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**PhD thesis submitted by RNDr. Daniel Růžek**  
**-Review report-**

To whom it might concern:

I was asked to review the PhD thesis entitled “Molecular aspects of epidemiology and pathogenesis of tick-borne encephalitis” submitted by Mr. RNDr. Daniel Růžek and to give report.

The thesis consists of 106 pages including an introduction and literature review to the research field at the beginning, an experimental section with six manuscripts forming an accumulative thesis, the proposed future lines of research and a summary. Included were also the CV of the PhD candidate and a list of recent publications and given presentations.

The introduction and literature review gives a good and comprehensive overview on the research field. Even though tick-borne encephalitis (TBE) has already been described in 1931 and knowledge concerning the TBE virus structure, molecular biology, ecological interaction and vaccination has increased enormously since then, there are still important questions remaining. Some of these questions have been clearly addressed in the presented thesis. The thesis focused on the (i) investigation of the molecular epidemiology of TBE virus; (ii) development of a rapid and simple multiplex RT-PCR for subtyping; (iii) comparative study of the susceptibility of different tick cell lines derived from vector and non-vector ticks; (iv) investigation of the microevolution of the TBE virus in laboratory animals and cell cultures; (v) studies on the pathogenesis of TBE by analysing the role of the host immune system and investigating the interaction of TBE virus with human neural cells.

Six well written manuscripts with sounding methodology, described in sufficient detail without the necessity for the reader for further consultation, are assembled in this thesis. 4 manuscripts have been published or are revised for International peer-reviewed journals with high impact and 2 manuscripts are un-



published to date. Doubtless the presented results are a significant contribution to the described field of research.

#### Manuscript 1 (Chapter II.1):

Although this unpublished manuscript will need some small modifications in the Material & Method part, spelling and structuring, it reveals new and important results concerning the phylogenetical and evolutionary changes within the viral E gene that are discussed with respect to the epidemic activity of the TBE virus. Also, conservative and semiconservative patterns of the 3' untranslated region were analysed suggesting functional importance of this genome region for virus growth and/or circulation under natural ecological conditions. Important to mention that the study presented here was the largest and most complex molecular epidemiological analysis of TBE by studying 34 TBE virus strains isolated in the Czech Republic during the period from 1948 to 2002.

#### Manuscript 2 (Chapter II.2):

In this manuscript the authors have developed for the first time a multiplex RT-PCR being able to discriminate between the different TBE virus subtypes: European, Siberian and Far Eastern. The assay is based on the unique combination of oligonucleotide primers which target the subtype-specific "signature" positions of the E-protein region in the TBE virus genome. The different subtypes are associated with different severity of the disease. Even though there is no specific drug treatment for the different subtypes available until today this method could be valuable for the early differential diagnosis of TBE infection in patients who present with a febrile illness following a tick bite. This is particularly true for those living in or coming from regions where more than one tick-transmitted disease is endemic and/or more than one TBE virus subtype are co-circulating. Moreover, the developed multiplex RT-PCR can be useful in studies focused on the molecular epidemiology of TBE virus to monitor the circulation of the subtypes in different areas of Europe and Asia.

#### Manuscript 3 (Chapter II.3):

In this manuscript Růžek et al. carried out a comparative study on the susceptibility of different tick cell lines to infection with TBE virus. This was done to answer the important question concerning the potential vectorial capacity of different tick species which have not been reported to transmit the virus as the principal ixodid vector.

#### Manuscript 4 (Chapter II.4):

This is the first study demonstrating that virulent and attenuated viruses may co-exist as quasispecies in the same TBE virus population and rapid conversion of neurovirulence during virus tick/mammal adaptation is mediated by selection from the quasispecies population rather than random mutagenesis during virus passage in the laboratory. Host-range adaptation is associated with mutations in the non-structural NS2B/NS3 proteins, with one mutation mapping close to the catalytic site of the virus serine protease. The presented results are an important contribution for the understanding of the molecular basis of TBE



virus circulation in natural foci and induction of population immunity in endemic regions.

