1 Short Communication: Investigation into the genetic diversity in Toll-like
2 Receptors 2 and 4 in the European badger *Meles meles*.

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**Abstract**

The Toll-like receptor (TLR) genes are a conserved family of genes central to the innate immune response to pathogen infection. They encode receptor proteins, recognise pathogen associated molecular patterns (PAMPs) and trigger initial immune responses. In some host-pathogen systems, it is reported that genetic differences, such as single nucleotide polymorphisms (SNPs), associate with disease resistance or susceptibility. Little is known about TLR gene diversity in the European badger (*Meles meles*). We collected DNA from UK badgers, carried out PCR amplification of the badger TLR2 gene and exon 3 of TLR4 and determined DNA sequences for individual badgers for TLR2 (n=61) and TLR4 exon 3 (n=59). No polymorphism was observed in TLR4. Three TLR2 amino acid haplotype variants were found. Ninety five percent of badgers were homozygous for one common haplotype (H1), the remaining three badgers had genotypes H1/H3, H1/H2 and H2/H2. By broad comparison with other species, diversity in TLR genes in badgers seems low. This could be due to a relatively localised sampling or inherent low genetic diversity. Further studies are required to assess the generality of the low observed diversity and the relevance to the immunological status of badgers.

**Keywords**

Toll-Like Receptor; Polymorphism; badger; *Meles meles;* TLR2; TLR4

43 Toll-like receptors (TLRs) are a family of proteins that target highly conserved molecules essential for parasite
44 and pathogen survival (Takeda and Akira, 2005). Single nucleotide polymorphisms (SNP) in TLR genes have
45 been linked to pathogen susceptibility in some host species. For example, there is a relationship between TLR
46 variation and *Borrelia afzelii* susceptibility (Tschirren et al., (2013)) and TLR polymorphism and susceptibility to
47 bovine tuberculosis (bTB) in both Chinese Holstein cattle (Sun et al. 2012) and water buffalo (Alfano et al., 2014).
48 Other studies have shown links between TLR variation and risks of cancer (Gomaz et al., 2012), diabetes (Liu et
49 al., 2012), asthma (Schwartz and Cook, 2005) and TB (Zhang et al., 2013) in humans.
50 TLR genes are conserved throughout evolution (Lu et al., 2008) and homologues are found across a wide
51 range of species (Vasselon and Detmers, 2002). The proteins that they encode have two broad domains; an
52 extracellular Leucine Rich Repeat (LRR) domain which recognises and binds certain pathogen associated
53 molecular patterns (PAMPs) and an intracellular Toll-Interleukin receptor homology domain (TIR). The LRR
54 displays variability which is thought to be driven by an evolutionary arms race with invading parasites and
55 pathogens (Roach et al., 2005). The TIR domain is highly conserved and functions to deliver intracellular signals
56 triggering an innate immune response. Studies on TLR variation have found a higher degree of variation within
57 the LRR domains, some of which appears to be generated by positive selection on amino acid diversity (Jann et
58 al., 2008, Werling et al., 2009). Polymorphisms in the LRR domain can be associated with enhanced susceptibility
59 to disease. For example, the change from arginine to glutamine at position 753 in the human TLR2 gene
60 increases susceptibility to staphylococcal infection (Lorenz et al., 2000), tuberculosis (Ogus et al., 2005),
61 rheumatic fever (Berdeli et al., 2005) and urinary tract infection (Tabel et al., 2007).
62 TLR population studies are of increasing interest for analysis of broad host responses to disease. This is
63 particularly the case in humans but relevant equivalent data is sparse in wildlife species. To the best of our
64 knowledge, there are no reported studies of TLR variation in European badgers (*Meles meles*) and there are no
65 reported DNA sequences of TLRs from this species. An investigation into badger immune molecules is pertinent
66 in the context of the role of the badger in the perpetuation of bTB in cattle in the UK and Republic of Ireland
67 (Krebs et al., 1997; Bhuachalla et al., 2015). TLR 2 and TLR 4 recognise glycosyl-phosphatidyl-inositol (GPI)
68 anchors, and thus may be relevant to both the pathogenesis of bTB infection and to other pathogens of the

