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Genetic polymorphisms in toll-like receptors 1, 2, and 4 in feline upper respiratory tract aspergillosis

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ABSTRACT

Fungal species in the genus *Aspergillus* are environmental saprophytes that can act as opportunistic pathogens of the nasal cavity and paranasal sinuses in humans, cats and other species. Upper respiratory tract aspergillosis (URTA) presents as non-invasive and invasive forms with the latter occurring almost exclusively in immunocompromised hosts. However, in domestic cats, invasive URTA affects apparently immunocompetent patients. A defect in innate immunity has been proposed as a predisposing factor in invasive feline URTA. Single nucleotide polymorphisms (SNPs) in pattern recognition receptor genes have been implicated in the pathogenesis of aspergillosis in humans. The aims of this study were to identify non-synonymous SNPs in the coding regions of toll-like receptors involved in the immune response to *Aspergillus* spp. and to compare the frequency of these SNPs between affected and control cats. The coding and flanking regions of *TLR1*, *TLR2* and *TLR4* were sequenced in 14 cats with URTA and the sequences were compared with those in 20 control cats without aspergillosis. In total, 23 non-synonymous SNPs were identified in *TLR1* (n = 11), *TLR2* (n = 3) and *TLR4* (n = 10). Differences in allelic frequency of non-synonymous SNPs between affected and controls were not identified either within breeds or overall or between non-invasive and invasive disease phenotypes. Although allelic frequency differed between cat breeds that are overrepresented for URTA and underrepresented breeds there was no association differences identified between affected cats and underrepresented breeds. The difference in allelic frequency of an INDEL point mutation identified in intron 1 of *TLR4*, between cats with non-invasive versus invasive aspergillosis approached significance (p = 0.054). While results from this study do not support a role for non-synonymous SNPs in the pathogenesis of feline URTA they do provide evidence that investigation for polymorphisms in non-coding regions of these genes and in other pattern recognition receptors are warranted.

1. Introduction

Aspergillus species are ubiquitous saprophytic fungi with worldwide distribution that can cause non-invasive or invasive forms of aspergillosis in many mammalian and avian species. Non-invasive upper respiratory tract aspergillosis (URTA) in domestic cats, sinonasal aspergillosis (SNA) typically involves colonisation by *Aspergillus fumigatus* (Barrs, 2018), shares clinical features with SNA in dogs and chronic erosive non-invasive fungal sinusitis in humans and carries a favourable prognosis with treatment (Day, 2009; Barrs and Talbot, 2014). By contrast, feline sino-orbital aspergillosis (SOA) is a highly invasive osteodestructive mycosis involving cryptic species in the *A. viridinutans* complex section *Fumigati* that is refractory to current treatments. Clinically, SOA in cats resembles chronic granulomatous fungal rhinosinusitis (CGFRS) in humans (Barrs and Talbot, 2014).

The host immune response to *Aspergillus* has been most thoroughly investigated using murine models and *in vitro* studies of human invasive pulmonary aspergillosis. Innate immunity is crucial to an effective host response to *Aspergillus* spp. with neutrophil recruitment and activation, and a Th1/Th17 response essential for clearance of infection (Lass-Florl et al., 2013). Pattern recognition receptors (PRRs) on phagocytes and sentinel cells (dendritic, epithelial and endothelial cells) in the sinonasal mucosa recognise pathogen-associated molecular patterns (PAMPs) on fungal elements (conidia and hyphae), resulting in

Abbreviations: BSH, British shorthair; CGFRS, chronic granulomatous fungal rhinosinusitis; PRR, pattern recognition receptor; SNA, sinonasal aspergillosis; SNP, single nucleotide polymorphism; SOA, sino-orbital aspergillosis; SSH, Scottish shorthair; Th, T-helper; TIR, Toll/interleukin-1 receptor; TLR, Toll-like receptor; URTA, upper respiratory tract aspergillosis

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Table 1
Primers designed and used for amplification of cDNA of feline *TLR1, TLR2* and *TLR4* based on Feline Genome Assembly v.8.

