

RESPONSE
official opponent
Doctor of Veterinary Sciences, Professor,
Head of epizootology department,
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Vitalii Ukhovskiy
for Liu Mingcheng's Thesis «Experimental research on pathogenesis of
Streptococcus suis infection», submitted for the degree of Doctor of
Philosophy from the field of knowledge 21 «Veterinary» in the specialty
211 «Veterinary medicine»

1. Relevance of the dissertation topic.

In today's conditions, among the causative agents of bacterial diseases in pigs, the role of pathogenic and opportunistic microorganisms, namely streptococcus, is increasing dramatically. *Streptococcus suis* is a zoonotic pathogen that causes diseases such as meningitis, pneumonia, endocarditis, polyserositis, arthritis, septicemia and abortion in pigs. A significant number of pigs in China's pig farms suffer from streptococcal infection, the infection is also registered in Ukraine and other countries of the world. In recent years, the incidence rate of streptococcal meningitis has been increasing significantly. Due to its widespread, the causative agent of swine streptococcosis, *Streptococcus suis*, is becoming increasingly resistant to antibiotics. The danger of introducing pathogenic strains into the farms calls for constant monitoring of these diseases and effective therapeutic measures. This pathogen is also dangerous for humans. Outbreaks of streptococcal infection among the population have been reported in China, characterized by serious complications in people of various ages and a high mortality rate. In order to consistently reduce the infectious morbidity and mortality of sick people, clinical guidelines recommend the antibacterial therapy. However, the effectiveness of pharmacological measures is quite low, which is reflected in the growth of regional morbidity and mortality rates. Antibacterial drugs used to treat patients are unable to penetrate the blood-brain barrier to reach the therapeutic target site, which is the key to controlling bacterial meningitis. Therefore, strengthening the prevention and control of streptococcal infection in pigs has become an urgent task. The solution for this scientific problem is based on the study of the pathogenesis of the streptococcal infection.

The prerequisite for the induction of meningitis is the penetration of *S. suis* through the blood-brain barrier and subsequent damage of the central nervous system. Endothelial cells of the brain microvessels are the main component of the blood-brain barrier, the dysfunction of which is associated with various neurological diseases. Therefore, the study of the interaction between *S. suis* (serotype 2) and microvascular endothelial cells of the brain have a great importance in the study of the pathogenesis of meningitis.

The above confirms that the dissertation work is devoted to an extremely urgent problem of today, namely the experimental study of the pathogenesis of

streptococcal infection in pigs and is an important stage in the development of a strategy to control streptococcal infection caused by *S. suis*.

2. Connection of work with scientific programs, topics, plans.

The materials of the dissertation research are the part of complex scientific studies of the department of epizootology and parasitology of the faculty of veterinary medicine of the Sumy National Agrarian University according to the thematic plan of the scientific research work «Optimization of the set of measures to prevent the occurrence and spread of infectious diseases of animals in the farms of the northeastern region of Ukraine», state registration number 0122U001254 (2022–2027). Experimental research on the topic of the dissertation was conducted in the period from 2019 to 2023 in a Department of Epizootology and Parasitology of Sumy National Agrarian University.

The dissertation is a fragment of the research programs of the National Natural Science Foundation of China «Project for the Development of Young Talents of Henan Province».

3. Scientific novelty of the obtained results.

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

- to experimentally substantiate the use of the brain microvascular endothelial cells (bEnd.3) for infection with *S. suis* serotype 2 (*S. suis* 2);
- to analyze the infection pathway of the brain microvascular endothelial cells (bEnd.3) by *S. suis* serotype 2;
- to investigate the mRNA levels and the protein levels of cytokines secreted by the infected cells (bEnd.3);
- to determine the protein content of cell supernatants;
- to study morphological changes in infected cells (bEnd.3) after to determine if pyroptosis occurred during infection by *S. suis* 2;
- to confirm the pathogenesis of meningitis of the streptococcal infection caused by *S. suis* serotype 2 (*S. suis* 2).

The dissertation student successfully completed all assigned tasks.

The following new scientific results were obtained in the dissertation: it was theoretically and experimentally established for the first time that pyroptosis is involved in the pathogenetic process of developing meningitis due to streptococcal infection of pigs caused by *S. suis*.

The use of the endothelial cells culture of brain microvessels (bEnd3) for infection with *S. suis* serotype 2 was experimentally substantiated in in vitro experiments. A study was conducted to determine the effectiveness of various schemes for infecting the cell culture (bEnd.3) with the zoonotic bacterial pathogen *Streptococcus suis* type 2. The optimal multiplicity of infection (MOI) was screened and the time and dose of an infection of the endothelial cells of the microvessels of the brain with the pathogen *S. suis* 2 was determined experimentally. For the first time, he conducted a study of genes related to the pyroptosis, based on the extraction of a total RNA from an infected microvessels endothelial cells culture of the brain of white mice and the subsequent transcription

into a complementary DNA (cDNA). The content of proteins in the supernatant of infected cells was determined experimentally. Molecular genetic methods for detecting cytokines released by infected cells were experimentally developed.

