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The review of the PhD thesis entitled „The role of *sanA* in *Salmonella* pathogenicity”

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Assistant supervisor: dr Rafał Kolenda

Formal basis: Uchwała Rady Naukowa Dyscypliny Weterynaria Uniwersytetu Przyrodniczego we Wrocławiu (letter No. MDDD0000.4101 of 30th January 2024).

1. Evaluation of the dissertation for compliance with the requirements of Ustawa z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz. U. 2018, poz. 1668, with amendments)

The structure of the presented work is typical for a dissertation based on the publication series. The series includes a single published and another pre-printed manuscript. Both are included in the dissertation and they are preceded by the introduction, research objectives, and hypotheses, and followed by summary and future prospects, and conclusions. The reviewed work contains also information on its structure, abbreviations used, abstracts in English and Polish, as well as citation list, supplementary materials and authors' statements. Altogether fits into 54 pages. Based on the aforementioned legal basis of the review, I assume that the form of the dissertation composed of an article and a pre-print fulfils the criteria for the publication series adopted by the University of Life Sciences in Wrocław.

The introductory chapter confirms the general theoretical knowledge of the PhD student in the field of veterinary sciences, specifically microbiology and molecular biology. It provides concise, but sometimes scarce information on *Salmonella* taxonomy and classification, zoonotic and antimicrobial resistance aspects, as well as factors responsible for pathogenicity and virulence, including the title figure of the studies – SanA protein. One may read also about the US approaches (CDC, FDA) to the gradual elimination of antibiotics from agriculture or antimicrobial resistance of *Salmonella* isolated in India or China (page 14). I wish more focus was given to the epidemiological situation in the EU and specifically in Poland. My concerns on the issue were elaborated further in the polemical part of the review. I appreciate the clear presentation of the complex course of *Salmonella* infection and its numerous factors including SPIs (although their composition across serovars is not as simple as described) and cell wall elements (outer and inner membranes, peptidoglycan, periplasm, etc.).

The addressed research problem refers to the role of *sana* in *Salmonella* pathogenicity. It was further specified in 7 study aims (formulated often as stages of the study i.e. generation of mutants, investigation of the role, etc.). The straightforward aims were expressed in 4 research hypotheses addressing SanA role in the cell membranes properties, their changes following SanA alternation, and the protein role in the infection, via i.e. regulation of the *Salmonella* pathogenicity island (SPI-1) expression. The identified research problem is original of utmost importance. The research outcomes provide relevant insight into one of the numerous factors contributing to *Salmonella* pathogenicity and virulence. My concerns about the congruence of the title and the contents is mentioned in the polemical part of the review.

Considering the above, I confirm that the doctoral dissertation submitted for review meets the requirements of Article 187 of the Act.

2. Evaluation of the dissertation scope and the approach adopted for the solution of the scientific problem

I appreciate the choice of the research problem. The validity of the research is confirmed with the still high incident rate of human salmonellosis and the role of *Salmonella* infections in animals as the major source of the foodborne zoonotic agent. Any insight into the background of pathogenicity of the pathogen is thus of relevance.

The research hypotheses were verified against the outcomes of a set of well-designed in vitro experiments described in both manuscripts. *Salmonella* Typhimurium strain (4/74WT) and its derivate mutants were used as a model. The adopted methodological approach included the generation of *sana* mutants and the evaluation of their features under harsh conditions challenge. I wish some justification for the serovar selection was given.

In the already published paper evaluated by disclosed reviewers, the authors provide experimental evidence for *sana* deletion resulting in increased membrane permeability, hydrophilicity, and enhanced resistance to peptidoglycan-targeting antimicrobials. This was achieved by *S. Typhimurium sana* mutant generation, their phenotype profile analysis and several assays targeting membrane permeability (i.e. ethidium bromide uptake). Furthermore, replication within macrophages was evaluated in the cell culture model. My major complaint refers to the unjustified generalisation of the findings – although *S. Typhimurium* strain was used as a model, the conclusions are formulated for the whole of *Salmonella*, a very diverse genus.

In the second, yet not reviewed manuscript, the Authors go further and evaluate the location of SanA protein and its interplay with pathogenicity island SPI-1. The manuscript is well structured, although the repetition of some information needs to be avoided in the final submission (i.e. study aim in the 1st paragraph of the results chapter; methods in the discussion; review-type description of own previous work in the discussion, interpretative description in the result chapter). Nevertheless, the Authors managed to determine the inner-membrane location of SanA using protein fractionation and hybridisation with the developed specific antibody. Based on the luciferase test they determined the peak of protein expression in bacteria entering stationary growth phase. In human and murine cell line models the authors studied the

course of infection and nutrient-dependence of the process. Finally, they indicate on SanA as a mediator for virulence genes located in SPI-1.

In general, the scientific and cognitive aspects of the dissertation as a whole, outweigh its practical values. From my epidemiological perspective, the latter might be also identified in a far perspective as an indication for technical measures for combat against foodborne intoxication. It is also highlighted by the Author in the final words on future prospects chapter, namely the development of therapeutic strategies, based on the interference into protein biosynthesis of the agent.

The overall picture drawn in both manuscripts offers solid proof for the hypotheses set and the achievement of the goals foreseen from the dissertation.

