Structural characterisation of Toll-like receptor 1 (TLR1) and Toll-like receptor 6 (TLR6) in elephant and harbor seals

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Abstract

Pinnipeds are a diverse clade of semi-aquatic mammals, which act as key indicators of ecosystem health. Their transition from land to marine environments provides a complex microbial milieu, making them vulnerable to both aquatic and terrestrial pathogens, thereby contributing to pinniped population decline. Indeed, viral pathogens such as influenza A virus and phocine distemper virus (PDV) have been identified as the cause of several of these mass mortality events. Furthermore, bacterial infection with mammalian Brucella sp. and methicillin-resistant Staphylococcus aureus strains have also been observed in marine mammals, posing further risk to both co-habiting endangered species and public health. During these disease outbreaks, mortality rates have varied amongst different pinniped species. Analyses of innate immune receptors at the host–pathogen interface have previously identified variants which may drive these species-specific responses. Through a combination of both sequence- and structure-based methods, this study characterises members of the Toll-like receptor (TLR) 1 superfamily from both harbour and elephant seals, identifying variations which will help us to understand these species-specific innate immune responses, potentially aiding the development of specific vaccine-adjuvants for these species.

Keywords: Toll-like receptor; Pinniped; Evolution; Zoonosis; Conservation;

1. Introduction

Marine mammals represent a diverse group of aquatic and semi-aquatic species understood to have evolved from land-based mammals (Arnason et al., 2006; Berta and Sumich, 1999; Boisserie et al., 2011; Harington, 2008). Modern pinnipeds are semi-aquatic carnivores most closely related to ursids and mustelids. Recent phylogenetic studies using morphological and molecular evidence support a pinniped monophyly which consists of three further monophyletic superfamilies: Phocidae ('true' or earless seals), Otariidae (seal lions and fur seals) and Odobenidae (walruses) (Arnason et al., 1995; Arnason et al., 2006; Higdon et al., 2007). The extant genus *Enaliarctos* has been hypothesised as the common ancestor of these three subfamilies, retaining many primitive features (Berta et al., 1989; Berta and Sumich, 1999). Unlike modern pinnipeds, skeletal modification in *Enaliarctos* suggests that it used both its hindlimbs and forelimbs in swimming, the former lost in Otarridae and the latter in Phocidae. Moreover, the skull of this animal also shows the presence of slicing carnassials, suggesting that it may have returned to land with its prey to ingest it (Higdon et al., 2007; Jones et al., 2015). Thus, this common ancestry may explain why these aquatic carnivores share a similar susceptibility to pathogens as land carnivores. Indeed, modern pinnipeds have a rich and interesting evolutionary history with terrestrial ancestors from multiple lineages thought to have returned separately to the aquatic environment. However, despite significant adaptive radiation, marine mammals are often subject to disease-related mass mortalities which are linked to terrestrial origins (Gulland and Hall, 2007; Kennedy et al., 2000; van Elk et al., 2012; Ward and Lafferty, 2004). However, while the communal nature of their habitat and a significant increase in ocean pollution are important factors in their disease susceptibility, transmission of pathogens from land-based mammals has more recently become a topic of considerable interest (Nymo et al., 2011). In particular, zoonotic pathogens such as *Staphylococcus spp*., *Leptospira spp*. and *Brucella spp*. have been identified in several aquatic species and while their recognition by innate immune receptors has been well studied in terrestrial mammals, little is known about their recognition in pinnipeds and cetaceans (Nymo et al., 2011; Pei et al., 2012; van Elk et al., 2012). Crucially, at this host-pathogen interface are pathogen recognition receptors, such as Toll-like receptors (TLRs), vital components of the immune system which shape the innate immune response (Cameron et al., 2008). Until now, understanding the selective pressures acting on innate immune proteins within this clade has been hindered by sequence availability.

