Abstract
of the dissertation work by Bakhit Muratovich Baimenov
on the topic: "Identification of *Staphylococcus aureus* and genetic markers of antibacterial resistance in food products using multiplex polymerase chain reaction (PCR)"
submitted for the degree of Doctor of Philosophy (PhD)
in the educational program 8D09102 – Veterinary Sanitation

1. General characteristics of the work. The dissertation is devoted to the development of a multiplex real-time PCR method for the simultaneous detection of *Staphylococcus aureus* (via the nuc gene) and antibiotic resistance genes (blaZ, ermC, tetK) in food products of animal origin. The method was tested on 87 *S. aureus* strains isolated from 1,680 samples and demonstrated high efficiency. The results are of practical importance for ensuring food safety and monitoring antibiotic resistance (ABR) in veterinary and food industry settings.

2. Relevance of the research topic. In addition to their high pathogenicity, staphylococci are well known for their pronounced resistance to almost all existing antibiotics, due to mutations or horizontal gene transfer. In recent decades, Kazakhstan, like many countries worldwide, has experienced a rapid spread of resistance among infectious disease pathogens to antibacterial agents (ABAs). According to research conducted from 2018 to 2022, among 146 *S. aureus* isolates obtained from food products in the Republic of Kazakhstan, 70.5% were resistant to at least one antibiotic. The most frequently detected resistance genes included blaZ (87.1%), mecA (45.2%), lnuA (40.7%), as well as ermC and tetK. In northern regions of Kazakhstan, a high percentage of resistant *S. aureus* and *Salmonella spp*. isolates from animals has been reported, particularly resistant to tetracyclines, nitrofurans, β-lactams, and macrolides.

Monitoring the spread of antibiotic resistance (ABR) is a strategic objective in veterinary medicine and includes surveillance of resistance among pathogens in livestock and animal-derived food products.

A promising approach in ABR diagnostics is the application of molecular genetic methods such as PCR and sequencing. The advantage of PCR lies in its independence from phenotypic characteristics and the rapid delivery of results, which is critically important for the prompt and accurate detection of *S. aureus* resistance and the selection of effective antimicrobial therapy.

Keywords: Staphylococcus aureus, antibiotic resistance, multiplex real-time PCR, genes nuc/blaZ/ermC/tetK, food safety.

3. Aim and Objectives of the Study

Aim: Identification of *Staphylococcus aureus* and its genetic resistance markers (blaZ, ermC, and tetK) to antibacterial agents in food products of animal origin using multiplex real-time polymerase chain reaction (PCR).

Objectives:

1. Isolation of *S. aureus* strains from food products of animal origin and investigation of their biological properties and antibiotic resistance.

2. Design, synthesis, and validation of primer and probe sequences for multiplex real-time PCR.

3. Optimization of real-time PCR conditions and evaluation of its analytical characteristics — specificity and sensitivity.

4. Identification of *S. aureus* isolates and resistance genes (blaZ, ermC, and tetK) using the developed primers and probes; assessment of concordance between phenotypic and genotypic identification methods.

4. Object and Subject of the Study

Object of the study: Antibiotic-resistant *Staphylococcus aureus* isolates obtained from food products of animal origin.

Subject of the study: Antibiotic resistance, the species-specific nuc gene, and the molecular resistance mechanisms (blaZ, ermC, tetK) of *S. aureus*; development and application of multiplex real-time PCR for quality control and safety assessment of animal-derived food products.

5. Research Methodology (Materials and Methods). The work was carried out at the Laboratory of Molecular Genetic Research and the Laboratory of Clinical Diagnostic, Microbiological Research and Biosafety of Biological Materials based at the Research Institute of Applied Biotechnology of the Autonomous Nonprofit Organization "KRU named after Akhmet Baitursynuly," during the period 2020–2023. During the study, 1680 samples were collected from local markets and 16 dairy farms in the Kostanay region. Isolation and identification of staphylococcal strains were performed in accordance with regulatory documents (MUK 4.2.1890-04, GOST 26809.1-2014, GOST 31746-2012, GOST 31467-2012, ST RK GOST R 51447-2010, GOST 30347-2016). Typing of strains was carried out by sequencing the 16S rRNA gene using the Sanger method. Antibiotic susceptibility was determined by the disk diffusion (DD) method according to EUCAST standards. Target gene searches were conducted using the NCBI database. For phylogenetic analysis, the MEGA 11 program was used; for primer property evaluation, the IDT OligoAnalyzer Tool; for nucleotide sequence analysis, NCBI BLAST; and for primer design, NCBI Primer-BLAST.

