Investigation into the genetic diversity in toll-like receptors 2 and 4 in the European badger Meles meles


http://dx.doi.org/10.1016/j.rvsc.2018.06.020

<table>
<thead>
<tr>
<th>Title</th>
<th>Investigation into the genetic diversity in toll-like receptors 2 and 4 in the European badger Meles meles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Publication title</td>
<td>Research in Veterinary Science</td>
</tr>
<tr>
<td>Publisher</td>
<td>Elsevier</td>
</tr>
<tr>
<td>Type</td>
<td>Article</td>
</tr>
<tr>
<td>USIR URL</td>
<td>This version is available at: <a href="http://usir.salford.ac.uk/id/eprint/47924/">http://usir.salford.ac.uk/id/eprint/47924/</a></td>
</tr>
<tr>
<td>Published Date</td>
<td>2018</td>
</tr>
</tbody>
</table>

USIR is a digital collection of the research output of the University of Salford. Where copyright permits, full text material held in the repository is made freely available online and can be read, downloaded and copied for non-commercial private study or research purposes. Please check the manuscript for any further copyright restrictions.

For more information, including our policy and submission procedure, please contact the Repository Team at: library-research@salford.ac.uk.
Short Communication: Investigation into the genetic diversity in Toll-like Receptors 2 and 4 in the European badger *Meles meles*.

Andrew M. Whiteoak\textsuperscript{a}, Justin Ideozu\textsuperscript{a}, Hadil Alkathiry\textsuperscript{a}, Alexandra J. Tomlinson\textsuperscript{b}, Richard J. Delahay\textsuperscript{b}, Sara Cowen\textsuperscript{c}, Elizabeth Mullineaux\textsuperscript{c}, Eamonn Gormley\textsuperscript{d}, Richard J. Birtles\textsuperscript{a}, Zhao-Rong Lun\textsuperscript{a,e,f} and Geoff Hide\textsuperscript{a,f}

\textsuperscript{a}Ecosystems and Environment Research Centre, School of Environment and Life Sciences, University of Salford, M5 4WT, UK

\textsuperscript{b}National Wildlife Management Centre, Animal and Plant Health Agency, Woodchester Park, Gloucestershire, GL10 3UJ, UK

\textsuperscript{c}Secret World Wildlife Rescue, New Road, East Huntspill, Highbridge, TA9 3PZ, UK.

\textsuperscript{d}School of Veterinary Medicine, Veterinary Science Centre, University College Dublin (UCD), Belfield, Dublin 4, Ireland.

\textsuperscript{e}State Key Laboratory of Biocontrol, School of Life Sciences; Key Laboratory of Tropical Diseases and Control of the Ministry of Education, Zhongshan Medical School, Sun Yat-Sen University, Guangzhou 510275, P.R. China

\textsuperscript{f}Biomedical Research Centre, School of Environment and Life Sciences, University of Salford, M5 4WT, UK.

Corresponding Author:

Professor Geoff Hide,
Ecosystems and Environment Research Centre and Biomedical Research Centre,
School of Environment and Life Sciences, University of Salford, M5 4WT, UK.
Tel: +44 (0) 161 295 3371;
Email: g.hide@salford.ac.uk
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
Toll-like receptors (TLRs) are a family of proteins that target highly conserved molecules essential for parasite and pathogen survival (Takeda and Akira, 2005). Single nucleotide polymorphisms (SNP) in TLR genes have been linked to pathogen susceptibility in some host species. For example, there is a relationship between TLR variation and *Borrelia afzelii* susceptibility (Tschirren et al., 2013) and TLR polymorphism and susceptibility to bovine tuberculosis (bTB) in both Chinese Holstein cattle (Sun et al., 2012) and water buffalo (Alfano et al., 2014).

Other studies have shown links between TLR variation and risks of cancer (Gomaz et al., 2012), diabetes (Liu et al., 2012), asthma (Schwartz and Cook, 2005) and TB (Zhang et al., 2013) in humans.

TLR genes are conserved throughout evolution (Lu et al., 2008) and homologues are found across a wide range of species (Vasselon and Detmers, 2002). The proteins that they encode have two broad domains; an extracellular Leucine Rich Repeat (LRR) domain which recognises and binds certain pathogen associated molecular patterns (PAMPs) and an intracellular Toll-Interleukin receptor homology domain (TIR). The LRR displays variability which is thought to be driven by an evolutionary arms race with invading parasites and pathogens (Roach et al., 2005). The TIR domain is highly conserved and functions to deliver intracellular signals triggering an innate immune response. Studies on TLR variation have found a higher degree of variation within the LRR domains, some of which appears to be generated by positive selection on amino acid diversity (Jann et al., 2008, Werling et al., 2009). Polymorphisms in the LRR domain can be associated with enhanced susceptibility to disease. For example, the change from arginine to glutamine at position 753 in the human TLR2 gene increases susceptibility to staphylococcal infection (Lorenz et al., 2000), tuberculosis (Ogus et al., 2005), rheumatic fever (Berdeli et al., 2005) and urinary tract infection (Tabel et al., 2007).

