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Referee's comments to PhD. thesis of Veronika Burešová, entitled "Function of the α_2 -macroglobulin protein family in the immune response of the tick *Ixodes ricinus*"

Due to transmission of a wide range of pathogens causing diseases of humans and animals, the medical and veterinary impact of ticks is very important. Finding of new measures to control ticks as well as tick-borne diseases is highly demanded. "Immunological" control, i.e. the development of vaccines that affect both the vector and pathogen and additionally block the transmission of pathogens and development of the disease is very promising, however, it requires understanding of both, the biological processes involved in tick immunity and on the host – vector - pathogen interface.

The submitted thesis of Veronika Burešová, a PhD. student of the Institute of Parasitology of the Biology Centre of the Academy of Sciences of the Czech Republic in České Budějovice, elaborated in the Laboratory of Vector Immunology and supervised by Dr. Petr Kopáček, focuses on molecules involved in the tick – pathogen interaction and thus its topic is very actual. The Laboratory where the thesis was elaborated has achieved high-priority results on mechanisms of tick – pathogen – host interactions and is among the world leading laboratories in this field of study.

The PhD. thesis is based on two original first-author papers published in recognized international journals – *Journal of Invertebrate Pathology* (IF 1.463) and *Developmental and Comparative Immunology* (IF 3.155), and is supplemented by two more papers published in high impact journals, *International Journal for Parasitology* (IF 3.392) and *Proceedings of the National Academy of Sciences* of the USA (IF 9.598), with important contributions of the candidate.

The thesis, including pages that contain copies of the first-author papers, is written on 56 pages and comprises introduction, characterization of proteins of the α_2 -macroglobulin family, brief description and characterization of the tick immune system, description of the published results and presentation of unpublished data, summary and list of up-to-date referred sources.

The Introduction is an overview of the invertebrate immune system and its role in pathogen recognition, activation of the defense pathways and clearance of invading pathogens. Proteins of the α_2 -macroglobulin family are important components of humoral and cellular immunity throughout the animal kingdom and are characterized in more detail, including their structure and functions. The last part of the Introduction summarizes recent knowledge on tick immune system which, in comparison to crabs or insects, has been explored to a lesser extent and, considering the capacity of ticks to transmit a wide array of microorganisms, represents an important area for future studies.

The first paper compares pathogenicity of *Chrysobacterium indologenes* in soft and hard ticks. The bacterium is a newly identified pathogen of the soft tick *Ornithodoros moubata*, causing lethal infection, while the bacterium is effectively cleared out from the gut of the hard tick *Ixodes ricinus*, indicating differences in the immune system of soft and hard ticks. However, in both tick species, phagocytosis of the bacterium by hemocytes is obvious. In the second paper IrAM, an α_2 -macroglobulin was identified in *I. ricinus*, cloned and its structure was predicted. Expression of IrAM was localised in tick hemocytes and its function in phagocytosis of *C. indologenes* was demonstrated.

The third part of the results is based on unpublished data on searching for genes coding proteins of the α_2 -macroglobulin family in the genome of *Ixodes scapularis*. Based on the data a phylogenetic analysis of tick thioester proteins was carried out, which revealed that, among animals, ticks are probably the only organisms that possess members of all main α_2 -macroglobulin groups. In addition to IrAM, further three α_2 -macroglobulins were characterized from *I. ricinus*.

I have no questions or critical comments to the published papers as they were subject to strict peer-review processes in respective journals.

Overall, the thesis is elaborated very precisely, there are only a few mistakes in spelling and style and the thesis fulfils international standards. I only miss clear stating of the aims of the thesis at the end of introduction (or in a separate subchapter), although aims are stated in the two papers which are basis of the thesis.

I have only two general questions for discussion:

Did the author perform data mining for proteins of the α_2 -macroglobulin family in gene or sialome databases (although not complete databases are available) of Metastriate ticks (e.g. *Boophilus microplus*, *Amblyomma variegatum*)? If yes, do Metastriate ticks also possess thioester proteins and of which groups?