	Forward (5'-3')	Reverse (5'-3')	Product length	Annealing temperature
TLR1				
a	TGTACCTGTATCAAGATGCTCT	TGTGCTGACTGATACATCCAA	710	58 °C
b	CAAAAACCTATCCCTGAAAACA	CCAGACACTGTGAAATTTTGG	884	58 °C
c	CAGCACAGCAGTAAGTCTGG	GCTGCTAAAAGCTCCACAGT	843	58 °C
d	TCAAAAACTGTGGAAACTTGG	CATGGAGACAAATCCTTAGGTC	914	58 °C
e	AGGGTTGGCCTGATTCTTAT	TCTGCATCACACATTCAAAA	846	58 °C
f	TGCTGCTTTTGGAAAAATGT	ATGGCCAAGAGAAAATCCTT	509	58 °C
TLR2				
a	CATTTGCCGCTCTGTTATTT	GTCCAAATGTTCGAGACTCC	477	58 °C
b	TGAATTACTGGACTGGACAATG	TCGCTAAAACTTCCATCAGTG	821	58 °C
c	CGAATTCTGAAAGTGGGAAA	TTCTGGCCACTGACAAGTTT	854	56.7 °C
d	TCGTTTCGTGATCTGAGTAGTG	GAGCAGGATCAACAGGAAAA	842	58 °C
e	CAGATGCCTCCTTCTTACCC	TTTAACCAAAAACCCTTCCTG	852	58 °C
f	TGGAGAACCTTATGGTCCAG	TGTTCCTTCGTGCTATTTCC	898	58 °C
TLR4				
Exon 1				
	CAGCGTTGCTTTGAATACAG	ATGAAATTCGGCAACTAGCA	362	54 °C
Exon 2				
	GGATGAAAGATGGTTGGATG	GGAGACTCCTGACATAAGAACG	397	58 °C
Exon 3				
a	TGTCACATCTGTGTGAAGAGC	AGATGCACCAGAGAAATGGT	793	54 °C
b	GACTTGTCCCTCAACCCTTT	TTTGGAAGGAAGTTGTCCTG	844	54 °C
c	GGTTAAGTTGGAAAGCCTTGA	ATACACCAGAACCACCACCA	852	60 °C
d	AACCTCTTCACTCCCTCCAG	CAGCAAGGCTTTTCTGAGTC	829	54 °C
e	TTTTCAGCTCTGCCTTCACT	GCATTCAATAGAAAAGGGAAAA	849	54 °C
f	CAAGGGCAGATGATTCAGTG	CATCAAGCGACAAAGTGATG	679	54 °C

phagocyte activation and induction of the adaptive immune response. The major PRRs of *Aspergillus* spp. are toll-like receptor (TLR)2, TLR4, dectin-1, mannose-binding lectin and DC-SIGN (Lass-Florl et al., 2013). However, ligand binding and pro-inflammatory signalling of TLR2 requires its heterodimeric association with TLR1 and/or TLR6 (Ozinsky et al., 2000).

In contrast to other forms of aspergillosis, particularly invasive aspergillosis, feline URTA, canine SNA, human chronic erosive non-invasive fungal sinusitis and CGFRS are reported in apparently immunocompetent hosts (Sharman and Mansfield, 2012; Denning and Chakrabarti, 2017; Barrs, 2018). An inherited defect in innate immunity has been proposed to contribute to these observed disease susceptibilities (Tomsa et al., 2003; Barrs and Talbot, 2014; Denning and Chakrabarti, 2017). In support of this contention, purebred cats of Persian-lineage are over-represented in cases of feline URTA (Barrs et al., 2012, 2015) and CGFRS is almost exclusively reported in people from North Africa, the Middle East and the Indian subcontinent (Hope et al., 2005).

Polymorphisms in several PRRs confer an increased risk of invasive pulmonary aspergillosis in human stem cell and organ transplant recipients. These include single nucleotide polymorphisms (SNPs) in *TLR1*, *TLR2, TLR3, TLR4*, *TLR5*, *TLR6*, *CLEC7A* (dectin-1), *pentraxin-3* and *CD209* (DC-SIGN) (reported odds ratios 1.25–5.6; reported hazard ratios 1.33–7.33) (Kesh et al., 2005; Bochud et al., 2008; Cunha et al., 2010; de Boer et al., 2011; Carvalho et al., 2012; Sainz et al., 2012; Grube et al., 2013; Cunha et al., 2014; Wojtowicz et al., 2015; Fisher et al., 2017; Skonieczna et al., 2017; White et al., 2018).

We hypothesized that polymorphisms in feline *TLR* genes may influence susceptibility to URTA in cats. The aim of this study was to assess whether genetic polymorphisms in toll-like receptor genes are associated with feline URTA. Three TLR genes, *TLR1*, *TLR2* and *TLR4* were selected for study based on their well characterised role in the host immune response to *Aspergillus* spp. and evidence that SNPs in these receptors are associated with an increased risk of invasive aspergillosis (Kesh et al., 2005; Bochud et al., 2008; de Boer et al., 2011; Lass-Florl et al., 2013; Skonieczna et al., 2017). Neither *TLR1* or *TLR6* has been characterised in cats and both are placed in the same region of the current feline reference genome. *TLR1* was chosen for further investigation as TLR2 detection of *A. fumigatus* involves both TLR1 and TLR6 in mice and TLR1 but not TLR6 in humans (Rubino et al., 2012).