TLR population studies are of increasing interest for analysis of broad host responses to disease. This is particularly the case in humans but relevant equivalent data is sparse in wildlife species. To the best of our knowledge, there are no reported studies of TLR variation in European badgers (*Meles meles*) and there are no reported DNA sequences of TLRs from this species. An investigation into badger immune molecules is pertinent in the context of the role of the badger in the perpetuation of bTB in cattle in the UK and Republic of Ireland (Krebs et al., 1997; Bhuachalla et al., 2015). TLR 2 and TLR 4 recognise glycosyl-phosphatidyl-inositol (GPI) anchors, and thus may be relevant to both the pathogenesis of bTB infection and to other pathogens of the...
badger such as *Toxoplasma gondii* (Anwar et al., 2006; Hide et al., 2009), *Eimeria melis, Isospora melis*, (Newman et al., 2001; Cottrell 2011), and *Trypanosoma pestanai*, (Lizundia et al., 2011; Ideozu et al., 2015). In the present study, we set out to investigate TLR2 and TLR4 genetic diversity in the European badger using a sample of animals from a well-studied population at Woodchester Park, in south-west England, alongside geographically distinct badgers from other UK locations.

For DNA extraction, blood samples were collected from 54 badgers captured as part of a long-term capture-mark-release study of badgers at Woodchester Park (e.g. Rogers et al., 1998; Delahay et al., 2000), Gloucestershire, England. Ethical approval, ethical practice and appropriate licensing of badger capture and examination were described previously (Ideozu et al., 2015). Additionally, a further 14 DNA extractions were performed on other blood samples provided by Secret World Wildlife Rescue, which were collected from badgers originating from eight geographically distinct locations in a 150 km radius of the Woodchester Park site. DNA was extracted from badger blood samples using a modification of a phenol-chloroform protocol (Morley et al., 2005; 2008; Ideozu et al., 2015). Appropriate measures were taken to prevent cross contamination in DNA extractions and subsequent PCR reactions (Williams et al., 2005; Bajnok et al., 2015).

Briefly, 1.0 ml of badger blood was centrifuged at 2500 RPM for 10 minutes and the recovered pellet was then incubated, by the addition of 400 µl lysis buffer (100 mM NaCl, 25 mM EDTA, 0.5% SDS, 20 mM Tris pH 8.0) and 100 µl proteinase K (20 mg/ml), at 56 °C overnight. Then 500 µl Tris buffered phenol-chloroform (pH 8.0) was added, mixed for 10 min before separation of phases by centrifugation for 10 min at 13,000 rpm. The phenol chloroform extraction was repeated twice more. Then 90 µl sodium acetate (3M pH 5.2) and 900 µl of 100% ethanol were added to the final supernatant and incubated overnight at -20 °C. Following centrifugation for 20 min at 13000g, the pellet was washed in 70% ethanol, centrifuged again and re-dissolved in 100 µl TE buffer (10 mM Tris pH 8.0, 1 mM EDTA). To design PCR primers, TLR nucleotide sequences were retrieved from PubMed-NCBI and ENSEMBLE, and TLR amino acid sequences from UniProt using database searches.

As no badger TLR gene information was available, primers suitable for TLR2 and TLR4 amplification from badgers were predicted from evolutionarily related species (ferret, *Mustela putorius furo*, cat *Felis catus*, dog *Canis lupus familiaris*, giant panda *Ailuropoda melanoleuca* and Pacific walrus *Odobenus rosmarus divergens*).
Badger TLR sequences were run through predictive modelling software using the PubMed position-specific iterated basic local alignment search tool (PSI-BLAST) to identify possible protein domains from known sequences.

