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### Review

of MSc. Mulugeta Aseratie Wondim's doctoral dissertation "Detection and Molecular Characterization of *Babesia* spp. and Tick-Borne Encephalitis Virus in Ticks from Poland and Ethiopia" accomplished in the Department of Infectious Diseases and Neuroinfections, Faculty of Medicine with the Division of Dentistry and Division of Medical Education in English, Medical University of Białystok, and supervised by Professor Anna Moniuszko-Malinowska (MD, PhD) and Associate Professor Justyna Dunaj-Małyszko (MSc, PhD)

Ticks are one of the most important vectors of human and animal infectious diseases worldwide. In comparison with other arthropods, they are characterized by a diversity of transmitted pathogens and a high ability to spread quickly and adapt to changing environmental conditions. The tick range expansion creates the need to monitor the threat posed to human and animal health by attacks of these arthropods in different regions of the world. For this reason, the PhD research on the Ixodidae fauna collected from vegetation in Central Europe and East Africa and on the transmitted tick-borne pathogens (TBPs) is relevant and important from both a scientific and practical point of view. These investigation results will help to identify tick species occurring in the study area and determine the frequency of occurrence of TBPs transmitted by these ticks. Such knowledge is indispensable for development

of optimal tick prevention methods and strategies for protection of animals and humans against the effects of tick parasitism, including tick-borne diseases. Particularly noteworthy is the fact that Professor Anna Moniuszko-Malinowska (MD, PhD)

and Professor Justyna Dunaj-Małyszko (MSc, PhD) the supervisors of the dissertation, are highly valued specialists in research on diseases transmitted by ticks and in development of standards for diagnosis of tick-borne diseases.

The dissertation submitted for evaluation is written in a very good style and has a clear layout typical for this type of work. It consists of 11 well-edited chapters, most of them with separate sub-chapters, all constituting a dissertation with high aesthetic value. The work has 99 pages and contains 12 tables and 16 figures.

In the first "Introduction" chapter, the Author synthetically presented the most important issues of tick ecology and biology (Acari: Ixodida: Ixodidae). The Candidate also described the morphological features of some Ixodidae representatives that are important for the systematics of these arthropods. He also reviewed the recent data on the pathogenesis, clinical features, and epidemiological status of babesiosis and tick-borne encephalitis in Europe (including Poland) and Ethiopia, where the research was conducted. Additionally, the Candidate provided information about the prevention and control of the presented tick-borne diseases. The content of this chapter perfectly introduces the reader to the subject of the doctoral dissertation and proves MSc. Mulugeta Aseratie Wondim's extensive knowledge of ticks and selected TBPs investigated during the PhD study.

In the second chapter "Aim", the Author precisely defined the following three research goals:

1. PCR-based detection and characterization of *Babesia* spp. and TBEV in ticks collected from Poland and Ethiopia,

3. Analysis of the potential predictors (temperature, humidity, developmental stage, and species) of detection of tick-borne pathogens in ticks collected from Poland and Ethiopia.

In the third chapter "Material and methods", the Author presented the rationale behind the choice of the study areas and characterized their topography, nature, and climate. The method for collecting ticks from vegetation (flagging method/dragging method) selected by the PhD Student is commonly used in this type of research around the world. During the collection of ticks in the field, the Candidate measured the air temperature and relative humidity in the habitats (please provide information about the equipment used for measurements and the accuracy of measurements of both parameters). The laboratory tasks consisted in morphological analyses of collected specimens, isolation of DNA and RNA from collected ticks, detection of DNA and RNA of selected tick-borne pathogens in the isolates (PCR and RT-PCR), and sequencing of amplicons. The analysis of the information presented in this chapter proved that the PhD student actively participated in all stages of the research process.

The sequences obtained by the Candidate were compared with sequences collected in the NCBI database and checked for homology and cover index.

In total, 995 ticks were collected (727 from Poland and 268 from Ethiopia). Eighty-five (9.51%) ticks out of 894 ticks tested for the presence of *Babesia* spp. were positive. 9.74% (N=61) of the 626 ticks from Poland and 8.96% (N=24) of the 268 ticks from Ethiopia were positive for *Babesia* spp.