2. Materials and methods

2.1. Samples

Data were collected from 34 cats comprising 14 cats with confirmed URTA and 20 controls. The case group included domestic crossbred cats (n = 9), British shorthair/Scottish shorthair (BSH/SSH; n = 3), a ragdoll and a Cornish rex. Diagnosis of URTA was made based on the presence of fungal hyphae on cytology or histology of tissue biopsies, positive fungal culture and molecular identification of the infecting isolate. Of the 14 affected cats 7 cats had SNA and 7 cats had SOA. The control group included breeds over-represented in cases of URTA: domestic crossbred cats (n = 8), Persian/Himalayan (n = 4) and BSH (n = 4). A group of Burmese cats (n = 4) was also included as URTA has not been reported in this breed. All control cats were examined by a veterinarian and had no signs of upper respiratory tract disease or systemic fungal infection. Ten of the cats in the control group were included in a previous study describing breed variations in *TLR4* (Whitney et al., 2019).

2.2. DNA extraction

Genomic DNA was extracted from EDTA-blood using the DNeasy® Blood & Tissue Kit (Qiagen, Hilden Germany) or saliva swabs using the manufacturer's protocol (DNA Genotek, Ottawa Canada).

2.3. PCR & sequence analysis

Primers were designed for the coding regions of feline *TLR1* and *TLR2* using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), the felCat8 reference genome (GCA_000181335.3) and known canine (NM_001146143.1; NM_001005264.3) and human (NM_003263.3; NM_001318790.1) mRNA sequences (Table 1). The coding regions were divided into overlapping segments < 850 base pairs. Previously reported primers

were used for sequencing *TLR4* (Table 1) (Whitney et al., 2019).

Polymerase chain reaction was performed using MyTaq™ DNA Polymerase (Bioline, UK), in a total volume of 25 µL according to the manufactures protocol. Each reaction comprised 1.25 units MyTaq™ DNA Polymerase, 5 µL MyTaq™ Reaction Buffer, 1 µL of 10µM forward and reverse primers, and 5–15 ng template DNA. Reactions were heated to 95 °C for 3 min, followed by 35 cycles of 95 °C for 15 s, annealing temperatures (Table 1) for 15 s and 72 °C for 1 min, then a further 72 °C for 7 min. PCR products were checked for yield and purity on a 1% w/v agarose gel and submitted to Macrogen Inc (Seoul, South Korea) for purification and Sanger sequencing using the PCR primers.

The sequence data was compared with the predicted feline *TLR1* (XM_003985440.3) and *TLR2* (XM_003984930.5) mRNA sequences and reported feline *TLR4* cDNA sequence (NM_001009223.1)(Asahina et al., 2003) using Sequencher® 5.4.6 (Gene Codes Corporation, Ann Arbor, USA). Protein sequences were predicted using ExPASy (https://web.expasy.org/translate/) and compared to the predicted feline *TLR1* (XP_003985489.3) and *TLR2* (XP_003984979.1) and reported feline *TLR4* (NP_001009223.1) protein sequences using Blast Local Alignment Search Tool (BLAST; blast.ncbi.nlm.nih.gov/Blast.cgi). The functional effects of the non-synonymous SNPs identified were assessed using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2).

2.4. Association analysis

Variant allele frequencies were calculated for each SNP using Microsoft® Excel 2010. Comparisons between cases and predisposed controls were analysed for all breeds together and for individual breeds; between predisposed controls and cats with each disease phenotype; between all cases, predisposed controls and Burmese cats; and between affected cats with SNA and SOA using Fisher's exact tests. Statistical significance was set at $p < 0.05$.

Six SNPs identified in *TLR4* for which all cats were homozygous for the alternative allele were not included in the association analysis.

2.5. Ethics

Ethics approval for this study was given by the University Animal Care and Ethics Committee; Approval no. N00/7-2013/3/6029 and 2015/902.

3. Results

3.1. Sequence analysis

Sequence analysis of the *TLR1* gene in all 34 cats revealed a total of 26 sequence variants (Table 2). Twenty-five variants were located in the coding region of *TLR1* and 11 changed the associated amino acid. One missense variant (c.1860 T > C) was predicted to be possibly damaging to the protein structure (p.S565R). One variant was located in the 3′ UTR of *TLR1* (Table 2).