To assess the analytical specificity and sensitivity of the method, control samples were used, including a specially developed recombinant positive control for PCR verification. A comparative analysis of the multiplex PCR efficiency (with determination of sensitivity, specificity, PPV/NPV, and kappa coefficient) and traditional phenotypic methods (microbiological studies and DD test) was conducted on 87 strains.

6. Scientific Novelty. For the first time, a multiplex real-time PCR (qPCR) was developed for the identification of *S. aureus* with simultaneous detection of antibiotic resistance genes (blaZ, ermC, and tetK). A region of the gene encoding the thermostable nuclease (nuc) was selected for *S. aureus* identification. The scientific rationale and experimental proof of its effectiveness for *S. aureus* identification were established. The analytical sensitivity of the reagent kit based on multiplex real-time PCR was from 10 DNA copies/μL, specificity was 100%, and reproducibility was 100%.

Significant levels of resistance to β-lactam antibiotics, tetracyclines, macrolides, and fluoroquinolones were demonstrated, and the prevalence of the blaZ, ermC, and tetK genes in *S. aureus* isolates was established. Statistically significant variations were found in the results of detecting the resistance genes blaZ, ermC, and tetK by antibiotic susceptibility testing and disk diffusion method.

New data were obtained confirming the necessity of monitoring resistance profiles of *S. aureus* isolates circulating in livestock farms to ensure the safety of animal-derived food products.

7. Key Points Submitted for Defense

1. The prevalence of *S. aureus* strains isolated from animal-derived food products that are resistant to antibacterial agents circulating in the Kostanay region;

2. The development of a multiplex PCR assay for the identification of *S. aureus* and genetic markers of resistance (blaZ, ermC, and tetK) to antibacterial agents in animal-derived food products;

3. Identification of *S. aureus* and genetic markers of resistance (blaZ, ermC, and tetK) to antibacterial agents in animal-derived food products using the developed multiplex real-time PCR assay.

8. Practical Significance. Based on the conducted research, the following have been developed and proposed:

- A multiplex real-time PCR-based kit for the detection of *S. aureus* and genetic markers of resistance (blaZ, ermC, and tetK) to antibacterial agents;

- Regulatory and technical documentation for the use of the kit in the detection of *S. aureus* and the determination of their antibiotic resistance loci using multiplex real-time PCR;

- Practical guidelines, a textbook, a methodological manual, and a collective monograph;

- A utility model patent;

- The results of the dissertation are used in teaching the courses "Microbiological Diagnostics of Farm Animal Diseases" and "Modern Methods of Animal Disease Diagnostics" at the Faculty of Agricultural Sciences of KRU named after Akhmet Baitursynuly, as well as in research activities at the laboratory of Zhangir Khan West Kazakhstan Technical University (implementation act dated 11.11.2024).

9. Main Research Findings in the Form of Conclusions

1. During the dissertation research period, 1680 samples of animal-derived food products were collected from retail outlets in the city and various farms in the Kostanay region. A total of 87 *S. aureus* strains were isolated. The detection of *S. aureus* in animal-derived food products may play an important role in understanding the health status of animals and the quality of the produced products.

2. The research results indicate a high level of resistance of *S. aureus* strains to various groups of antibacterial agents (ABAs). The highest proportion of *S. aureus* strains showed susceptibility to β-lactam antibiotics – up to 100%, followed by tetracyclines and fluoroquinolones – up to 95.4%, and macrolides – up to 60.92%. The lowest number of resistant strains was found to sulfonamides – 21.84%, and aminoglycosides – 27.59%. Among the 87 *S. aureus* strains: 3 (3.44%) were resistant to two groups of ABAs; 17 (19.54%) – to three; 46 (52.87%) – to four; 19 (21.83%) – to five; and 2 (2.29%) – to six groups. The ABAs with the highest activity against *S. aureus* included gentamicin, kanamycin, and neomycin, whereas ampicillin, tetracycline, and ciprofloxacin showed reduced activity.

3. A multiplex real-time PCR assay was developed for the rapid identification of *S. aureus* with simultaneous detection of resistance genes (blaZ, ermC, and tetK) to

antibacterial agents. The effectiveness of this method for detecting *S. aureus* and the resistance genes (blaZ, ermC, and tetK) in food products and biological material of animal origin was scientifically validated and experimentally confirmed. The analytical sensitivity of the assay was from 10 DNA copies/μL, with 100% specificity and 100% reproducibility.