Two overlapping primer sets were designed and optimised to amplify 1909 bp of the 2238 bp third exon of TLR4 (TLR4.1F 5'CTGTATCTCTTTTCCCTAGGTGTGA3', 5'GTTTAGGAGCGAGCCAAATG3' nucleotide 1 to 1110 (see accession number KR780356), annealing 56°C; TLR4.2F 5'TTACCTGTATTTTCTTATAC3', TLR4.2F 5' GAANCCCTCCTGGAT3', nucleotide 1071 to 1909 (see accession number KR780356), annealing 50°C. This fragment covers all of the leucine rich repeat regions of TLR4. Three overlapping primer sets were designed and optimised to amplify the single 2358 bp exon of TLR2 in its entirety (TLR22SF, 5'ATGTACGGTGTYTGTGGACA3', TLR22SR 5'GTCRCTGAGATCCAAATATTC3', nucleotide 1 to 1110, annealing 60°C; TLR22XF 5'GATGGAAGTTTTAGCGAACTTGTG3', TLR22XR 5'TCCGAGTGAAAGACAGG3', nucleotide 789 to 1643, annealing 56°C; TLR22F 5'GCTCCTGTGAATTCCTGTCTTTC3', TLR22R 5'GCCGTGTCAGAATAAGCTACC3', nucleotide 1613 to 2435 (77 bases beyond the stop codon), annealing 64°C. Each PCR Reaction contained 0.1 volume 10x NH4 buffer (Bioline, UK), 2 mM MgCl2, 1 mM dNTP (Bioline UK), 0.5 µM forward primer and 0.5 µM reverse primer and 2.5 units BioTaq Polymerase (Bioline, UK). PCR amplification was carried out using a Stratagene Robocycler, denaturing for 40 secs at 94°C, annealing as specified in Table 1, depending on the primer combination, and extension at 72°C for 1 min 30 secs. The number of PCR cycles also varied for each primer set as shown in Table 1. All PCRs were run with 10 minutes denaturing at 94 °C before cycling and 5 minutes extension at 72 °C at the end of the final cycle. Amplified DNA was examined by gel electrophoresis using 1% agarose then checked for purity and quantity using a nanodrop spectrophotometer (Thermo Fisher Scientific, UK). PCR products were adjusted to 10 ng/µl and sequenced by Sanger sequencing (SourceBioscience, Cambridge) using both forward and reverse primers and a consensus sequence was generated. Sequence graphics were examined by eye, using the program FinchTV (https://digitalworldbiology.com/FinchTV), to check correct base calling and identify heterozygous bases (identified as two overlapping peaks of, usually, a smaller amplitude at the same point on the electropherogram). All polymorphisms and heterozygotes were rechecked by
resequencing at least three times. Investigation of predicted structures of TLRs were carried out by sequence alignment with example mammalian species (Program, Clustal Omega [www.ebi.ac.uk] and Leucine Rich Repeats (LRRs) were predicted using LRR Finder (http://www.lrrfinder.com/lrrfinder.php)).

From Woodchester Park and other locations, 59 badgers were sequenced (accession number KR780356) across exon 3 of TLR4 (1909 bp of the 2238 bp third exon) which covers the LRR region of TLR4. No polymorphisms were found in the TLR4 gene. Analysis of the predicted protein sequence shows good identity to TLR4 from other species (ferret, 92%; human, 73%; bovine 76%; mouse 62%). Eighteen LRRs are predicted compared to 20 for the other species. The badger sequence is missing LRRs 7,8,19 (relative to the human sequence) but has an additional predicted LRR before LRR1 (human).

Sixty-one badgers, from Woodchester Park and other locations, were sequenced across the entire 2355 bp of the single exon TLR2 gene. Low genetic diversity was found. In addition to the wild type sequence, Haplotype 1 (H1), two single nucleotide polymorphisms were found in TLR2 (H2 and H3) and both were non-synonymous missense mutations (Table 1) (Accession numbers KR780353 (TLR2-H1), KR780354 (TLR2-H2), KR780355 (TLR2-H3)). The three haplotypes generated two homozygous genetic types, one of which (the wild type H1/H1 n=58) predominated over the other (H2/H2 n=1). Two heterozygote haplotype combinations were seen (H1/H2, n=1; H1/H3, n=1). One out of 52 (2%) of the badgers from the Woodchester Park study showed variation from the majority consensus TLR2 sequence (heterozygous H1/H3), while 2/9 (22%) badgers from the other regions showed TLR2 sequence variation (H1/H2 or H2/H2).

Analysis of the predicted protein sequences showed good identity to TLR2 from other species (ferret, 91% human, 76%; bovine 75%; mouse 67%). Nineteen LRRs (including the LRR C-terminal) are predicted compared to 20 for the other species. The badger sequence is missing LRR 5 (relative to the human sequence).

