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Review report on doctoral thesis of MVDr. Miray Tonk, MSc.:
Characterization and functional analysis of defensins from the ticks *Ixides ricinus* and *Ixides scapularis*

The thesis deals with detailed characterization of representatives of antimicrobial peptides from defensin class – six of them were identified in *I. ricinus* and two in *I. scapularis*. The ticks are well-known vectors of significant diseases, thus, the study of their immune system represents an excellent topic with important theoretical and practical impacts. The present thesis consists of two parts: the first one represents classical form with a common structure (Introduction, Materials and Methods, Results and Discussion), the second one comprises 2 publications. One publication has already been published in a prestigious journal, and the second one has been submitted for publication. Both papers summarize results from the first part of the thesis. As the papers have passed/are passing via strict evaluation process, I will focus on the first part of the thesis.

The text is well-written and well-presented which documents both research and narrative skills of the author. In the Introduction the author describes basic data from the tick biology and focuses on detailed characterization of antimicrobial peptides preferably defensins. In Materials and Methods section all used methods are described and well characterised including details of biochemical and molecular analyses. The results are documented by tables and good-quality figures mostly showing the structure and activity of characterized defensins. This part of the thesis is terminated by detailed discussion, and by final remarks clearly summarizing the obtained results.

General questions and notes:
- page 5 and also below: You are considering to use defensins as potential antibiotics. Is not there a problem with immunogenicity of the defensin molecules in the practical use?
- page 10: Is there any structural and functional similarity between the tick and insect defensins? Please, comment it.
- page 13: You are mentioning here that *I. scapularis* possesses 25 scapularisins. Why so big number? Is there any specialisation among them? Please, comment it.
- page 25: Which volume of haemolymph are you able to take out from one tick?
- page 34-36: Antibacterial assays. Why did you test selected defensins against selected bacteria and did not do all combinations?
- page 38: What do you mean by "insect-type" of disulphide bridges in defensins molecules (see also question to page 10)?
- page 61: Why lipocalin is mentioned here? Lipocalins are known in insect as transporters of small hydrophobic molecules.
- page 63: It is shown here that various defensins are expressed in various tick organs – why? Please, comment it.
- page 65 and also below: What is a function of defensins in plants?

- You are the first author of both presented papers, however, several co-author are in the list as well (which I consider to be normal), nevertheless, could you briefly specify and describe your share and your responsibility in the papers, please?

Formal reservations:
- There is a list of abbreviations at the beginning of the thesis, which is very useful for a reader, however, not all abbreviations used in the text are present in the list
- page 32: A sentence should not start with a number. See: 350 µl of…
- Pagination is missing in the second paper, which complicates orientation within the paper

Summary and conclusion:
Miss Miray Tonk has been trained in and used a range of techniques, some of which demand considerable care. With help of them she obtained and interesting results with significant theoretical, and potential practical impact. With this "background" she has prepared comprehensive and interesting thesis. I consider the thesis entirely fulfilling demands of high quality doctoral work and I recommend it to be accepted.

České Budějovice, 15th June 2014

Dalibor Kodrík
reviewer

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Opponent's report

Characterisation and functional analysis of defensins from the ticks *Ixodes ricinus* and *Ixodes scapularis*

MVDr. Miray Tonk, MSc.

MVDr. Miray Tonk, MSc. has submitted her PhD dissertation entitled: "Characterisation and functional analysis of defensins from the ticks *Ixodes ricinus* and *Ixodes scapularis*".

