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Identification and expression of Toll-like receptors 1–10 in selected bovine and ovine tissues

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Abstract

Members of the Toll-like receptor (TLR) family are vital to immune function through the sensing of pathogenic agents and initiation of an appropriate immune response. More specifically, tissue and cell specific TLR expression patterns have been correlated with the ability to respond to various pathogenic challenges. Bovine sequence exists for 4 of the 10 human TLR Reference Sequences and no ovine TLR sequence has been reported. The main goal of this study was to determine if homologues of human TLRs 1–10 exist within the cattle and sheep. Subsequent to this, quantitative real time PCR assays were to be developed to produce transcript expression profiles in cattle skin and sheep gut-associated lymphoid tissue, as these epithelial tissues are the primary sites of host/pathogen interactions for numerous pathogens. Our findings show that homologues of human TLRs 1–10 do indeed exist within both cattle and sheep, with respective bovine and ovine homologues sharing at least 95% nucleotide sequence identity and 83–90% identity to the corresponding human Reference Sequences. Conservation of the amino acid sequence between homologous ruminant and human TLRs ranged between 84 and 97%. Quantitative real time PCR (qPCR) assays confirmed expression of all 10 TLRs within ovine jejunum, Peyer's patch and mesenteric lymph nodes. While in bovine skin all TLRs apart from TLR6 were detected. The most abundant TLR transcripts within the ovine jejunum were TLRs 3, 5 and 6, while TLRs 6, 7 and 10 were abundant in both ovine Peyer's patch and mesenteric lymph node. In bovine skin TLRs 2 and 7 were most abundant. In all tissues tested TLR4 expression was at the lower limit of detection.
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1. Introduction

Toll-like receptors (TLRs) are a highly conserved group of proteins that have been identified in organisms as diverse as insects (Lemaitre et al., 1996) and mammals (Medzhitov et al., 1997). Within mammals, the TLR family is proposed to contain at least 10 members (Beutler, 2004). The TLRs provide

Abbreviations: TLR, Toll-like receptor; PAMP, pathogen associated molecular pattern; EST, expressed sequence tag; PP, Peyer's patch; MLN, mesenteric lymph node; LPS, lipopolysaccharide; CpG DNA, unmethylated dinucleotides; LRR, leucine rich repeats; TIR, Toll/interleukin-1 signalling domain

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the host with a means of discriminating foreign from self and, as such, they function at the earliest stages of immune development (Kopp and Medzhitov, 1999). In addition, the TLRs are able to specifically identify pathogenic agents from commensals. Factors such as the location and abundance of each TLR are intimately involved in this process (Cario et al., 2002; Abreu, 2003; Kelly and Conway, 2005). Recognition of foreign molecules is based on the detection of molecular patterns that are indicative of entities likely to cause the host harm. These pathogen associated molecular patterns (PAMPs) comprise molecules such as lipopolysaccahride (LPS), flagellin, dsRNA and (unmethylated dinucleotides) CpG DNA.

Variations in TLR abundance have been associated with altered immune responsiveness. For example, TLR4 knockout mice (producing no TLR4) have been shown to resist chronic nematode infection whereas mice producing functional TLR4 routinely develop chronic infections (Helmby and Grencis, 2003). In order to gain an understanding of how responsive tissues and cells are likely to be at detecting pathogens TLR mRNA expression patterns have been determined in humans (Zarember and Godowski, 2002; Hornung et al., 2002), mice (Pruett et al., 2004; Kurt-Jones et al., 2004; Kokkinopoulos et al., 2005), fish (Jault et al., 2004; Meijer et al., 2004) and chickens (Iqbal et al., 2005).

As reported by Werling and Jungi (2003) in their review of TLR analysis in production species, there is only limited information published on expression levels of bovine TLRs, and nothing reported for ovine TLRs. The majority of work on bovine TLRs concentrates on TLRs 2 and 4. Of the mammalian TLR family these are the best studied and have been implicated in the recognition of Gram positive and Gram negative bacteria, with components of the bacterial cell wall or membrane serving as PAMPs (Takeuchi and Akira, 2002). Bovine TLRs 2 and 4 have been mapped to chromosomes 17 and 8, respectively (White et al., 2003a) and TLR9 to chromosome 22 (Goldammer et al., 2004). TLR4 has also been haplotype mapped in order to identify potential areas of variation that might affect an individual's disease resistance ability (White et al., 2003b). More recently, real time PCR tests have been developed for TLRs 2, 4 and 9 allowing transcript abundance to be measured (Eicher et al., 2004; Goldammer et al., 2004; Werling et al., 2004). Such tests are vital for gaining an insight into immune function and the acquisition of immunity.