In the present study, we use interspecifically conserved regions within the carnivore lineage to amplify and characterize members of the TLR1 superfamily from both harbor seals (*Phoca vitulina*) and elephant seals (*Mirounga angustirostris*). Furthermore, using the recently published genomes of the Weddell seal (*Leptonychotes weddellii*) and Pacific walrus (*Odobenus rosmarus*), we have identified both species- and taxon-specific variants which provide a unique insight into the driving forces behind susceptibility during recent mass mortality events.

2. Materials and methods

2.1. Sample preparation

Whole blood samples were collected from three harbour seals and three elephant seals (Permit No. 932-1905-00/MA-009526) at The Marine Mammal Center (Sausalito, California). Genomic DNA (gDNA) was isolated from the samples using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. Concentration and purity of extracted DNA were determined using the NanoDrop ND-1000 (Thermo Fisher Scientific).

2.2. Amplification, cloning and sequencing of harbor and elephant seal TLR1 and TLR6

At the time of this study, pinniped genome sequences were not available. Therefore, primers were designed to amplify interspecifically conserved regions encoding TLRs 1, 2, 6 and 10 in closely related mammals utilising wobble bases at sites of ambiguity (Table S1). The target sequences were amplified using Easy-A enzyme mastermix (Agilent Technologies) in 50 µl reactions and carried out using thermocycler (Eppendorf; Mastercycler Pro) conditions outlined in Table S2. PCR products were then purified using the MinElute PCR purification kit (Qiagen) according to the manufacturer's protocol. Sequencing of the purified PCR products was performed by DNA Sequencing & Services (University of Dundee) using the corresponding primers.

2.3. Sequence analysis

Contigs were assembled in CLC Main Workbench v6.8.3 (CLC bio, Denmark) and validated using BLAST (Altschul et al., 1990) whilst LRRfinder2.0 (Offord and Werling, 2013) was used to identify leucine-rich repeats (LRRs) and predict domain structure from the translated sequences. Multiple sequence alignment of the seal TLR1 and TLR6 amino acid sequences to 47 mammalian orthologs (Table S3) was performed using ClustalW2 (Thompson et al., 2002) followed by phylogenetic reconstruction of the TLR1 and TLR6 evolutionary histories. Evolutionary analyses were performed in MEGA6 (Tamura et al., 2013) using the Maximum Likelihood method based on the JTT matrix-based model using a discrete Gamma distribution way to model evolutionary rate differences among sites. For TLR1 only, the rate variation model allowed for some sites to be evolutionarily invariable. In both analyses, positions with less than 95% site coverage were eliminated, yielding a total of 586 and 533 positions in the TLR1 and TLR6 datasets respectively. Bootstrap consensus trees for both TLR1 and TLR6 were inferred from 1000 replicates, collapsing those branches corresponding to partitions reproduced in less than 50% of the bootstrap replicates.

2.4. Comparative modelling and molecular dynamics simulations

MODELLER version 9.10 (Fiser and Sali, 2003) was used to generate 100 comparative models from each of the translated TLR6 seal sequences. The resolved structure of murine TLR6 (PDB: 3A79) was used as the template for both the harbour and elephant seal TLR6 amino acid sequences. For each species, the five models with the lowest discrete optimized protein energy (DOPE) score were validated using PROCHECK (Laskowski et al., 1998), Verify3D (Eisenberg et al., 1997), ERRAT (Colovos and Yeates, 1993) and ProQ (Wallner and Elofsson, 2003). Models with residues in disallowed regions, Verify3D score < 80% or ProQ LGscore < 4 were excluded and optimal models representing the harbour and elephant seal TLR6 protein structures were selected from the remaining models. Molecular dynamics (MD) simulations were performed in GROMACS 4.5.5 (Pronk et al., 2013). Harbour and elephant seal TLR6 models were prepared for MD simulation under the GROMOS96 43a1 force field. Models were placed into a cubic box maintaining a distance of 1nm (10 Å) between the box edge and the model surface. The system was solvated using the simple point charge (SPC) 216 three-point solvent

