 animals were infected with the MAP carrying Val220Met mutation.
[c] All animals carrying these mutations in TLR2 and TLR4 were MAP positive (100% MAP positivity), thus the calculation of OR was not possible.
[d] Apart from the mutations presented above the missense mutations in TLR1, TLR2 and TLR4 genes were found with no significant effect (statistically) on the susceptibility to the MAP infection: TLR1: T357G (Asp119Glu), G358C (Ala120Leu), C359T (Ala120Leu), C360G (Ala120Leu), G418A (Glu140Lys), T431A (Ile144Asn), G448A (Gly150Ser), C508T (Pro170Ser), and T601A (Phe201Ile). TLR2: C2037G (Phe679Leu), A2090G (His697Arg), A2117G (Asn706Ser), A2126G (Lys709Arg), A2233G (Ile745Val), and A2276G (Lys759Arg). TLR4: T854G (Ile285Arg), T855G (Ile285Arg), C859A (Gln278Thr), A860C (Gln278Thr), T862A (Phe288Ile), C864T (Phe288Ile), G880A (Asp294Asn), A881R (Asp294[Asn,Ser]), A883R (Lys295[Glu,Lys]), T1011G (Ile337Met), A1051C (Ser351Arg), A1088R (Asp363[Asp,Gly]), A1090G (Ile364Ala), T1091Y (Ile364[Val,Ala]), C1097S (Thr366[Thr,Ser]), G1105A (Glu369Lys), T1108A (Phe370Thr), T1109C (Phe370Thr), and C1111G (Gln371Glu).