Among ticks, *Ixodes* spp. are vectors of the widest range of microorganisms, including viruses, various bacteria and protozoa, whereas co-infections with more pathogens are also quite frequent. What does the author think can be the basis of this outstanding vector competence of *Ixodes* ticks?

In conclusion, I would like to state that I enjoyed reading this thesis.

The thesis of Veronika Burešová fulfils all requirements set for PhD. theses and I can fully recommend it for defense and fulfillment of the academic degree "*Philosophiae doctor*" (PhD).

Bratislava, 20.4.2009


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RNDr. Mária Kazimírová, CSc.



**Referee's comments to PhD. thesis of Mgr. Veronika Burešová
"Function of the α_2 -macroglobulin protein family in the immune
response of the tick *Ixodes ricinus*"**

Submitted PhD. thesis of Veronika Burešová, a PhD. student of the Faculty of Science of the University of South Bohemia in České Budějovice, was elaborated in the Institute of Parasitology of the Biology Centre of the Academy of Sciences under the supervision of RNDr. Petr Kopáček, CSc. The Laboratory of Vector Immunology successfully focuses on molecular identification of factors important in tick innate defense and regulation of their expression.

The PhD. thesis of Veronika Burešová is based on two published first-author papers in respected journals, supplementary unpublished data and two papers on closely related topics. The research articles are always briefly introduced and the major results are highlighted.

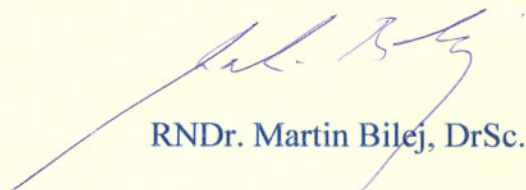
The Introduction is very short, however it provides sufficient information on innate immune system of invertebrates, the role of protein of α_2 -macroglobulin family and finally, on the immune system of ticks. Some inaccuracies in the Introduction are probably due to the tendency to generalize. For example, the second paragraph on page 1, I agree that LPS is characteristic for Gram-negative bacteria but it is not as obvious discrimination in the case of induction of Toll and Imd pathways in *Drosophila*. On page 2, I do not agree with the statement that invertebrates have an open circulatory system etc. Nevertheless, I believe that it is due to the effort to simplify the introduction and devote more space to the major topic, i.e. α_2 -macroglobulin family.

I have appreciated to read the published papers and since I do know that they have been rigorously reviewed prior the publication I would like to ask only three questions.

- The paper published in *J. Invertebr. Pathol.* describes differences in pathogenicity of *Chryseobacterium indologenes* in soft tick *Ornithodoros moubata* and hard tick *Ixodes ricinus*. Apparently hard ticks possess more efficient defense system as compared to soft ticks. On the other hand, phagocytosis in soft tick seems to be faster and stronger. How do you explain this observation? What is the mechanism of pathogenicity of *C. indologenes*, is it due to a release of some toxins?
- The paper in *Dev. Comp. Immunol.* shows convincingly the impairment of phagocytosis in IrAM-silenced hard ticks. Do you have similar data in soft ticks (the cellular response seems to be stronger)?
- I am missing a part with general discussion and perspectives – what are the future plans in your research?

In conclusion, Veronika Burešová has proven the ability of independent research and conceptual work and I can fully recommend her thesis for the fulfillment of the academic degree PhD.

Prague, April 25, 2009



RNDr. Martin Bilej, DrSc.