model, adding counter ions for neutralization. Energy minimisation was conducted using the steepest descent method for a maximum of 50,000 cycles or until the maximum force (F_{max}) of the system was less than 1,000 kJ mol^{-1} nm^{-1}. Equilibration of each system was performed for 100 picoseconds (ps) in a temperature coupling bath set at 300 Kelvin using a modified Berendsen thermostat and 0.1 ps time constant followed by Parrinello-Rahman pressure coupling maintained at 1 bar using a 1 ps time constant with water compressibility of $4.5e^{-5}$ bar^{-1}. The MDS production run was conducted for 10 nanoseconds (ns) with a 2 femtosecond (fs) time step at a temperature of 300 Kelvin and pressure of 1 bar with snapshots collected every 2 ps. Root mean square deviation between structures was calculated using PDBeFold (Krissinel and Henrick, 2004). Structural illustrations for this publication were subsequently generated in PyMOL version 1.5.0 (Schrödinger).

3. Results and Discussion

In the present study, we compare sequences of TLRs involved in the interaction with pathogens of seals (Surendran et al., 2012) to identify potential pinniped specific selective pressure. Prior to the publication of genomic sequences for both the Weddell seal and Pacific walrus, previous studies have utilized interspecifically conserved regions from carnivores to amplify pinniped genes of interest (McCarthy et al., 2011), clearly indicating a close relationship between these species. Whilst in the present study primers were designed upon such regions to amplify the single exons encoding TLRs 1, 2, 6 and 10 from both harbor and elephant seals, variation within the primer-targeted regions of elephant seal TLR1 and both harbour and elephant seal TLR2 and TLR10 resulted in the absence of amplicons for these genes. However, partial nucleotide sequences of 1758bp and 1605-1608bp were amplified from three harbor seals encoding TLR1 (hsTLR1) and TLR6 (hsTLR6) and three elephant seals encoding TLR6 (esTLR6), respectively (GenBank:KP744360-KP744368). Domain analysis of the putative peptide sequences using LRRfinder2.0 (Offord and Werling, 2013) identified 15 complete leucine-rich repeats (LRRs) from the extracellular domain spanning LRR6 to LRR20 (TLR1: residues 4-379, TLR6: residues 18-389) as well as a C-terminal LRR capping domain (TLR1: residues 380-419, TLR6: residues 390-431), a transmembrane helix (TLR1: residues 420-444, TLR6: residues 432-454) and an intracellular TIR domain (TLR1: residues 445-586, TLR6: residues 455-536) (Fig. S1 and Fig. S2). Subsequent sequence comparison of the putative peptides to both the Weddell seal and Pacific walrus reveals that hsTLR1, hsTLR6 and esTLR6 share greater than 95% sequence identity between orthologs.

To further elucidate the evolutionary history of TLRs from marine mammals, phylogenetic analyses were conducted using the hsTLR1, hsTLR6 and esTLR6 peptide sequences coupled with those from 47 mammalian TLR1 and TLR6 peptides respectively (Fig. S3A and Fig. S3B). In agreement with previous studies, the evolutionary relationships (Fig. S3) confirmed a monophyletic pinniped lineage consisting of walruses (Odobenidae), fur seals/sea lions (Otariidae) and true seals (Phocidae) (Fyler et al., 2005). Strong bootstrap support was observed for distinct TLR6 lineages (Fig. S3B) for the northern, Arctic and sub-Arctic species such as the harbor seal (Phocinae) and the more widely spread southern species native to the Antarctic and northern Pacific (Monachinae) which include the elephant seal and Weddell seal (Fyler et al., 2005). Within the phocine lineage, eight variants were identified between hsTLR6 and esTLR6 at positions L189P (LRR6), G219E (LRR8), I286V (LRR10), T352I (LRR12), E398K(LRR14), N304D (LRR17), V575M (LRRCT) and A592T (TIR) as well as four further variants E184K (LRR6), R258K (LRR9), R420G (LRR15) and V605I (transmembrane helix) with respect to TLR6 from the Weddell seal (GenBank: XP_006732367.1). Interestingly, in harbor seals position 398 is occupied by the negatively charged glutamic acid whilst in all other known pinnipeds the positively charged lysine is found at this site (Fig. S2).