In turn, 1.17% (N=7) of the 601 ticks were coinfecting with both *Babesia* spp. and TBEV, while only one of the TBPs mentioned was detected in 14.31% (N=86) of ticks. The prevalence of *Babesia* spp. in ticks has shown a staggering pattern. The Author found that the overall prevalence of *Babesia* spp. in *I. ricinus* and *D. reticulatus* was 9.51% (9.59% and 9.83%, respectively). The sequencing analysis of *Babesia* spp. amplicons revealed *B. microti* in 70.59% (60/85) of the samples with a mean homology of 87.56%, which is in the range of 82.29-100%. In addition to *B. microti*, *Theileria velifera*

8.24% (7/85), *B. capreoli* 4.71% (4/85), *B. venatorum* and *B. canis* - each 3.53% (3/85), as well as *Theileria mutans* 2.35% (2/85) were also detected via sequencing.

As demonstrated by multivariable logistic regression, *I. ricinus* was 93.7% less likely to harbour TBEV (1/291) compared to *D. reticulatus* (38/453) ( $p= 0.007$ ). Adult ticks (OR=23.66) were more likely to be infected with TBEV ( $p< 0.001$ ). Ticks collected during an ambient temperature of 15-17°C were 95.8% less likely to harbour TBEV than those collected at an air temperature lower than 15°C ( $p< 0.001$ ).

All TBEV detected in *I. ricinus* and *D. reticulatus* from eastern and north-eastern Poland showed a homology of 165/167 (98.8%) similarity with the tick-borne encephalitis virus Hypr polyprotein gene, complete cds Sequence ID: U39292.1 (European subtype).

Co-infection of *Babesia* spp. with other TBPs may hamper accurate diagnosis, resulting in inappropriate treatment of tick-borne infections. Monitoring co-infections in ticks is also advisable because it is postulated that tick-borne infections are polymicrobial.

In the "Discussion" chapter, the Author compares his research data with the results obtained by scientists in various regions of the world. This chapter is a comprehensive and state-of-the-art analysis, with the Author demonstrating his knowledge of issues in the field of tick biology as well as the environmental determinants and epidemiology of tick-borne diseases. The Author's great caution in evaluating the results of his research and pointing out all study limitations is noteworthy.

The seventh chapter of the dissertation is a summary presenting all the necessary information about the purpose, methods, and results of the research as well as conclusions. It is followed by "Funding and ethical consent disclosures" (chapter 8).

The **Conclusions** chapter is a synthetic summary of the main achievements of the study presented as three well-grounded conclusions corresponding to the objectives of the dissertation.

The **bibliography** comprises 177 English-language papers related to the topic addressed in the dissertation and published in reputable scientific journals. The references include the most important publications on the investigated problem. The study closes with a List of tables (chapter 10) and a List of figures contained in the dissertation (chapter 11).

As a reviewer, I would like to point out some inaccuracies I came across during the analysis of the dissertation, which should be corrected during the preparation of the results for publication in a scientific journal.

#### Title, Aims

- It needs to be clarified in the title and objective 1 that the tick testing for TBEV applies only to specimens collected in Poland, which is the endemic area of its occurrence, and the quantitative and qualitative analysis of *Babesia* spp. isolates obtained from the ticks apply to ticks collected in both countries (continents).

#### Introduction

##### 1.2. Tick biology

##### Page 11

- The first sentence should be reworded as it suggests that all ticks belong to the Ixodidae.
- In the case of the given taxonomy, both family and subfamily are Ixodidae. Therefore, please change the subfamily to Ixodinae.
- In most cases, females are larger than males, but this does not apply to all species of ticks, e.g. in the case of *D. reticulatus* usually, the opposite is true. Within tick species there may be large variations in size of the sexes.

- When the main parts of the tick body are mentioned, it should be stated that the larger posterior part of the body containing the internal organs is called the opisthosoma.

#### Page 15

- "Eggs get hatched and moult into larvae with enough blood meal" should be reworded because eggs do not moult. Instead, I suggest: "Larvae develop inside the eggs until they are ready to hatch."
- Moulting is a process taking place after the feeding of larvae and nymphs. After completing feeding, the larvae moult to the nymphs. Afterwards, the nymphs complete feeding and moult to either a female or male.

