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New defensins from hard and soft ticks: Similarities, differences, and phylogenetic analyses

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ABSTRACT

Despite the importance of ticks as vectors of disease very little is known about their immune system. Antimicrobial peptides, including defensins (phylogenetically ancient antibacterial peptides) are major components of innate immunity in ticks that have been shown to provide protection against gram-negative and gram-positive bacteria, fungi, viruses and protozoan parasites. With the aim of studying the evolution of the genes involved in tick defense, we identified the preprodefensin genes from four *Ornithodoros* tick species (*O. papillipes*: isoforms A, B, and D; *O. tartakovskyi* and *O. puertoricensis*: isoforms A and B; *O. rostratus*: isoform A) and from two *Dermacentor* tick species (*D. reticulatus* and *D. marginatus*: one isoform) not previously described. Phylogenetic analyses revealed that *Ornithodoros* defensin isoforms (A, B, C, and D) form 4 separate clades, while hard tick defensins are divided into several branches based on particular tick species.

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1. Introduction

After mosquitoes, ticks occupy the second place in the abundance of transmission of arthropod-borne diseases and hence the worldwide focus on tick research. However, ticks are not only the passive vectors of pathogens. Tick–host–pathogen interactions are rather complicated and the understanding of the complex dynamics between vector and host during disease pathogenesis is under extensive study. Like other arthropods, ticks have innate immunity that is nonspecific and lacks memory. The data about the tick innate immune system are still limited.

Ticks are able to recognize invading pathogens due to molecules like lipopolysaccharides (LPS), peptidoglycans (PGN) or glucans present on pathogens surface. Recognition and the reaction to these stimuli are provided by cellular and humoral pathways of immunity. The cellular pathway is represented by hemocytes. These cells support clearing of pathogens in the haemolymph through phagocytosis, nodulation and encapsulation (Gillespie et al., 1997). Hemocytes are also very efficient in the synthesis of many antimicrobial peptides, which are released into the fluids and tissues of ticks. Cellular and humoral immunity pathways cooperate closely. Humoral immune responses consist of three main types: humoral encapsulation, hemagglutination and the rapid and transient synthesis of a set of potent antimicrobial peptides (AMPs) that can attack the invading microorganisms following tick blood feeding, infection or trauma. Several AMP groups have already been characterized in ticks.

Lysozymes have been shown to have bactericidal effects in several tick species and work in concert with other antimicrobial peptides to kill invaders. They are able to lyse bacteria by cleaving β-1, 4 glycosidic bonds

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occurring in peptidoglycans of bacteria cell wall (Qasba and Kumar, 1997). Soft tick, *Ornithodoros moubata*, over-expressed chicken (c) type lysozyme during blood ingestion (Grunclová et al., 2003). Haemocoelic inoculations of *Dermacentor variabilis* with *Borrelia burgdorferi* also induced up-regulation of a lysozyme-like protein (Johns et al., 2000).

Cystatins are the next large group of antimicrobial proteins found in wide variety of living species like plants and animals. Cystatins, reversible inhibitors of papain-like cysteine proteases, have recently been shown to function in innate immune responses of ticks. Cystatin genes were found to be expressed in *O. moubata*, *Haemaphysalis longicornis* and *Rhipicephalus* (*Boophilus*) *microplus* (Grunclová et al., 2006; Lima et al., 2006; Zhou et al., 2006). Blood feeding and LPS injection increased cystatin expression in *H. longicornis*. It was shown that cystatins were able to inhibit *Babesia bovis* growth in culture (Zhou et al., 2006).

Among the naturally occurring antimicrobial peptides, defensins form a unique family of cysteine-rich cationic peptides. Defensins have been isolated from mammals, insects and plants and they serve as effector molecules of innate immunity, providing an efficient initial defense against infectious pathogens. Many defensin-like antimicrobial peptides have been identified in scorpions (Cociancich et al., 1993; Ehret-Sabatier et al., 1996), mussels (Charlet et al., 1996; Hubert et al., 1996) and several tick species including *Ixodes scapularis*, *Amblyomma americanum*, *D. variabilis*, and *R. microplus* (Ceraul et al., 2003; Fogaca et al., 2004; Hynes et al., 2005; Todd et al., 2007). More than one defensin isoform has been identified in *O. moubata*, *Ixodes ricinus*, *Amblyomma hebraeum* and *H. longicornis* ticks (Nakajima et al., 2003a; Lai et al., 2004; Rudenko et al., 2005, 2007; Zhou et al., 2007). Defensins are produced as prepropeptides; functional mature defensins are cationic peptides with molecular mass up to 4 kDa, usually containing 6 cysteine residues that form characteristic intra-molecular disulphide bridges.