In this study we planned to determine if homologues of the 10 TLRs identified in humans existed in cattle and sheep, as only 4 TLRs had been previously identified in cattle. Partial fragments of putative TLRs were amplified from cDNA, cloned and sequenced. Using the newly obtained sequence further primers were designed, for use in real time PCR assays, in order to confirm transcription of each TLR and produce tissue specific mRNA abundance profiles. We have focussed on tissues of the ovine gut and bovine skin as these epithelial surfaces are involved in the primary interactions between host and pathogen in numerous situations.

2. Materials and methods

2.1. Identification of bovine TLR orthologs

The MegaBlast, blastn (nucleotide query versus nucleotide database) or blastx (translated query versus protein database) programs, with default settings, (Altschul et al., 1990) were used to identify bovine sequences, contained within the GenBank database (http://www.ncbi.nlm.nih.gov/), showing identity to each of the 10 human TLR Reference Sequences. The GenBank database contains genomic and transcribed sequences of bovine origin. Accession numbers for input human Reference Sequences are listed in Table 1.

Table 1
GenBank accession numbers for human Reference Sequences (RefSeq) and bovine TLRs and region of identity

TLR	Input human RefSeq	Bovine Blast hit	Region of identity to human RefSeq	
			Nucleotide	Protein
1	NM_003263	BF230168	665–1079	130–268
2	NM_003264	NM_174197	1840–2261	537–704
3	NM_003265	AY124007	2494–2799	797–906
4	NM_138554	AB056444	374–781	69–205
5	NM_003268	BE753283	2804–3064	720–820
6	NM_006068	AB020807	1522–2202	486–711
7	NM_016562	BM255019	2506–2900	790–921
8	NM_016610	AY642125	2729–3118	830–959
9	NM_017442	AJ509825	1013–1325	123–228
10	NM_030956	ti510876751	551–758	23–90

2.2. RNA extraction and cDNA synthesis

Skin snips (8 mm diameter) were removed from the neck of three 12-month-old Hereford heifers with biopsy punches (Kai Medical, Japan). Following euthanasia and necropsy, 1 cm square sections of ovine jejunum, Peyer's patch and mesenteric lymph node gut tissues were removed from three 6-month-old Romney Merino sheep. All work was performed in accordance with guidelines set out by the CSIRO Livestock Industries Animal Ethics Committee. All tissues were snap frozen in liquid nitrogen immediately following removal. Animals were not housed in pathogen-free surroundings.

For each RNA preparation, 250 mg of frozen tissue was removed and wrapped in aluminium foil and stored at −80 °C until required. The wrapped tissue was then dipped into liquid nitrogen and hammered in order to disrupt the tissue. This material was scraped into QIAGEN RNeasy lysis buffer (Qiagen, Germantown, MD) and immediately homogenised with 3 × 5 s bursts of an Ultra Turrax (Labortechnik, Staufen, Germany) mini probe. RNA was extracted from this homogenised material following the manufacturer's instructions using the RNAeasy midi kit (Qiagen). To determine quantity and ensure integrity of extracted RNA, absorbance was determined at 260 and 280 nm and samples were analysed via agarose gel electrophoresis. Samples were treated with DNase I (Qiagen), twice, during the extraction procedure and again using Turbo DNA-free DNase I (Ambion, Austin, TX) following completion of the RNAeasy protocol, in order to remove genomic DNA.

cDNA was prepared in a 20 μl reaction volume using 2 μg of total RNA per reaction. Random hexamers (Promega, Annandale, Australia) were used to prime the complementary strand and Invitrogen (Carlsbad, CA) Superscript III was used to manufacturer's specifications in the reverse transcription reaction. At the completion of the reaction cDNA was diluted 1/5 in water and stored frozen at −80 °C until required.