Eighteen polymorphisms were identified in the coding region of the feline *TLR2* gene, including three non-synonymous SNPs (Table 3). One of these (c.3289A > G) was predicted to be probably damaging to the protein structure of the receptor. An additional polymorphism was identified within intron 1 (g.2554A > T). One SNP, (c.3363C > T) occurred in a single domestic crossbred cat from the control group that was heterozygous at this location.

Analysis of the feline *TLR4* gene revealed 22 SNPs (Table 4). Ten of the 15 SNPs in the coding region were non-synonymous. Seven SNPs were located in untranslated segments of the DNA including the 3′ UTR (n = 5) and intron 1 (n = 1). All of the cats were homozygous for the variant allele for 6 of the SNPs identified (c.564 T > C, c.1057C > T, c.1099 G > A, c.1140C > T, c.2261 G > A, c.2770A > G). One additional SNP (c.2969A > G) occurred as a heterozygous genotype in a single affected domestic crossbred cat.

3.2. Association studies

No significant difference in allelic frequency at any of the locations on any gene was found between the cats with URTA and the predisposed control group (domestic crossbred, Persian/Himalayan, BSH/SSH). There was also no significant difference in allelic frequencies between the predisposed control group and either of the feline URTA phenotypes group (SNA or SOA) (Table S1).

The same analysis was conducted between affected and unaffected cats within two groups (domestic crossbred, BSH/SSH). Although allelic frequencies differed between breed groups there was no significant difference in frequency between phenotypes within breed groups (Tablets S2 and S3).

There were no significant differences in the allelic frequencies in the non-synonymous SNPs identified in *TLR1, TLR2* and *TLR4* between affected cats and Burmese control cats. However, 5 non-synonymous SNPs in *TLR1* (c.1048 G > A, c.1222A > C, c.1802C > T, c.1860 T > G, c.2471A > G), occurred with significantly different allelic frequencies between Burmese cats and all other breeds combined (Table S4). For one SNP in *TLR2* (c.2752A > G) and another in the UTR 3′ region of *TLR4* (c.2546C > A), the variant allele occurred with higher frequency in Burmese cats compared to all other control cats combined (Table S4).

The allelic frequencies of non-synonymous SNPs in *TLR1, TLR2* and *TLR4* in cats with SNA compared to cats with SOA were not significantly different (Table S5). However, the difference in allelic frequency approached significance ($p = 0.0542$) for the nucleotide deletion in intron 1 of *TLR4*, with the alternative allele occurring with higher frequency in cats with the non-invasive form of disease.

4. Discussion

This study identified 24 non-synonymous SNPs in the coding regions of feline *TLR1* (n = 11), *TLR2* (n = 3) and *TLR4* (n = 10). Breed differences in allelic frequency were detected for 7 SNPs, but no associations with disease phenotype, either within or between breeds, were identified. These findings are similar to those in dogs with SNA in which no differences in alternative allele frequency were identified for non-synonymous SNPs in *TLR2*, *TLR4* or *TLR9* in affected and unaffected dogs within breed groups (Mercier et al., 2014).

Polymorphisms in *TLRs* are most frequently observed in the extracellular domain and with variation mainly occurring between race or breed in humans and animal species. It has been proposed that this variation is associated with evolutionary pressure of different geographic environments and subsequent variation in microbial environments (Smirnova et al., 2001; Werling et al., 2009; Ioana et al., 2012). The results of human studies investigating the role of *TLR* polymorphisms in the pathogenesis of aspergillosis have been variable. This may, in part, be associated with differences in patient groups, disease phenotype or the TLR gene investigated.

The possibly damaging SNP identified in *TLR1* occurred in all breeds tested and all Burmese cats were homozygous for the alternate allele. The resultant amino acid substitution lies within the leucine rich repeat C-terminal domain of the receptor. This region of the TLR1 structure is believed to play a crucial role in Toll signalling (Bell et al., 2003). Thus, cats with this this allele may have alterations in the function of TLR1, however, additional investigations are required to investigate this further.

The amino acid encoded by the probably damaging SNP in *TLR2* lies within the intracellular Toll/interleukin-1 receptor (TIR) domain. The TIR domain of TLRs is involved in intracellular signalling and gene expression. Recruitment of TIR-containing adaptors regulates specificity of the TLR response (Vogel et al., 2003). Abnormalities in this pathway may result in a dysregulated or inappropriate immune response. All Burmese cats tested were homozygous for the reference allele at this position.

Table 2
Sequence variants, their location and effect detected in coding regions feline *TLR1*.