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Finally, the third part of the thesis described the yet unpublished data on the evolutionary analyses of the family of α_2 -macroglobulins in *I. scapularis* and in *I. ricinus*. At least 9 proteins belonging to this family were identified in *I. scapularis*, out of which IrAM8 and 9 represent a new sub-clade in the family of invertebrate TEPs. RNAi silencing of *IrAM2*, *IrAM5* and *IrAM6* demonstrated their involvement into regulation of phagocytosis against *C. indologenes* but not *Borrelia* spirochetes nor *Staphylococcus* spp. The candidate also participated in the study of two other aspects of immune responses in ticks. The first study was on the asparaginyl endopeptidase (legumain) in the gut of the hard tick *I. ricinus*, results of which were published in 2007 in the International Journal of Parasitology. The second study focused on the role of iron metabolism in tick reproduction and immunity and was published in PNAS in 2009.

Given the high quality of experimental work, reflected by four publications in the high-end specialized international journals, and the overall important contribution of the candidate to our understanding of immune responses in invertebrates, I strongly recommend this thesis for oral presentation.

Sincerely,

Elena A. Levashina
Directeur de Recherche au CNRS,
HHMI International Research Scholar

Elena Levashina, Ph.D.



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Strasbourg, April 22, 2009

Report on the Thesis of Veronika Buresova
“Function of the α_2 -macroglobulin protein family in the immune response of the tick *Ixodes ricinus*”

Presented to the University of South Bohemia, Faculty of Science, Department of Molecular Biology and Biochemistry

The focus of this thesis is analysis of functional roles of thioester-containing proteins (TEPs) in ticks. Although, TEPs are playing important roles in immune system of a diverse range of animals, the molecular mechanisms of activation, specificity of binding and exact effector mechanisms are not fully understood. Therefore the topic of this thesis is not only of great medical and economical importance but also provides a significant step forward in our understanding of molecular evolution and function of this intriguing protein family. The written report of the thesis is presented in two major chapters: Introduction and Results and Discussion, and is based on 3 major projects: (i) Characterization of *Chryseobacterium indologenes* as a model tick pathogen; (ii) Identification and characterization of IrAM – an α_2 -macroglobulin from *Ixodes ricinus* and its role in immunity; and (iii) Unpublished data.

The candidate has done a remarkable amount of skilful work first in development of tools for functional gene analysis, more specifically – the establishment of a bacterial model to study antibacterial immune responses in ticks, and in identification and characterization of new members of the thioester-protein family in two tick species. The work is well written with only few spelling mistakes and is illustrated with excellent diagrams.

The introduction provides a brief general overview of the immune system in invertebrates, with a particular emphasis on the structure and function of thioester-containing proteins. Although the majority of particular features are presented in this part, I would like to discuss with the candidate in more details the mechanisms of activation of thioester-containing proteins, the topic which received lots of interest over the last months.

The first part of Results describes the identification and characterization of the Gram-negative bacterium *C. indologenes*, which efficiently infected and killed two distinct tick species that showed differential susceptibility to the same microbe. Moreover, the candidate demonstrated that several immune reactions, including the phagocytosis, were required for efficient elimination of the bacterium, therefore establishing a powerful model for analysis of immune responses in ticks. The results of this part were published in the Journal of Invertebrate Pathology in 2006, and the candidate is the first author of this publication.

The second part of the results builds on the established model of infection and addresses the role of the tick α_2 -macroglobulin (IrAM) in phagocytosis in the hard tick *I. ricinus*. First the primary structure of IrAM was established and revealed the presence of an alternative splicing of the “bait region”, a feature found in *Drosophila* and *Aedes* insects. The candidate then developed an RNAi-based approach to inactivate expression of IrAM and demonstrated the function of this protein in phagocytosis of *C. indologenes*. Interestingly, efficiency of phagocytosis was also dependent on the bacterial metalloprotease, which is believed to be required for crossing of the tick midgut epithelium. The results of this part were published in 2009 in the Journal of Developmental and Comparative Immunology, and the candidate is the first author of this paper. I would like to discuss with the candidate the potential mode of action of this bacterial virulence factor and its interaction with IrAM.

Elena Levashina, Ph.D.