2.3. Amplification and cloning of TLRs

Primers were designed to bovine TLR sequences, using the Primer3 software (Rozen and Skaletsky, 2000). Resulting primer sequences are shown in Table 2. These primers were used in a PCR consisting of 30 cycles of: 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. Reactions were performed in 20 μl total volume and contained 10 ng of either bovine or ovine cDNA, 200 pmol of TLR-specific primers and 3 mM $MgCl_2$. Other reagents were added as recommended for use with Invitrogen *Taq* DNA polymerase.

Amplified fragments were visualised on an agarose gel and purified using the QIAquick gel purification system (Qiagen). Purified fragments were cloned into pGemT-Easy following the manufacturer's instructions (Promega). Plasmid DNA was extracted from several colonies using a Qiagen Miniprep kit and sequenced with the ABI (Foster City, CA) BigDye terminator kit version 3.1. Sequences obtained were used to generate alignments recorded in Table 3.

2.4. Quantitative real time PCR

Primers were designed for TLRs 1–10, based on cloned bovine and ovine sequence, as well as for bovine and ovine GAPDH using Primer Express

Table 2
Sequence of primers used in PCR and GenBank accession numbers of nucleotide sequences from which they were derived

TLR	Primer sequence 5′–3′ forward/reverse	Size (bp)	Bovine sequence	Ovine sequence
1	CTGCCCATATGCCAAGAGTT/AAACCAACTGGAGGATCGTG	421	AY957622	AY957612
2	GCTCCTGTGACTTCCTGTCC/CCGAAAGCACAAAGATGGTT	501	AY957623	AY957613
3	GAGGCAGGTGTCCTTGAACT/GCTGAATTTCTGGACCCAAG	327	AY957624	AY957614
4	AGGCAGCCATAACTTCTCCA/GGTTGAGTAGGGGCATTTGA	410	AY957625	AY957615
5	AACGCTTTGCTCAAACACCT/ACCCTCTGATGGACTGATGC	301	AY957626	AY957616
6	ACTGACCTTCCTGGATGTGG/GCACCACTCACTCTGGACAA	679	AY957627	AY957617
7	ACTCCTTGGGGCTAGATGGT/GCTGGAGAGATGCCTGCTAT	180	AY957628	AY957618
8	GTTATGTTGGCTGCCCTGG/GTTCTCATCCATTAGCCTCTGC	393	AY957629	AY957619
9	CTGGAGGAGCTGAACCTGAG/TGGTTGTAGGACAGCAGCAG	320	AY957630	AY957620
10	GGCACAGGGTTAGGAAAACA/GAGATTGTGGTGGGCAAAGT	246	AY957631	AY957621

Table 3
Identity, expressed as a percentage, of bovine and ovine TLR sequences to each other and to the human Reference Sequence

TLR	Bovine or ovine V human		Bovine V ovine nucleotide
	Nucleotide (%)	Protein (%)	
1	83	84	98
2	86	94	95
3	90	96	99
4	85	89	97
5	87	92	96
6	87	93	97
7	87	91	97
8	89	97	99
9	84	85	97
10	88	88	99

(ABI). Primer sequences used in quantitative real time PCR (qPCR) and the expected size of amplicons are listed in Table 4. Only final primer pairs are recorded in the Table. Any primers that did not function under standard assay conditions to generate a unique product were eliminated and new primers were designed.

Each qPCR was performed in a total volume of 5 μl and contained 900 nM of each primer and 10 ng of cDNA in addition to the commercially available SYBR Green Master Mix (ABI). To ensure cDNA samples were not contaminated with genomic DNA, reactions were set up using 10 ng of non-reverse transcribed RNA in place of cDNA. Failure to generate a detectable signal signified the sample as DNA free.

Each qPCR was performed in quadruplicate, using standard conditions in an ABI Prism 7900HT Sequence Detection System. Reaction efficiency was determined for each gene using 10-fold dilutions of plasmid containing the cloned amplicon (Table 4). Dissociation curves were generated to ensure a single amplicon had been produced.