To determine whether this alteration may be of functional relevance, hsTLR6 and esTLR6 sequences were comparatively modelled based on the resolved structure of murine TLR6 (PDB: 3A79). Strong validation scores from ERRAT (73.63 ± 0.94), ProQ (6.68 ± 0.26) and Verify3D (89.81 ± 5.85) were observed for both hsTLR6 and esTLR6 modelled structures which, following relaxation into their native state through molecular dynamics shared a target-template root mean square deviation (RMSD) of 1.59Å. Interestingly, upon electrostatic surface potential overlay it became clear that a significant region of positive surface charge on esTLR6 (Fig. 1B) results from the presence of lysine in contrast to the glutamine found in hsTLR6 (Fig. 1A). Previous studies have identified the region comprising LRRs 9-14 as essential for ligand recognition in members of the TLR1 superfamily (Jin et al., 2007; Jin and Lee, 2008; Kang et al., 2009). However, whilst E398K does not co-locate with the resolved TLR6:TLR2 dimerization site or the murine TLR6 Pam_2CSK_4 binding site (PDB: 3A79) (Kang et al., 2009) it is possible that secondary binding sites exist for which this residue may determine ligand specificity or affinity. Of greater significance, however, are those residues in both TLR1 (Fig. 1C) and TLR6 (Fig. 1D) which are unique to the pinniped and phocine lineages (Table 1). In particular, position 317 in TLR1 is occupied exclusively by threonine in pinnipeds and instead by serine, asparagine, arginine or glycine in other mammals. In humans, this corresponds to position 313 and is occupied by a glycine residue whose backbone oxygen is known to form a hydrogen bond to the backbone nitrogen of lysine at position three of Pam_3CSK_4 (Fig. 1E) in the TLR1:TLR2 crystal structure (PDB: 2Z7X) (Jin et al., 2007). Notably, this position Moreover, this site was identified as being subject to positive selection in primates (Wlasiuk and Nachman, 2010) and thus is likely to be crucial in determining species-specific pathogen recognition.

In summary, we have characterized novel pinniped TLR1 and TLR6 genes which, together with currently available genome sequences of other marine mammals, suggest key sites that may be the driving force behind pinniped- and species-specific innate immune responses.

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Table 1. Species- and taxon-specific residues within the TLR1 and TLR6 pinniped lineage

Gene	Position	Lineage specificity	Within lineage	Other lineages
TLR1	252	*p.vit*, *l.wed* and *o.ros*	T	N,S,K
TLR1	258	*p.vit*	M	I,V
TLR1	317	*p.vit*, *l.wed* and *o.ros*	T	S,N,G,R
TLR1	326	*p.vit*, *l.wed* and *o.ros*	L	I,T,V
TLR1	333	*p.vit* and *l.wed*	H	Q,K,I
TLR1	438	*p.vit*	L	I,M,A,V,T
TLR1	485	*p.vit*	D	A,T,M,V
TLR1	491	*p.vit*, *l.wed* and *o.ros*	D	G,E
TLR1	498	*p.vit*	K	I,T,V,L,A
TLR1	693	*p.vit*, *l.wed* and *o.ros*	R	K
TLR6	182	*p.vit*, *m.ang* and *l.wed*	S	Y,F,H,Q,N
TLR6	189	*m.ang*	P	L
TLR6	231	*p.vit*, *m.ang* and *l.wed*	N	D,E,Y,S,K,G
TLR6	322	*p.vit*, *m.ang* and *l.wed*	V	L
TLR6	352	*m.ang*	I	T,A,S,M
TLR6	490	*p.vit*, *m.ang*, *l.wed* and *o.ros*	D	G,E
TLR6	560	*p.vit*, *m.ang* and *l.wed*	G	D,E,A,H

Alignments of 47 mammalian TLR1 and TLR6 peptide sequences to hsTLR1, hsTLR6 and esTLR6 were used to identify pinniped-, phocine- and species-specific residues within the pinniped lineage. Positions correspond to TLR1 (GenBank:XP_006732366) and TLR6 (GenBank:XP_006732367) amino acid sequences from the Weddell seal (*Leptonychotes weddelli*). Lineage abbreviations are: p.vit (harbor seal: *Phoca vitulina*), m.ang (elephant seal: *Mirounga angustirostris*), l.wed (Weddell seal: *Leptonychotes weddelli)* and o.ros (Pacific walrus: *Odobenus rosmarus*).

