

REVIEW

by Professor Rumen Veselinov Karakolev, PhD, from the National Diagnostic Scientific Research Veterinary Medical Institute /NDNIVMI/ – IRL Veliko Tarnovo, reviewer of a dissertation for the award of an educational and scientific degree "Doctor" in the professional field of Biological Sciences, scientific specialty Microbiology 01.06.12

Topic: "DISTRIBUTION AND CHARACTERISTICS OF ENTEROPATHOGENIC STRAINS OF YERSINIA ENTEROCOLITICA ISOLATED FROM SWINE"

Author of the dissertation: Maya Angelovska from the Institute of Microbiology "Stefan Angelov," Bulgarian Academy of Sciences.

Biographical Information about the Candidate

The author of the presented dissertation for the award of an educational and scientific degree "Doctor" in the field of Biological Sciences (specialty "Microbiology," 01.06.12) is Maya Angelovska. She was born on May 2, 1982. She completed her secondary education in Bitola, North Macedonia. From 2000 to 2006, she studied at Sofia University "St. Kliment Ohridski" and earned a Bachelor's degree in Molecular Biology and a Master's degree in Molecular Biology - Virology. From 2006 to 2007, she worked as a specialist in biology-virology at the Institute of Microbiology "Stefan Angelov" at the Bulgarian Academy of Sciences. Since 2012, she has been a specialist-microbiologist at the same institute. The candidate's scientific interests are related to conventional methods for pathogen detection, methods for the isolation and identification of foodborne pathogens, molecular methods for the detection and typing of pathogenic bacteria, molecular confirmation of virulent factors, determination of antibiotic sensitivity (microtiter method and agar diffusion methods), and more. Maya Angelovska has participated in numerous international scientific congresses and conferences and has worked as a member of scientific teams on projects funded by the Scientific Research Fund of the Ministry of Education and Science.

Evaluation of the Dissertation

Structure

The dissertation on the topic "DISTRIBUTION AND CHARACTERISTICS OF ENTEROPATHOGENIC STRAINS OF YERSINIA ENTEROCOLITICA ISOLATED FROM SWINE" consists of 157 pages. It follows the commonly accepted requirements and includes the following sections: Introduction - 3 pages, Literature Review - 45 pages, Objectives - 1 page, Materials and Methods - 14 pages, Results - 42 pages, Discussion of the Results - 16 pages, Conclusions - 1 page, Contributions - 1 page, and References - 20 pages. The dissertation contains 26 figures and 16 tables. The reference list includes 294 literature sources.

Relevance

Yersinia enterocolitica has garnered continuous interest within the international scientific community as it causes zoonotic infections known as yersiniosis in humans and animals. It is the third most significant disease among the most common ones in European Union countries. Therefore, the topic of the presented dissertation is highly relevant, considering that pigs are a primary source and asymptomatic carriers of *Yersinia* in their tonsils and Peyer's patches. During slaughtering, conditions are created for environmental contamination, and opportunities for *Yersinia* to enter the food chain.

Literature Review

A comprehensive review of literature sources related to the issues examined in the dissertation has been conducted, spanning a total of 45 pages. The literature review is purposeful, extensive, and analytical, encompassing both local and foreign authors. It includes recent literature sources, but it does not neglect older authors with foundational research on specific topics. This demonstrates a strong understanding of the literature, an ability to analyze and interpret literary data, and provides the necessary foundation for the author's own research. Towards the end of this section, there is an analysis of unresolved issues related to the evaluation of the distribution and qualitative determination of *Y. enterocolitica* from slaughtered pigs. This allows the candidate to precisely formulate the goals and objectives of their own research.

Goals and Objectives

The author aims to investigate the distribution of enteropathogenic strains of *Y. enterocolitica* isolated from slaughter-age pigs using conventional and modern molecular methods. Additionally, the goal is to develop a rapid and cost-effective protocol for the qualitative determination of *Y. enterocolitica* in samples directly from the tonsils and feces of slaughter pigs. To achieve this goal, eight distinct tasks have been defined, all of which are correctly formulated and logically follow the experimental design chosen to accomplish the selected objective.

Materials and Methods

The research was conducted using modern laboratory methods. It began with the gold standard for pathogen detection in food (BDS EN ISO 10273:2003) and then transitioned to molecular biology tests, including biotyping, serotyping, plasmid profiling, antibiotic sensitivity determination, and loop-mediated isothermal amplification (LAMP). The methods are clearly and precisely described, offering an objective means of achieving the established goals and objectives and the expected results.

Results, Discussion, Conclusions, and Contributions

The obtained results align with the defined objectives and are presented clearly and comprehensively. The dissertation is richly illustrated with an adequate number of tables and figures that provide valuable information. The discussion of the results is competently and

thoroughly performed, demonstrating analytical thinking and the ability to make comparisons, highlight new scientific findings, and showcase the author's contributions.

Regarding the main goal of the study, which is to investigate the distribution of enteropathogenic strains of *Y. enterocolitica* isolated from pigs and develop laboratory methods for their detection, the author examined a total of 601 samples from slaughtered pigs in a facility serving various regions in Bulgaria. From these samples, a pair of tonsils was aseptically collected, individually packaged, and labeled. After the removal of the intestines, 189 feces samples were collected from the same number of pigs for slaughter, ensuring a correspondence between the numbers of collected tonsils and feces. All samples originate from seven swine farms for fattening, covering four different geographical areas of Bulgaria.