For each of the three biological samples gene expression was quantified by normalizing each target TLR against the reference gene, GAPDH, through the Q-GENE statistical analysis package (Simon, 2003). Independent tests have shown GAPDH to be

Table 4
Sequence of primers used in quantitative real time PCR

TLR	Primer name	Sequence	Size (bp)	Efficiency (*E*)
1	TLR1-2F	CCCACAGGAAAGAAATTCCA	208	1.80
	TLR1-2R	GGAGGATCGTGATGAAGGAA		
2	TLR2-2F	ACGACGCCTTTGTGTCCTAC	192	1.95
	TLR2-R	CCGAAAGCACAAAGATGGTT		
3	TLR3-2F	GAGGCAGGTGTCCTTGAACT	329	1.96
	TLR3-2R	GCTGAATTTCTGGACCCAAG		
4	TLR4-2F	ACTGACGGGAAACCCTATCC	208	1.98
	TLR4-2R	CAGGTTGGGAAGGTCAGAAA		
5	TLR5-3F	AAAACCACATCGCCAACATC	191	1.98
	TLR5-3R	CATCAGATGGAACTGGGACA		
6	TLR6-2F	CAAAGCAGGGAACAATCCAT	206	1.95
	TLR6-2R	CCACAATGGTGACAATCAGC		
7	TLR7-3F	ACTCCTTGGGGCTAGATGGT	180	1.92
	TLR7-3R	GCTGGAGAGATGCCTGCTAT		
8	TLR8-3F	TCCACATCCCAGACTTTCTACGA	150	1.80
	TLR8-3R	GGTCCCAATCCCTTTCCTCTA		
9	TLR9-2F	CTCGTATCCCTGTCGCTGAG	210	1.98
	TLR9-2R	CACCTCCGTGAGGTTGTTGT		
10	TLR10-4F	TCTGCCTGGGTGAAGTATGA	190	1.87
	TLR10-4R	AATGGCACCATTCAGTCTGG		
GAPDH	GAPDH-F	CCTGGAGAAACCTGCCAAGT	200	1.93
	GAPDH-R	GCCAAATTCATTGTCGTACCA		

constantly expressed in all samples. Four technical replicates were performed on each biological sample. The mean of the three values was determined and the mean result is expressed in the form of mean normalised expression (MNE) ± standard error (S.E.).

3. Results

3.1. Identification of bovine and ovine TLR sequences

Bovine orthologs of human TLRs 1–10 were identified within the GenBank database through Blast searches. Accession numbers for all sequences and the region of nucleotide identity shared between each human Reference Sequence and its corresponding bovine Blast hit are listed in Table 1 (ti510876751 can be found through the GenBank Trace archive link). Bovine sequence obtained through Blast analysis was then used to design primers targeting each of the 10 TLRs genes. Primer sequences are listed in Table 2. These primers successfully amplified the 10 TLRs genes from both bovine and ovine cDNA. Amplified TLR fragments were cloned and sequenced and accession numbers obtained for these sequences are listed in Table 2. The partial nucleotide sequences obtained for each bovine and ovine TLR were then aligned to the corresponding region of the relevant human nucleotide sequence and to each other in order to determine identity. As shown in Table 3 there is at least 95% sequence identity between respective bovine and ovine TLR nucleotide sequences. The identity of each cloned bovine and ovine TLR to the relevant human reference sequence ranges between 83 and 90% (Table 3). Translation of the bovine or ovine nucleotide sequences and alignment to the human TLR protein shows 84–97% of the amino acid sequence is conserved (Table 3).

3.2. TLR abundance determined in ovine gut tissues using qPCR

Highly specific primers capable of amplifying the bovine and ovine versions of each TLR in qPCR were designed based on the cloned bovine and ovine sequences. Primer sets are shown in Table 4. Fig. 1 displays the expression level of each TLR determined by qPCR in ovine jejunum, Peyer's patches (PP) and Mesenteric lymph nodes (MLN). Transcriptional expression of each of the10 TLRs was detected in the three ovine gut-associated tissues analysed. TLRs 3 and 6 were the most abundant of the TLRs in the jejunum, whereas TLRs 6, 7 and 10 were most abundant in the PP and MLN. Apart from TLRs 3 and 5, that are most abundant in the jejunum, all other TLRs are more abundant in the PP or MLN.