4. A total of 87 *S. aureus* strains and their genetic resistance markers (blaZ, ermC, and tetK) were identified using the developed real-time PCR assay. Among the 87 *S. aureus* strains, 2 (2.3%) did not carry any of the tested resistance genes, 18 (20.69%) had one gene, 39 (44.83%) had two genes, and 28 (35.18%) carried all three. Overall, 85 strains (97.7%) possessed at least one of the investigated resistance genes.

5. To evaluate the performance and compliance of the PCR test, concordance between phenotypic and genotypic methods of identification was assessed. According to the analysis results, the diagnostic sensitivity of the PCR test for the blaZ gene was 80.43%, specificity – 92.68%, PPV – 92.50%, NPV – 80.85%, and the kappa value was 0.73, indicating discrepancies in the classification of isolates as susceptible/resistant. The diagnostic sensitivity of the PCR test compared to the disk diffusion (DD) method for the tetK gene was 87.95%, specificity – 100%, PPV – 100%, NPV – 28.57%, and the kappa value was 0.40, indicating a substantial discrepancy between methods in classifying isolates as susceptible/resistant. The diagnostic sensitivity of the PCR test compared to the DD method for the ermC gene was 81.54%, specificity – 36.36%, PPV – 79.1%, NPV – 40%, and the kappa value was 0.18, indicating a significant discrepancy in the classification of isolates as susceptible/resistant. The thermonuclease gene nuc was detected in 100% of cases, with diagnostic sensitivity, specificity, PPV, and NPV all at 100%, and a kappa coefficient of 1, indicating perfect agreement between methods.

6. A laboratory prototype of a multiplex real-time PCR assay kit was developed to enhance the speed and accuracy of *S. aureus* identification and detection of resistance genes, aimed at ensuring the quality and safety control of animal-derived food products.

10. Relevance to Research Projects and Government Programs. The work was carried out within the framework of two scientific and technical programs:

BR24992785-OT-24 "Organization and implementation of comprehensive research to ensure the sustainable development of the agro-industrial complex of the Kostanay region with the establishment of a research and technology center" (2024–2027);

BR10764944 "Development of analytical control methods and monitoring of food safety," project: "Development of multiplex real-time PCR for the detection of *Staphylococcus aureus* and Streptococcus agalactiae in dairy products and determination of antibiotic resistance loci" (2020–2023).

11. Reliability and Justification of the Obtained Results

The reliability and validity of the obtained results are confirmed by the use of new molecular genetic methods conducted on certified equipment in an accredited laboratory. The conclusions of the dissertation are novel and based on the results of original research.

12. Information on Publications Based on the Main Results. On the topic of the dissertation, 16 scientific and educational-methodological works have been published, including:

- 11 articles, among them: 2 in international scientific journals indexed in Scopus (Veterinary World doi:10.14202/vetworld.2023.1815-1820; Ecology, Environment and Conservation

https://www.envirobiotechjournals.com/issues/article_abstract.php?aid=11014&iid=322&jid=3) with percentiles of 80 and 15; 7 articles in journals of the Committee for Control of Education and Science of the Ministry of Education and Science of the Republic of Kazakhstan (КОКСНВО МНВО РК), including 3 articles in national scientific publications with assigned DOIs (DOI: 10.52578/2305-9397-2022-1-1-3-12; DOI: 10.56339/2305-9397-2022-3-1-105-114; DOI: 10.52578/2305-9397-2023-2-2-144-153);

- 2 articles in the proceedings of international conferences;
- 1 utility model patent №7828 dated 17.02.2023;
- 1 chapter in a collective monograph;
- 3 educational-methodological publications.

13. Description of the Doctoral Candidate's Contribution. The author participated in all stages of the research: reviewing and analyzing literature sources, sample collection, determination of staphylococcal resistance to antibacterial agents, a set of molecular genetic studies, analysis and interpretation of research results, and statistical data processing.

14. Volume and Structure of the Dissertation. The dissertation is presented on 187 pages of typed text and includes the following sections: introduction, literature review, original research, research results, summary and evaluation of research findings, list of references, and appendices. The work contains 11 formulas, 59 figures, 29 tables, 18 appendices, and 419 references.