

REVIEW

the official reviewer of Zhike Liu's dissertation work «Construction of multiplex PCR assay based on the *citE2* gene to identify *Salmonella pullorum* and its effector *SteE* in pathogenicity and immunity», which was submitted for obtaining the scientific degree of Doctor of Philosophy at the one-time special council at Sumy National Agrarian University, branch of knowledge 21 – «Veterinary medicine», specialty 211 – «Veterinary medicine».

1. The relevance of the PhD thesis topic

Salmonella enterica serovar Pullorum (*S. Pullorum*) is an avian-specific pathogen that causes severe diarrhea (pullorum disease) and systemic fatal diseases with high mortality among chickens under 2–3 weeks old. Infected adult chickens' present reproductive tract abnormalities without severe clinical symptoms. Pullorum disease causes significant economic losses to the poultry industry worldwide, especially in developing countries. *S. Pullorum* can escape the host immune system via special effectors, thereby enhancing its intracellular survival and growth in chickens. In addition, *Salmonella* effectors are translocated into host target cells via the *Salmonella* pathogenicity island-2 encode type III secretion system (T3SS2), which can manipulate host immune signaling cascades. Therefore, the host immune response is closely correlated with *Salmonella* effector levels. Due to long-term unjustified use of antibiotics, bacterial resistance has increased, and antibiotic treatment disrupts the normal homeostasis of the body and intestinal flora. The problems caused by long-term, large-scale use of antibiotics are becoming more and more serious, and the development of effective and safe substitutes for antibiotics is inevitable and urgent. Therefore, there is a need to urgently search for an alternative to antibiotics.

Salmonella infection drives the polarization of M1/M2 macrophages. The M1 or M2 macrophages then induce T lymphocytes to produce Th1 or Th2, respectively, which leads to further amplification of M1 or M2 responses in a positive feedback loop, thereby stabilizing the main immune phenotype in different environments of infection or inflammation. The Th1/Th2 balance plays a vital role in the host's immune response. IFN- γ and IL-12 produced by Th1 cells increase

the anti-viral/microbial effects, thereby causing host damage and inflammation, whereas IL-4 and IL-10 produced by Th2 cells enhance humoral immune responses and contribute to tissue repair and remodeling. *S. Pullorum* regulates the immune response toward a Th2 response, which is associated with the M2 phenotype of macrophages, and contributes to intracellular carriage in infected chickens. Therefore, the macrophage phenotype can indirectly reflect the type of T cell response, which is associated with the immune regulatory function of the Th1/Th2 cytokines.

So, the topic of the dissertation is extremely relevant because the PhD thesis is devoted to providing development and scientific justification of the prevention and accurate detection of *S. Pullorum*, and clarifies the mechanism of persistent *S. Pullorum* infection in chickens. *S. Pullorum* is a host-specific pathogen, causes severe economic losses to the chicken farms, and also can induce an anti-inflammatory response in chickens. A rapid and accurate method can help us make corresponding prevention and control measures in clinical and food samples. Additionally, *S. Pullorum* effectors with anti-inflammatory function can be a unique drug candidate for targeting salmonellosis.

Connection of the PhD thesis with scientific programs, topics, plans
Work carried out in according with the main directions of scientific research of the National Natural Science Foundation of China-Henan Joint Fund (Grant Number U1904117), Key Science and Technology Program of Henan Province (Grant Number: 21210210100 and 212102110009), Sumy National Agrarian University and Henan Institute of Science and Technology within the framework of scientific programs of research work. The materials of the dissertation work are part of comprehensive scientific research of the Department of Veterinary Expertise, Microbiology, Zoohygiene and Safety and Quality of Livestock Products of the Sumy National Agrarian University according to the following thematic plans of research works: "System of monitoring methods of control and veterinary and sanitary measures, regarding the quality and safety of livestock products in diseases of infectious etiology" (state registration No. 0114U005551, 2014-2019);

"Forecasting the risks of cross-border introduction and spread of particularly dangerous animal diseases and the development of scientifically based disinfection systems based on innovative import-substitutable highly effective means" (state registration No. 0115U001342, 2018-2023).

2. *The degree of validity and reliability of scientific statements, conclusions and recommendations formulated in the dissertation*

Evaluating the main results of the dissertation work submitted for defense, there is a need to emphasize their theoretical validity and the applicant's orientation to a clear formulation of his own position. The use of Construction of multiplex PCR assay based on the *citE2* gene to identity *Salmonella pullorum* and its effector *SteE* in pathogenicity and immunity received great attention in the scientific community in the last decades.