3.3. TLR abundance determined in bovine skin using qPCR

Fig. 2 displays the expression level of each TLR determined in bovine skin using the primer sets in Table 4. Expression of the TLR6 transcript was not detected in bovine skin and TLR4 was at the lower limit of detection. Expression of TLRs 1, 2, 3, 5, 7, 8,

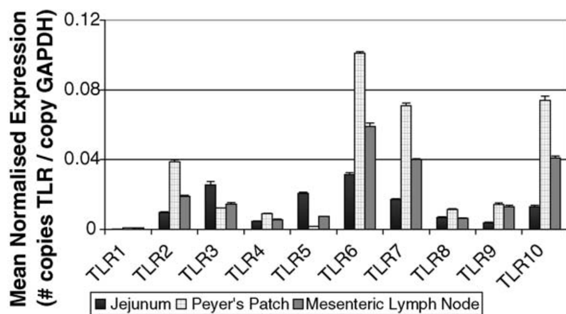

Fig. 1. Relative transcript abundance of TLRs 1–10 determined in ovine gut-associated tissues. Data shown is an average of $n = 3$ biological replicates ± standard error. There were four technical replicates performed for each biological sample.

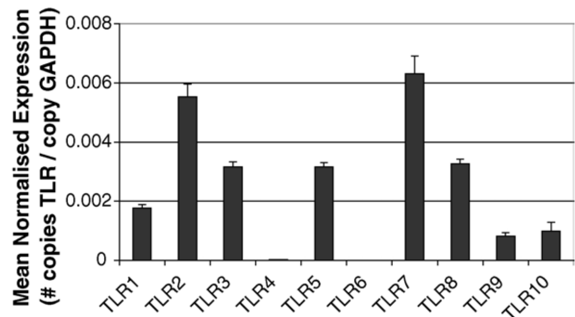

Fig. 2. Relative transcript abundance of TLRs 1–10 determined in bovine skin. Data shown is an average of $n = 3$ biological replicates ± standard error. There were four technical replicates performed for each biological sample.

9, and 10 was confirmed, with TLRs 2 and 7 being the most abundant TLR transcripts found in the skin.

4. Discussion

TLRs are of great interest to the research community due to their ability to recognize pathogens and initiate development of an immune response. While at least 10 TLRs have been described in humans only 4 have been described previously in cattle and none in sheep. This work has confirmed that homologues of each of the 10 characterised human TLR genes exist within cattle and sheep. We have also confirmed that the TLR genes are transcribed as each gene was amplified in PCR using bovine or ovine cDNA, as template, and also detected by quantitative real time PCR assays. Alignment of the corresponding cloned bovine and ovine TLR nucleotide sequences shows they share at least 95% identity. Due to this high sequence identity only bovine sequence is shown aligned to the human sequence as alignment of the ovine sequences generates an identical result. These alignments show the ruminant and human TLRs share 83–90% nucleotide identity and 84–97% amino acid identity. Previously cloned bovine TLR2 and TLR4 were reported to show 77 and 72% identity to human TLR amino acid sequence (Werling and Jungi, 2003). Theses values are much lower than determined here although, direct alignment of nucleotide sequences for TLR2 and TLR4 determined here and those determined previously (AF310951 and AF310952) show only a single nucleotide difference in either comparison (data not shown), indicating the difference might be due to only partial sequences being aligned here.

TLR expression profiles are suggestive of an individual's ability to respond to challenge. For this reason TLR expression profiles were determined in epithelial tissues that form the interface between host and pathogen for numerous pathogens.

Transcript expression of each of the 10 TLRs was confirmed in the three ovine gut-associated tissues analysed by qPCR. Apart from TLRs 3 and 5, all other TLRs were more abundant in the Peyer's patches or Mesenteric lymph nodes then they were in the jejunum. This result suggests there are more TLR transcripts per cell or more cells expressing TLRs in both the PP and MLN. It is interesting that TLRs 3 and 5 are relatively more abundant in the jejunum suggesting gut epithelial cells possibly express these molecules. However, the expression of TLRs relative to GAPDH is quite low, ranging from 10 to 100 copies of TLR transcript per 1000 copies of GAPDH, suggesting only a small subset of cells express the TLRs or that only a few copies are expressed per cell. Similar expression profiles for TLRs 3 and 5 have been shown previously in the human small intestine (Zarember and Godowski, 2002) and intestinal epithelial cells (Cario and Podolsky, 2000). If the abundance of these TLRs is indicative of a heightened ability to respond to the appropriate PAMP it seems likely that the jejunum or gut epithelium is very sensitive to potential viral and motile bacterial infections, through the ability of TLR3 to recognize dsRNA and TLR5 to recognize flagellin. Differences in relative abundance may also correlate to the sensitivity with which each TLR recognises its target PAMP.