Both SNPs occurred in the extracellular LRR domain. Figure 1 shows the location of SNPs in relation to the overall TLR2 structure. Haplotype 2 (C1007T) causes a missense mutation amino acid change from a threonine to an isoleucine (T336I). Haplotype 3 (C1722G) causes a missense mutation amino acid change from a glutamine to a histidine (Q574H). There was no difference between the predicted LRRs in H1, H2 and H3. The structural or immunological outcomes of these changes have not been elucidated.
In this study, we report the first TLR sequences from European badgers. There is low gene variation in both TLR2 and TLR4 with the latter being invariant in the study cohort. However, population level data on the variation in either TLR2 or TLR4 genes in wildlife species is scarce. In comparison to other studies conducted, haplotype diversity in the badger was lower than that in other species, for example, TLR 2 from bank voles (Tschirren et al., 2013), TLR4 from grey partridges (Vinkler et al., 2015), pigs (Palermo et al., 2009) and both TLRs from water buffalo (Alfano et al., 2014), humans (Mukherjee et al., 2014). Furthermore, studies on other TLRs have also shown greater diversity in other species than in the badger genes reported here (e.g. TLR11 and TLR12, Woodmice (Morger et al., 2014); TLR 1 and TLR9, cattle (Sun et al., 2012)). There is a growing body of evidence to suggest that TLR variation, may have some influence on resistance or susceptibility to infection, for example in Borrelia infection in wild rodents (Tschirren et al., 2013, Tschirren 2015) and bTB susceptibility in cattle (Sun et al., 2012). Studies show that populations with low genetic diversity are less adaptable to exposure to pathogens (e.g. Kaslow et al., 2008; Chapman et al., 2012). The restricted genotype range in the two badger genes, examined here, raises questions about susceptibility to infection.

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

Acknowledgements

We thank the field team at Woodchester Park for live capture and sampling of badgers, Defra who fund the study and the landowners for continuing co-operation. We also thank Secret World Wildlife Rescue for their support and provision of veterinary samples. We acknowledge the financial support of OSAPND (Nigeria), MARG, British Society for Immunology, Wellcome Trust, University of Salford, British Society for Parasitology and American...
Society of Microbiology. ZRL's laboratory is supported by grants from National Natural Science Foundation of China (No.31272305 and No.31472058). None of the funding agencies had any influence over the generation, analysis or interpretation of the data reported here.

**Conflict of Interest**

The authors declared no conflict of interest.
References


Bajnok J, Boyce K, Rogan MT, Craig PS, Lun ZR, Hide G (2015) Prevalence of Toxoplasma gondii in localised populations of Apodemus sylvaticus is linked to population genotype not to population location. Parasitology 143:680-690


Naturally occurring Toll-like receptor 11 (TLR11) and Toll-like receptor 12 (TLR12) polymorphisms are not associated with *Toxoplasma gondii* infection in wild wood mice. Infection Genetics and Evolution 26:180-184

Evidence that primary infection of charollais sheep with *Toxoplasma gondii* may not prevent foetal infection and abortion in subsequent lambings. Parasitology 135:169-173

Significant familial differences in the frequency of abortion and *Toxoplasma gondii* infection within a flock of Charollais sheep. Parasitology 131:181-185


Toll-like receptor 4 genetic diversity among pig populations. Animal Genetics 40:289-299

The evolution of vertebrate Toll-like receptors. Proceedings of the National Academy of Sciences, USA 102:9577-9582

Movement of badgers (*Meles meles*) in a high density population: individual, population and disease effects. Proceedings of the Royal Society Series B 265:1269-1276

Polymorphisms of the Toll-like receptors and human disease. Clinical Infectious Diseases 41:403-407


Figure Legends

Fig. 1: Schematic diagram of the amino acid structure of the badger TLR2 gene showing SNP locations. Spotted area = LRR domain; Hatched region = TIR signalling domain; The positions of variant amino acids T336I (C1007T) and Q574H (G1722C) are marked at the base of the gene image by upward pointing arrows (▲). A 300 base-pair scale is marked horizontally along the top for reference.

Table 1: DNA sequence polymorphisms in badger TLR2

This table shows the three haplotypes found, indicating position of changes and types of mutation.
### Table 1. DNA sequence polymorphisms in Badger TLR2

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>Nucleotide Variation</th>
<th>SNP Type</th>
<th>Amino Acid Variation</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1 (Wild Type)</td>
<td>Position 1007 ACA</td>
<td>Position 1722 CAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2</td>
<td>Position 1007 ACA &gt; ATA</td>
<td>Transition</td>
<td>Position 336; Threonine (T) &gt; Isoleucine (I)</td>
<td>Missense</td>
</tr>
<tr>
<td>H3</td>
<td>Position 1722 CAG &gt; CAC</td>
<td>Transversion</td>
<td>Position 574; Glutamine (Q) &gt; Histidine (H)</td>
<td>Missense</td>
</tr>
</tbody>
</table>
Figure 1

C1007T  G1722C

Extracellular region  Intracellular region