As indicated by the title and based on a more detailed inspection of the content, the main topic concerns defensins of the hard-bodied ticks *Ixodes ricinus* and *Ixodes scapularis*. These antimicrobial peptides were recognized within the genome, identified by sequencing, synthesized and further structurally, functionally, and phylogenetically characterized in this study. Six novel defensin genes (DefMT2, DefMT3, DefMT4, DefMT5, DefMT6, and DefMT7) from *I. ricinus* and two (Scapularisin-3 and Scapularisin-6) from *I. scapularis* were reported. These defensins show a high degree of evolutionary diversity. The transcriptional expression pattern in salivary glands, midguts, ovaries, haemolymph and Malpighian tubules was diverse. Some were found to have tissue-specific expression while others were ubiquitously expressed. Three of the novel defensins were characterised at the genomic level; two contain introns and one was an intronless gene. Finnally, the synthesized defensins were functionally characterised. They showed activity against fungi and Gram+ bacteria. Thus, from these molecular and structural analyses and antimicrobial assays it was properly concluded that they are antimicrobial peptides belonging to the family of defensins.

The thesis is formally arranged in a classical way consisting of eight chapters as follows: *Introduction* (20 pages), *Project objectives* (4 particular aims), *Materials and methods* (15 pages), *Results* (22 pages), *Discussion and conclusions* (9 pages), *Final remarks* (1 page), *Published and submitted manuscripts* (44 pages), and *References* (more than 165 citations). The dissertation is accompanied by 17 figures and 9 tables that are conveniently listed also at the beginning of the thesis together with the list of *Abbreviations* used. It should be worth to mention that MVDr. Miray Tonk, MSc. has published 2 papers in journals indexed in *Current Contents*, i.e. *Gene* (2012 IF=2.196), *PlosOne* (2012 IF=3.730), and submitted one more to *Parasites and Vectors* (2012 IF=3.246).

In fact, there are not any significant issues to be criticized since the thesis, in addition to its high scientific level, was written well even from the formal point of view. It is easy to read, follow and understand. The individual parts of results being appropriately illustrated and also separated from each other. The thesis, of course, contains also some formal errors that - in my opinion – if more attention is paid to final reading, could be eliminated. These are, for example, words „microrganisms (page 6)" and "*Candina albicans* (page 11)" in *Chapter 1*, „dublicate" (page 35) in *Chapter 3*, „DNAwere" (page 37) in *Chapter 4*, „rectanges" (page 43) *in Figure 6B*, „valuesmore"

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and „themain" (page 46) in *Figure 9,* "fromthis" (page 62) in *Chapter 5 o*r the expression „the vector of several diseases of animals" (page 3) in *Chapter 1*, etc. There are also slight differences in writing the references (in abbreviation of journals, position of the year in which the work was published, etc.). Furthermore, in my opinion, there are abbreviations (^{o}C, DNA,bp, kDa, M, mM, ml, PCR, etc)(page viii) which are too common to be present in the list of abbreviations. On the other hand frequently used words as „fat body or salivary gland tissues", etc can be abbreviated.

As an opponent of this thesis I have the following questions:

(1) Could you please summarize briefly how the results you achieved can be practically used, or what do they simply indicate? What is your idea why some of the defensins can be find in all tissues and some are tissue-specific? Could you stress *pros and cons* of the defensins you identified and tested?

(2) It was suggested that mode of action of defensins involve electrostatic interaction with the bacterial membrane by introducing voltage-dependent channels into bacterial walls, leading to permeability changes or pore formation. Could you please comment why for example the DefMT3 has no activity against any of the G- or G+ bacteria species used in the antimicrobial assays except of *Multicoccus luteus*?

(3) As you have mentioned in page 7: „Mature defensins are cyclic peptides possessing a pattern of six paired cysteine residues in their primary structure with three or four disulphide bridges, forming conserved cysteine-stabilised α-helix and β-sheet structural motifs that are crucial for the antimicrobial activity". How did you confirmed that the chains of synthesized defensins are linked with a covalent bond that generate a ring? How do you feel about DefMT7 which lacks the antiparallel β strands?

I am pleased to point out that the author has clearly demonstrated her ability to work as a scientist and the presented thesis fulfils unambiguously the requirements for this type of work in this branch of science.