Overall, the results determined in the gut tissues are in agreement with those determined elsewhere and reflect the relative abundance of TLRs determined in human intestinal myofibroblasts (Otte et al., 2003) and chicken jejunum (Iqbal et al., 2005). More specifically the low relative abundance of TLRs 1, 2 and 4 determined in the jejunum matches previous reports that TLRs 2 and 4 are barely detectable in the gut (Cario and Podolsky, 2000) and TLR1 lowly abundant in intestinal myofibroblasts and intestinal tissue (Otte et al., 2003; Zarember and Godowski, 2002). When compared to the jejunum, TLRs 2, 6, 7 and 10 are more abundant in the PP and MLN suggesting an abundance of cells shown previously to express these TLRs including, monocytes, B cells and T cells (Hornung et al., 2002; Kokkinopoulos et al., 2005). TLR10 expression has been confirmed in all gut-associated tissues studied here. In other work TLR10 was not detected in intestinal myofibroblasts (Otte et al., 2003) but expression has been confirmed in cells such as dendritic cells (Hornung et al., 2002).

The bovine skin snips contained the three distinct regions of skin including the outermost layer or epidermis, the underlying dermis and a small amount of subcutaneous tissue. TLR expression has been confirmed in a number of skin cell types in humans, mice and chickens. Cell types include keratinocytes (skin epithelial cells) (Kawai et al., 2002; Mempel et al., 2003; Baker et al., 2003), Langerhans cells (dendritic cells unique to the epidermis) (Takeuchi et al., 2003), dermal dendritic cells (Gilliet et al.,

2004), skin derived mast cells (Matsushima et al., 2004), and Schwann cells that are associated with the peripheral nervous system (Oliveira et al., 2003). Comprehensive searches for all TLRs were not performed in every cell type and again most publications have focussed on TLRs 2 and 4. However, in this work we have determined expression of TLRs 1–10. TLR7 was found to be the most abundantly expressed TLR in bovine skin. Similarly TLR7 has been found to be abundant in the skin of fish (Jault et al., 2004) but no expression was detected in human keratinocytes (Mempel et al., 2003) or chickens (Iqbal et al., 2005). Such variations in the relative abundance of TLRs may reflect the different pathogenic agents unrelated species are likely to encounter. TLR2 was also abundant in bovine skin which is inconsistent with TLR2 expression profiles in other epithelial tissues but is consistent with relative abundance seen in LPS treated mouse liver (Liu et al., 2000) and human peripheral monocytes (Sabroe et al., 2002; Hornung et al., 2002). Given TLR2 and TLR7 are abundant in bovine skin implies this region is armed with receptors capable of responding to Gram positive bacterial infection through TLR2 and viral infection through TLR7, although the association of TLR7 with viral infection is less well defined (Crozat and Beutler, 2004). TLR6 was the only TLR not detected in bovine skin by qPCR. Similarly no TLR6 expression was detected in human keratinocytes (Mempel et al., 2003) and very low levels, relative to the other TLRs were detected in chicken skin (Iqbal et al., 2005). As was seen in the ovine gut TLR4 was barely detectable in bovine skin and this pattern appears to be representative in a range of epithelial tissues from different animals.

In summary we have confirmed the presence of TLRs1–10 in both bovine and ovine tissues. Real time PCR assays were developed for each of the TLRs and these were used to quantify expression in a range of epithelial tissues relevant to challenge. Assays such as these will be vital to improving our understanding of the early events controlling immunological development in livestock.

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References

Abreu, M.T., 2003. Immunologic regulation of Toll-like receptors in gut epithelium. Curr. Opin. Gastroenterol. 19, 559–564.

