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.

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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 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 were TLR1/F 5'-GGAGACAGCATCTGATGAGCCAGAGAAAGAGAA and TLR1/R 5'-GTCTCATCCACGGGCCACTCCA, while, TLR4 oligo sequences were TLR4/F 5'-GGGACTGTGCAACCTGACCA and TLR4/R 5'-GGAGATACTTATGGGGAAAGAGAA and TLR2/R 5'-CTTCTTCCCCCACTACTCTCACAGGA. PCR conditions for TLR2 were TLR4/F 5'-GGAGACGCTGGACACTTCAAACC and TLR4/R 5'-GTCTCATCCACGGGCCACTCCA, while, TLR4 oligo sequences were TLR4/F 5'-GGAGACTGTGCAACCTTCACTCAACC and TLR4/R 5'-GTCTCAATCCAGGGCCACCTCA, 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).
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) 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, 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).

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

<table>
<thead>
<tr>
<th>Breed</th>
<th>Number of animals tested</th>
<th>Number of MAP infected animals (%)</th>
<th>Prevalence of MAP infected animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slovak spotted cattle</td>
<td>10</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Slovak spotted cattle × Holstein</td>
<td>16</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Polish red</td>
<td>64</td>
<td>5</td>
<td>3.13</td>
</tr>
<tr>
<td>Holstein</td>
<td>136</td>
<td>27</td>
<td>16.45</td>
</tr>
<tr>
<td>Pinzgauer</td>
<td>247</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Slovakian Simmental</td>
<td>206</td>
<td>103</td>
<td>64.56</td>
</tr>
<tr>
<td>Dark brown Carpathians</td>
<td>32</td>
<td>24</td>
<td>15</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>711</strong></td>
<td><strong>160</strong></td>
<td><strong>22.50</strong></td>
</tr>
</tbody>
</table>
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: LxxLxLxxN, wherein ‘x’ is any amino acid, ‘L’ is Leu, Ile,
Table 2

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nucleotide substitution</th>
<th>Aminoacid change</th>
<th>Genotype frequency of the mutated amino acid</th>
<th>Allele frequency of the mutated amino acid</th>
<th>OR#number of animals carrying mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>G658A</td>
<td>Val220Met</td>
<td>0.03</td>
<td>0.03</td>
<td>3.459#21</td>
</tr>
<tr>
<td>TLR2</td>
<td>A2038G</td>
<td>Ile680Val</td>
<td>0.02</td>
<td>0.02</td>
<td>-#14</td>
</tr>
<tr>
<td>TLR4</td>
<td>G892Y</td>
<td>Gly298[Arg,Trp]</td>
<td>0.02</td>
<td>0.01</td>
<td>-#14</td>
</tr>
<tr>
<td></td>
<td>G895A</td>
<td>Asp299Asn</td>
<td>0.02</td>
<td>0.02</td>
<td>-#14</td>
</tr>
<tr>
<td></td>
<td>G1165A</td>
<td>Gly389Ser</td>
<td>0.02</td>
<td>0.02</td>
<td>-#14</td>
</tr>
<tr>
<td></td>
<td>T1167C</td>
<td>Gly389Ser</td>
<td>0.02</td>
<td>0.02</td>
<td>-#14</td>
</tr>
</tbody>
</table>

\[ OR: Odds ratio. \]
\[ \# number of animals infected with the MAP carrying mutation. \]
\[ All animals carrying these mutations in TLR2 and TLR4 were MAP positive (100% MAP positivity), thus the calculation of OR was not possible. \]
\[ a Apart from the muttations presented above the missense mutations in TLR1, TLR2 and TLR4 genes were found with no significant effect (statistically) on the susceptibility to the MAP infection: TLR1: T357G (Asp119Glu), G358C (Ala120Leu), C359T (Ala120Leu), C360G (Ala120Leu), G418A (Glu140Lys), T431A (Ile144Asn), G448A (Gly150Ser), C508T (Pro170Ser), and T601A (Phe201Ile). TLR2: C2037G (Phe679Leu), A2090G (His697Arg), A2117G (Asn706Ser), A2233G (Ile745Val), and A2276G (Lys759Arg). TLR4: T854G (Ile285Arg), T855G (Ile285Arg), C859A (Gln278Thr), A860C (Glu278Thr), A881R (Asp294[Asn, Ser]), A883R (Lys295[Glu, Lys]), T1011G (Ile337Met), A1051C (Ser351Arg), A1088R (Asp363[Asp, Gly]), A1090G (Ile364Ala), T1091Y (Ile364[Val, Ala]), C1097S (Thr366[Thr, Ser]), G1105A (Glu369Lys), T1108A (Phe370Thr), and C1111G (Gln371Glu). \]
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 (LxxLxxN9thL) (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 LxxLxxLxxN 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 (Fig. 3).

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

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.

Acknowledgement

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