Manuscript 5 (Chapter II.5):

This revised manuscript presents a study which expands the current knowledge of the antiviral immune response during TBE infection and the understanding of the TBE pathogenesis. It could be demonstrated that CD4+ T-cells may have some immunomodulating function while CD8+ T-cells play a role in the immunopathology. However, in the absence of an immune response the virus itself might induce direct damage. The results could provide the basis for a rational therapeutic strategy against TBE. The methodology described in this manuscript is very sophisticated.

Manuscript 6 (Chapter II.6):

This unsubmitted manuscript describes for the first time the application of human neural cells in TBE research and shows (i) that TBE virus replicates more efficiently in neural cells than in cells of extraneural origin; (ii) different modes of ultrastructural changes in neuroblastoma and glioblastoma/ medulloblastoma cells; (iii) apoptosis as well as necrosis of neural cells following TBE virus infection. Important to mention that the described methodology is very ambitious.

Following I will answer the questions recommended by the Committee for PhD studies in Molecular and Cell Biology and Genetics, Faculty of Science, University of South Bohemia for the review of this PhD thesis:

- 1. Is an original intellectual input of the student apparent from the thesis?**

Clearly yes, according to the presented results the PhD candidate has done a very good work. He has performed important contributions and improvement of methodology in the area of TBE virus research which has been also acknowledged in International peer-reviewed journals with high impact.

- 2. In the referee's opinion, did the student work independently in his/her experiments as well as in their interpretation and writing?**

I know the PhD candidate very well from conferences, laboratory visits and as reviewer of some of his published manuscripts. So, I can definitely answer this question with yes.

- 3. Is the project original and what is its main outcome?**

Yes, the project done provides answers to remaining questions in TBE research and definitely expands our knowledge in the molecular aspects of epidemiology and pathogenesis of TBE as indicated above in the commentaries for the presented manuscripts.

4. Would this thesis earn PhD at the referee's own Institution? – Please, answer Yes or No.

Yes

5. How would the referee evaluate this student relative to all PhD candidates he/she has previously known: among top 10%, among top 25%, within the top 50%, among the worst 20%?

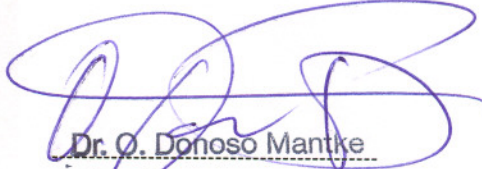
Among top 25%

6. Please, do not grade the thesis, only state whether or not it meets criteria for PhD.

This thesis would meet all criteria to earn a PhD in Germany and probably would receive a excellent grade.

25.09.2008

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Berlin at



Dr. O. Donoso Mantke  
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Signature

**Nationales Konsiliarlabor f. FSME  
am Robert Koch-Institut  
Nordufer 20  
D-13353 Berlin**



Dear Mr. Jindra,

Please find my referee report for the Dissertation Thesis of Mr. RNDr. Daniel Ruzek for the degree of Philosophiae Doctor attached to this letter. Mr. Ruzek carries my strongest recommendation.

Best regards

Dr. Christian Beuret

**Referee**

**Christian Beuret**

Molecular biologist / Virologist, Dr.sci.med.

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## Referee Report

Applicant Mr. RNDr. Daniel Ruzek  
Biology Centre, Institute of Parasitology  
Academy of Sciences of the Czech Republic  
University of South Bohemia

Thesis Molecular aspects of epidemiology and pathogenesis of TBE

I first met Mr. Ruzek at our Institute in may 2008, as we invited him to give a presentation about his research topics. I was really impressed by his work and his publications (already published, in press or submitted) research papers and these, together with his presentation, clearly demonstrate to me his research potential. The reason for contacting the TBEV-working group, where Mr. Ruzek is doing research, is that we actually have an ongoing research grant supporting a PhD-student conducting a national tick screening for various tick-borne diseases in Switzerland. Mr. Ruzek was a precious help for setting up our TBEV diagnostics.