Altschul, S.F., Gish, W., Miller, W., Meyers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Baker, B.S., Ovigne, J.M., Powles, A.V., Corcoran, S., Fry, L., 2003. Normal keratinocytes express Toll-like receptors (TLRs) 1 2 and 5: modulation of TLR expression in chronic plaque psoriasis. Br. J. Dermatol. 148, 670–679.

Beutler, B., 2004. Inferences, questions and possibilities in Toll-like receptor signalling. Nature 430, 257–263.

Cario, E., Gerken, G., Podolsky, D.K., 2002. For whom the bell tolls!"—innate defense mechanisms and survival strategies of the intestinal epithelium against lumenal pathogens Z. Gastroenterol. 40, 983–990.

Cario, E., Podolsky, D.K., 2000. Differential alteration in intestinal epithelial cell expression of Toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. Infect. Immun. 68, 7010–7017.

Crozat, K., Beutler, B., 2004. TLR7: a new sensor of viral infection. Proc. Natl. Acad. Sci. U.S.A. 101, 6835–6836.

Eicher, S.D., Mcmunn, K.A., Hammon, H.M., Donkin, S.S., 2004. Toll-Like receptors 2 and 4, and acute phase cytokine gene expression in dexamethasone and growth hormone treated dairy calves. Vet. Immunol. Immunopathol. 98, 115–125.

Gilliet, M., Conrad, C., Geiges, M., Cozzio, A., Thurlimann, W., Burg, G., Nestle, F.O., Dummer, R., 2004. Psoriasis triggered by toll-like receptor 7 agonist imiquimod in the presence of dermal plasmacytoid dendritic cell precursors. Arch. Dermatol. 140, 1490–1495.

Goldammer, T., Zerbe, H., Molenaar, A., Schuberth, H.-J., Brunner, R.M., Kata, S.R., Seyfert, H.-M., 2004. Mastitis increases mammary mRNA abundance of {beta}-defensin 5, Toll-like-receptor 2 (TLR2), and TLR4 but not TLR9 in cattle. Clin. Diagn. Lab. Immunol. 11, 174–185.

Helmby, H., Grencis, R.K., 2003. Essential role for TLR4 and MyD88 in the development of chronic intestinal nematode infection. Eur. J. Immunol. 33, 2974–2979.

Hornung, V., Rothenfusser, S., Britsch, S., Krug, A., Jahrsdorfer, B., Giese, T., Endres, S., Hartmann, G., 2002. Quantitative expression of Toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J. Immunol. 168, 4531–4537.

Iqbal, M., Philbin, V.J., Smith, A.L., 2005. Expression patterns of chicken Toll-like receptor mRNA in tissues, immune cell subsets and cell lines. Vet. Immunol. Immunopath. 104, 117–127.

Jault, C., Pichon, L., Chluba, J., 2004. Toll-like receptor gene family and tir-domain adapters in *Danio rerio*. Mol. Immunol. 40, 759–771.

Kawai, K., Shimura, H., Minagawa, M., Ito, A., Tomiyama, K., Ito, M., 2002. Expression of functional Toll-like receptor 2 on human epidermal keratinocytes. J. Dermatol. Sci. 30, 185–194.

Kelly, D., Conway, S., 2005. Bacterial modulation of mucosal innate immunity. Mol. Immunol. 42, 895–901.

Kokkinopoulos, I., Jordan, W.J., Ritter, M.A., 2005. Toll-like receptor mRNA expression patterns in human dendritic cells and monocytes. Mol. Immunol. 42, 957–968.

Kopp, E.B., Medzhitov, R., 1999. The Toll-receptor family and control of innate immunity. Curr. Opin. Immunol. 11, 13–18.

Kurt-Jones, E.A., Sandor, F., Ortiz, Y., Bowen, G.N., Counter, S.L., Wang, T.C., Finberg, R.W., 2004. Use of murine embryonic fibroblasts to define Toll-like receptor activation and specificity. J. Endotoxin Res. 10, 419–424.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., Hoffmann, J.A., 1996. The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 86, 973–983.

Liu, S., Salyapongse, A.N., Geller, D.A., Vodovotz, Y., Billiar, T.R., 2000. Hepatocyte toll-like receptor 2 expression in vivo and in vitro: role of cytokines in induction of rat TLR2 gene expression by lipopolysaccharide. Shock 14, 361–365.