For the first time, he investigated the stages of pyroptosis development in endothelial cells of brain microvessels infected with *S. suis* 2 (bEnd.3).

With the help of an electron microscope, the changes of morphological characteristic of pyroptosis were established for the first time in the *S. suis* 2 infected culture of endothelial cells of brain microvessels (bEnd.3).

4. Scientific and practical significance.

The pathogenesis of meningitis due to the streptococcal infection caused by the zoonotic pathogen *S. suis* serotype 2 has been theoretically and experimentally substantiated. Based on in vitro studies, a stepwise process of the pathogenetic processes of the pyroptosis development in the microvessels endothelial cells culture of the mice brain infected with *S. suis* 2 has been established (bEnd.3). The obtained research results can be used in the development of vaccines for swine streptococcal infection caused by *S. suis* serotype 2 and targeted therapeutic agents in the veterinary and human medicine to prevent pathological changes in the brain by similar processes.

The main results of the dissertation work are presented in the scientific and practical recommendations «*Streptococcus suis* infection (Etiology, Epidemiology, Laboratory diagnosis, Prevention and Treatment)», protocol № 18, dated 29.05. 2023, authors: Mingcheng Liu, Oksana Kasianenko. The obtained research results and conclusions were implemented in the educational process during the teaching of the disciplines: «Veterinary technologies for the prevention of infectious diseases of animals», «Epizootology and infectious diseases», «Anti-epizootic measures in animal husbandry» at the department of epizootology and parasitology in the training of specialists for the degree of higher education «Master» from the field of knowledge 21 «Veterinary» in the specialty 211 «Veterinary Medicine» at the Sumy National Agrarian University.

The obtained research results are recommended for implementation in the educational process during the study of the educational component «Veterinary Microbiology» at the Henan Institute of Science and Technology, China.

5. Completeness of presentation of the dissertation material in scientific publications.

Based on the materials of the dissertation, 13 scientific works were published, including 5 articles in specialized scientific publications of Ukraine, 7 theses of reports in collections of conference materials, 1 scientific and practical recommendations.

6. The degree of validity of scientific statements.

Liu Mingcheng's dissertation is a completed scientific work on which he have worked during 2019–2023 years, it fully meets the set goal and objectives of the research. The results of his own research obtained by the doctoral student are properly substantiated and logically compared with the data of other researchers. The conclusions are formulated on the basis of the obtained practical data, which prove the author's awareness of the problem under study.

The results presented in the dissertation determine the modern ideas about the pathogenetic processes of the development of meningitis due to streptococcal infection caused by *S. suis*.

7. The structure and content of the dissertation, its completeness and compliance with the established requirements for design.

The dissertation consists of an introduction, Literature review on the topic and choice of research directions, objects and methods, results of own research and summary and analysis of results, conclusions, production proposals, literature review (222 names), applications. The work is laid out on 129 sheets of computer text, contains 5 tables, 30 figures.

In the introduction, the author substantiated in detail the relevance of the conducted research, taking into account modern knowledge on the topic to which the dissertation is devoted; clearly defines the object and subject of research; lists the research methods used by him; objectively highlights the scientific novelty and practical significance of the obtained data; determines the personal contribution, indicates the place and period of approval of scientific research and the number of publications prepared on the topic of the work.

Chapter 1 «Literature review on the topic and choice of research directions» is informative and analytical, corresponds to the purpose of the research, written at a high scientific and methodical level with the use of a sufficient number of primary sources. In general, this section of the dissertation is quite voluminous, written competently and reflects the author's ability to critically analyze the literature data and draw objective conclusions from them.

Chapter 2 «Objects and methods». Reasons, stages and conditions of conducting experiments. This section provides specific methods of research, namely: molecular genetic (polymerase chain reaction in real time), microbiological (microscopy, electron microscopy), analytical (Western blot method for detecting specific proteins in cells), immunological (ELISA method for detection of expression of individual genes in cell supernatants), and statistical (processing of research results).

Chapter 3 «Results of own research». The results of experimental studies conducted by the acquirer are presented here. The materials of this part of the dissertation work are presented in the form of 10 subsections.

In subsection 3.1 «Bacterial count» was conducted to determine the level of adsorption cell culture of different breeds *Streptococcus suis* under the influence of waves of different lengths: 450 nm, 570 nm, 600 nm, 650 nm.

