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SHORT COMMUNICATIONS

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Initial Sequencing and Tissue Distribution of Toll-like Receptor 3 mRNA in White-tailed Deer (*Odocoileus virginianus*)

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ABSTRACT: Toll-like receptor (TLR) 3 recognizes double-stranded RNA (dsRNA) and activates a signal transduction pathway that results in the release of a variety of chemokines and cytokines and apoptotic activity. Variability in TLR3 expression may play an important role in disease susceptibility of white-tailed deer (WTD; *Odocoileus virginianus*) to bluetongue and epizootic hemorrhagic disease viruses, which are dsRNA viruses. Because little is known about TLR3 in WTD, our objective was to sequence WTD TLR3 mRNA and to determine baseline levels of tissue expression. A 209-base pair sequence of TLR3 mRNA was obtained from WTD peripheral blood mononuclear cells. Dot blots confirmed that the sequence obtained was part of total WTD mRNA. Variable expression or ligand binding of TLR3 may contribute to observed susceptibility differences between populations of WTD, so the level of TLR3 in small intestine, skin, spleen, heart, cecum, rumen, lymph node, lung, kidney, and liver from WTD fawns ($n=2$) was analyzed using real-time reverse transcriptase-polymerase chain reaction. Tissue expression of TLR3 mRNA relative to the housekeeping gene β-actin was highest in spleen, heart, skin, and lung.

Key words: Disease susceptibility, *Odocoileus virginianus*, real-time reverse transcriptase-polymerase chain reaction, Toll-like receptor 3, TLR3, white-tailed deer.

Toll-like receptors (TLRs) are a type 1 integral membrane protein family involved in innate and adaptive immunity. Presently, 12 TLRs in mice and 10 TLRs in humans have been identified (Tabeta et al., 2004). Unique pathogen-associated molecular patterns (PAMPs) of viruses, bacteria, protozoa, and other pathogens are recognized by different members of this receptor family and evoke antimicrobial responses (Ashkar et al., 2004; Bowie and Haga, 2005; Bell et al., 2006; O'Neill, 2006). When the PAMP is recognized by the surface portion of the TLR, the cytosomally localized Toll/interleukin (IL)-1 (TIR) domain of each receptor elicits specific cellular responses, for example, the activation of transcription factors such as nuclear factor-κB or mitogen-activated protein kinases and various chemokines and cytokines such as interferon-β and IL-8 (Takeda and Akira, 2005; O'Neill, 2006; Bode et al., 2007).

Unlike other TLRs, which are expressed on the cell membrane or intracellularly, TLR3 is expressed on both endosomes and on the cell membrane (Vercammen et al., 2008). Toll-like receptor 3 recognizes PAMPs such as viral double-stranded (ds)RNA, mRNA, single-stranded (ss)RNA viruses, and sequence-independent small interfering RNA (siRNA) (Choe et al., 2005; Marshall-Clarke et al., 2007). The TLR3 also uses a unique signaling adaptor molecule from other TLRs. Whereas myeloid differentiation primary response gene 88 (MyD88) signaling is common to most TLRs, TLR3 only uses TIR-domain-containing adapter-induced interferon-β for signaling. This receptor is involved in infection by a variety of animal viruses such as bovine rotavirus, viral hemorrhagic septicemia virus in fish, and rabies virus (Jackson et al., 2006; Novoa et al., 2006; Aich et al., 2007) and has the potential to be involved in infection by other dsRNA viruses or ssRNA viruses with dsRNA intermediates, such as rinderpest virus,

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canine distemper virus, influenza, and foot and mouth disease virus. The siRNA knockdown of TLR3 during infection with viruses such as porcine reproductive and respiratory syndrome virus (Sang et al., 2008) and Karposi's sarcoma-associated herpes virus (West and Daman, 2008) have illustrated the importance of TLR3 in mediating immune and inflammatory responses.