Mr Ruzek's written dissertation proposal is very professional; it is detailed and well thought out and presented. The English writing is well, and has a confident grasp of the pertinent literatures.

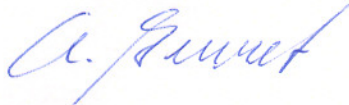
The work performed covers many aspects of TBEV research. The use of a unique sample collection over 50 years in Central Europe delivers an unrivalled insight in the surprising minor molecular evolution of TBE viruses. The outcome is a solid basis for the second work, the designing of a multiplex RT-PCR diagnostics. Classical virology work was covered by the comparison of different tick-cell lines and for the first time in TBEV research, extensive experiments in human neural cells revealing very interesting data.

The study of a defined TBEV strain with peculiar biological properties to postulate a theory for the emergence of highly virulent variant out of circulating quasispecies instead of new mutants opens new strategies for the understanding of emergent viral diseases. The final

unique study of the pathogenesis of TBE reveals the detrimental role of the host's immune system mediated through CD8 T-cells, the massive cytopathic changes and the atypical involvement of both, the necrotic and the apoptotic mechanisms for cell death.

The final chapter proposing future research underlines Mr. Ruzek's widespread understanding in molecular epidemiological and immunopathological research and rises some very interesting questions. Mr. Ruzek carries my strongest recommendation for the degree of a Philosophiae Doctor (PhD.).

Best regards



Dr. Christian Beuret

### **Referee**

#### **Christian Beuret**

Molecular biologist / Virologist, Dr.sci.med.

Federal Department of Defence,  
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# **RNDr. Daniel Růžek: Molecular aspects of epidemiology and pathogenesis of tick-born encephalitis**

Presented PhD thesis consists of an introductory part, three already published papers, two submitted and/or to be submitted manuscripts and one unpublished paper.

## **General comments:**

The introductory part is well arranged, readable and gives nice and comprehensive introduction to the TBEV field. If I try to raise at least some criticism, I would say, that a reader would appreciate a list of used abbreviations as well as some short introduction or a commentary preceding each manuscript included. I also understand that all the papers contain their own discussion, nevertheless, in my view, a more detailed discussion presenting all the experimental data framed into the current knowledge of the topic would be advisable and should not be simply substituted with just a short note on future prospects in TBEV research within the authors home research group. However, author probably followed a common faculty practice and recommendation.

## **Specific comments:**

Three of the manuscripts presented, had been reviewed in the reputable scientific journals in a course of their acceptance. One manuscript has been already resubmitted after revision. Reviewer of the thesis thus has a quite easy job because most of the work has already been done by others. I will list my specific comments according to the corresponding chapters.

## **Introduction:**

- p2: Can author explain or hypothesize why there is such a high increase of the tick-born encephalitis incidence followed with remarkable decrease in mortality after 1945 year?
- p6-7: From the introductory note, it seems to be clear that a route of the TBEV production in insect cells differs from that in mammalian cells and that these life-cycle-differences may also influence the virion surface formation. However, according the last Daniel's manuscript: "Morphological changes in human neural cells...", at least in human UKF-NB4 neuroblastoma cells a substantial fraction of the TBEV virions can be seen free in cytoplasm, like in insect cells. Daniel also shows in other his manuscript that virus adaptation from insect to mammals occurs mainly by selection of pre-existing quasispecies, which can be more virulent than the parental strains. The question is, if there is any difference in an infectivity and ability to enter the host cells when comparing transmission between homologous hosts versus transmission between heterologous hosts, that means between insects and mammals and *vice versa*. Did author observe any kind of budding on cytoplasmic membrane in neuroblastoma cells?
- p7: Author summarizes taxonomy of TBEV within the introductory chapter and touches this issue several more times throughout the work. Can author say his opinion about the vaccination against TBEV, summarize differences and similarities of the vaccines used in the Czech Republic and discuss a possible degree of protection against other than European TBEV subtypes or even against other similar flaviviruses?
- p8: Taking into the account the presented data, I cannot agree with the author's explanation of the remarkable increase of TBE incidence from the beginning of nineties. Instead of proclaimed impact of the global raise of the temperature I would rather connect - at least in the countries of the former socialist block - the increased number of TBE cases to the political changes and resulting changes in farming, forestry and gamekeeping. The



