National Institute of Allergy and Infectious Diseases

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Evaluation of `s Ph.D. thesis

In this letter, I give my assessment of the Ph.D. thesis by M.Sc Sazzad Mahmood, titled "Exploration of the tick-Borrelia molecular interactions by employing the transcriptomic approaches".

The thesis presents original research results in the area of ticks and tick-borne diseases. It focuses on differential gene expression in *Ixodes ricinus* salivary gland and midgut, induced in the nymphal stage upon infection by *Borrelia afzelii*. Genes that were regulated upon infection were further validated by quantitative PCR and their biological importance for tick feeding and *Borrelia afzelii* transmission was evaluated either by RNA interference or vaccination trials.

M.Sc Sazzad Mahmood`s thesis is comprised by a first-authored paper published at Frontiers in Immunology and a second co-authored paper published at Scientific Reports. Undoubtedly, research presented here has quality and fills a gap trying to understand the modulation of tick gene expression upon pathogen infection. In summary, my conclusion is that the PhD thesis of M.Sc Sazzad Mahmood presents original research results of large importance and I recommend without hesitation the thesis for the defense. Below I give a few suggestions of what might have been improved or would in my opinion deserve more attention in his thesis.

In general, the thesis is written well, with occasional minor grammatical and spelling mistakes. These are, however, minor issues which can be easily corrected.

1. General Introduction

Second paragraph, last sentence: "the most predictable indicator of a dense tick population is the presence of many deer in the forest". This is true for some tick species, not for all.

Third paragraph, first sentence: please spell out EU.

Third paragraph, fourth sentence: "and uses an anticoagulant to prevent the clotting of blood". Although we do not know the exact dynamics of secretion, it is most likely that more than one molecule is secreted to modulate the blood coagulation, e.g inhibitors of platelet aggregation, inhibitors of factor Xa, thrombin, factor XIa, etc.

Fourth paragraph, fourth sentence: "cement-like" instead of "cement" like. Please check through the thesis.

1.1 Midgut impacts on Borrelia

First paragraph, seventh sentence: albumin and hemoglobin are not the unique proteins ingested during feeding.

1.2 Salivary gland as the exit point

Second paragraph, second sentence: "production of the cement cone" instead of "the cement protein".

Fifth paragraph, second sentence: the study by Kern et al., 2011 does not describe immunomodulatory properties of the cement cone. Similarly, the next sentence, studies by Mans, 2013 and Kern et al., 2011 are not the most appropriated. Mans, 2013 is not listed at References. I would suggest going through the thesis and review if all references are listed at References.

Fifth paragraph, fourth sentence: there is an extra space when referencing Chinery, 1993.

Fifth paragraph, fifth sentence: would be important to expand the discussion about the role of glycine-rich proteins for the cement cone formation. Please see:

https://pubmed.ncbi.nlm.nih.gov/29258831/

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2901319/

2. Tick as a disease vector

For a thesis, would be important to describe the importance of ticks as vectors of pathogens in a historical and chronological perspective, e.g describing the identification of the link between ticks and *Babesia bigemina* (back in 1893). If description specifically of *Ixodes* ticks is desired, I would recommend adjusting the text accordingly.

As the thesis focuses on *Ixodes ricinus* and *Borrelia afzelii*, would be informative including a little more about the biology and wildlife cycle of these organisms in Europe (e.g natural reservoirs, enzootic cycle, seasonality, etc).

First paragraph, second sentence: reference is italicized.

Fourth paragraph, second sentence: reference is italicized.

Sixth paragraph: "*Borrelia miyamotoi*, a relapsing fever spirochete, was first described in *Ixodes persulcatus* in Japan (Fukunaga et al., 1995). In 2001, the ability of *I. scapularis* to transmit *B. miyamotoi* while feeding, and to pass spirochetes transovarially, was demonstrated under laboratory conditions (Fukunaga et al., 1995). A decade later, *B. miyamotoi* was recognized as a human pathogen in a report of 46 cases from Russia (Fukunaga et al., 1995)." It is stated "A decade later" but is referencing the paper from the same year as stated in the previous reference.

3. Lyme Disease

Second paragraph: "erythema migrans" instead of "Erythema migrans"

Fourth paragraph: "*Ixodes ricinus*" instead of "*Ixodes Ricinus*"

Histopathology

Missing an important contribution on the field:

https://pubmed.ncbi.nlm.nih.gov/29099929/

Would be better to introduce a sentence before starting the description of Stages 1-3.

3.4 Evaluation

"Treatment can be started as the first preference without a proper diagnosis if the patients are from an endemic area or display the classical EM rash". This is not consensus among doctors, even from endemic regions.

5.2 Borrelia transmission:

"On the other hand, *B. afzelii* establishes the colony in the tick midgut with the help of a protein-protein interaction involving the tick midgut protein TROSPA and the spirochete lipoprotein OspA (Figlerowicz et al., 2013)." Would be important to discuss (if know) about the dynamic expression of OspA, OspB and OspC homologs in *B. afzelii* during tick feeding.

Table 1 and Table 2 are missing important studies published on this topic.

Article 1

How is the dynamic of expression of OspA, OspB and OspC homologs in *B. afzelii* and TROSPA in *Ixodes ricinus* during tick feeding?

Ticks used in this study were collected from the field. How do you control for the presence/absence of other pathogens?

How many ticks were tested to check the percentage of infected nymphs?

Why biological replicates were not used for the MACE analysis? Did the collected RNA from 150-220 nymphs was enough for library preparation? If so, why not dividing these ticks in three replicates?

How DEGSeq deals with data without replicates?