Confirmation of the scientific argumentation and validity of the provisions, conclusions and recommendations of the dissertation work is a logical sequence in the setting and execution of tasks according to the purpose of the research. The dissertation contains the necessary theoretical, methodological, methodical and analytical studies, properly covered in the relevant sections.

The scientific validity and reliability of the results presented in the dissertation work is ensured by the creative use of scientific approaches, systematic study and generalization of the results of scientific developments by scientists.

Based on the results of theoretical and experimental studies provide a scientific reference for the prevention and accurate detection of *S. Pullorum* in chicken farmers. The developed multiplex PCR system had high sensitivity and specificity, and could be a valuable tool for the clinical diagnosis of *S. Pullorum*, which is an important pathogen in chickens. In addition, *steE* was associated with the persistent infection of *S. Pullorum*, which modulated Th1/Th2-related immune responses by STAT3/SOCS3 and NF- κ B axis and could be a unique drug candidate for targeting salmonellosis. According to the results of research, 14 scientific papers were published, including: 3 articles in professional editions of

Ukraine, 2 articles in Web of Science or Scopus scientific-metric publication, and 9 abstracts of reports at scientific and practical international conferences.

Scientific novelty of scientific provisions, conclusions and recommendations formulated in the dissertation

Having familiarized ourselves with the PhD student' work, we can note that the work really has the necessary novelty. Consequently, the topic of the PhD thesis is current and relevant. The PhD thesis established the theoretical basis of prevention and treatment of *S. Pullorum* in chickens. This is the first multiplex PCR method based on the *citE2* gene and the intergenic sequence of SPS4_00301–SPS4_00311 was established for the accurate detection of *S. Pullorum* in clinical and food samples. Confirmed the pathogenicity associated with *steE* in *Salmonella Pullorum*. These results dissecting the molecular mechanism of *steE* in regulated the balance of Th1/Th2 cytokines during *S. Pullorum* infection for the first time. This thesis established the theoretical basis of prevention and treatment of *S. Pullorum* in chickens. This is the first multiplex PCR method based on the *citE2* gene and the intergenic sequence of SPS4_00301–SPS4_00311 was established for the accurate detection of *S. Pullorum* in clinical and food samples. Confirmed the pathogenicity associated with *steE* in *Salmonella Pullorum*. These results dissecting the molecular mechanism of *steE* in regulated the balance of Th1/Th2 cytokines during *S. Pullorum* infection for the first time.

Practical value of the obtained research results.

The research results presented by the PhD thesis have theoretical and practical significance.

Based on the results of theoretical and experimental studies provide a scientific reference for the prevention and accurate detection of *S. Pullorum* in chicken farmers. The developed multiplex PCR system had high sensitivity and specificity, and could be a valuable tool for the clinical diagnosis of *S. Pullorum*, which is an important pathogen in chickens. In addition, *steE* was associated with the persistent infection of *S. Pullorum*, which modulated Th1/Th2-related immune

responses by STAT3/SOCS3 and NF- κ B axis and could be a unique drug candidate for targeting salmonellosis.

The main provisions of the PhD thesis were included in the guidelines according to prevention and detection of *Salmonella* and the mechanism via which *steE* inhibits the host inflammatory response by STAT3/SOCS3 and NF- κ B axis, approved by the Academic Council of SNAU (Protocol № 5, dated 29.12.2019).

The PhD thesis materials are included in the syllabus, work program of courses "Veterinary microbiology", "Veterinary medicine" for masters of the Faculty of Veterinary Medicine of Sumy National Agrarian University, and are used in distance learning of students based on the platform «Moodle».

And for the courses "Veterinary microbiology" and "Veterinary medicine" for masters of the Henan Institute of Science and Technology (HIST).

No doubt that the theoretical developments of the author can be implemented in production: to establish the production of a diagnostic test system, and are also essential for the improvement of measures to combat salmonellosis in chickens.

The completeness of the presentation of scientific provisions, materials, conclusions and recommendations of the dissertation in published scientific works, counted according to the topic of the dissertation

Zhike Liu took part in the implementation of scientific programs based on PhD thesis, and conceived and designed experiments. Setting objectives, discussing the results, forming conclusions were conducted together with tutors. PhD student analyzed the data as well as interpretation of the data on the topic of the dissertation, and conducted experimental research using modern methods and software's with co-authors of scientific papers. The applicant wrote dissertation and published articles in which the main material of the PhD thesis. The author thanks all participants in the manuscript for kindly help.