To conclude, the submitted doctoral dissertation, in my opinion, represents a comprehensive piece of work by a young scientific personality supervised by excellent supervisor, Prof. RNDr. Libor Grubhoffer, CSc. I therefore without any hesitation strongly recommend to accept the thesis and, after a successful defence, to award MVDr. Miray Tonk, MSc. with the relevant scientific title in the field of study "Parasitology".

Bratislava, May 13th, 2014

Ing. Ludovit Skultety, Ph.D.

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Review of the PhD thesis from Ms Miray Tonk

Berlin, 16 June 2014

Dear Prof. Ditrich,

Herewith my review of the PhD thesis by Ms Miray Tonk.

This PhD thesis, entitled 'Characterisation and functional analysis of defensins from the ticks *Ixodes ricinus* and *Ixodes scapularis*', focuses on components of the innate immune system from two tick species of great medical and veterinary importance. The identification and partial functional characterisation of six novel antimicrobial peptides (AMPs) from *I. ricinus* was recently published as a paper in Gene. A manuscript describing the second part of the candidates' studies, which focused on the functional characterisation of two AMPs from *I. scapularis*, was submitted to Parasites and Vectors. Both journals have a good standing in their fields, as evidenced by their impact factors of 2.196 and 3.25. In addition, the candidate also contributed to a manuscript published in PLoS ONE by Cabezas-Cruz et al. in 2013.

I find the thesis worthy to be defended; several minor comments and suggestions for questions that could be asked to the candidate during the thesis defence can be found below.

A brief introduction to *I. ricinus* and *I. scapularis*, their importance and the tick innate immune system is presented in Chapter 1. A minor comment could be given here on the fact that *Ixodes ricinus* is falsely implicated as a vector of *Ehrlichia canis* on page 3, whereas its vector role for *Babesia divergens* and *Babesia microti* is not mentioned, despite the zoonotic relevance of these piroplasms.

Q1: on page 6, it is written that 'AMPs are excellent candidates for developing novel therapeutic agents complementary to conventional antibiotic therapy.' Can the candidate give examples of AMPs that have been successfully commercialised in the past? Which properties should the ideal AMP have from a therapeutic point of view?

Q2: I noted that there is some contradiction in the thesis regarding the mode of action of defensins: it is mentioned that several mechanisms are responsible (page 10), but it is later stated that the actual antimicrobial mechanism is, in fact, unclear (page 19). Could the candidate elaborate on the mode of action of AMPs and how it differs from the mechanisms of action from conventional antibiotics?

Q3: is it known how AMP production is activated and controlled in ticks?

Q4: certain defensins have been associated with borreliacidal activity (paragraph 1.3.2.4, page 11). It is interesting to note that these particular defensins have been isolated from *D. variabilis*, a tick species that does not transmit *Borrelia* spp. To which extent do AMPs play a role in the vector competence of ticks for certain pathogens? And which other factors could play a role in determining the vector competence of a tick species for a certain pathogen?

Chapter 3 describes the employed materials and methods. Minor comment: details such as city and country should have been provided for the Fraunhofer Institute for Molecular Biology and Applied Ecology on page 36.

Q5: The fat body is known to produce antimicrobial compounds (Taylor, 2006), but this tissue was not included in the expression profile studies (page 24). Why not?

Q6: could the candidate explain why different initial optical densities were used for different bacteria in the growth inhibition assays?

The results are presented in Chapter 4 and discussed in Chapter 5.

Q7: what could explain the observed difference in activity of the identified defensins against several bacteria in the functional assays (page 51-58) compared to their predicted activity (page 60)?

Q8: on which grounds were Scapularisin-3 and Scapularisin-6 selected from the 25 known Scapularisins for further analysis within the framework of this thesis?

Future perspectives of research on AMPs are presented in Chapter 6, a chapter that could have been more elaborate. Chapter 7 contains a list of the published and submitted manuscripts. These two manuscripts can be found from page 70 onwards.

I would finally like to congratulate the candidate on the work which she performed for this PhD thesis and wish her all the best in her future endeavours.

Yours sincerely,

Ard Nijhof, DVM PhD

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