69 badger such as *Toxoplasma gondii* (Anwar et al., 2006; Hide et al., 2009), *Eimeria melis, Isospora melis*,
70 (Newman et al., 2001; Cottrell 2011), and *Trypanosoma pestanai*, (Lizundia et al., 2011;Ideozu et al., 2015). In
71 the present study, we set out to investigate TLR2 and TLR4 genetic diversity in the European badger using a
72 sample of animals from a well-studied population at Woodchester Park, in south-west England, alongside
73 geographically distinct badgers from other UK locations.
74 For DNA extraction, blood samples were collected from 54 badgers captured as part of a long-term capture-
75 mark-release study of badgers at Woodchester Park (e.g. Rogers et al., 1998; Delahay et al., 2000),
76 Gloucestershire, England. Ethical approval, ethical practice and appropriate licensing of badger capture and
77 examination were described previously (Ideozu et al., 2015). Additionally, a further 14 DNA extractions were
78 performed on other blood samples provided by Secret World Wildlife Rescue, which were collected from
79 badgers originating from eight geographically distinct locations in a 150 km radius of the Woodchester Park
80 site. DNA was extracted from badger blood samples using a modification of a phenol-chloroform protocol
81 (Morley et al., 2005; 2008; Ideozu et al., 2015). Appropriate measures were taken to prevent cross
82 contamination in DNA extractions and subsequent PCR reactions (Williams et al., 2005; Bajnok et al., 2015).
83 Briefly, 1.0 ml of badger blood was centrifuged at 2500 RPM for 10 minutes and the recovered pellet was then
84 incubated, by the addition of 400 μl lysis buffer (100 mM NaCl, 25 mM EDTA, 0.5% SDS, 20 mM Tris pH 8.0)
85 and 100 μl proteinase K (20 mg/ml), at 56 $^{o}$C overnight. Then 500 μl Tris buffered phenol-chloroform (pH 8.0)
86 was added, mixed for 10 min before separation of phases by centrifugation for 10 min at 13,000 rpm. The
87 phenol chloroform extraction was repeated twice more. Then 90 μl sodium acetate (3M pH 5.2) and 900 μl of
88 100% ethanol were added to the final supernatant and incubated overnight at -20 $^{o}$C. Following centrifugation
89 for 20 min at 13000g, the pellet was washed in 70% ethanol, centrifuged again and re-dissolved in 100 μl TE
90 buffer (10 mM Tris pH 8.0, 1 mM EDTA). To design PCR primers, TLR nucleotide sequences were retrieved
91 from PubMed-NCBI and ENSEMBLE, and TLR amino acid sequences from UniProt using database searches.
92 As no badger TLR gene information was available, primers suitable for TLR2 and TLR4 amplification from
93 badgers were predicted from evolutionarily related species (ferret, *Mustela putorius furo,* cat *Felis catus,* dog
94 *Canis lupus familiaris,* giant panda *Ailuropoda melanoleuca* and Pacific walrus *Odobenus rosmaus divergens*).