The secondary and tertiary structures of tick defensins show similarity to a wide range of membrane potential modulators, such as scorpion neurotoxins, snake safaratoxins and plant γ-thionins (Froy and Gurevitz, 1998). The mechanism of killing bacteria by tick defensins is through depolarization of cytoplasmic membrane (Nakajima et al., 2003b). Generally, tick defensins showed antibacterial activity against many gram-positive bacteria (e.g. *Bacillus cereus*, *Enterococcus faecalis*), but some isoforms have also revealed the potential activity against gram-negative bacteria (*Escherichia coli*), yeasts (*Pichia pastoris*) and protozoa (Nakajima et al., 2003b; Tsuji et al., 2007). For example, longicin, a defensin from tick *H. longicornis*, demonstrated the ability to inhibit the proliferation of merozoites in erythrocytes infected with *Babesia equi* by killing the parasites and, in *in vivo* experiment, longicin induced the reduction of parasitemia in animals infected with *Babesia microti* (Tsuji et al., 2007).

Ticks encounter a large diversity of pathogens, therefore they had to develop multiple antimicrobial factors. With the growing problem of pathogenic organisms that are resistant to conventional antibiotics, ticks are becoming fruitful sources of novel pharmaceutical substances for infection treatment. As there is a real clinical need for a novel class of antibiotics, derivates of naturally occurring AMPs represent the challenge for research and development.

2. Materials and methods

2.1. Tick samples

Dermacentor marginatus, *Dermacentor reticulatus*, *Ornithodoros papillipes*, *Ornithodoros tartakovskyi*, *Ornithodoros puertoricensis*, and *Ornithodoros rostratus* female ticks were provided by the internal tick facility of the Biological Centre, Institute of Parasitology, Academy of Science of the Czech Republic. Uninfected ticks were fed on adult guinea pigs (infection free animals reared under strict hygiene regulations of the Central Commission for the Protection of Animals (§21, section 3e, law 246/1992)) as it was described previously (Rudenko et al., 2005). Briefly, three unfed females and one male (per animal) were placed into the cell, attached to the shaved area on the guinea pig back until they had fully engorged. Ticks were removed from the animals and kept at 4 °C for another 7 days.

2.2. Total RNA isolation

Total RNA was isolated from the pool of six female ticks from each species mentioned above. Ticks were washed in ethanol and distilled water to eliminate surface contaminants. Total RNA was purified from homogenate of ticks using TRI reagent (Sigma, USA) according to manufacturer's recommendation.

2.3. cDNA synthesis

Single strand cDNA was prepared from total RNA with random primers (0.2 μg per reaction) using RevertAid H Minus First Strand cDNA Synthesis Kit (MBI Fermentas, Lithuenia). Five micrograms of total RNA was used per each reaction. Synthesis of the first strand cDNA was carried out at 42 °C for 60 min according to manufacturer's recommendations.

2.4. Designing of primer sets and polymerase chain reaction (PCR)

Defensin primers were designed according to nucleotide alignments of published sequences (Genbank accession numbers: AB041813, AB041814, AB041815, and AB041816) of genera *Ornithodoros* and *Dermacentor*, respectively. Based on conserved regions the following primer pairs were synthesized (Generi-Biotech, Czech Republic): *Ornithodoros* primers 5′-ATG AAC AAG YTS TTC ATT G-3′ and 5′-TCA GTA ACA WTT ACA TGT C-3′; *Dermacentor* primers 5′-ATG CGC GGA CTT TGC ATC-3′ and 5′-TTA ATT CCT GTA GCA GGT GC-3′.

To amplify the target gene, the single strand cDNA (150 ng per reaction) was used. Amplification was performed with the use of GoTaq DNA polymerase (5 U/μl, USA) in 20 μl of total volume of PCR reaction. PCR conditions were as follows: 5 min at 96 °C, followed by 35

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