Table S1. Primers and amplification conditions used to amplify harbor and elephant seal TLRs 1, 2, 6 and 10

Gene	Primer direction	Primer sequence	PCR annealing temperature (°C)	Product size
TLR 1	Forward	GGGTTGAGTGCCACACAGTT (20)	54.7	1848bp
	Reverse	CTTTGGGCCATTSCAARTAAG (20)		
TLR 2	Forward	ACTTMTCATCCTCCTGGTTCAG (22)	57.0	1987bp
	Reverse	AMCCAAAACCCTTCCTGCTG (20)		
TLR 6	Forward	TGCCCATCTGTAACCAATTTG (21)	51.7	1710bp
	Reverse	AGTTGGGAGACAGAACAAAGAT (22)		
TLR 10	Forward	AYAAYCTCCTTTYTCAACTCCA (22)	50.8	2235bp
	Reverse	TCAGAGAKATTGCAGAACCT (20)		

Primers were designed based on interspecifically conserved regions in the single exons encoding carnivore TLR1, 2, 6 and 10 putative proteins using the canine genome as a reference. Both forward and reverse primers are shown in a 5' to 3' orientation. Wobble bases included at sites of ambiguity are denoted as follows: W=A/T, S=C/G, R=A/G, K=G/T, M=A/C, Y=C/T.

Table S2. Thermocycler conditions for amplification of harbor and elephant seal TLRs 1, 2, 6 and 10

Step	Temperature	Time	Cycles
Initial denaturation	94°C	1 min	1
Denaturation	94°C	1 min	
Annealing	*See Supplementary Table 1*	1 min	30
Extension	72°C	*See Supplementary Table 1*	
Final extension	72°C	7 min	1

Target sequences were amplified using Easy A enzyme mastermix (Agilent Technologies) in 50µl reactions and carried out using the thermocycler (Eppendorf; Mastercycler Pro) conditions in this table. PCR products were subsequently purified using MinElute PCR purifcation kit (Qiagen) according to the manufacturer's protocol. Sequencing of the purified PCR product was then carried out by DNAseq (University of Dundee) using the corresponding primers.

Table S3. Nucleotide sequences used for phylogenetic analysis of mammalian TLRs

Inherited Blast Name	Common name	Accession
bats	big brown bat	XM_008140359
bats	Brandt's bat	XM_005877435
bats	mouse-eared bat	XM_006759337
bats	little brown bat	XM_006088512
bats	black flying fox	GU045600
bats	large flying fox	ENSPVAT00000016705
carnivores	giant panda	ENSAMET00000020308
carnivores	dog	NM_001146143
carnivores	cat	XM_003985440
carnivores	Weddell seal	XM_006732303
carnivores	ferret	XM_004814006
carnivores	Pacific walrus	XM_004396165
carnivores	Amur tiger	XM_007098862
carnivores	polar bear	XM_00870139
even-toed ungulates	yak	KF776518
even-toed ungulates	zebu	EU006645
even-toed ungulates	wild yak	XM_005897339
even-toed ungulates	cow	NM_001046504
even-toed ungulates	water buffalo	XM_006066109
even-toed ungulates	Wild Bactrian camel	XM_006185604
even-toed ungulates	goat	NM_001285605
even-toed ungulates	sheep	NM_001135060
even-toed ungulates	Tibetan antelope	XM_005960989
even-toed ungulates	pig	NM_001031775
insectivores	star-nosed mole	XM_004681492
insectivores	western European hedgehog	XM_007522803
insectivores	European shrew	XM_004608066
odd-toed ungulates	southern white rhinoceros	XM_004418976
odd-toed ungulates	horse	NM_001256899
odd-toed ungulates	Przewalski's horse	XM_008520700
placentals	sloth	ENSCHOT00000005293
placentals	Cape golden mole	XM_006878192
placentals	nine-banded armadillo	XM_004459377
placentals	small Madagascar hedgehog	XM_004703351
placentals	Cape elephant shrew	XM_006903585
placentals	African savanna elephant	ENSLAFT00000008548
placentals	Aardvark	XM_007953531
placentals	hyrax	ENSPCAT00000005241
placentals	Florida manatee	XM_004391229
placentals	northern tree shrew	ENSTBET00000006228
placentals	Chinese tree shrew	XM_006169244
primates	white-tufted-ear marmoset	ENSCJAT00000001513
primates	sooty mangabey	EU204931
primates	green monkey	XM_008017649
primates	gorilla	NM_001279584

