

Response

the official opponent 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. Relevance of the topic of the work.

Zoonotic diseases continue to be a threat to human health, leading to significant economic losses every year. So, for example, during the two years of the COVID-19 pandemic (which began in December 2019), caused by the SARS-CoV-2 virus, about 694 million people in the world fell ill, and the pandemic claimed more than 7 million human lives. That is, zoonoses are an extremely serious problem that has socio-economic significance.

Among zoonoses, salmonellosis remains one of the most urgent problems of infectious pathology for the field of veterinary medicine and has sanitary-epidemiological and socio-economic significance.

EFSA and ECDC reports present the results of zoonoses monitoring in European countries. So, for example, the 2020 report provides statistical reporting data on zoonoses and zoonotic agents in humans, food products, animals and feed for 27 EU member states and nine non-EU countries. Thus, over the past five years, it was established that campylobacteriosis and salmonellosis were the most frequently registered among zoonoses in humans, respectively; *Salmonella spp.* was the leading etiological factor in food-borne disease outbreaks.

The system of monitoring zoonotic pathogens in the EU countries functions taking into account the concept of «One Health», the monitoring results are a scientific basis for adjusting measures to control the spread of pathogens.

The above confirms that the dissertation work is devoted to an extremely urgent problem today - the development of a method for detecting *S. pullorum*, and the elucidation of the mechanism of persistent *S. pullorum* infection in chickens, which causes significant economic losses for poultry farms.

2. The degree of validity of the conclusions and recommendations of the scientific provisions formulated in the work.

The results of the research were reported and approved at the annual scientific seminars and conferences of the teaching staff and postgraduate students of the Sumy National Agrarian University of the Faculty of Veterinary Medicine (2019-2023); 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).

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.

The materials of the dissertation work were obtained within the framework of comprehensive scientific research of the department of veterinary expertise, microbiology, zoohygiene and safety and quality of animal husbandry products of the Sumy National Agrarian University according to the following thematic plans of research works: "System of monitoring methods". control and veterinary-sanitary measures regarding the quality and safety of livestock products for diseases of infectious etiology" (state registration number 0114U005551, 20142019); "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 number 0115U001342, 20182023).

Also, scientific research was carried out in accordance with the research directions of the National Natural Science Foundation of China-Henan Joint Fund (grant number U1904117), the Key Scientific and Technical Program of Henan Province (grant number: 21210210100 and 212102110009), Sumy National Agrarian University and Henan Institute of Science and technologies within the framework of scientific programs of research work.

The aim of the study was to isolate *S. pullorum* from sick chickens with suspected pullorosis and to determine resistance to antimicrobial drugs, as well as to develop a multiplex PCR for early diagnosis of *S. pullorum* in clinical samples. Study of the role of the *S. pullorum* *SteE* effector in pathogenicity and immunity, as well as the mechanism of *steE* regulation of the development of the inflammatory process.

To achieve the main goal of the study, it was necessary to solve a number of interrelated tasks:

1. Isolate *S. pullorum* from sick chickens and determine the antibiotic profile.
2. Develop a multiplex PCR method using *citE2*
3. Analysis of the association of the T3SS2 effector *SteE* with the virulence of *S. pullorum*.
4. Assess the role of *steE* in *S. pullorum*-induced inflammation and elucidate the molecular mechanism of persistent *S. pullorum* infection in chickens.

The tasks set before the dissertation were completed in full.

Within the scope of dissertation research, the author isolated *S. pullorum* cultures that were resistant to amoxicillin, sulfamethazine, tetracycline, and iprofloxacin, but sensitive to ceftriaxone, ceftiofur, and kanamycin.

The author established that the *citE2* gene is specific for *S. pullorum*, and using the *citE2* and *SPS4_00301-SPS4_00311* genes, a multiplex PCR was developed, the sensitivity of which was 6.25 pg/ μ L and 104 CFU/mL for genomic DNA and *S. pullorum* cells. The effectiveness of the proposed diagnostic was confirmed in an experiment with artificially contaminated *S. pullorum*, *S. enteritidis* eggs and 69 samples of anal swabs.

The next stage of the work consisted in identifying the mechanisms of the pathogenesis of pullorosis infection using the *S. pullorum* strain constructed by the author with the deletion of the virulence marker *steE* - *S. pullorum* ($\Delta steE$).

It has been experimentally demonstrated that the presence of *steE* enhances the infection and virulence of *S. pullorum* by regulating the inflammatory response, which may provide a basis for the development of new therapeutic agents.

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).

Based on the materials of the dissertation, methodological recommendations «Construction of multiplex PCR assay based on the *citE2* gene to identify *Salmonella pullorum* and its effector *SteE* in pathogenicity and immunity».

We recommend using the materials of the dissertation work when studying the courses «Veterinary microbiology», «Veterinary immunology» for masters of the Faculty of Veterinary Medicine of Sumy NAU. And for the courses «Veterinary microbiology» and «Veterinary immunology» for masters of the Henan Institute of Science and Technology (HIST).

Scientific novelty of the obtained results. The doctoral student proposed an efficient method of multiplex PCR based on the *citE2* gene and intergenic sequence *SPS4_00301-SPS4_00311*, which was created for accurate detection of *S. pullorum* in clinical and food samples. Pathogenicity associated with *steE* in *Salmonella pullorum* was experimentally confirmed. The obtained results substantiate the molecular mechanism of *steE* in the regulated balance of Th1/Th2 cytokines during *S. pullorum* infection. The results of the thesis laid the theoretical foundations for the prevention and treatment of *S. pullorum* in chickens.

Practical significance of the obtained results. 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 persistent *S. pullorum* infection, which modulated Th1/Th2-related immune responses via STAT3/SOCS3 and the NF- κ B axis and could be a unique drug candidate for the treatment of salmonellosis.

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. 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. Namely:

- Do you think it is possible to use the deletion strains you obtained to create vaccines against pulorosis?
- Are mutant strains used at all for the production of vaccines, in particular against salmonellosis?
- In what cases is it possible to use deletion mutants for the production of vaccines?

3. Approval of research results, completeness of the statement of scientific provisions, conclusions, recommendations formulated in the work.

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

4. Regarding the compliance of the dissertation with the established requirements.

The content of Zhike Liu's scientific research corresponds to the specialty 211 – «Veterinary Medicine». The work was performed at the appropriate level and meets the requirements for the preparation of dissertations and the Procedure for awarding the scientific degree of Doctor of Philosophy, approved by the Resolution of the CMU of January 12, 2022 No. 44.

5. Significance for science and practice of the results obtained by the author of the dissertation and ways of their use.

Thus, the dissertation showed that with the help of bacteriological studies, the determination of the antibiotic profile and the use of the multiplex PCR system proposed by the author, it is possible to significantly improve the effectiveness of anti-epizootic measures, identify the source of the pathogen and control the spread of resistant salmonella clones in chicken flocks. In addition, the author confirmed the role of *steE* in the pathogenesis of *S. pullorum* infection. The obtained data are essential for improving measures to combat salmonellosis in chickens.

The dissertation student mastered modern research methods, which include bacteriological, molecular-genetic, immunological and statistical methods.

6. 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 CMU 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».

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