Position (gDNA)[¥] Chrom B1	Sequence change (cDNA) .[$]	Location	Protein [†]	Predicted effect (estimated probability)	Domestic crossbred genotypes	Persian genotypes	BSH genotypes	Burmese genotypes	Affected genotypes
g.175521749 T > C	c.291 T > C	Exon 2	Silent	–	T/T, T/C, C/C	T/C/, C/C	T/T, T/C	T/T	T/T, T/C, C/C
g.175521780A > G	c.322A > G	Exon 2	I53V	Benign (0.001)	A/A, A/G	A/A	A/A	A/A	A/A, A/G, G/G
g.175521831A > G	c.373A > G[*]	Exon 2	I70V	Benign (0.004)	G/A, G/G	G/G	G/G	G/G	G/G
g.175521833A > C	c.375A > C	Exon 2	Silent	–	A/A, A/C	A/A	A/A	A/A	A/A, A/C, C/C
g.175522155 G > A	c.697 G > A[*]	Exon 2	A178T	Benign (0.002)	G/A, A/A	A/A	A/A	A/A	A/A
g.175522280 T > C	c.822 T > C[*]	Exon 2	Silent	–	T/C,C/C	C/C	C/C	C/C	C/C
g.175522506 G > A	c.1048 G > A	Exon 2	D295N	Benign (0.008)	G/G, G/A, A/A	G/A, A/A	G/G, G/A	G/G	G/G, G/A, A/A
g.1755522587C > T	c.1129C > T[*]	Exon 2	H322Y	Benign (0.000)	C/T, T/T	T/T	T/T	T/T	T/T
g.175522596 G > A	c.1138 G > A[*]	Exon 2	E325K	Benign (0.000)	G/A, A/A	A/A	A/A	A/A	A/A
g.175522680A > C	c.1222A > C	Exon 2	T353P	Benign (0.001)	A/A, A/C, C/C	A/C, C/C	A/A, A/C	A/A	A/A, A/C, C/C
g.175522772A > G	c.1314A > G	Exon 2	Silent	–	A/A, A/G	A/A	A/A	A/A, G/G	A/A, A/G
g.175522943 G > T	c.1485 G > T[#]	Exon 2	Silent	–	G/G, G/T	G/G	G/G	G/G	G/G
g.175523057 T > C	c.1599 T > C	Exon 2	Silent	–	T/T, T/C, C/C	T/C, C/C	T/T, T/C	T/T	T/T, T/C, C/C
g.175523066C > T	c.1608C > T	Exon 2	Silent	–	C/C, C/T, T/T	C/T, T/T	C/C, C/T	C/C, T/T	C/C, C/T, T/T
g.175523228 G > A	c.1770 G > A	Exon 2	Silent	–	G/G, G/A	G/G	G/G	G/G	G/G, G/A, A/A
g. 175523260C > T	c.1802C > T	Exon 2	A546V	Benign (0.008)	C/T, T/T	C/T, T/T	C/C, C/T	T/T	C/C, C/T, T/T
g.175523318 T > G	c.1860 T > G	Exon 2	S565R	Poss damaging (0.812)	T/G, G/G	T/G, G/G	T/T, T/G	G/G	T/T, T/G, G/G
g.175523324 G > A	c.1866 G > A	Exon 2	Silent	–	G/A, A/A	G/A, A/A	G/G, G/A	A/A	G/G, G/A, A/A
g.1755234435C > A	c.1977C > A[#]	Exon 2	Silent	–	C/C, C/A	C/C	C/C	C/C	C/C
g.175523510A > C	c.2052A > C[#]	Exon 2	K629N	Benign (0.252)	A/A	A/A	A/A	A/A	A/A, A/C
g.175523717C > T	c.2259C > T	Exon 2	Silent	–	C/C, C/T	C/C, C/T	C/T, T/T	C/C	C/C, C/T, T/T
g.175523819A > G	c.2361A > G	Exon 2	Silent	–	A/G, G/G	A/G, G/G	A/A, A/G	G/G	A/A, A/G, G/G
g.175523929A > G	c.2471A > G	Exon 2	H769R	Benign (0.012)	A/A	A/A	A/A	A/A, G/G	A/A, A/G
g.1755234855T > C	c.2397T > C	Exon 2	Silent	–	T/T	T/T	T/T	T/T, C/C	T/T, T/C
g.175523957A > G	c.2499A > G[#]	Exon 2	Silent	–	A/A	A/A	A/A	A/A	A/A, A/G
g.175524133InsT	c.2618InsT[#]	3'UTR	–	–	:/:	:/:	:/:	:/:, T/T	:/:

[¥] Numbering refers to accession number: NC_018726.3.
[$] Numbering refers to accession number: XM_003985440.3.
[†] Numbering refers to accession number: XP_003985489.3.
[#] SNP only identified in a single, different, cat at each position. The affected cats were heterozygous for the polymorphism at 4/5 positions.
[*] 33/34 cats were homozygous for the variant allele at these positions. A single domestic crossbred control cat was heterozygous at all of these positions.