primates	human	NM_003263
primates	common gibbon	EU488847
primates	crab-eating macaque	XM_005554674
primates	Rhesus monkey	NM_001130424
primates	northern white-cheeked gibbon	ENSNLET00000021750
primates	small-eared galago	XM_003803952
primates	pygmy chimpanzee	NM_001279215
primates	chimpanzee	NM_001130465
primates	olive baboon	ENSPANT00000028966
primates	Sumatran orangutan	XM_009239872
primates	Bornean orangutan	AB445621
primates	cotton-top tamarin	EU488856
primates	Bolivian squirrel monkey	XM_003927513
primates	Philippine tarsier	XM_008053017
rabbits and hares	American pika	XM_004579325
rabbits and hares	rabbit	XM_002709270
rodents	guinea pig	XM_003471538
rodents	long-tailed chinchilla	XM_005391996
rodents	kangaroo rat	ENSDORT00000003539
rodents	thirteen-lined ground squirrel	XM_005319910
rodents	lesser Egyptian jerboa	XM_004658825
rodents	golden hamster	XM_005074382
rodents	prairie vole	XM_005359255
rodents	house mouse	NM_030682
rodents	blind mole rat	XM_008833223
rodents	degu	XM_004642668
rodents	prairie deer mouse	XM_006974597
rodents	Norway rat	NM_001172120
whales and dolphins	minke whale	XM_007178823
whales and dolphins	Yangtze River dolphin	XM_007449409
whales and dolphins	killer whale	XM_004266234
whales and dolphins	sperm whale	XM_007102319
whales and dolphins	bottlenosed dolphin	XM_004321018

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Figure 1: Evolutionary history of pinniped TLR1 and TLR6. The evolutionary histories of 50 mammalian TLR1 (A) and 53 mammalian TLR6 (B) amino acid sequences were inferred using the Maximum Likelihood method based on the JTT matrix-based model. In both analyses, a discrete Gamma distribution was used to model evolutionary rate differences among sites and all positions with less than 95% site coverage were eliminated. In addition, for the TLR1 analysis the rate variation model allowed for some sites to be evolutionarily invariable. Bootstrap consensus trees were inferred from 1000 replicates with branches corresponding to partitions reproduced in less than 50% bootstrap replicates collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Evolutionary analyses were conducted in MEGA6.

