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Short communication

The role of toll-like receptor polymorphisms in susceptibility to canine distemper virus

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ABSTRACT

Canine distemper virus (CDV) is a global multi-host pathogen of wildlife. Toll-like receptors (TLR) are key recognition structures of the innate immune system. To investigate host susceptibility to CDV, the presence of non-synonymous single nucleotide polymorphisms (SNPs) in the coding regions of TLR 2, 3, 4, 7 and 8 genes were investigated in two recent CDV outbreaks in South Africa. The first case consisted of five lions (*Panthera leo*), diagnosed with CDV. Four of the lions died following exposure to the virus. The second case consisted of six African wild dogs (*Lycaon pictus*) with CDV and one surviving African wild dog. TLR diversity showed a higher rate of polymorphism in the African wild dogs within each of the TLR loci compared to lions. A single amino acid change (Met527Thr) within the leucine rich repeat of TLR2 was observed in the single surviving lioness. This alteration resulted in a non-polar to polar group change, potentially influencing the expression and function of TLR2. No specific amino acid alterations could be associated with CDV susceptibility in the African wild dogs. This study provides a critical starting point in elucidating the mechanism involved in host immunity and therefore susceptibility towards CDV infection.
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Infectious diseases are increasingly recognised as a potential threat to the conservation and biological diversity of wildlife (Daszak et al., 2000; Smith et al., 2009). To fully understand this threat and to implement successful management strategies for wildlife populations, knowledge of host ecology, pathogen characteristics, and host-pathogen interactions are required (Joseph et al., 2013). Information on the complex interactions between pathogen and host is however difficult to study and is often lacking for wildlife diseases. An example is the infectious viral disease canine distemper (CD) caused by the canine distemper virus (CDV; family *Paramyxoviridae*; genus *Morbillivirus*). First isolated from domestic dogs (*Canis familiaris*, family Canidae) in 1905 (Carré, 1905), CDV has subsequently been shown to infect a wide range of non-domestic carnivores, as well as some non-human primates (Beineke et al., 2015). Canine distemper virus infection ranges from subclinical to severe systemic disease, characteristically exhibiting lympho-, neuro- and epitheliotropism (Beineke et al., 2009; Iwatsuki et al., 1995; von Messling et al., 2004), resulting in the infection of the lymphatic, respiratory, endocrine, digestive, urinary, cutaneous, skeletal and central nervous systems (Lempp et al., 2014; von Messling et al., 2004). Survival of infection provides lifelong immunity in domestic dogs (Appel and Summers, 1995).

The ability of CDV to infect various animals from different species and its broad host range has been a significant area of research interest. The past two decades have accrued several publications on CDV's ability to infect a wide range of canine and non-canine carnivorous hosts with a focus on the mechanisms involved in viral entry of a host cell and how it relates to host range specificity (Cuthill and Charleston, 2013; Ludlow et al., 2014; Nikolin et al., 2012; Ohishi et al., 2014). Other factors influencing host range, such as the ability of a host to respond to viral infections have, however, not been explored in detail for CDV. Clinical and pathological characteristics of CDV infection in a variety of species largely resembles the disease in dogs, however, mortality and morbidity may vary greatly among different species infected (Beineke

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et al., 2015). Observed differences in the infection rate are especially evident in felids, with only 49% of reported records of infected with CDV presenting with clinical disease (Martinez-Gutierrez and Ruiz-Saenz, 2016).

Research on the immune responses in wildlife has thus far been generally conducted on the major histocompatibility complex (MHC), a multigene family crucial to the adaptive immune response of vertebrates (Acevedo-Whitehouse and Cunningham, 2006). However, immunity is a complex system and studies have revealed that approximately half of genetic variability for resistance to infection is reliant on non-MHC immune-relevant genes such as cytokines and toll-like receptors (TLRs) (Castro-Prieto et al., 2011; Jepson et al., 1997). Toll-like receptor molecules are a first line of defence against a variety of pathogens, including bacteria, protozoa, fungi and viruses (Melchjorsen et al., 2005; Uematsu and Akira, 2006). They can be expressed either on the cell surface or membrane compartments of immune (macrophages, dentritic cells, mast cells, eosinophils, neutrophils, B lymphocytes) and non-immune (epithelial, endothelium, cardio-myocytes and adipocytes) cells (Jin and Lee, 2008; Takeda et al., 2003). Toll-like receptors encode Type I transmembrane glycoproteins consisting of cytoplasmic, transmembrane, and extracellular regions. The cytoplasmic region of the TLR is related to the interleukin-1 receptor family, designated Toll-IL-IR. These sequences are highly conserved between species and are required for initiating intracellular signalling and inducing biological responses towards specific microorganisms (Akira et al., 2006; Kawai and Akira, 2005; Takeda et al., 2003; Xu et al., 2000). Leucine-rich repeats (LRRs) within the ectodomain of TLRs are responsible for directly interacting with microbes and microbial components (Uematsu and Akira, 2007).