Matsushima, H., Yamada, N., Matsue, H., Shimada, S., 2004. TLR3-, TLR7-, and TLR9-mediated production of proinflammatory cytokines and chemokines from murine connective tissue type skin-derived mast cells but not from bone marrow-derived mast cells. J. Immunol. 173, 531–541.

Medzhitov, R., Preston-Hurlburt, P., Janeway Jr., C.A., 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 388, 394–397.

Meijer, A.H., Gabby Krens, S.F., Medina Rodriguez, I.A., He, S., Bitter, W., Ewa Snaar-Jagalska, B., Spaink, H.P., 2004. Expression analysis of the Toll-like receptor and tir domain adaptor families of zebrafish. Mol. Immunol. 40, 773–783.

Mempel, M., Voelcker, V., Kollisch, G., Plank, C., Rad, R., Gerhard, M., Schnopp, C., Fraunberger, P., Walli, A.K., Ring, J., Abeck, D., Ollert, M., 2003. Toll-like receptor expression in human keratinocytes: nuclear factor kappaB controlled gene activation by *Staphylococcus aureus* is toll-like receptor 2 but not toll-like receptor 4 or platelet activating factor receptor dependent. J. Invest. Dermatol. 121, 1389–1396.

Oliveira, R.B., Ochoa, M.T., Sieling, P.A., Rea, T.H., Rambukkana, A., Sarno, E.N., Modlin, R.L., 2003. Expression of Toll-like receptor 2 on human Schwann cells: a mechanism of nerve damage in leprosy. Infect. Immun. 71, 1427–1433.

Otte, J.M., Rosenberg, I.M., Podolsky, D.K., 2003. Intestinal myofibroblasts in innate immune responses of the intestine. Gastroenterology 124, 1866–1878.

Pruett, S.B., Zheng, Q., Fan, R., Matthews, K., Schwab, C., 2004. Acute exposure to ethanol affects Toll-like receptor signaling and subsequent responses: an overview of recent studies. Alcohol 33, 235–239.

Rozen, S., Skaletsky, H., 2000. Primer3 on the WWW for general users and for biologists. Methods Mol. Biol. 132, 365–386.

Sabroe, I., Jones, E.C., Usher, L.R., Whyte, M.K., Dower, S.K., 2002. Toll-like receptor (TLR)2 and TLR4 in human peripheral blood granulocytes: a critical role for monocytes in leukocyte lipopolysaccharide responses. J. Immunol. 168, 4701–4710.

Simon, P., 2003. Q-Gene: processing quantitative real-time RT-PCR data. Bioinformatics 19, 1439–1440.

Takeuchi, O., Akira, S., 2002. Genetic approaches to the study of Toll-like receptor function. Microb. Infect. 4, 887–895.

Takeuchi, J., Watari, E., Shinya, E., Norose, Y., Matsumoto, M., Seya, T., Sugita, M., Kawana, S., Takahashi, H., 2003. Down-regulation of Toll-like receptor expression in monocyte-derived Langerhans cell-like cells: implications of low-responsiveness to bacterial components in the epidermal Langerhans cells. Biochem. Biophys. Res. Commun. 306, 674–679.

Werling, D., Jungi, T.W., 2003. TOLL-like receptors linking innate and adaptive immune response. Vet. Immunol. Immunopathol. 91, 1–12.

Werling, D., Hope, J.C., Howard, C.J., Jungi, T.W., 2004. Differential production of cytokines reactive oxygen and nitrogen by bovine macrophages and dendritic cells stimulated with Toll-like receptor agonists. Immunology 111, 41–52.

White, S.N., Kata, S.R., Womack, J.E., 2003a. Comparative fine maps of bovine Toll-like receptor 4 and Toll-like receptor 2 regions. Mamm. Genome 14, 149–155.

White, S.N., Taylor, K.H., Abbey, C.A., Gill, C.A., Womack, J.E., 2003b. Haplotype variation in bovine Toll-like receptor 4 and computational prediction of a positively selected ligand-binding domain. Proc. Natl. Acad. Sci. U.S.A. 100, 10364–10369.

Zarember, K.A., Godowski, P.J., 2002. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. J. Immunol. 168, 554–561.