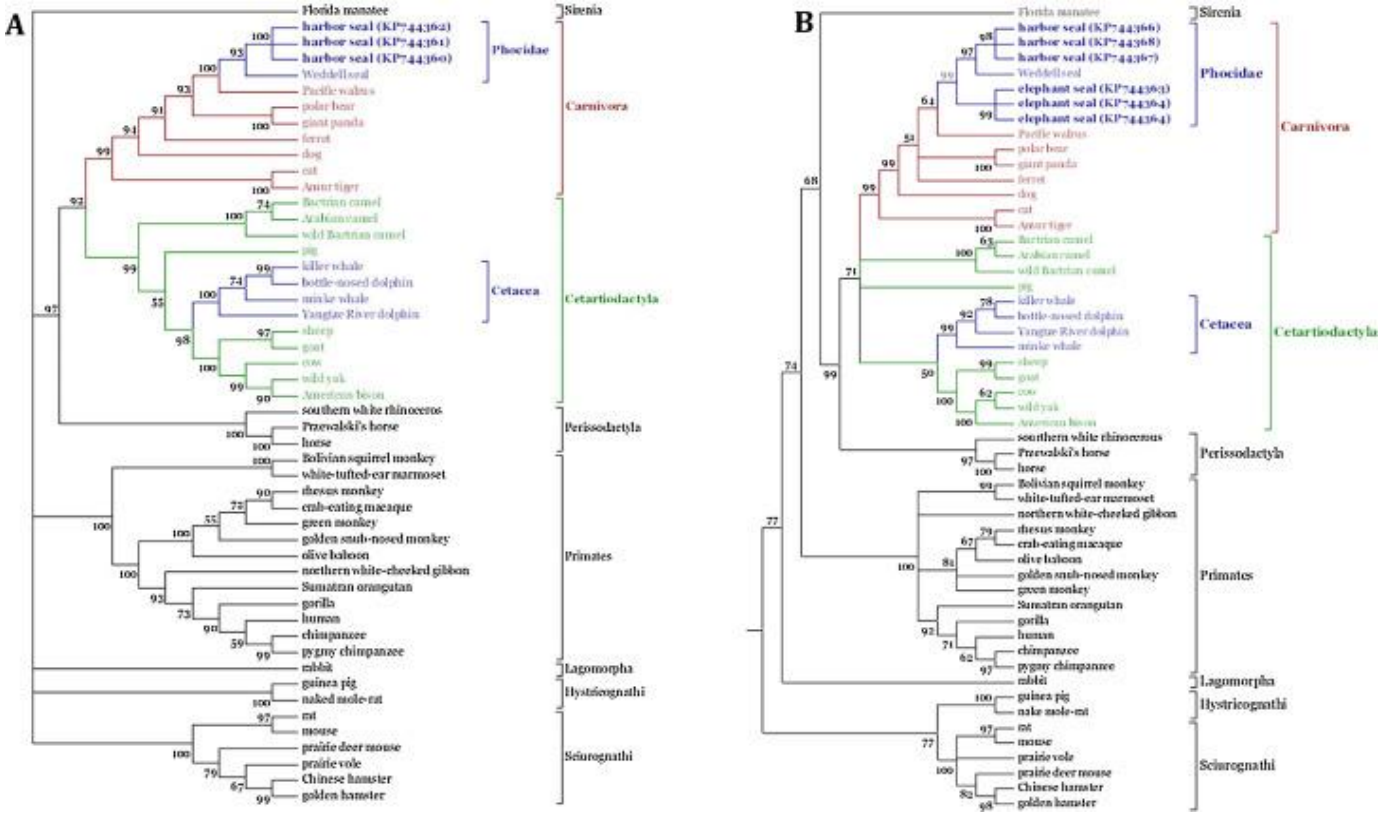

Figure 2: Analysis of pinniped TLR1 superfamily variations reveal a loss of negative surface potential at E398K in TLR6 and pinniped-specific site under adaptive selection in TLR1. Comparative models of hsTLR6 (A) and esTLR6 (B) were generated using murine TLR6 (PDB: 3A79) as a template. Of the eight variants identified between hsTLR6 and esTLR6, E398K (circled in yellow) was the only variant observed to result in the alteration of electrostatic surface potentials. Moreover, pinniped- and phocine-specific variants were also identified which have been superimposed onto the human TLR1:TLR2:Pam3CSK4 (PDB: 2Z7X) (C) and murine TLR6:TLR2:Pam2CSK4 (PDB: 3A79) (D) resolved structures. The cartoon structure of TLR2 is represented in grey, the relevant ligand in magenta and human TLR1 and murine TLR6 in blue and green respectively. The identified sites are shown as yellow spheres with a magnified resolution of the human TLR1 ligand-binding residue G313 which forms a hydrogen bond to Pam3CSK4 (E).