#### Page 16

Sentences to be reworded:

- Engaged female ticks lay thousands of eggs and die as Ixodide ticks follow a single gonotrophic cycle (22,37).

It should be: Engorged female ticks lay thousands of eggs and die as Ixodidae follow a single gonotrophic cycle (22,37).

- "Mated female ticks ahead of their feeding can take as much as 100 times their size to help lay their eggs." I explain that females of Ixodidae feed once and ingest more than one hundred times their body mass in blood. Please change the word "size" to "body mass in blood".

#### Materials and methods

- In the tick collection section, the device used to measure the temperature and relative humidity of the air during collections and the accuracy of recording ( $\pm$ ) of both parameters should be specified.
- Concerning the identification of the species of collected ticks, please provide information on the systematic key/keys used when classifying the examined specimens into species, developmental stage, and sex.

- "Before extraction of nucleic acid, ticks were kept at 4°C" - to obtain the highest quality of the isolate, it is recommended that the material from which nucleic acids are not isolated within 48 hours should be stored at -20°C or even -70 °C. What was the storage time of the samples at 4°C, and in what form and temperature were the ticks transported from Ethiopia to Poland? In what way may the storage temperature have influenced the results (if at all)?

- As in the case of the species collected in Poland, it would be worth indicating the developmental stages and sex of tick species collected in Ethiopia and the developmental stages and sex of African ticks in which TBPs were detected.

- In the case of Figure 14, the source and information about any modifications introduced by the Author should be provided.

- the following sentence should be moved to the Results or Conclusions section (point 2): "For *Babesia* spp., the difference between ticks of Poland and Ethiopia was conducted however, there was not enough evidence to conclude there was a statistically significant difference between the two countries *Babesia* spp. detection rate"

## Results

- Returning to the subject of tick identification, please indicate the key used for the identification of the species *Amblyomma gamla* and *Amblyomma vergienatum*. Is *Amblyoma gamla* not a mistaken entry for *Amblyomma gemma*, which commonly occurs in Ethiopia?

## Conclusions

Conclusion no 2: The information concerning the detection of *Theileria mutans* and *T. velifera* in ticks from both studied countries is repeated.

## Discussion

- Page 60, sentence "A study has reported 15.53% *B. bigemina* and 6.17% of cattles in southern part of Ethiopia while this study has detected only one tick infected with *B. bigemina* (145)", should be completed as follow:

- "A study has reported 15.53% of *B. bigemina* and 6.17% of *B. bovis* in cattle in the southern part of Ethiopia, while this study has detected only one tick infected with *B. bigemina* (145).

## References

- In the case of reference no. 87, only the second author Gebregergs Tesfamarym is included. Please add the names of the other authors as follows:

Alemu Hamsho, Gebregergs Tesfamarym, Gurara Megersa, and Mulisa Megersa

Concurrently, I would like to emphasize that the comments mentioned above do not diminish the value of the dissertation.

Summing up the assessment of the thesis, I conclude that the aim of the doctoral dissertation has been outlined by the PhD Student clearly and is consistent with the content of its individual parts organized with a clear and logical approach to the investigated research problems. The choice of the research methodology proves the Candidate's ability to use relevant techniques to achieve the goals. Mr. Mulugeta Aseratie Wondim also showed a very good knowledge of the scientific literature covering the issues discussed in the dissertation. The assumed aims of the study have been fully achieved, and the conclusions from the experiments presented by the Author concisely in three points correspond to the goals.



## **Final conclusions**

I hereby state that MSc. Mulugeta Aseratie Wondim' doctoral dissertation submitted for the review meets all requirements specified in Art. 187 of the Act of 20 July 2018 Law on Higher Education and Science (i.e. Journal of Laws of 2022, item 574). Therefore, I recommend that the Senate of the Medical University of Bialystok should admit MSc. Muluget Aseratie Wondim for the subsequent stages of the doctoral proceedings.

Associate Professor Katarzyna Bartosik (MSc, PhD)

*Katarzyna Bartosik*