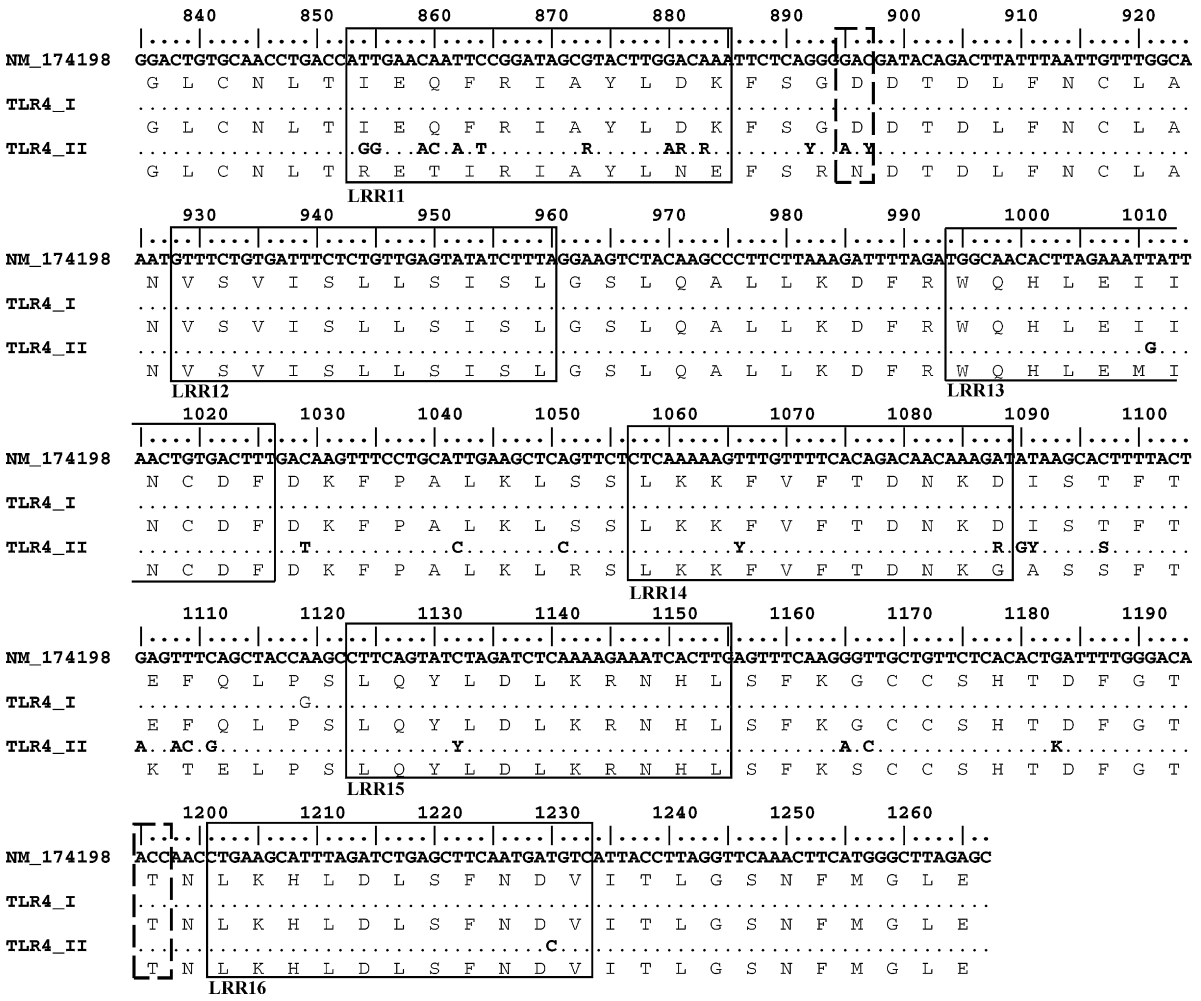

Fig. 3. Mutations in the central part of bovine TLR4 ectodomain. Alignment of nucleotide and amino acid sequences of TLR4 ECD (LRR11–LRR16) is presented. LRRs are bracketed (LRR11-nts: 853–885, LRR12-nts: 928–960, LRR13-nts: 994–1026, LRR14-nts: 1057–1089, LRR15-nts: 1123–1155 and LRR16-nts: 1201–1233). Well known mutations Asn299 and The399 are bracketed with dashed lines.

Val or Phe, and 'N' is Asn, Thr, Ser or Cys. In the present study a novel Val220Met mutation was observed in LRR10 motif at the 9th amino acid position (L*xx*L*x*L*xx*N9th *x*L) (Fig. 1). The presence of methionine at the 9th position may disrupt hydrogen bonds in the LRR loop structure that may cause the reduced recognition of PAMPs (Matsushima et al., 2005). The association between mutation at 9th amino acid position in LRR motif and poorly-differentiated gastric adenocarcinomas was reported recently (Ohara et al., 2006). The increased incidence of MAP infection in cattle bearing the Val220Met mutation (OR-3.459) was also observed in this study.

TLR2 recognizes mycobacterial cell wall lipoproteins and lipopolysaccharides (Schwandner et al., 1999). Functional studies confirmed that TIR domain plays a crucial role in MyD88 mediated downstream signaling. The TIR structure has a large conserve patch region (Xu et al., 2000). Mutations (Arg677Trp, Pro681His and Arg753Gln) in TIR conserve region may abolish immune response to lipopolysaccharide (Ben-Ali et al., 2004; Berdeli et al., 2005; Xu et al., 2000). Although previously described mutations (Arg677Trp, Pro681His and Arg753Gln) in TLR2-TIR domain were not observed in the present study, some novel mutations were found (Fig. 2). Ile680Val mutation, one residue upstream to Pro681His, was associated with increased MAP susceptibility in Holstein cows (Table 2).

TLR4 ECD consists of 23 LRR motifs which recognize bacterial lipopolysaccharide (Hoshino et al., 1999; Poltorak et al., 1998). The central part of ECD plays a key role in PAMP recognition (Matsushima et al., 2007). However, LRR motifs in the central part of ECD (11–14 LRR motifs) are prone to mutations causing irregularities in LRR motif structure. In this study, LRR11 was the most irregular LRR motif (deviation from the L*xx*L*x*L*xx*N*x*L structure) in TLR4 gene. Mutations (Asp299Gly and Thr399Ile) associated with hyporesponsiveness to bacterial infections are located near the central ECD region (Garza-Gonzalez et al., 2007). Instead of Asp299Gly, a missense mutation Asp299Asn was found in MAP infected animals in this study. Similarly, a transversion mutation (G892C/T; Gly298Arg, Trp) was associated with MAP infection

(Table 2). Transversion mutations usually change the chemical structure of ECD that might have an augmentative effect on hyporesponsiveness to PAMPs.

LRR motif forms a loop structure and the juxtaposition of several loops produces solenoid-like shapes of ECD. The LRR consensus motif forms the inner core of horseshoe shaped ECD, while extra LRR regions form convex surfaces (Bell et al., 2003). Irregularities in the convex surface, for example a mutation at the 4th residue downstream from the LRR motif, may affect PAMP binding onto the TLR horseshoe (Bell et al., 2003). The well known TLR4 mutation, Asp299Gly, is one of the best examples of the mutation at 4th residue downstream from LRR11 (Fig. 3).

In summary, a noteworthy association between TLR mutations and increased susceptibility to MAP infection was found. Considering the highly conserved nature of TLRs among mammals, these novel mutations may play a significant role in MAP infection not only in cattle but also in other mammals including humans.

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