Thirteen mammalian TLR members have been identified, each responsible for selectively recognising distinct invariant microbial structures (Hopkins and Sriskandan, 2005). Of these, only six TLRs have been implicated in viral recognition in mammals by means of distinctive pathogen-associated molecular patterns that include glycoproteins (TLR2, TLR4), double stranded RNA (TLR3), single stranded RNA (TLR7, TLR8) and unmethylated viral CpG DNA (TLR9) (Boehme and Compton, 2004; Mogensen and Paludan, 2005). Polymorphisms in TLR genes are associated with the variability of a hosts immune response against specific pathogens (Bharti et al., 2014; Bochud et al., 2007; Heng et al., 2011; Saçkesen et al., 2005; Xue et al., 2010). TLR2 is able to initiate an immune response by recognising glycoproteins from various viruses including measles virus (MeV), human cytomegalovirus and herpes simplex virus type 1 (Bieback et al., 2002; Compton et al., 2003; Kurt-Jones et al., 2004), whereas West Nile virus triggers an inflammatory response via TLR3 (Wang et al., 2004). TLR4 was shown to be involved in the innate immunity of mice to respiratory syncytial virus (Haynes et al., 2001; Kurt-Jones et al., 2000), while TLR7 and TLR8 are able to detect viral guanosine- and uridine-rich single stranded RNA of Sendai virus, human immunodeficiency virus and influenza virus (Beignon et al., 2005; Diebold et al., 2004; Heil et al., 2003; Melchjorsen et al., 2005). TLR9 has been demonstrated to induce antiviral responses via CpG DNA of viruses such as herpes simplex virus type 1 and type 2 and murine cytomegalovirus (Lund et al., 2003; Tabeta et al., 2004).

No published studies have investigated the involvement of TLRs in the immune response of animals susceptible to CDV. The aim of this study was to characterise viral-associated TLRs using samples obtained from two separate case and control groups from recent CDV outbreaks in lion and African wild dog populations in South Africa. We hypothesise that single nucleotide polymorphisms (SNPs), which potentially influence the expression and function of TLRs, contribute to differential infection outcomes.

All biological materials used in the present study were collected for diagnostic purposes and were stored at the Biobank of the National Zoological Gardens of South Africa (NZG). Samples from two case studies of recent CDV outbreaks from two privately owned reserves within South Africa were included. These consisted of a pride of lions from Welgevonden Nature reserve and a single pack of African wild dogs from Tswalu Kalahari Reserve that succumbed to CDV in December 2015 to May2016. Ethical approval was obtained from the Animal Ethics Committee (V072-14), University of Pretoria, South Africa and the NZG Research, Ethics and Scientific Committee (NZG/RES/P/001/F/07). Samples were obtained under a Section 20 permit (NZG/RES/P14/26) from the Department of Agriculture, Forestry and Fisheries, South Africa. Toll-like receptors were selected based on their known involvement in viral recognition in mammals and included TLR2, TLR3, TLR4, TLR7 and TLR8 (Haynes et al., 2001; Kurt-Jones et al., 2000; Wang et al., 2004). Three of these TLRs (TLR2, TLR7 and TLR8) have additionally been reported to be involved in human measles virus (MeV) infection (Bieback et al., 2002; Clifford et al., 2012). Bieback et al. (2002) further reported that the haemagglutinin (H) protein of wild-type strains of MeV activates cellular signalling via TLR2 and that a single amino acid alteration in the H protein was sufficient to eliminate agonistic activity. Canine distemper virus shares clinicopathological features with the paramyxovirus MeV (de Vries et al., 2014) and although TLRs have been studied for MeV, they have not yet been characterised for CDV. Thus, similar to Mev, a polymorphism that alters the structure of TLR2 affecting interactions with the H protein may with other factors determine the outcome of infection of individuals exposed to CDV. Primers previously developed for use in felids and hyenids (Flies et al., 2014; Ignacio et al., 2005) were used (Appendix A Table A1). Genomic DNA from blood and tissue samples was extracted using the MagMAX^{TM}-96 DNA Multi-Sample kit (Ambion), according to the manufacturer's protocol. Conventional polymerase chain reaction (PCR) was carried out at an annealing temperature of 55–58 °C using DreamTaq Green PCR Master Mix (Thermo Fisher Scientific Inc.) Successful PCR products were subsequently purified with Exonuclease I and FastAP (Thermo Fisher Scientific Inc.). Purified gene fragments were sequenced, in both the forward and reverse directions, using the BigDye Terminator v3.1 Cycle sequencing kit and visualised on a 3500 Genetic Analyser (Applied Biosystems). Sequence chromatograms were edited and assembled in BioEdit Sequence Alignment Editor v7.0.9.0 (Hall, 1999). .Synonymous and non-synonymous SNP variations were determined by translating the TLR gene nucleotide sequences to the longest open reading frames. The identity and integrity of the respective amino acid sequences were confirmed by standard protein BLAST (blastp as implemented on the National Centre for Biotechnology Information platform). Amino acid variations were visually inspected using BioEdit v.7.0.9.0 (Hall, 1999). The possible association between TLR non-synonymous SNPs and differential infection outcomes was assessed in lions and African wild dogs during a CDV outbreak. Diagnosis of CDV was made on the basis of typical clinical signs and histopathology, immunohistochemical staining of formalin fixed paraffin embedded samples and PCR amplification of the H-gene of the virus (data not shown). In December 2015, the carcasses of three lions were observed on the Welgevonden reserve. Upon post mortem examination no clear cause of death could be determined. However, in the weeks that followed other lions in the reserve were observed with severe seizures (a neurological symptom associated with CDV). Blood tests and post mortem analyses confirmed the presence of CDV. One lioness showed no clinical signs and has consistently tested negative for CDV, however, serological evidence suggested that she had been exposed to the virus. She was kept in isolation and monitored closely for two months before being released back into the reserve after all subsequent tests (including the screening of cerebrospinal fluid) were all negative for CDV. None of the lions had been vaccinated against CDV. In the second case, three African wild

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