Some genes selected for RNAi are upregulated at the unfed stage (Contig_7059 and 30818). How did you check for the protein translation turnover in these unfed ticks?

Article 2

This is stated at the introduction: "In spite of this, it has been shown in a mouse model that *B. afzelii*-infected ticks need to feed for longer than 24 h to establish infection. *B. afzelii* is presumably transmitted through the saliva of the feeding tick, although alternative routes of infection have been proposed."

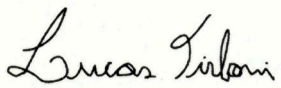
However, on the thesis introduction it is stated: "Transmission of *B. burgdorferi* through the tick salivary gland is a well-established and proven route (Kurokawa et al., 2020). But there is another unconfirmed hypothesis in the case of the *I. ricinus* / *B. afzelii* system. This route was suggested by Pospisilova et al. (2019) for *B. afzelii* transmission and was called as 'active reverse migration'. Here the Borrelia travels from the tick midgut to the mouthparts for transmission instead of crossing the midgut barrier and invading the salivary gland. This theory was strongly supported by the lack of any trace of Borrelia in the salivary gland by microscopy over the different feeding stages." Could you please clarify this?

Two times on the text is referenced this study as "an unprecedent" source of information for the transcriptome of *Ixodes ricinus*. How about the studies by Perner et al., 2018, Kotsyfakis et al., 2015, Schwarz et al., 2014 and 2013?

"Ideal vaccine candidates are highly conserved and expressed in multiple biological replicates, which is the case for the three selected transcripts". How about being expressed when ticks are feeding on different hosts? Specially the target for vaccine development and not only on animal models.

What are your thoughts correlating the lack of protection when immunizing hosts against the lipocalin (gene 2) and the abundance and/or redundance of lipocalins during tick feeding?

Sincerely,



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6th March 2021

Re.: Report for Ph.D. Thesis by Sazzad Mahmood, University of South Bohemia (Faculty of Science), Czech Republic - "Exploration of the tick-*Borrelia* molecular interactions by employing the transcriptomic approaches"

Dear Dr Doležal,

This PhD thesis by Sazzad Mahmood explores the molecular interactions between *Borrelia afzelii*, the main agent of Lyme borreliosis in Europe, and the sheep tick *Ixodes ricinus*, its principal vector. The studies presented therein combine transcriptomic approaches of gene expression in key tick tissues with RNA interference of upregulated transcripts in the tick midgut, vaccination against tick salivary proteins in mice, and assessment of these interventions on *Borrelia* infection success in the mammalian host.

The presented studies are highly novel in analysing *B. afzelii* rather than *B. burgdorferi s.s.* in the tick vector and constitute important steps forward in identifying prophylactic or therapeutic targets for Lyme Disease control. The experiments are rigorously performed and appropriately interpreted for the most part.

I have no hesitation in concluding that this thesis would have met the criteria for award of a PhD in the British system even its format is quite different to that of a British thesis. While we do not grade PhD thesis in the UK, it is likely to have passed with minor corrections only.

Yours sincerely,

Ben Makepeace.

Dr Ben Makepeace

UNIVERSITY OF LIVERPOOL

Examination questions for Sazzad Mahmood.

1. On p. 7, it is stated that "In the following year, for the first time in Europe, morphologically and antigenically similar spirochetes were detected in *Ixodes dammini* (Burgdorfer, 1984)." Please clarify this statement. I do not believe it is correct as *I. dammini* is a synonym for *I. scapularis*, which is only found in the US.
2. On p.7, it is stated that "In 1996, *I. scapularis* was experimentally confirmed as a vector of *E. phagocytophila*, and *P. leucopus* was shown to be a competent reservoir (Mather et al., 1989)." I believe the reference here is incorrect.
3. On p. 11, the candidate refers to "Other *Ixodes* 'subspecies' as vectors". In fact, several tick species can transmit LD in Europe. Please list these.
4. The introduction focuses heavily on human diseases transmitted by *I. scapularis* in the US. However, *I. ricinus* is the vector of *B. afzelii*. Which other human diseases are vectored by *I. ricinus*?
5. The other main agent of LD in Europe is *B. garinii.* How does the epidemiology and pathogenesis of this agent of LD differ from that of *B. afzelii*?
6. On p. 16, it is stated that recent research has challenged the view that *B. afzelii* is transmitted via the salivary glands. Is there any evidence that *B. afzelii* interacts with Salp15? If *B. afzelii* does not infect SG, how does this affect the conclusions of the Trentelman *et al.* paper?
7. Which bacterial symbiont is found in most *I. ricinus* individuals? Where is it located in the tick?
8. In both publications, can the candidate offer reasons why biological validation failed for most transcripts?
9. Does the candidate have suggestions regarding how "contig 30818" from Mahmood *et al.* could be further characterised?
10. In the Trentelman *et al.* paper, was folding of the recombinant vaccine candidates examined? If not, how could this be done?
11. Why was Freund's adjuvant chosen for the vaccination experiments? What type of immune response does this adjuvant tend to generate? What other adjuvants could be evaluated?
12. In the Conclusion on p. 80, it is stated that "The experimental design for the salivary transcriptome had only a single time point at 24 hours after starting nymphal feeding (24h)." The Trentelman paper seems to present transcript data from fully-fed SG too. Please clarify.
13. In the Conclusions on p. 81, it is stated that "In our study, we have shown that knock-down of cytochrome p450 induced the transmission of significantly more *Borrelia* in murine ear at the third week of tick repletion". This does not seem to tally with the data presented in the Mahmood *et al.* paper. Please clarify.