The most important viral disease of white-tailed deer (WTD) is hemorrhagic disease (HD), which is caused by two closely related dsRNA orbiviruses, bluetongue and epizootic hemorrhagic disease viruses (Thomas et al., 1974). Although these viruses can cause significant morbidity and mortality in WTD, it has been shown that different populations of WTD vary in susceptibility to epizootic hemorrhagic disease virus (EHDV), potentially due to variation in innate immunity (Gaydos et al., 2002). We hypothesize that differences in expression or allelic forms of TLR3 may lead to differences in viral recognition to the TLR3 in WTD and may help explain differences in disease susceptibility within the species. Currently, no information is available on TLR3 in WTD; therefore, the objective of this research was to amplify and sequence deer TLR3 mRNA and evaluate baseline expression of TLR3 in various tissues.

Initially, we attempted to amplify WTD TLR3 mRNA from peripheral blood mononuclear cells (PBMCs). Blood was collected from 5-mo-old fawns in sodium heparin tubes before euthanasia. Blood was spun at 200×G for 10 min at 4 C, and the supernatant containing the platelets was removed and replaced with Dulbecco's phosphate-buffered saline (DPBS) without $MgCl_2$ and $CaCl_2$. Cells were then spun at 400×G for 10 min at 4 C, and the buffy coat was removed and layered on Ficolite (density, 1.077; Atlanta Biological Supply Company, Atlanta, Georgia, USA). After centrifugation at 1300×G for 30 min at 4 C, the PBMC layer was removed and washed with DPBS, centrifuging at 400×G for 10 min at 4 C. Cells were resuspended in minimal essential medium (MEM) with nonessential amino acids supplemented with an antibiotic/antimycotic (Sigma-Aldrich, St. Louis, Missouri, USA), 2 mM L-glutamine (Sigma-Aldrich), and 10% fetal bovine serum incubated at 37 C in 5% CO_2, and then counted.

To evaluate baseline tissue expression of TLR3, tissue samples were collected postmortem from two 5-mo-old captive-raised fawns. Tissues included skin, heart, lung, cecum, small intestine, brain, spleen, spiral colon, rumen, liver, kidney, abomasum, and prescapular lymph node. Tissues were stored in 1 ml of RNA-Bee (Tel-Test, Friendswood, Texas, USA) or without RNA-Bee at −20 C before RNA extraction. The RNA was extracted from tissues using RNA-Bee (Tel-Test) following the manufacturer's instructions and quantified using an Ultraspec 3000 (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Hemi-nested reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to obtain a partial sequence of WTD TLR3 mRNA from PBMC RNA. The PBMC TLR3 mRNA was amplified with primers BOVTLR3R/F (1 μM; Table 1) in a primary RT-PCR reaction. One μl of this product was used in a subsequent PCR reaction, which used primers BOVTLR3R and TLR3F (1 μM; Table 1). The initial RT-PCR used the Titan One Tube RT-PCR System (Roche Applied Science, Penzberg, Germany) following the manufacturer's instructions and a PTC-100 Programmable Thermal Controller (MJ Research, Waltham, Massachusetts, USA). The housekeeping gene β-actin was used as a positive control for amplification reactions. β-Actin specific primers (Table 1) were developed from partially sequenced WTD β-actin mRNA (GenBank EU656109). β-Actin-specific primer final concentration was 0.4 μM. The creation of a cDNA copy was performed at 50 C for 30 min followed by denaturation at 94 C for 2 min fol-

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TABLE 1. Primer table. See text for additional details.

Primer	Forward	Reverse	Reference
BOVTLR3[a]	5′-GAGGCAGGTGTCCTTGAACT-3′	5′-GCTGAATTTCTGGACCCAAG-3′[c]	Menzies and Ingham, 2006
TLR3[a]	5′-AACAGCATCAGAAGGAGGCAG-3′[b,c]	5′-TGCTGAACTGCATGGTGAAC-3′[b]	McGuire et al., 2006
WTDTLR3	5′-GCATTTACCCCGTTCTTTCTG-3′[d]	5′-ACTATGCAAAAAGATTCAAGGTT-3′[d]	This study[e]
WTDβact	5′-CCGCACTACTGGTATTGT-3′[d]	5′-AGAGCTTCTCCTTGATGTC-3′[d]	This study[e]

[a] Primers used to obtain a 209-base pair sequence of white-tailed deer Toll-like receptor 3 through hemi-nested reverse transcriptase-polymerase chain reaction (RT-PCR).

[b] Primers used in the original RT-PCR reaction.

[c] Primers used in PCR reaction after RT-PCR reaction.