95 Badger TLR sequences were run through predictive modelling software using the PubMed position-specific
96 iterated basic local alignment search tool (PSI-BLAST) to identify possible protein domains from known
97 sequences.
98 Two overlapping primer sets were designed and optimised to amplify 1909 bp of the 2238 bp third exon of TLR4
99 (TLR44.1F 5'CTGTATCTCTTTTCCCTRTAGGTGTGA3', 5'GTTTAGGAAGCGAGCCAAATG3' nucleotide 1 to
100 1110 (see accession number KR780356), annealing 56°C; TLR44.2F 5'TTACCTTGATATTTCTTATAC3',
101 TLR44.2F 5' GAANCCTTCCTGGATGAT3', nucleotide 1071 to 1909 (see accession number KR780356),
102 annealing 50°C. This fragment covers all of the leucine rich repeat regions of TLR4. Three overlapping primer
103 sets were designed and optimised to amplify the single 2358 bp exon of TLR2 in its entirety (TLR22SF,
104 5'ATGTCACGTGTTYTGTGGACA3', TLR22SR 5'GTCRCTGAGATCCAAATATTCTA3', nucleotide 1 to 1110,
105 annealing 60°C; TLR22XF 5'GATGGAAGTTTTAGCGAACTTGTG3', TLR22XR
106 5'TCCCGAGTGAAAGACAGGAAT3', nucleotide 789 to 1643, annealing 56°C; TLR22F
107 5'GCTCCTGTGAATTCCTGTCTTTC3', TLR22R 5'GCCGTGTCAGAATAAGCTACCAC3', nucleotide 1613 to
108 2435 (77 bases beyond the stop codon), annealing 64°C. Each PCR Reaction contained 0.1 volume 10x NH4
109 buffer (Bioline, UK), 2 mM $MgCl_2$, 1 mM dNTP (Bioline UK), 0.5 µM forward primer and 0.5 µM reverse primer
110 and 2.5 units BioTaq Polymerase (Bioline, UK). PCR amplification was carried out using a Stratagene
111 Robocycler, denaturing for 40 secs at 94°C, annealing as specified in Table 1, depending on the primer
112 combination, and extension at 72°C for 1 min 30 secs. The number of PCR cycles also varied for each primer
113 set as shown in Table 1. All PCRs were run with 10 minutes denaturing at 94 °C before cycling and 5 minutes
114 extension at 72 °C at the end of the final cycle. Amplified DNA was examined by gel electrophoresis using 1%
115 agarose then checked for purity and quantity using a nanodrop spectrophotometer (Thermo Fisher Scientific,
116 UK). PCR products were adjusted to 10 ng/µl and sequenced by Sanger sequencing (SourceBioscience,
117 Cambridge) using both forward and reverse primers and a consensus sequence was generated. Sequence
118 graphics were examined by eye, using the program FinchTV (https://digitalworldbiology.com/FinchTV), to check
119 correct base calling and identify heterozygous bases (identified as two overlapping peaks of, usually, a smaller
120 amplitude at the same point on the electropherogram). All polymorphisms and heterozygotes were rechecked by

121 resequencing at least three times. Investigation of predicted structures of TLRs were carried out by sequence
122 alignment with example mammalian species (Program, Clustal Omega www.ebi.ac.uk) and Leucine Rich
123 Repeats (LRRs) were predicted using LRR Finder (http://www.lrrfinder.com/lrrfinder.php).
124 From Woodchester Park and other locations, 59 badgers were sequenced (accession number KR780356) across
125 exon 3 of TLR4 (1909 bp of the 2238 bp third exon) which covers the LRR region of TLR4. No polymorphisms
126 were found in the TLR4 gene. Analysis of the predicted protein sequence shows good identity to TLR4 from other
127 species (ferret, 92%; human, 73%; bovine 76%; mouse 62%). Eighteen LRRs are predicted compared to 20 for
128 the other species. The badger sequence is missing LRRs 7,8,19 (relative to the human sequence) but has an
129 additional predicted LRR before LRR1 (human).
130 Sixty-one badgers, from Woodchester Park and other locations, were sequenced across the entire 2355 bp
131 of the single exon TLR2 gene. Low genetic diversity was found. In addition to the wild type sequence, Haplotype
132 1 (H1), two single nucleotide polymorphisms were found in TLR2 (H2 and H3) and both were non-synonymous
133 missense mutations (Table 1) (Accession numbers KR780353 (TLR2-H1), KR780354 (TLR2-H2), KR780355
134 (TLR2-H3)). The three haplotypes generated two homozygous genetic types, one of which (the wild type H1/H1
135 n=58) predominated over the other (H2/H2 n=1). Two heterozygote haplotype combinations were seen (H1/H2,
136 n=1; H1/H3, n=1). One out of 52 (2%) of the badgers from the Woodchester Park study showed variation from
137 the majority consensus TLR2 sequence (heterozygous H1/H3), while 2/9 (22%) badgers from the other regions
138 showed TLR2 sequence variation (H1/H2 or H2/H2).
139 Analysis of the predicted protein sequences showed good identity to TLR2 from other species (ferret, 91%
140 human, 76%; bovine 75%; mouse 67%). Nineteen LRRs (including the LRR C-terminal) are predicted compared
141 to 20 for the other species. The badger sequence is missing LRR 5 (relative to the human sequence).
142 Both SNPs occurred in the extracellular LRR domain. Figure 1 shows the location of SNPs in relation to the
143 overall TLR2 structure. Haplotype 2 (C1007T) causes a missense mutation amino acid change from a threonine
144 to an isoleucine (T336I). Haplotype 3 (C1722G) causes a missense mutation amino acid change from a
145 glutamine to a histidine (Q574H). There was no difference between the predicted LRRs in H1, H2 and H3. The
146 structural or immunological outcomes of these changes have not been elucidated.