A number of SNPs were identified in the 5′UTR, 3′UTR and intronic regions of the receptor genes. Polymorphisms in these regions may influence initiation of translation or post-transcriptional gene expression (Hube and Francastel, 2015). The difference in allelic frequency in the intronic base pair deletion in *TLR4* between cats with SNA and cats with SOA approached significance. However, the differences in phenotype may also be associated with an alternate immune defect, difference in virulence between the primary causative *Aspergillus* spp. or level of pathogen exposure (Barrs, 2018).

A recent study of human paediatric patients with haematological malignancies demonstrated an association between intronic SNPs in *TLR2* and *CLEC6A* (dectin-2) and development of aspergillosis (Skonieczna et al., 2017). Furthermore, intronic polymorphisms in *TLR4* have been identified that affect disease phenotype and outcome in bacterial sepsis (Chantratita et al., 2017).

While a defect in innate immunity was not identified in the cats investigated, PRRs are involved in the immune response to *Aspergillus* spp. infection, and genetic variation in some of these increases the risk of infection in humans. Unlike in the canine study *TLR9* was not selected for investigation as SNPs in this gene have been predominantly associated with allergic and colonising forms of aspergillosis in humans (Carvalho et al., 2008).

The felCat9 reference genome (GCF_000181335.3) places *TLR6* in the same region as *TLR1*. Similarly, in the felCat8 reference genome (GCA_000181335.3), although *TLR*6 is located approximately 30kbp upstream from *TLR1*, this sequence of 1444bp is 87.9% homologous to *TLR1*. The *TLR6-TLR1-TLR10* gene cluster has been described in a number of mammalian species (Beutler and Rehli, 2002; Opsal et al., 2006), with all three genes located within close proximity on the same chromosome. Although *TLR6-TLR1-TLR10* are not yet fully characterised in the cat, it is assumed that similar homology exists. Thus it is possible that some DNA sequences in this study may be derived from *TLR6* or *TLR10*.

During sequence analysis and comparison between the sequenced cDNA and reference gDNA an approximately 700bp section DNA with 99% homology to a section of *TLR2* was identified approximately 11 kbp upstream from the *TLR2* gene. This appears to be a duplication of a segment of exon 2 similar to the *TLR2* pseudogene that has been reported in a number of other species including dogs and humans (Roach et al., 2005; Huang et al., 2011). Six point mutations, including one non-synonymous SNP were identified in this section of sequence in the current study, but as some of the primers occurred within this region it is unknown as to whether these mutations occurred within the gene or the pseudogene.

Some anomalies in the exonic flanking regions of the genes investigated resulted in difficulties in sequencing the sections of DNA in which they were included. In *TLR1* a 21bp microsatellite located at g.175521533 interfered with the forward sequencing of section a, while an INDEL at g.175524232 prevented reverse sequencing of section f. However, as neither of the affected regions fall within the coding sequence of the gene, their occurrence did not interfere with acquisition and interpretation of results. Their proximity to the coding region precluded the possibility of designing alternate primer pairs to exclude them from the amplified sequences. Similarly, as has been previously

Table 3
Sequence variants, their location and effect detected in coding regions feline *TLR2*.