[d] Primers developed for real-time RT-PCR.

[e] Developed using LightCycler® (Roche) software.

lowed by 35 cycles of 94 C for 10 sec, 60 C for 30 sec, and 68 C for 45 sec. A final elongation step was performed at 68 C for 10 min. The PCR reaction was run using GeneAmp PCR kit (Applied Biosystems, Roche, Branchberg, New Jersey, USA) following the manufacturer's instructions. Initially, the DNA was denatured for 2 min at 94 C followed by 30 cycles of 94 C for 1 min, 50 C for 2 min, and 72 C for 3 min. Products were visualized and analyzed using 2% agarose 1× Tris-acetate EDTA (TAE) buffer, pH 8.4 (0.04 M Tris base, 0.002 M disodium EDTA, and glacial acetic acid) gels and ethidium bromide at a final concentration of 0.143 μg/μl. Amplicons were extracted from gels using the QIAquick gel extraction kit (QIAGEN, Valencia, California, USA) and submitted to The University of Georgia Sequencing and Synthesis Facility (Athens, Georgia, USA) for sequencing.

A dot-blot was used to confirm the obtained PCR product was part of WTD RNA. Nitrocellulose membranes previously dotted with total RNA from WTD heart tissue and UV cross-linked were prehybridized in Dig Easy Hyb (Roche Applied Systems, Indianapolis, Indiana, USA) at 37 C for 30 min. Primers TLR3DEER R/F (primers designed specifically for WTD TLR3 from known mRNA sequence; Table 1) were used in a RT-PCR reaction with 1 μl of digoxigenin deoxyuridine diphosphate (Dig-dUTP) and 1 μl of template RNA to create a WTD TLR3-specific probe. After prehybridization, membranes were hybridized overnight shaking at 37 C with 3–8 μl of digoxigenin-labeled probe to 1 ml Dig Easy Hyb. The Dig-dUDP probes were denatured at 95 C for 10 min and quenched on ice before incorporation in the hybridization solution. After hybridization, membranes were washed two times with 2× sodium chloride-sodium citrate (SSC) buffer with 0.1% sodium dodecyl sulfate for 5 min. Two 15-min stringency washes were performed at 68 C in 0.5% SSC. Digoxigenin was detected using colorimetric and chemiluminescent tech-

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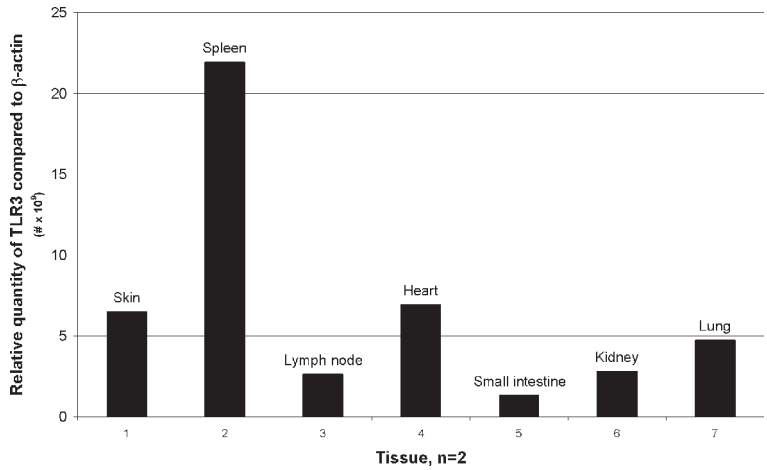


FIGURE 1. Relative expression of white-tailed deer toll-like receptor (TLR) 3 mRNA in various tissues from two white-tailed deer. Relative expression was determined by quantitative reverse transcriptase-polymerase chain reaction comparing TLR3 to β-actin. All samples were run in duplicate and compared with a standard curve.

niques. For the colorimetric technique, membranes exposed to nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (XPhos; Roche Diagnostics, Mannheim, Germany) were incubated in the dark at room temperature until color change was observed on membrane. For chemiluminescence, CDP-*Star* (Roche Diagnostics)-treated membranes were exposed to film for periods of 1, 5, and 30 min and 1, 2, and 24 hr.