147 In this study, we report the first TLR sequences from European badgers. There is low gene variation in both
148 TLR2 and TLR4 with the latter being invariant in the study cohort. However, population level data on the variation
149 in either TLR2 or TLR4 genes in wildlife species is scarce. In comparison to other studies conducted, haplotype
150 diversity in the badger was lower than that in other species, for example, TLR 2 from bank voles (Tschirren et
151 al., 2013), TLR4 from grey partridges (Vinkler et al., 2015), pigs (Palermo et al., 2009) and both TLRs from water
152 buffalo (Alfano et al., 2014), humans (Mukherjee et al., 2014). Furthermore, studies on other TLRs have also
153 shown greater diversity in other species than in the badger genes reported here (e.g. TLR11 and TLR12,
154 Woodmice (Morger et al., 2014); TLR 1 and TLR9, cattle (Sun et al., 2012)). There is a growing body of evidence
155 to suggest that TLR variation, may have some influence on resistance or susceptibility to infection, for example
156 in *Borrelia* infection in wild rodents (Tschirren et al., 2013, Tschirren 2015) and bTB susceptibility in cattle (Sun
157 et al., 2012). Studies show that populations with low genetic diversity are less adaptable to exposure to pathogens
158 (e.g. Kaslow et al., 2008; Chapman et al., 2012). The restricted genotype range in the two badger genes,
159 examined here, raises questions about susceptibility to infection.

160 However, lack of variation could be explained by restricted sample size and geographical spread. Indeed,
161 TLR2 variation is heavily influenced by geographic dispersion (Tschirren, 2015). The TLR data presented here
162 are consistent with a study based on a wider set of data (microsatellite loci; mitochondrial genes) that shows
163 lower genetic diversity amongst European badgers in western regions of Europe (e.g. Britain) (Frantz et al.,
164 2014). Future studies should be aimed at gaining a broader perspective of the role of innate immune genes in
165 infection in badgers and investigation of the variability of TLRs over a wider geographical range.

166

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168

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177 **Conflict of Interest**

178 The authors declared no conflict of interest.

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183 **References**

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283

284 **Figure Legends**

285

286 **Fig. 1: Schematic diagram of the amino acid structure of the badger TLR2 gene showing SNP locations.**

287 Spotted area = LRR domain; Hatched region = TIR signalling domain; The positions of variant amino acids T336I

288 (C1007T) and Q574H (G1722C) are marked at the base of the gene image by upward pointing arrows (☗). A

289 300 base-pair scale is marked horizontally along the top for reference.

290

291 **Table 1: DNA sequence polymorphisms in badger TLR2**

292 This table shows the three haplotypes found, indicating position of changes and types of mutation.

293

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295 Table 1. DNA sequence polymorphisms in Badger TLR2

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| Haplotype | Nucleotide Variation | SNP type | Amino Acid Variation | Mutation |
|---|---|---|---|---|
| H1<br>(Wild Type) | Position 1007 ACA<br>Position 1722 CAG | | | |
| H2 | Position 1007<br>A**C**A > A**T**A | Transition | Position 336;<br>Threonine (T)<br>>Isoleucine (I) | Missense |
| H3 | Position 1722<br>CA**G** > CA**C** | Transversion | Position 574;<br>Glutamine (Q)<br>> Histidine (H) | Missense |

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Figure 1