Position (gDNA)[¥] Chrom B1	Sequence change (cDNA)[$]	Location	Protein [†]	Predicted effect (estimated probability)	Domestic crossbred genotypes	Persian genotypes	BSH genotypes	Burmese genotypes	Affected genotypes
g.76025800 A > T	–	*Intron 1*	–	–	*A/A, A/T*	*A/A*	*A/A*	*A/T, T/T*	*A/A, A/T*
g.76025752 G > A	c.1099C > T	Exon 2	R3C	Benign (0.026)	C/C	C/C	C/C, C/T	C/C	C/C, C/T
g.76025455 G > A	c.1398C > T	Exon 2	Silent	–	C/C, C/T	C/C	C/C	C/T, T/T	C/C, C/T
g.76025213A > G	c.1638 T > C	Exon 2	Silent	–	T/T	T/T	T/T, T/C	T/T	T/T, T/C
g.76025198C > T	c.1653 G > A	Exon 2	Silent	–	G/G	G/G	G/G, G/A	G/G	G/G, G/A
g.76024913 T > C	c.1938A > G[#]	Exon 2	Silent	–	A/A, A/G	A/A, A/G	A/A	A/A	A/A, A/G, G/G
g.76024880 G > A	c.1971C > T[#]	Exon 2	Silent	–	C/C, C/T	C/C, C/T	C/C	C/C	C/C, C/T
g.76024619 G > T	c.2232C > A	Exon 2	Silent	–	C/C	C/C	C/C	C/C	C/C, C/A
g.76024259C > T	c.2592 G > A	Exon 2	Silent	–	G/G, G/A	G/G, G/A	G/G, G/A	G/G	G/G, G/A, A/A
g.76024214A > C	c.2637 T > G	Exon 2	Silent	–	T/T, T/G, G/G	T/T, T/G	T/G, G/G	T/G, G/G	T/G, G/G
g.76024166 G > A	c.2685C > T	Exon 2	Silent	–	C/C, C/T	C/C, C/T	C/C	C/C, C/T, T/T	C/C, C/T
g.76024099 T > C	c.2752A > G	Exon 2	I554V	Benign (0.012)	A/A, A/G, G/G	A/A, A/G, G/G	A/A, A/G	A/G, G/G	A/A, A/G, G/G
g.76024052 G > A	c.2799C > T	Exon 2	Silent	–	C/C, C/T, T/T	C/C, C/T, T/T	C/C	T/T	C/C, C/T, T/T
g.76023830 G > A	c.3021C > T	Exon 2	Silent	–	C/C, C/T	C/C	C/C	C/T, T/T	C/C, C/T
g.76023710 T > C	c.3141A > G	Exon 2	Silent	–	A/A, A/G, G/G	A/A, A/G	A/A, A/G	A/G, G/G	A/A, A/G, G/G
g.76023572 G > A	c.3279C > T	Exon 2	Silent	–	C/C, C/T	C/C, C/T	C/C	C/C	C/C, C/T, T/T
g.76023562 T > C	c.3289A > G	Exon 2	I733V	Prob. damaging (0.992)	A/A, A/G	A/A, A/G	A/A, A/G	A/A	A/A, A/G, G/G
g.76023488 G > A	c.3363C > T	Exon 2	Silent	–	C/C, C/T	C/C	C/C	C/C	C/C
g.76023385C > T	c.3466 G > A	Exon 2	Silent	–	G/G, G/A, A/A	G/G, G/A	G/G	A/A	G/G, G/A, A/A

[#] One segment of DNA (section c) containing two of the reported polymorphisms was unable to be amplified and sequenced from a single control BSH cat. Genotypes for intronic sequence reported as variants in gDNA and presented in *italics*.
[¥] Numbering refers to accession number: NC_018726.3.
[$] Numbering refers to accession number: XM_003984930.5.
[†] Numbering refers to accession number: XP_003984979.1.

Table 4
Sequence variants, their location and effect detected in coding regions feline *TLR4*.