The main provisions of the dissertation were reported and discussed and approved at a meeting of: BTRP Ukraine 2021 International BioThreat Reduction Symposium, (29 June - 2 July, 2021, Kyiv, Ukraine); Chinese Association of

Animal Science and Veterinary Medicine, 2022 Academic Forum (14-16 August, 2022. Foshan, China); The III Scientific and Practical International Distance Conference «Microbiological and Immunological Research in Modern Medicine», (March 24, 2023, Kharkiv, Ukraine); The XI International Scientific and Practical Conference «Problems of the development of science and the view of society», (March 21-24, 2023, Graz, Austria); Chinese Association of Animal Science and Veterinary Medicine, 2023 Academic Forum (12-15 May, 2023. Yangzhou, China).

The structure and content of the dissertation

The dissertation work of Zhike Liu's is a logically constructed study, which consistently describes approaches to Construction of multiplex PCR assay based on the *citE2* gene to identity *Salmonella pullorum* and its effector *SteE* in pathogenicity and immunity.

The PhD thesis is well structured and Mr. Zhike Liu's quoted an appropriate number of bibliography sources. Furthermore, this thesis is well written in clear and concise manner. The figures and schemes are properly. The language is comprehensive and coherent while errors and inaccuracies are relatively rare.

The dissertation is set out on 133 pages of computer text. It consists of an annotation, introduction, 4 chapters, conclusions, a list of sources used and 2 annexes. The main body of the dissertation contains 7 tables, 45 figures. The list of references includes the name of 294 sources.

The design of the dissertation in terms of structure, language and presentation style meets the requirements for the design of dissertation works, which are approved by the order of the Ministry of Education and Culture of Ukraine dated 12.01.2017 No. 40 with changes and additions introduced by the order of the Ministry of Education and Culture of Ukraine dated 31.05.2019 No. 759. The materials of the dissertation clearly highlight the scientific and practical results obtained during the research.

Academic integrity

Violations of academic integrity (academic plagiarism, self-plagiarism, fabrication, falsification) in the dissertation work on scientific papers, which highlight the main scientific results of the applicant's dissertation research, were not detected.

Specific comments and questions:

For some published articles, supplementary materials are available that was not included. Supporting information should be placed directly after the respective manuscript.

I am missing an outlook about further applications, strategies to improve the performance or a paragraph that presents directions for further research.

In summary, the PhD thesis represents original and high level of scientific work. The conducted experiments are well arranged and methods are clearly described. The results are very well presented and the explanations are reasonable as well as suitable and focused on the relevant topics.

In general, positively evaluating the work of the dissertation researcher Zhike Liu, I consider it possible to inquire about the author's opinion regarding the results presented in the dissertation work:

1. For what purpose did you use the Western blot analysis in your research?
2. Why was *S. Pullorum* used as a model pathogen?
3. What is the novelty of your results?

The stated debatable provisions and certain shortcomings do not significantly affect the overall positive assessment of the dissertation work. I would like to emphasize the comprehensive interdisciplinary character of the research project covering the research fields of Construction of multiplex PCR assay based on the *citE2* gene to identify *Salmonella pullorum* and its effector *SteE* in pathogenicity and immunity. Mr. Zhike Liu's has proving his excellent analytical skills and the high quality of his work which is demonstrated by peer reviewed publications in reputable journals so far, altogether attesting very good performance and writing skills. In my opinion, the PhD thesis by Zhike Liu's fulfills all requirements for obtaining the PhD degree.

4. Conclusion.

Dissertation Zhike Liu: «Construction of multiplex PCR assay based on the *citE2* gene to identify *Salmonella pullorum* and its effector *SteE* in pathogenicity and immunity», which was submitted for obtaining the scientific degree of Doctor of Philosophy to the one-time special council at the Sumy National Agrarian University, issued in accordance with the order of the Ministry of Education and Science of Ukraine dated January 12, 2017 No. 40 «On Approval of Requirements for Dissertation» and the Ministry of Education and Science of Ukraine dated May 31, 2019 No. 759 with changes and additions, in terms of relevance, scientific novelty and practical significance of the obtained results, it fully meets the requirements stipulated. The procedure for awarding the degree of Doctor of Philosophy approved by the Resolution of the Cabinet of Ministers of Ukraine dated 12.01.2022 No. 44, and its author deserves the award of the educational and scientific degree of Doctor of Philosophy in the field of knowledge 21 «Veterinary Medicine» in the specialty 211 «Veterinary Medicine».

Official reviewer

Doctor of Veterinary Sciences.

Professor of the Department of

Veterinary Examination, Microbiology,

Zoohygiene and Safety and Quality

of Livestock Products

of Sumy National Agrarian University

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Hanna FOTINA