During the author's own research, the pathogen was isolated and identified using BDS EN ISO 10273:2003, with species identification through sequencing of the 16S rRNA genes. Biotyping, serotyping, and plasmid profiling were also performed, along with the determination of the isolates' biochemical and pathogenic characteristics. Genetic diversity and antibiotic susceptibility of *Yersinia* were examined, and a strain bank and their DNA were established.

To optimize the LAMP protocol, 32 previously identified and known strains were used. For pathogen detection using the optimized LAMP protocol, genomic DNA isolated from 30 pig samples (tonsils and feces) was used as the matrix. Isolated DNA served as a template for ail gene detection through conventional PCR.

The results show that the ail gene was detected in only eight DNA samples (8/30) isolated directly from tonsils and four DNA samples (4/30) isolated directly from feces. Positive samples for pathogenic *Y. enterocolitica* by PCR detection were 12. LAMP analysis results did not show discrepancies between the obtained LAMP products when analyzed by staining and electrophoresis. Of the thirty tonsil samples examined, a positive LAMP product for the phoP gene was detected in twenty-five samples (25/30). As a result of their own research, the author found the presence of *Y. enterocolitica* in 6.7% of slaughtered pigs in various regions of Bulgaria. It is noted that geographical location does not influence the presence of the pathogen. *Y. enterocolitica* was isolated only during the cold period from October to March. Another major finding indicates that only *Y. enterocolitica* biotype 4/serotype O:3 was isolated from the examined pigs. High carriage of virulence determinants among *Y. enterocolitica* strains isolated from slaughter pigs was observed. Three resistance profiles to commonly used antibiotics were identified, along with the presence of multi-resistant *Y. enterocolitica* strains. The isolated strains were tested for susceptibility to 15 antibiotics from 8 classes and one unclassified antibiotic. Impressively, all 43 tested strains were sensitive to gentamicin, ceftriaxone, amikacin, and ciprofloxacin and exhibited a universal resistance profile to bacitracin, ampicillin, cefamandole, and novobiocin. Two strains displayed multi-resistance, being additionally resistant to three other antibiotics: tetracycline, nalidixic acid, and chloramphenicol. Based on the presented results, three resistance profiles were identified among the isolated *Y. enterocolitica*. A predominant genotype of *Y. enterocolitica* was demonstrated to be prevalent throughout the five-year study period.

A LAMP protocol for the direct detection of the *phoP* gene in enteropathogenic *Y. enterocolitica* in tonsils and feces of slaughter pigs was optimized.

In summarizing the scientific data obtained, it is worth noting that the reviewed dissertation is executed at a high level of professionalism and scientific rigor. It features clear and precise scientifically substantiated conclusions and findings. It is evident that pigs from the slaughterhouse are a source of enteropathogenic *Y. enterocolitica*, which, once introduced into the food chain, can cause infections in humans. Routine detection of the pathogen in food samples is carried out through ISO 10273:2003, which is time-consuming and less sensitive. Therefore, the introduction of new, faster, and more sensitive methods that directly detect pathogens in food or samples from pigs is necessary. The author demonstrates that LAMP, with all its advantages, justifies its use as a rapid diagnostic method in low-budget laboratories and field conditions.

The dissertation comprises nine conclusions and three original contributions.

Publications Related to the Dissertation

Maya Angelovska has presented 2 publications related to the dissertation. One of these publications is in the journal "Antibiotics," indexed in Scopus, Web of Science, and other databases, and has a high impact factor (IF of 4.8 for 2022). Maya Angelovska is the lead author in both publications, which demonstrates her abilities in independent and collaborative work, reflecting a focused research activity related to the development of the dissertation. The author has also provided a list of 12 participations in international scientific forums related to the dissertation.

Recommendations for Practice

The scientific data obtained regarding the optimization of the LAMP protocol for the direct detection of the *phoP* gene in enteropathogenic *Yersinia enterocolitica* in tonsils and feces of slaughterhouse swine are presented as recommendations for practice. These findings are of interest to both human and veterinary laboratory practice and for food safety laboratory control.

Abstract

The presented abstract is correctly structured, illustrated with the necessary figures and tables, comprising 50 pages, and clearly reflects the objectives, tasks, results, conclusions, and contributions of the dissertation.

Conclusion

Maya Angelovska's dissertation on the topic "DISTRIBUTION AND CHARACTERISTICS OF ENTEROPATHOGENIC STRAINS OF *YERSINIA ENTEROCOLITICA* ISOLATED FROM SWINE" meets the necessary requirements of the Higher Attestation Commission for Scientific Research and represents an in-depth scientific development with significant original

contributions to microbiology, human medicine, veterinary medicine, and the safety of animal-derived food products.

This gives me a basis to express my positive assessment of the dissertation, the presented scientific work, and the candidate's scientific activities.

Considering the aforementioned motives, I recommend to the respected members of the scientific jury to vote positively for the awarding of the educational and scientific degree of "Doctor" in the field of Biological Sciences, scientific specialty 01.06.12 Microbiology, with code 4.3.

October 12, 2023

Veliko Tarnovo

Prepared by:

(Prof. Dr. Rumen Karakolev)