In subsection 3.2 «The preparation, identification and culture of brain microvascular endothelial cells». The results of research on preparation for infection and immunofluorescent identification of the culture of endothelial cells of brain microvessels.

In subsection 3.3 «Screening of optimal multiplicity of infection» was presented the results of the study of the relative expression of cytokines from the culture of cells during different times after infection with *S. suis*. Experiments have established the optimal conditions for infection of brain endothelial cell culture.

Subsection 3.4 «Cellular total RNA extraction and reverse transcription» presents data on the development of a method for RNA extraction by the Trizol method from endothelial cells of brain microvessels infected with *S. suis* (serotype 2) isolates. A study on the assessment of the quality of extracted nucleic acids is given. The obtained data provide an experimental basis for the further work.

Subsection 3.5 «QPCR detection» presents research, the results of which confirm the presence of pyroptosis in an infected cell culture based on the use of the fluorescent quantitative PCR method.

In subsection 3.6 «Western blot detection» presented data of experimental studies regarding detection of protein expression of cytokines that are associated with pyroptosis, such as IL-18, IL-1 β , caspase-1, GSDMD and GSDME. These studies were conducted by experimental method Western blot. In subsection 3.7 «ELISA detection» experimental studies are presented to determine the expression of genes and protein concentrations in the supernatant of cells of experimental groups and control.

In subsection 3.8 «Lactate dehydrogenase Detection» PhD student received research results that confirmed cellular pyroptosis of the infected cells. He founded out that the integrity of the cell membrane is broken. The PhD student established that lactate dehydrogenase is released from the cells, which is an indicator of cytotoxicity.

In subsection 3.9 «Electron microscopy observation» presents the results of studies of morphological changes in endothelial cells of brain microvessels infected by *S. suis* 2, which are characteristic of pyroptosis.

In the subdivision 3.10 «Conclusions to Chapter 3» the PhD student have presented a logically structured, deep and comprehensive analysis and discussion of the obtained results. It summarizes the obtained data, compares them with the available data of the literature and emphasizes the fact that the author brought something new to this problem.

Chapter 4 «Summary and analysis of results». In this section, the author meaningfully and objectively argues the experimental and scientific facts he has accumulated, comparing them with the results of research by other scientists.

The conclusions consist of 6 points, contain concise information about the results of the research, are structured according to the tasks set in the dissertation, stem from the results of own research and are based on the experimental data presented in the dissertation. Proposals for production are proposed by the PhD student, properly designed and documented.

So, in general, this section is presented professionally, competently and it characterizes the PhD student as a comprehensively prepared and erudite scientist.

8. Discussion clauses and remarks to the dissertation.

Giving a general assessment of the dissertation work of Liu Mingcheng, one should point out the identified individual shortcomings, inconsistencies, and also ask some debatable questions that need clarification:

Evaluating the thesis work of Liu Mingcheng positively, I consider it necessary to express some remarks and discussion questions, as well as to point out some identified shortcomings:

1. It is recommended to show with an arrow the morphological changes in cells at different stages of pyroptosis established by electron microscopy in the photos 3.25, 3.26, 3.27, 3.28, which are described in the dissertation work.
2. The text contains the abbreviation SS2 (p. 94), photos 3.16, 3.17, 3.18, 3.19, 3.20, 3.21, 3.22, 3.23, 3.24; it would be write «S. suis 2».
3. The abbreviation «SSU method» on page 112 is not included in the list of conditional abbreviations.
4. The name of the pathogen agent «S.suis2» should be highlighted in italics font on page 115.
5. In the course of bacterial infection, how to determine the infection dose and infection time?
6. How do you select the genes to be tested?

9. General conclusion

Liu Mingcheng's Thesis «Experimental research on pathogenesis of Streptococcus suis infection», which was submitted for defense to the specialized academic council for obtaining the degree of Doctor of Philosophy in field 21 «Veterinary» in the specialty 211 «Veterinary Medicine» according to its relevance, scientific and theoretical level, the main results of validity, the main provisions and results published in professional publications, the novelty of the statement and practical significance meet the requirements of the order of the Ministry of Education and Culture of Ukraine № 40 of January 12, 2017 «On approval of requirements for the preparation of a dissertation» and the Resolution of the Cabinet of Ministers of Ukraine of January 12, 2022 № 44 «On approving the Procedure for awarding the degree of Doctor of Philosophy and canceling the decision of the one-time specialized academic council of a higher education institution, scientific institution on awarding the degree of Doctor of Philosophy» with changes introduced in accordance with Resolution of the Cabinet of Ministers № 341 dated 03.21.2022.

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Signature of the Head of epizootology department,
of the SSRILDVSE Vitalii Ukhovskiy I assure

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Larisa MOROZ