3. Polemics and critical comments

3.1. Substantive matters

a/ Salmonella taxonomy, differentiation, and source of information

I am dissatisfied with the content of the chapter presenting *Salmonella* taxonomy. Although species and subspecies are accurately described, the concept of serovar is not elucidated after the information on their occurrence in humans and animals. Further, quite ancient differentiation into phagotypes and biovars is mentioned (although the latter categorisation is still valid for some serovars such as Gallinarum – Pullorum). The Author omits i.e. currently used “gold standard” method – sequence type differentiation. Further, the Author underlines the adoption of that nomenclature just by one of the national institutions (CDC). The information on the annual *Salmonella* nomenclature update is fake (if so, why Supplement No 44 dated back to 2001 is cited, and not the most recent one). The citation (#3) is an opinion paper published in 2000, whilst the most recent White-Kauffmann-Le Minor scheme (8th edition, 2008) or some of the later supplements are omitted in the bibliography. I strongly disagree with Fig. 1. content. Even a superficial reading of the article #3 indicates that its content does not refer to categorization into typhoid and non-typhoid serovars, as one might understand from the inclusion of the citation in Fig. 1 title. Further, non-typhoid serovars are neither limited to *S. enterica* subsp. *enterica* nor adapted just for humans (i.e. even *S. Typhi* can infect Primates). Thus, I am confused about the Author’s understanding and differentiation of typhoid *Salmonella* infections in humans and animals and the host-adaptation of certain serovars. Also, for some years already the terms warm- and cold-blooded animals have been replaced with homeotherms and poikilotherms, respectively.

b/ selection of the study object

Although the variety of *Salmonella* is mentioned, the Author does not explain why *Salmonella* Typhimurium was selected as the study model. The direct transposition of the results and conclusions from just one serovar into the whole genus seems to be inappropriate. Such over-interpretation is consequently pursued in the narrative part of the dissertation, and both of the manuscripts. In all cases, it leads to the incongruence of the title and the contents. Therefore, I strongly suggest modification of the 2nd manuscript title before submission for publication. In my opinion “SanA is an inner membrane protein mediating early stages of *Salmonella*

Typhimurium infection” reflects better the content. The thesis title should therefore read as follows: “The role of *sanA* in *Salmonella* Typhimurium pathogenicity” or “The role of *sanA* in *Salmonella* pathogenicity – *Salmonella* Typhimurium model”. Consequently, all the conclusions should refer to *S. Typhimurium*. Since the protein is “the hero” I wonder if “SanA” is even more appropriate.

c/ recognition of the epidemic situation in the region

I hardly understand the reasons why the thesis refers to overseas data and does not mention European ones. Does it mean Europe, including Poland, is so favourable that *Salmonella* does not occur in livestock and humans? Is the problem of ESBL-producing bacteria relevant only for China (citation #25). I do believe the EU approach to antimicrobial prudent use is more restrictive than FDA rules. I do not agree that a decrease in *Salmonella* incidents has been observed since 2008. Those discrepancies need to be elucidated briefly during the public defence.

3.2. Technical remarks

My first remark refers to the terminology used throughout the dissertation, the paper and the pre-printed manuscript. Although it is not relevant in common sense, the academic perspective does not allow for alternative use of the terms “antibiotic” and “antimicrobial”. The first frame does not cover sulphonamides occurring throughout the thesis, also quinolones are questionable. I strongly argue for the consequent use of the term “antimicrobial”.

In my opinion, the typesetting and dissertation text makeup compromise commonly accepted rules. The chapters should start from odd pages. Page numbers should be given at outer page margins or centred. Although “orphan letters” are not considered typographic errors in English, the rule should not be neglected in Streszczenie.

The horizontal layout of table S1 is neither convenient nor results from the width of the columns.

The abbreviations introduced in the list should be either consequently used throughout the dissertation or deleted as redundant. As far as I noticed “Amp” has never been used in the body of the thesis, except for the paper already published in *Frontiers in Microbiology*. Instead, the full term “ampicillin” is used. The same for AMP, BCA, CCCP, CFU, and possibly, the others. Some acronyms are misleading i.e. WHO stands for World Health Organisation, but not WHO Collaborating Centre. Also, acronyms are used but not explained (i.e. FACS)

Besides prudent and precise citation issues mentioned above and usage of the most recent and updated citations, care should be paid to reference formatting (i.e. unnecessary duplication of the journal in #3; redundant publisher names in #16, #18, #19, #24, #32, #74, #75, etc.). The respectful authors should be spelt accurately (i.e. 2nd author of #10).

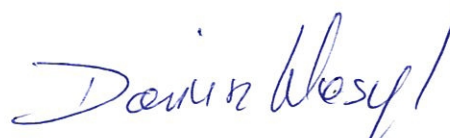
I would also prefer co-authors' declarations regarding their contribution to the development of the articles instead of statements undersigned just by the thesis Author and the Supervisor.

Since the dissertation does not fully prove general theoretical knowledge in veterinary sciences, and considering the substantive matters listed above, I will be happy to learn during public defence PhD student's opinion on the following issues:

- Is there any difference between typhoid diseases and the host-specificity of some *Salmonella*?
- From an ecological perspective, what could be the rationale for the epidemic success of *S. Enteritidis* observed in the second half of the XX century?
- Based on the European data, how would you understand epidemic trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2020?
- How would you explain the following acronyms: WOA, EMA, ECDC, ESVAC, AMEG, JIACRA?

4. Summary and recommendation

The doctoral dissertation submitted for review entitled „The role of *sanA* in *Salmonella* pathogenicity”, supervised by Professor Krzysztof Grzymajło, with the assistance of Dr Rafał Kolenda, fulfils the requirements of Ustawa z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz. U. 2018, poz. 1668, with amendments), precisely Art. 187, and may be further processed for awarding the doctoral degree in the field of veterinary sciences.


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