Real-time RT-PCR was used to determine tissue levels of TLR3 using WTD TLR3-specific primers (WTDTLR3; Table 1) and housekeeping gene, β-actin, primers (WTDβact; Table 1). Real-time RT-PCR was performed using a Stratagene Mx3000P® QPCR System (Stratagene, La Jolla, California, USA). Brilliant® II SYBR® Green QRT-PCR Master Mix kit, 1-Step (Stratagene) was used following the manufacturer's instructions. Primers were used at a final concentration of 150 nM and total RNA at a final concentration of 0.1 nM. All samples were run in duplicate under the following cycling parameters: 50 C for 30 min and 10 min at 95 C followed by 40 cycles of 95 C for 30 sec, 46 C for 1 min, and 72 C for 30 sec. A dissociation cycle followed the RT-PCR reaction with 1 min at 95 C, 30 sec at 55 C, and 95 C for 30 sec. Quantities were determined by comparing relative quantities of TLR3 mRNA to β-actin mRNA to their respective calibrators. Calibrators were designed from WTD synthetic RT-PCR-amplified DNA fragments containing the sequence for TLR3 and β-actin, respectively. Standard curve analysis was conducted to verify that the calibrators were being amplified correctly. Data analysis was performed using Mx3000 software (Stratagene).

Hemi-nested RT-PCR amplified a 209-base pair piece of WTD TLR3 mRNA (EU656110). This fragment has 96% sequence homology to cow (AY957624), 95% sequence homology to sheep (AY957614) and water buffalo (EU005237), and 89% sequence homology to African bush elephant (*Loxodonta africana*, DQ360412). Dot blots confirmed that the amplified fragment was part of total WTD mRNA. Northern blotting was attempted to confirm molecular size but was unsuccessful, which could be due to the size of the sequenced portion or the secondary structure of the sequence and low expression of intracellularly localized TLR3.

By quantitative real-time RT-PCR, tis-

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sues with highest TLR3 mRNA expression relative to β-actin mRNA were skin, spleen, heart, and lung (Fig. 1). Interestingly, the tissues that seem to have the highest baseline TLR3 expression are typically involved in hemorrhagic disease in WTD. Skin is the site of primary virus entry via the bite of an infected *Culicoides* (Monath and Guirakhoo, 1996); spleen is the primary site of secondary viral replication (Pini, 1976; MacLachlan et al., 1990) in which viruses can reach high titers (Stallknecht, unpubl. data); and heart and lung are where virus secondarily replicates in endothelial cells, resulting in significant lesions in these tissues (Fletch and Karstad, 1971; Howerth and Tyler, 1988; Howerth et al., 1988; Brodie et al., 1998).

Toll-like receptor 3 is expressed relatively abundantly in sheep alveolar macrophages, keratinocytes of skin, and jejunum (Chang et al., 2009); cow skin and macrophages (Menzies and Ingham, 2006); and water buffalo lung, testis, and skin (Dhara et al., 2007). Although expression patterns for TLR3 in healthy tissues are similar in many ruminants, the response to bluetongue viruses (BTV) and epizootic hemorrhagic disease viruses is markedly different. For example, WTD, cow, and sheep are all infected by BTV, but only WTD and sheep typically show clinical signs of infection (McLaughlin et al., 2003). In response to BTV, the lung vasculature becomes highly permeabilized in both deer and sheep, whereas such a response is not observed in cattle (McLaughlin et al., 2003). It is proposed that mediators of inflammation such as IL-1, interferons, and various chemokines are responsible for the observed difference (DeMaula et al., 2001). Thus, differential expression or structure of TLR3 between ruminant species may contribute to variant activation of mediators, resulting in the disparate susceptibility patterns. This also might explain variation in susceptibility to EHDV and BTV between populations of WTD.

In conclusion, we were able to partially sequence WTD TLR3 mRNA using primers originally designed for cattle (Menzies and Ingham, 2006; McGuire et al., 2006). Complete sequencing of WTD TLR3 mRNA was unsuccessful using a variety of techniques, including restriction enzymes and cloning, inverse RT-PCR, and degenerate primers. The partial sequence obtained was used to develop a quantitative RT-PCR assay, which can be used in future studies designed to determine the role of TLR3 in the pathogenesis of bluetongue and epizootic hemorrhagic disease in WTD.

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