Position (gDNA)[¥] Chrom D4	Sequence change (cDNA) [$]	Location	Protein [†]	Predicted effect (estimated probability)	Domestic crossbred genotypes	Persian genotypes	BSH genotypes	Burmese genotypes	Affected genotypes
g.79270717 C > A	–	–	–	–	*C/C, C/A*	*C/C, C/A*	*C/C*	*C/C*	*C/C, C/A*
g.79270898 DelC	–	*Intron1*	–	–	*C/C, C/T, T/T*	*C/T, T/T*	*C/C, C/T*	*C/C*	*C/C, C/T, T/T*
g.79275280A > C	c.172A > C	Exon2	K57Q	Benign (0.010)	A/A, A/C, C/C	A/A	A/C, C/C	A/A	A/A, A/C, C/C
g.79278952	c.564 T > C[#]	Exon 3	Silent	–	C/C	C/C	C/C	C/C	C/C
g.79279200 G > A	c.812 G > A	Exon 3	G270E	Benign (0.001)	G/G	G/G, G/G	G/G	G/G, G/A	G/G, G/A
g.79279213A > C	c.825A > C	Exon 3	K274N	Benign (0.001)	A/A, A/C	A/A	A/C, C/C	A/A	A/A, A/C, C/C
g.79279445	c.1057C > T[#]	Exon 3	P352S	Benign (0.001)	T/T	T/T	T/T	T/T	T/T
g.79279468C > T	c.1080C > T	Exon 3	Silent	–	C/C, C/T	C/C, C/T	C/T, T/T	C/C, C/T, T/T	C/C, C/T, T/T
g.79279487	c.1099 G > A[#]	Exon 3	A366T	Benign (0.010)	A/A	A/A	A/A	A/A	A/A
g.79279528	c.1140C > T[#]	Exon 3	Silent	–	T/T	T/T	T/T	T/T	T/T
g.79279552 G > C	c.1164 G > C	Exon 3	L387F	Benign (0.002)	G/G	G/G, G/C	G/G	G/G, G/C	G/G, G/C
g.79279770A > G	c.1382A > G	Exon 3	Q460R	Benign (0.005)	A/A, A/G	A/A	A/G, G/G	A/A	A/A, G/G
g.79280301 G > T	c.1913 G > T	Exon 3	G637V	Benign (0.004)	G/G	G/G, G/T	G/G	G/G, G/T	G/G, G/T
g.79280328 T > C	c.1940 T > C	Exon 3	F646S	Benign (0.119)	T/T	T/T, T/C	T/T	T/T, T/C	T/T, T/C
g.79280368 G > A	c.1980 G > A	Exon 3	Silent	–	G/G	G/G, G/A	G/G	G/G	G/G, G/A
g.79280599C > T	c.2211C > T	Exon 3	Silent	–	C/C	C/C, C/T	C/C	C/C	C/C, C/T
g.79280649	c.2261 G > A[#]	Exon 3	G753E	Benign (0.002)	A/A	A/A	A/A	A/A	A/A
g.79280934C > A	c.2546C > A	UTR 3'	–	–	C/C, C/A, A/A	C/C, C/A	C/A, A/A	C/C	C/C, C/A, A/A
g.79281158	c.2770A > G#	UTR 3'	–	–	G/G	G/G	G/G	G/G	G/G
g.79281219InA	c.2831InA	UTR 3'	–	–	:/:, :/A, A/A	:/:, :/A	:/A, A/A	:/:	:/:, :/A, A/A
g.79281357A > G	c.2969A > G	UTR 3'	–	–	A/A	A/A	A/A	A/A	A/A, A/G
g.79281452C > T	c.3064C > T	UTR 3'	–	–	C/C	C/C, C/T	C/C	C/C, C/T	C/C, C/T

Genotypes for non-exonic sequence reported as variants in gDNA and presented in italics.
[¥] Numbering refers to accession number: NC_018726.3.
[$] Numbering refers to accession number: NM_001009223.1.
[†] Numbering refers to accession number: NP_001009223.1.
[#] 100% cats tested were homozygous for variant allele.

reported (Whitney et al., 2019), the base pair deletion identified in *TLR4* intron 1 (g.154) affected the reverse sequencing of the DNA fragment including exon 1. However, due to the high quality of forward sequences, the lack of variations identified within the exon and the non-coding nature of this region, additional primers were not designed to overcome this.

The inflammatory cell population in the nasal mucosa cats with URTA has in increased concentration of plasma cells which is more reflective of a T helper (Th)2 response than the cell-mediated Th1 and Th17 response that is required to confer protective immunity against invasive aspergillosis (Whitney et al., 2016). Furthermore, an abnormality in T-cell subsets has been identified in a non-immunocompromised human with CGFR and attributed to a possible dysregulation in cytokine production (Lujber et al., 2003). Further investigation into the role of cytokine production and expression in the pathogenesis of invasive fungal rhinosinusitis in humans and cats is warranted.

The small sample size is a limitation of this study and may have resulted in associations between SNPs in *TLR1*, *TLR2* and/or *TLR4* and URTA being identified. As this was the first report of SNPs and associated allelic frequencies in feline *TLR1* and *TLR2*, insufficient data was available to determine samples sizes prior to the study. Based on the allele frequencies determined in this study, post-hoc power studies using UCSF Clinical & Translational Science Institute sample size calculators (www.sample-size.net/sample-size-proportions) indicated that at least 250 affected cats and controls would be required to identify a statistical difference in allele frequencies for at least 50% of the SNPs identified. Similarly, 210 affected cats (105 from each disease phenotype) would be required to identify differences in allelic frequencies between the two groups.

Novel point mutations in the feline *TLR1* and *TLR2* genes are reported here. One mutation in each gene was predicted to have a deleterious effect on protein structure and may therefore influence its function. However, despite variations in allelic frequency between breeds no associations between polymorphisms in the coding sequences of *TL1, TLR2* or *TLR4* and the development of feline URTA aspergillosis were identified. A number of other PRRs, as well as cytokines, are involved in the host immune response to *Aspergillus*. Abnormalities in expression of any of these may result in immune dysfunction and increased susceptibility to URTA. Genome wide association studies are indicated to identify candidate genes in cats with URTA.

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Declaration of Competing Interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2019.109921.

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