reason is that: 1<sup>st</sup> it is curious that global warming shows such a strong effect suddenly from the beginning of nineties when destruction of the former socialist block has occurred, 2<sup>nd</sup> the highest TBE incidence in the Czech Republic was reported in 2006 year, when we experienced exceptionally cold and long winter as can be seen from the data of the Czech Hydrometeorological Institute – see below a mean temperatures in the South Bohemia region in winter 2005/2006 taken from the CHI website (<http://www.chmi.cz/meteo/ok/indexe.html>)

Mean temperatures in winter 2005 / 2006 compared with the long-term normal 1961–1990

	November 2005	December 2005	January 2006	February 2006	March 2006
T	1,4	-2,0	-6,2	-3,0	-0,1
N	2,4	-1,2	-2,8	-1,3	2,3
O	-1,0	-0,8	-3,4	-1,7	-2,4

T: Monthly mean air temperature (° C)

N: Long-term normal 1961–1990 (° C)

O: Deviation from long-term normal (° C)

Would it be possible that higher incidence of TBE correlates rather than with global warming with increased number of reservoir hosts and their reduced fitness after the hard winter?

**Růžek D *et al*, 2008, Molecular epidemiology of tick-borne encephalitis virus in Central Europe (since its discovery up to the present, 1948-2002)**

The manuscript is focused on comparison and classification of TBEV strains according their differences in protein E and 3'-UTR region. I agree with author that other regions, especially in the connection to his own work on NS2B/NS3 region, should be sequenced and analyzed, as announced in the future prospects chapter. One of the current conclusions of the study is that TBEV exhibits minor genetic variations within a period of 50 years in the region of the Czech Republic. All the isolates underwent at least one passage on suckling mouse brain. However, author's own results published in Růžek D. *et al*, 2008, *Virology* 374, 249-255 show that just one round of virus propagation in brains of suckling mice can establish stringent selective pressure. Could author discuss that briefly?

**Růžek D *et al*, 2008, Rapid subtyping of tick-borne encephalitis virus isolates using multiplex RT-PCR. *J Virol Methods*. 2007 Sep;144(1-2):133-7. Epub 2007 Jun 4.**

My major concern is that the method was tested only on the very limited number of isolates from all three major subtypes. Did author or somebody else use it really for TBEV subtyping later on? Was it tested on more isolates? If so, did you use a complementary method for TBEV subtyping? If so, how was the specificity of the suggested test when challenged by the well-established ones?

**Růžek D *et al*, 2008 CD8+ T-cells mediate immunopathology in tick-borne encephalitis.**

This is really an excellent study shedding new light on the pathology of TBEV. Description of panel E, Fig 1 is missing. According panel D it seems that high-virulent

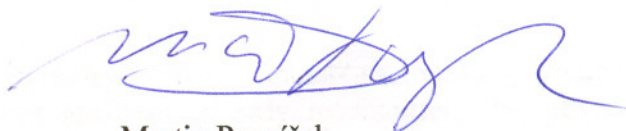
strain of TBEV was used. If so, what would have happened if low-virulent strain had been used in the SCID+CD4 experiment?

**Růžek D *et al*, 2008, Morphological changes in human neural cells following tick-borne encephalitis virus infection.**

In this manuscript Daniel shows that TBEV replicates more efficiently in human neural cells than in extraneural cell, which, he says: "...helps to explain why the virus tends to involve infection of the CNS rather than any other organs." I would expect that such a virus population should ensure that new biting ticks can be infected. Can author explain or hypothesize how the TBEV virions can get back from the brain to the bloodstream?

Daniel Růžek presents an excellent work containing important data of general interest. All of his already published manuscripts appeared in the respectable scientific journals. I was really delighted to read Daniel's thesis and fully recommending it without any hesitation to be accepted as his PhD dissertation.

September 30, 2008



Martin Pospíšek